Metabolic engineering strategies to increase *n*-butanol production from cyanobacteria

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Abstract

The development of sustainable replacements for fossil fuels has been spurred by concerns over global warming effects. Biofuels are typically produced through fermentation of edible crops, or forest or agricultural residues requiring cost-intensive pretreatment. An alternative is to use photosynthetic cyanobacteria to *directly* convert CO_2 and sunlight into fuel. In this thesis, the cyanobacterium *Synechocystis* sp. PCC 6803 was genetically engineered to produce the biofuel *n*-butanol. Several metabolic engineering strategies were explored with the aim to increase butanol titers and tolerance.

In papers I-II, different driving forces for *n*-butanol production were evaluated. Expression of a phosphoketolase increased acetyl-CoA levels and subsequently butanol titers. Attempts to increase the NADH pool further improved titers to 100 mg/L in four days.

In paper III, enzymes were co-localized onto a scaffold to aid intermediate channeling. The scaffold was tested on a farnesene and polyhydroxybutyrate (PHB) pathway in yeast and in *E. coli*, respectively, and could be extended to cyanobacteria. Enzyme co-localization increased farnesene titers by 120%. Additionally, fusion of scaffold-recognizing proteins to the enzymes improved farnesene and PHB production by 20% and 300%, respectively, even in the absence of scaffold.

In paper IV, the gene repression technology CRISPRi was implemented in *Synechocystis* to enable parallel repression of multiple genes. CRISPRi allowed 50-95% repression of four genes simultaneously. The method will be valuable for repression of competing pathways to butanol synthesis.

Butanol becomes toxic at high concentrations, impeding growth and thus limiting titers. In papers V-VI, butanol tolerance was increased by overexpressing a heat shock protein or a stress-related sigma factor.

Taken together, this thesis demonstrates several strategies to improve butanol production from cyanobacteria. The strategies could ultimately be combined to increase titers further.

Key words: cyanobacteria, metabolic engineering, biofuels, butanol, synthetic scaffold, CRISPRi, solvent tolerance.

Sammanfattning

Den ökade medvetenhet kring effekterna av global uppvärmning har stimulerat utvecklingen av hållbara alternativ till fossila bränslen. Biobränslen produceras traditionellt sett via jäsning av ätbara grödor eller av restavfall från skogs- och jordbruksindustrin. Det senare kräver en kostsam förbehandling innan jäsning är möjlig. Ett alternativ är att använda fotosyntetiska cyanobakterier för att omvandla CO_2 och solljus *direkt* till bränsle. I den här avhandlingen har cyanobakterien *Synechocystis* sp. PCC 6803 genmodifierats för att producera biobränslet *n*-butanol.

I *Paper I-II* utvärderades olika drivkrafter för produktion av *n*-butanol. Uttryck av ett fosfoketolas ökade nivåerna av acetyl-CoA och följaktligen mängden producerad butanol. Ansträngningar för att öka tillgängligheten av NADH höjde butanolmängden ytterligare, till 100 mg/L inom fyra dagar.

I *Paper III* co-lokaliserades enzymer på en slags dockningsstation för att underlätta intermediärtransport. Dockningsstationen testades på syntesvägar till farnesen och polyhydroxybutyrat (PHB) i jäst respektive *E. coli*, och skulle kunna appliceras även i cyanobakterier. Co-lokalisering av enzymer ökade farnesenproduktionen med 120%. Påkoppling av en liten affinitetsdomän på enzymet för vägledning till dockningsstationen ökade dessutom produktionen farnesen och PHB med 20% respektive 300% även i frånvaro av dockningsstation.

I *Paper IV* implementerades genrepressionstekniken CRISPRi i *Synechocystis* för att möjliggöra genrepression av flera gener samtidigt. Med hjälp av CRISPRi kunde fyra gener simultant nedregleras 50-95%. Metoden kommer att underlätta repression av syntesvägar som står i konkurrens till butanolsyntesvägen.

Butanol är toxiskt vid höga koncentrationer, vilket hämmar tillväxt och därmed även butanolproduktion. I *Paper V-VI* ökades toleransen mot butanol genom att överuttrycka ett värmechocksprotein eller en stressrelaterad sigmafaktor.

Sammanfattningsvis demonstreras ett flertal strategier för att öka butanolproduktionen från cyanobakterier i denna avhandling. Strategierna kan i framtiden kombineras för att öka produktionen ytterligare.

List of publications and manuscripts

This thesis is based on the following articles or manuscripts, referred to in the text by their Roman numerals (I-VI). The articles can be found in the appendix.

I. **Anfelt, J**., Kaczmarzyk, D., Shabestary, K., Renberg, B., Uhlén, M., Nielsen, J., Hudson, E.P. Genetic and nutrient modulation of acetyl-CoA levels in Synechocystis for n-butanol production. *Microb. Cell Fact.* **14**, 167 (2015).

II. **Anfelt, J**., Shabestary, K., Hudson, E. P. Complementary effects of ATP, acetyl-CoA and NADH driving forces increase butanol production in *Synechocystis* sp. PCC 6803. *Manuscript.*

III. Tippmann, S., **Anfelt, J**., David, F., Rand, J. M., Siewers, V., Uhlén, M., Nielsen, J., Hudson, E. P. Affibody scaffolds improve sesquiterpene production in Saccharomyces cerevisiae. *Manuscript.*

IV. Yao, L., Cengic, I., **Anfelt, J**. & Hudson, E. P. Multiple gene repression in cyanobacteria using CRISPRi. *ACS Synth. Biol.* ***5,*** *207–212* (2016).

V. **Anfelt, J**., Hallstrom, B., Nielsen, J. B., Uhlen, M. & Hudson, E. P. Using transcriptomics to improve butanol tolerance in Synechocystis sp. PCC 6803. *Appl. Environ. Microbiol.* **79**, 7419–7427 (2013).

VI. Kaczmarzyk, D., **Anfelt, J**., Särnegrim, A., Hudson, E. P. Overexpression of sigma factor SigB improves temperature and butanol tolerance of Synechocystis sp. PCC6803. J Biotechnol. **182–183**, 54–60 (2014).

Contributions to the papers

Paper I
Main responsible for planning and execution of laboratory experiments. Wrote the manuscript together with coauthors.

Paper II
Main responsible for planning and execution of laboratory experiments, as well as manuscript writing.

Paper III
Performed all FRET experiments and scaffold evaluations in *E. coli*. Contributed to project design and manuscript writing.

Paper IV
Contributed to selecting targets for gene repression, and the evaluation of dCas9 expression.

Paper V
Main responsible for planning and performing all laboratory experiments. Wrote the manuscript together with coauthors.

Paper VI
Contributed to project design, cloning of constructs, flow cytometry based tolerance evaluation, and manuscript editing.

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Thesis outline

This thesis gives an overview of techniques used in metabolic engineering of microorganisms for the production of biofuels, with particular focus on photosynthetic bacteria as synthesis hosts. Several of the presented strategies were applied in the appended publications and manuscripts, upon which the content of this thesis is based, with the aim to increase production of the biofuel *n*-butanol in cyanobacteria.

Chapter I gives a general introduction to microbial biofuel production. Previous efforts and the current state of the field are described, and the benefits of photosynthetic production hosts as well as the use of *n*-butanol as gasoline replacement are discussed.

In **Chapter II**, specific pathway optimization techniques, and methods to practically implement these, are described. The end of the chapter summarizes the work presented in papers I-IV, where many of the optimization strategies were applied. These include novel ways to increase substrate availability and enable multiplex gene repression in cyanobacteria.

Chapter III focuses on the toxicity effects imposed by high butanol concentrations, and methods to increase tolerance. The end of the chapter summarizes the work presented in papers V-VI, where different engineering strategies were applied to increase butanol tolerance in cyanobacteria. Paper V serves as the first published demonstration of increased tolerance of cyanobacteria to butanol.

I. MICROBIAL BIOFUEL PRODUCTION

The use of microorganisms for production of alcohols and other solvents has a long history. Already thousands of years BC, alcoholic beverages such as beer and wine were produced through fermentation of sugars into ethanol by yeast[1]. Ethanol has since been the dominating fermentation product industrially. In the early 1900s, the bacterium *Clostridium acetobutylicum*, capable of acetone-butanol-ethanol (ABE) fermentation, was isolated and used for large-scale production of acetone for cordite manufacturing during World War I and II. The large amount of butanol formed in the process was initially considered an unwanted by-product, but would later be used as solvent in car lacquer. During the 1950s, ABE fermentation was outcompeted by petroleum refining[2]. Microbial synthesis of bioproducts has however regained interest recently, with the increased awareness of global warming and the need for sustainable alternatives to petroleum-derived fuels, chemicals and plastics. Great advances in the fields of metabolic engineering, systems biology and synthetic biology over the last two decades have enabled the design of superior production hosts, so called cell factories, which are genetically modified to synthesize a product of interest at high titers and productivities. Microbial synthesis has been demonstrated for numerous compounds, including chemicals, biofuels, food additives and pharmaceuticals[3,4]. While most of these have only been produced on laboratory scale, several examples of commercial production exist; Gevo's yeast fermentation for production of isobutanol (used as biofuel, solvent and for plastics and rubber manufacturing), Amyris's conversion of sugarcane to squalane (emollient in cosmetics) by yeast, DuPont's production of 1,3-propanediol (solvent and chemical building block) from corn glucose in *E. coli*, and Evolva and International Flavor & Fragrances Inc.'s vanillin synthesis in yeast, to name a few[3] (Figure 1).

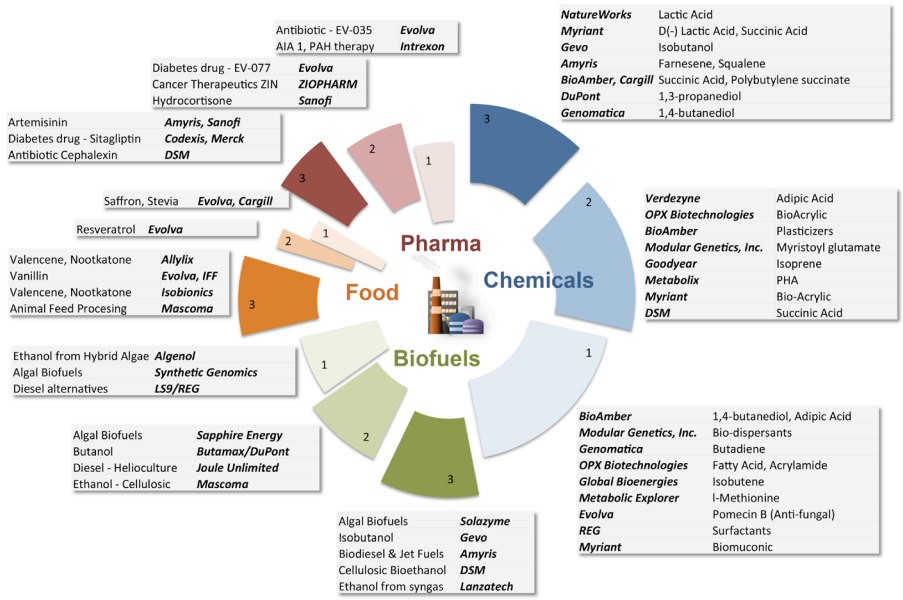

Figure 1. Overview of companies using engineered microorganisms for the synthesis of chemical compounds. Numbers represent the current stage of progress: 1) small-scale laboratory development, 2) pilot-scale, 3) commercial scale. Figure reprinted from Jullesson et al.[3] with permission from Elsevier.

Selection of production host

Production of bioproducts such as biofuels can theoretically be realized in various host organisms. Each microorganism has its own benefits and disadvantages as production host, and with the continuously expanding toolbox for genetic manipulation we are no longer constrained to natural producers of a certain compound, but can instead pick out desirable traits from several organisms and combine them into a single host. In practice, some traits are more easily engineered than others, and this will influence the choice of starting strain. When selecting the production host, several factors need to be considered:

(1) **The feedstock.** What carbon source(s) can the host strain utilize, and what carbon sources are available and feasible to use? Glucose and starch are readily

metabolized by most industrial microbes, including *Saccharomyces cerevisiae* (baker's yeast), *Escherichia coli* and *Clostridium* species, and are commonly used feedstocks in large-scale fermentation processes[5]. Biofuels produced from food crops are often referred to as *first generation biofuels* (Figure 2), where sugarcane ethanol in Brazil and corn ethanol in USA have dominated the market during the last decade[6]. Fuel production from edible crops is however controversial for its competition with the food industry for arable land, leading to rising food prices[7]. Some first generation biofuels also suffer from a low net energy gain. For instance, corn ethanol only yields 25% more energy than what is consumed in the production process, whereas the net energy gain for soybean biodiesel is close to 100%[8]. Additionally, feedstock can account for over two thirds of the total production cost of first generation biofuels, making the process vulnerable to fluctuations in feedstock price. Keeping feedstock costs low is of particular importance for low-value products like fuel and bulk chemicals. In contrast, second generation biofuels – derived from inedible plant biomass – can be produced with feedstock costs constituting 30-50% of total costs[9], and with net energy gains exceeding 500%[10]. The feedstocks are often derived from agricultural or forest residues, or from energy crops preferably grown on marginal land, and are rich in lignin, hemicellulose and cellulose. Although the lignocellulosic material in itself is relatively cheap, the microbial fuel producer cannot utilize the sugar content before the lignocellulose has undergone a cost-intensive pretreatment process[6]. Despite this, several examples of pilot-plant and commercial scale plants for cellulosic ethanol exist. Beta Renewables opened the world's first plant for second-generation biofuels in 2012 in Crescentino, Italy[7], where pretreated hemicellulose and cellulose are enzymatically hydrolyzed to free sugars which are further fermented to ethanol by yeast. Cellulose is a polysaccharide solely consisting of glucose units, which can be metabolized directly by baker's yeast. Hemicellulose on the other hand contains a mixture of 5- and 6-carbon sugar units, but only the latter can be used by *S. cerevisiae* naturally. Efficient conversion of hemicellulose to ethanol thus requires either genetic modification of the cell factory to allow C5 utilization, or the use of a natural C5-metabolizing organism such as the yeast *Pichia*[11].

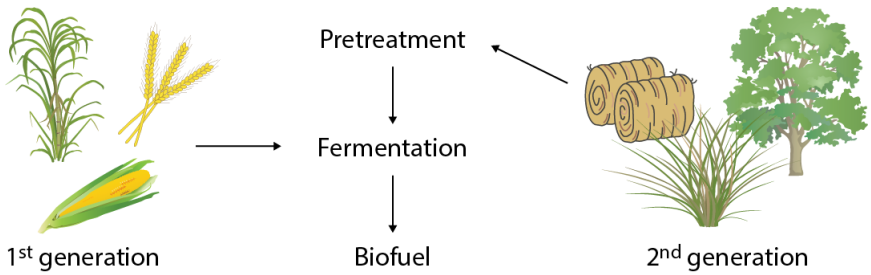

Figure 2. Feedstocks used for first and second generation biofuels. 1st generation biofuels are derived from edible crops such as corn, sugarcane and cereals. 2nd generation biofuel feedstocks comprise energy crops and residues from agricultural and forest industry.

An alternative to hydrolysis of hemicellulose and cellulose for release of carbohydrate feedstock is thermochemical gasification of the lignocellulosic biomass to syngas (synthetic gas). The exact composition of syngas depends on the biomass composition and the gasification process, but main components are CO, H_2 and sometimes also CO_2. These gases can be utilized as fermentation feedstock for e.g. ethanol synthesis by acetogenic bacteria, typically from *Clostridia* species[12]. The main challenges of syngas fermentation are low tolerance to inhibitors present in the gas[13] as well as poor solubility of CO and H_2 in the liquid culture, limiting productivity[13,14]. Overall, production of second-generation biofuels is in general still associated with higher production costs compared to first generation biofuels, on the order of 30%[12].

The examples discussed above are all based on plant matter. The *direct* use of CO_2 as carbon source for biofuel production is an alternative strategy. Acetogens can fix CO_2 from syngas through the Wood-Ljungdahl pathway, and further convert it to short-chain products such as acetate or ethanol. Synthesis of longer-chain carbon molecules is however not possible due to ATP limitation[19]. Photosynthetic organisms like cyanobacteria and algae also fix CO_2, but their ability to use sunlight as energy source enables high ATP production from the light reactions, and the repertoire of products that are metabolically feasible to synthesize is thus not constrained to short-chain products. The CO_2 can be fixed directly from the atmosphere or from CO_2-rich

flue gas and converted to fuel or fuel precursor, thus minimizing feedstock cost while avoiding competition for arable land. Some microalgae strains naturally accumulate lipids during nutrient limited growth conditions, which can be extracted and chemically converted to biodiesel[15]. The product extraction step requires de-watering and cell lysis and is energy intensive. It can however be avoided by genetically engineering the host to synthesize a product that can be secreted (Figure 3). Examples of this has been demonstrated for several biofuels, including isobutanol[16], ethanol[17] and isoprene[18]. Commercial production of cyanobacteria biofuels is anticipated to be realized within the next two years by companies Algenol and Joule unlimited. Both companies use CO_2 from industrial gas to cultivate cyanobacteria genetically modified to produce and secrete ethanol, targeting productivities of up to 25,000 gallons/acre/year – 10- and 60-fold higher than those of cellulosic and corn ethanol, respectively. In this thesis, the cyanobacteria strain *Synechocystis* sp. PCC 6803 was genetically engineered to produce *n*-butanol – an attractive gasoline replacement – directly from CO_2, light and water. The low-cost, fossil independent and readily available feedstock combined with the natural secretion of butanol, avoiding costly cell harvesting for product extraction, makes cyanobacterial butanol synthesis an attractive candidate as a sustainable biofuel production system.

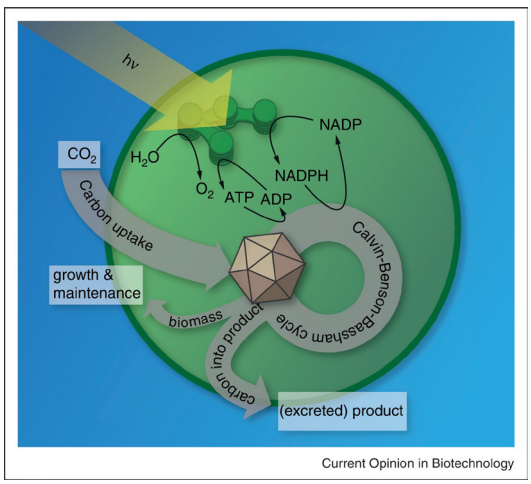

Figure 3. Biofuel production from cyanobacteria. Incoming light is used in the thylakoids to split water into oxygen and hydrogen ions. The resulting electrons are transported through the photosystems, enabling generation of NADPH and ATP, which are required for CO_2 fixation in the Calvin-Benson-Bassham cycle. Metabolic engineering allows the partitioning of fixed carbon into an excretable product, such as butanol or ethanol. Figure reprinted from Savakis et al.[153] with permission from Elsevier.

(2) **The product.** When selecting a host based on final product, there are two routes to choose from: either use a native producer, which can be further optimized through genetic engineering and/or modulation of growth conditions, or introduce the heterologous synthesis pathway into a non-native producer that possesses other attractive traits. Which choice results in higher productivity will vary from case to case. Starting from a native producer can have several advantages. For instance, the native producer may have a high tolerance to the product of interest. The native *n*-butanol producing *Clostridium acetobutylicum* can grow unhindered at butanol concentrations of 5 g/L (and, with reduced growth rate, up to 12 g/L)[20], while the growth rate of cyanobacterium *Synechocystis* sp. PCC 6803 drops significantly already at *n*-butanol concentrations above 1 g/L[21]. The underlying factors affecting tolerance are complex and challenging to engineer in a predictable manner (discussed in more detail in chapter III). Increasing the tolerance, e.g. through gradual adaptation to higher concentrations, may therefore require less time investment when starting from a microbe with inherently high tolerance to the product of interest. In some cases, the titer from the native producer can reach a sufficient level just through optimization of the growth conditions, hence avoiding genetic modifications completely. This is more common in the food and health food industry, where for instance algae species are used commercially for production of food colorants or anti-oxidant and vitamin A-rich carotenoids[22]. Working with wild-type strains not only reduces the strain optimization time, but also simplifies the scale-up process since potential release of genetically modified organisms into the surrounding is not a concern that needs to be addressed. Another advantage of native producers is that the pathway enzymes are well adapted to the intracellular environment. Many enzymes derived from strict anaerobes are known to be oxygen-sensitive and may have significantly reduced activity if expressed under aerobic or photoautotrophic conditions[23].

Although beneficial in some cases, there are several situations where the use of a native producer is inappropriate. The synthesis pathway of interest may for instance be tightly regulated, which hinders efficient production. Simply transferring the genes encoding the pathway enzymes into a different host, lacking these regulatory mechanisms, can circumvent this problem. It is also crucial that the cell factory can

be cultivated easily and with sufficient rate to reach high productivity. The high growth rate (doubling time of 20 min in LB media[24]) as well as the ability to grow both aerobically and anaerobically are two of the features making *E. coli* popular as cell factory.

(3) **The molecular toolbox.** The initial establishment of a functional biofuel pathway in a new host is in itself not necessarily difficult, provided that efficient tools for gene transfer are available. However, extensive optimization of the microbial host through metabolic engineering is typically required to reach economically feasible titers and yields. Deleting competing pathways could for instance increase carbon flux through the pathway of interest. The genetic modification process is greatly simplified by the availability of a well-established molecular toolbox. *Escherichia coli* and *Saccharomyces cerevisiae* are by far two of our most well-characterized microbes; both are easily transformable, their genomes have been sequenced and introduced into computational metabolic models, and several promoters and other genetic elements have been designed and/or evaluated to enable fine-tuning of protein expression levels. For this reason, *E. coli* and *S. cerevisiae* are the most common cell factories both in large-scale and lab-scale applications. Other common, well-characterized production hosts include *Clostridia* sp., *Bacillus* sp., *Corynebacterium* sp. and *Pseudomonas* sp[25]. Among unicellular cyanobacteria, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 and, more recently, *Synechococcus* sp. PCC 7002 have become the model organisms. Although the molecular tools are not as far developed as for *E. coli* and *S. cerevisiae*, the toolbox has expanded rapidly during the last few years enabling modulated expression levels and multiplex gene knockdowns[26–28]. Tools for optimization of cyanobacterial production systems are further reviewed in chapter II and exemplified in paper IV where CRISPRi was for the first time implemented in a cyanobacteria host, enabling fast and inducible gene repression of up to four genes simultaneously.

Metabolic routes to biofuel

The development of tools for genetic engineering, in combination with the constantly expanding collection of sequenced genomes, has enabled identification and implementation of various pathways for microbial biofuel synthesis. Both gaseous fuels, such as H_2 or ethylene, and different categories of liquid fuels can be synthesized in this way. **Figure 4** summarizes metabolic pathways to some of the promising biofuel candidates. Aromatic hydrocarbons as well as straight or branched short-chain compounds like isopropanol and butanol are suitable additives or replacements for gasoline. In contrast, biodiesel is typically composed of straight medium- to long-chain hydrocarbons (C9-C23)[29]. Although the possible routes to biofuels of varying chemical properties are many, they all originate from a handful of core metabolites found in the central metabolism which is almost perfectly conserved among all living organisms[30]. These include glyceraldehyde 3-phosphate (G3P), phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA (**Figure 4**), as well as TCA cycle intermediates succinyl-CoA and α-ketoglutarate[4]. G3P and PEP can be produced from glucose through glycolysis or, as in photosynthetic organisms, from CO_2 fixed in the Calvin cycle. The extended usage of a few core metabolites as starting substrate for multiple end products (not limited to fuel compounds) enables the development of platform strains with elevated levels of these metabolites. The platform strains serve as advantageous starting strains for introduction and optimization of product pathways derived from the abundant central metabolite. This concept was demonstrated in paper I, where overexpression of a phosphoketolase increased the acetyl-CoA pool in *Synechocystis*, with subsequent increase in *n*-butanol titer. Phosphoketolase overexpression is hence also a promising strategy for increasing titers of other acetyl-CoA derived products, such as fatty acids, alkanes, acetone and isopropanol.

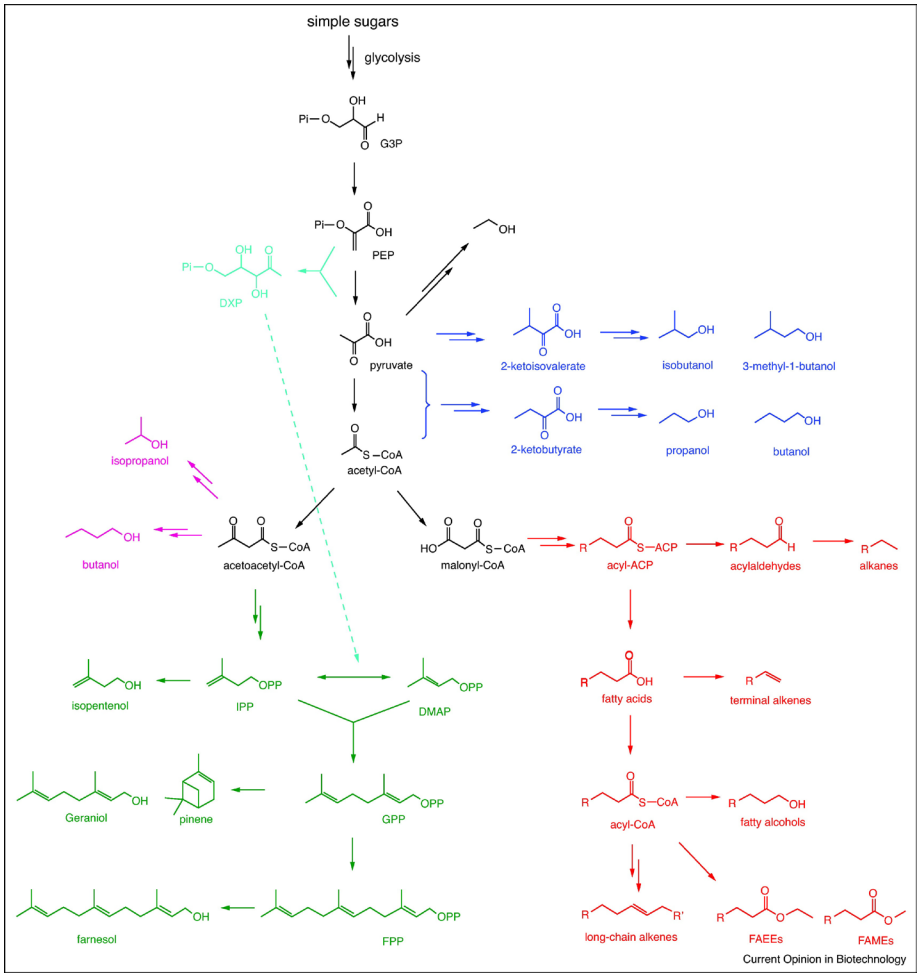

Figure 4. Engineered metabolic routes to a selection of advanced biofuels. Colors represent different pathway subgroups: central metabolism (black), 2-keto acid pathways (blue), fatty acid pathways (red), terpene and isoprenoid pathways (green), fermentative pathways to short-shain alcohols (purple). Double or dashed arrows correspond to multiple reaction steps. G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; DXP, 2-C-methy-D-erythritol-4-phosphate; ACP, acyl carrier protein; IPP, isopentenyl-diphosphate; DMAP, dimethyl- allylphosphate; GPP, geranyl-diphosphate; FPP, farnesyl-pyrophosphate; GGPP, geranylgeranyl-pyrophosphate; FAME, fatty acid methyl ester; FAEE, fatty acid ethyl ester. Figure reprinted from Zhang et al.[154] with permission from Elsevier.

n-Butanol – a promising replacement for gasoline

The short-chain alcohol ethanol has so far dominated the market for biofuels. The reason for this is not a possession of extraordinarily good biofuel characteristics, but rather the relative ease to produce large quantities for a reasonable price. In fact, ethanol is far from optimal for fuel applications for several reasons. First, ethanol is hygroscopic (i.e. it attracts water) and corrosive, making it unsuitable for transportation in pipelines and for use in higher concentrations than 1:10 ethanol-gasoline blends in modern gasoline engines. Second, the short carbon chain reduces the energy density of ethanol by 35% compared to gasoline (from 33 to 21 MJ/L, Figure 5). These disadvantages are easily overcome by simply adding two extra carbons to the alcohol chain. *n*-Butanol is less hygroscopic and corrosive than ethanol, allowing much higher concentrations in gasoline blends without requiring engine modifications. Additionally, the energy content (29 MJ/L) is more similar to that of gasoline. The lower vapor pressure also makes *n*-butanol safer to handle and easier to store[31].

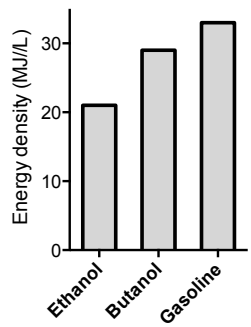

Figure 5. Energy densities of ethanol and butanol in relation to gasoline.

Microbial *n*-butanol synthesis has traditionally been achieved through the previously mentioned acetone-butanol-ethanol fermentation pathway, which is native to several *Clostridia* species and has been extensively studied during the last century. The fermentation process is biphasic, starting with an acidogenic phase where ATP generation is accompanied by accumulation of acetate and butyrate. The resulting low pH induces the second, solventogenic, phase where the acetate and butyrate are metabolized into acetone, ethanol and *n*-butanol[32] (Figure 6). In order to decrease product separation costs, attempts to engineer *Clostridia* for homobutanol fermentation has been made, increasing the butanol percentage of total solvent from 65% to 88%. Complete elimination of acetone and ethanol production has however been unsuccessful[13]. Engineering to improve *Clostridia* strains have long been

hampered by the lack of genetic tools. Although methods for gene deletions have recently improved significantly, genetic engineering remains challenging. This is partly due to the complex regulatory system and the incomplete understanding of the biphasic fermentation process[33]. In this thesis, a chimeric version of the *Clostridia n*-butanol pathway was introduced in *Synechocystis* sp. PCC 6803, lacking the regulatory mechanisms of native ABE fermentative microbes. The synthesis pathway for storage polymer polyhydroxybutyrate (PHB) in this host contains a thiolase and acetoacetyl-CoA reductase for the conversion of acetyl-CoA to 3-hydroxybutyryl-CoA. Hence, expression of three heterologous enzymes was sufficient to complete the synthesis route from CO_2 to *n*-butanol in *Synechocystis* (paper I).

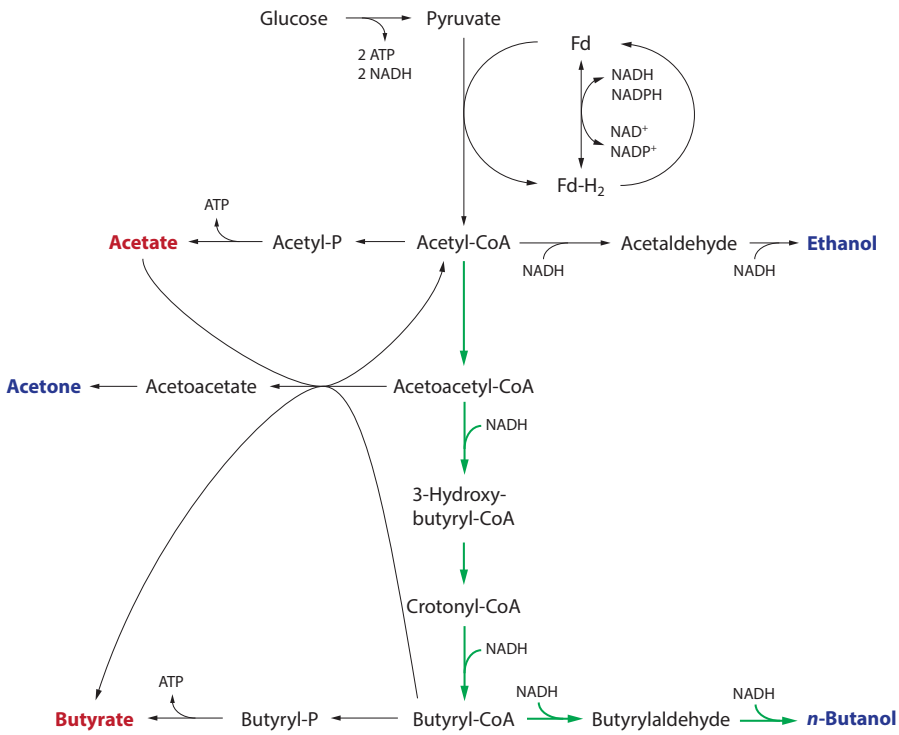

Figure 6. Schematic representation of acetone-butanol-ethanol fermentation pathways in *Clostridium acetobutylicum*. Products of the acetogenic and solventogenic phases are colored red and blue, respectively. Part of the *n*-butanol pathway (green arrows) from *C. acetobutylicum* was used in cyanobacteria in this thesis.

II. PATHWAY OPTIMIZATION

In order to reach the high rates, titers and yields required for commercial production of a microbial biofuel, extensive optimization of host metabolism, growth conditions and the scale-up process is necessary. The main focus of this thesis is on genetic engineering strategies for increased biofuel production, and to some extent also the impact of growth media composition.

The host engineering process is typically an iterative cycle of design and execution of genetic modifications, with subsequent evaluation of host performance. Identification of suitable targets for genetic modifications, i.e. what genes to be knocked out or upregulated, has traditionally been a manual process partly based on local pathway knowledge and human intuition. The overwhelming complexity of metabolic networks has stimulated the development and implementation of genome-scale metabolic models, which can be used for computational prediction of metabolic rate (flux) distributions and identification of genetic modifications for increased product formation[34]. These models are thus helpful tools in the host design step of the iterative engineering process. Introduction of the selected gene modifications is followed by quantification of the product of interest. One round of optimization is typically not enough to reach the desired titers and productivities, and efforts to identify bottlenecks in the metabolic system are therefore necessary. These can include quantification of transcript and protein levels, indicating what pathways and reaction steps are active, and metabolomics studies for identification of intermediate accumulation. Means to circumvent the current bottlenecks will then be addressed in the second round of the optimization cycle (Figure 7)[35]. This chapter will present some of the available molecular tools and strategies for increasing biofuel production, as well as their applications in the papers of this thesis, with special emphasis on *n*-butanol synthesis and photosynthetic production hosts.

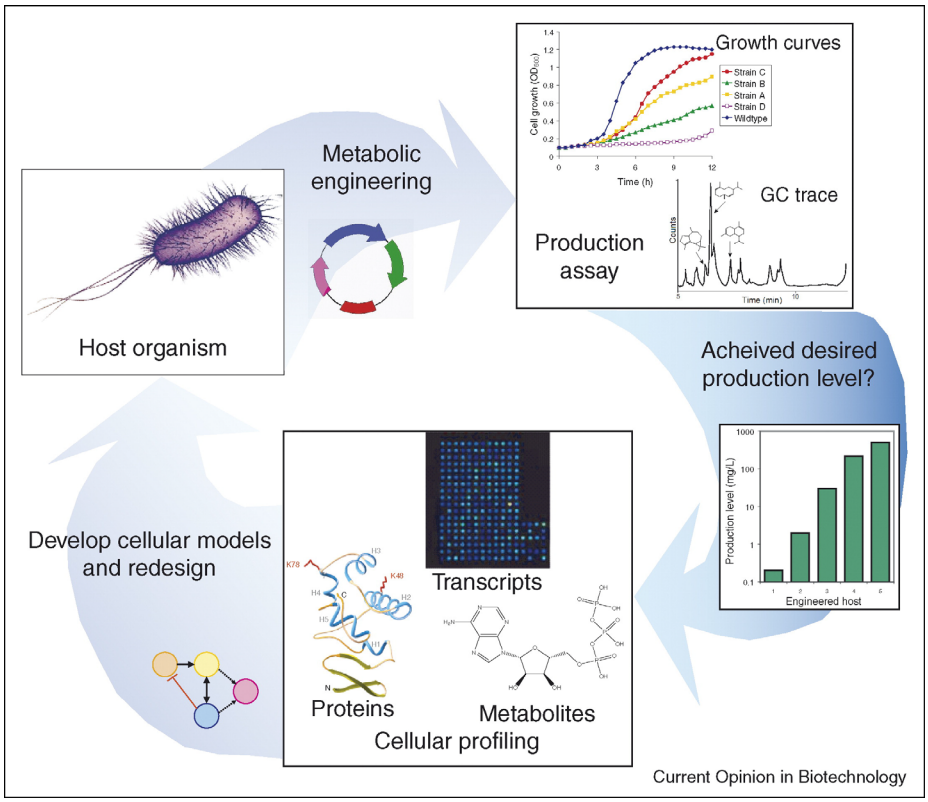

Figure 7. The iterative cycle of host engineering for increased biofuel production. The impact of genetic modifications on host performance is evaluated e.g. in terms of productivity and titer. Bottlenecks can be identified trough flux analysis and omics (proteomic, transcriptomic, metabolomic) measurements, and used as guidance for further engineering efforts. Figure reprinted from Mukhopadhyay et al.[35] with permission from Elsevier.

Flux prediction using genome-scale modeling

The rapid development of fast and cost-efficient sequencing methods has turned genome sequencing of microorganisms into a standard procedure, with the first bacterial genome sequence published in the mid 1990s[36]. This became the starting point for the construction of genome-scale models (GEMs) as a means to increase the understanding of metabolic networks and guide metabolic engineering efforts[34]. GEMs have since then been continuously refined and used to predict and optimize production of various compounds, including biofuels, in model organisms like *E. coli* and *S. cerevisiae*, but also in other microbes of industrial relevance[37,38]. Genome-scale models are stoichiometric representations of all possible biochemical reactions inside a cell and can be assembled with the help of databases such as KEGG and EXPASY, in order to link genotype to function, combined with information retrieved from literature. The models can be used to guide metabolic engineering strategies by predicting what modifications are likely to improve strain characteristics and thus worthwhile evaluating experimentally. Incorporation of experimental data allows continuous refinement of the model and is necessary to increase the accuracy of its predictions. Constraint-based reconstruction and analysis (COBRA) methods can then be applied to predict maximum theoretical growth and product yields, as well as gene deletions and regulatory changes necessary to increase product yield further[39].

Flux balance analysis

Flux balance analysis (FBA) is the oldest and most widespread COBRA method and is used to predict the rates at which metabolites flow through different parts of the metabolic network, i.e. the metabolic flux distribution[34]. FBA is based on the flux balance equation:

$$\mathbf{S} \cdot \mathbf{v} = 0$$

where **S** is the so called stoichiometric matrix comprising the stoichiometric coefficients for all metabolic reactions (with the metabolites listed as rows and the reactions as columns), and **v** is a vector containing the reaction rates. If metabolic reactions are considered being faster than the rate of growth and environmental changes, the system can be assumed to be in steady-state, i.e. all metabolites are produced in equal rate as they are being consumed, and the flux balance equation is hence set to zero[40]. This allows the prediction of the rates of all reactions in the cell. The flux balance equation is typically underdetermined since the number of reactions normally exceeds the number of metabolites, and multiple solutions therefore exist. The **S** matrix however imposes some constraints on the number of possible solutions, and further constraints can be introduced by setting upper and lower limits to the allowed rates of some of the reactions, such as substrate uptake. By setting an objective function, e.g. biomass formation in the case of growth rate prediction, FBA can identify the flux distribution(s) that maximizes the objective within the allowed solution space (Figure 8)[41]. Similarly, the objective can be set to predict the flux distribution for maximized biofuel production. FBA has become an attractive tool since it only requires information about metabolic reaction stoichiometry and a few strain-specific parameters, such as maximal substrate uptake rate and the metabolic requirements for maintenance reactions and biomass synthesis[42]. The simplicity of FBA is however both its strength and its weakness; the lack of kinetic data prevents the estimate of metabolite concentrations, the predictions are limited to steady-state growth conditions, and regulatory effects are typically not considered[34]. Despite this,

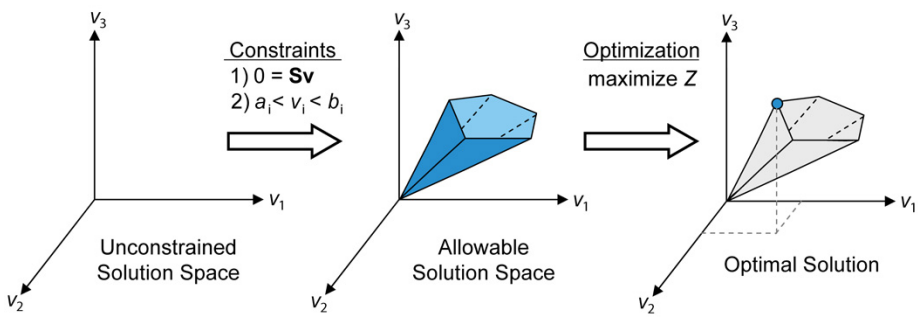

Figure 8. The concept of flux balance analysis. At steady-state, the product of the stoichiometric matrix (**S**) and the vector containing all cellular reaction rates (**v**) equals zero, constraining the number of allowed reaction rates to a limited solution space. By setting an objective function, e.g. maximize productivity, the specific reaction rates that result in highest theoretical productivity can be calculated. Figure reprinted from Orth et al.[41] with permission from Nature Publishing Group.

FBA has been successfully applied to guide metabolic engineering for production of various metabolites, including cofactors and short-chain alcohols[43,44].

Genome-scale modeling of cyanobacteria

The use of genome-scale models in cyanobacteria is still in its infancy. During the last few years, several GEMs have been developed and updated for *Synechocystis 6803* [45–48] in particular, but also for *Synechococcus* 7942 [49], *Synechococcus* 7002 [50], and *Cyanothece* sp. ATCC 51142 [51]. The photoautotrophic nature of cyanobacteria poses additional challenges for model construction compared to modeling of heterotrophic organisms. These include the metabolic representation of diurnal growth (i.e. the shift between light and dark conditions), residual respiratory activity during oxygenic photosynthesis, and light-dependent formation of reactive oxygen species (ROS). Knowledge gaps concerning enzyme specificity and directionality, as well as the presence of many non-annotated genes, also complicate model construction, in particular for dark metabolism where experimental data for model validation is scarce[46]. For instance, not until 2011 was it discovered that most cyanobacteria do in fact have a closed TCA cycle, in contrast to previous belief, but with the conventional 2-oxoglutarate dehydrogenase reaction replaced by the reactions of 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase[52]. The TCA cycle is hence incorrectly represented by an incomplete version in all but the newest cyanobacteria models, and it is possible that other important reaction mechanisms are yet to be identified in these hosts.

In paper I, we applied FBA on the most recent genome-scale model[46] of *Synechocystis* 6803, which addresses some of the previously overseen photosynthesis-specific mechanisms, in order to predict the effect of a phosphoketolase enzyme on butanol productivity during photoautotrophic growth. An increased butanol production was predicted by FBA and was also observed experimentally.

Modulation of enzyme expression and activity

Biofuel production in non-native producers requires introduction of foreign genes, either into the genome or on a replicating plasmid, to create a complete synthesis pathway. The expression levels of these enzymes should be high enough to allow fast synthesis of the final product, while being carefully balanced to avoid build-up of pathway intermediates or imposing a metabolic burden from protein overproduction. Accumulation of pathway intermediates not only indicates inefficient catalysis but can also be directly harmful to the cell. For example, the intermediate acetoin causes an acute toxicity response in *Synechococcus* sp. PCC7942 at approximately 250 times lower concentrations than does the pathway final product 2,3-butanediol[53]. Intermediate accumulation may also trap important coenzymes like CoA in its bound form, hindering new pathway substrate to be generated. This was proposed to limit *n*-butanol production in *Synechococcus* strains where butyryl-CoA accumulation was accompanied by decreased acetyl-CoA and increased pyruvate levels[54]. Enzyme expression levels are commonly modulated by varying promoter strengths and RBS sequences, and through codon optimization. The selection of inducible, orthogonal promoters that are functional in cyanobacteria has long been limited. However, several aTc- and IPTG-inducible promoters with wide dynamic range were recently developed for both *Synechocystis* and *Synechococcus* species[26,27,55]. Some of these are both inducible and fully repressible – an important feature utilized in paper IV where an inducible gene knockdown method (CRISPRi) was implemented in *Synechocystis*. Efforts to control expression levels through modulation of the RBS sequence have also been demonstrated. In contrast to results from model organisms like *E. coli*, predicted expression levels from different RBS prediction softwares have so far correlated poorly with observed expression levels in cyanobacteria[26,56].

Increased enzyme expression can be an efficient strategy to increase biofuel productivities and titers, as seen in a previous study where ethanol titers were increased from 1.1 to 5.5 g/L by the introduction of a second copy of the genes encoding pyruvate decarboxylase and alcohol dehydrogenase in a modified *Synechocystis* strain[57]. Increased enzyme expression through replacement of a moderately strong promoter (P_{psbA2}) with a stronger one (P_{trc}) also increased *n*-

butanol titers 3-fold in paper I. Overexpression however serves little purpose if the enzyme activity remains low. This has been an obstacle for the implementation of *n*-butanol synthesis in heterologous hosts, cyanobacteria in particular, since several of the *Clostridia* enzymes are oxygen sensitive and may thus have reduced activity in an oxygen-producing cell. *Clostridia* butyryl-CoA and aldehyde dehydrogenases, both believed to be oxygen sensitive, have been successfully replaced by oxygen-tolerant alternatives that typically also have had a different cofactor preference[23,58]. An oxygen-tolerant pathway diverting acetyl-ACP from fatty acid synthesis to *n*-butanol has also been developed as a potential replacement of the CoA-dependent pathway[59].

Metabolite availability

Reaching high productivity from heterologous pathways is sometimes complicated by the absence of natural driving forces. Short-chain biofuels like ethanol, isopropanol and isobutanol have been produced recombinantly at high titers of 40-50 g/L within two to three days, but all three pathways contain an irreversible decarboxylation step that effectively pulls flux towards product formation[60–62]. The *Clostridia n*-butanol pathway completely lacks irreversible reaction steps but still enable high butanol titers in the native host since well-balanced acetone-butanol-ethanol fermentation (typically in the ratio of 3:6:1 in *C. acetobutylicum*) is essential for the NADH recycling and ATP generation required for anaerobic growth[63]. Thus, production of *n*-butanol in a new host strongly benefits from the introduction of artificial driving forces that stimulate butanol formation. Such driving forces can be established e.g. through an increased substrate pool, introduction of one or more irreversible or energetically favored steps (such as CO_2-release or ATP hydrolysis), continuous removal of product, increased cofactor availability and/or removal of competing cofactor recycling reactions in order to couple production to growth.

Substrate availability

The first step of the traditional *n*-butanol pathway, condensation of two acetyl-CoA into acetoacetyl-CoA, has a strong thermodynamic preference for the reverse reaction. Despite this, high titers of *n*-butanol have been reached using this thiolase-mediated reaction in *E. coli,* both at aerobic (8.6 g/L)[64] and anaerobic (30 g/L)[65] conditions. The polymer PHB, which can accumulate to up to 90% of dry cell weight, is formed in a three-step pathway also starting with the condensation of two acetyl-CoA units, followed by a reduction to 3-hydroxybutyryl-CoA and polymerization into polyhydroxybutyrate[66] (Figure 9), demonstrating that the thiolase reaction allows for high titers if proper driving forces are in place.

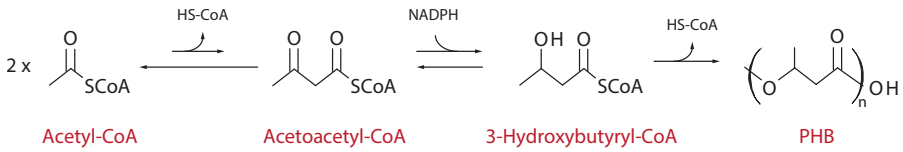

Figure 9. The polyhydroxybutyrate (PHB) biosynthesis pathway.

In paper I, we investigated whether an increased acetyl-CoA pool could serve as a driving force to push flux through the *n*-butanol pathway in cyanobacteria. An increased acetyl-CoA concentration should make the thiolase-mediated condensation reaction more thermodynamically favorable (Table 1). The pathway used in paper I contains a *trans*-enoyl-CoA reductase (Ter) from *T. denticola*, replacing the oxygen-sensitive butyryl-CoA dehydrogenase (Bcd) from *Clostridia.* The Ter-catalyzed reaction only uses NADH as reducing agent, in contrast to Bcd which also requires ferredoxin and flavoprotein, and has been shown to be irreversible[58]. Although the irreversibility of the crotonyl-CoA reduction substantially increased butanol titers in *E. coli*, production was improved an additional 10-fold by the deletion of competing pathways, creating an acetyl-CoA and NADH driving force for butanol synthesis[65].

Table 1. Estimated changes in the reaction Gibbs energy for the condensation of two acetyl-CoA into acetoacetyl-CoA at different intracellular acetyl-CoA:acetoacetyl-CoA ratios. Predications were calculated using eQuilibrator 2.0[67], with the intracellular pH and ionic strength set to 7.8 and 0.2 M, respectively. CoA levels were equal to acetoacetyl-CoA concentrations in this example.

Acetyl-CoA:Acetoacetyl-CoA	Estimated $\Delta_r G'$ (kJ/mol)
1	25.9 ± 1.7
10	14.5 ± 1.7
50	6.5 ± 1.7
100	3.1 ± 1.7
200	- 0.3 ± 1.7

Cofactor availability

Most of the commonly used fermentative pathways to biofuel and chemicals originate from obligate or facultative anaerobic microbes. Fermentation is necessary for the oxidation of NADH to NAD^+ needed in glycolysis. The involved enzymes thus typically have a strong preference for NADH over NADPH. In *E. coli*, the NADPH/NADH ratio has been determined to approximately 0.3 [68]. The reducing landscape in cyanobacteria however looks quite different, where the light reactions generate NADPH and ATP during photoautotrophic growth, and NADPH/NADH ratios range between 1-7 [69–71]. The low NADH availability risks limiting bioproduct titers, as demonstrated for lactic acid[72] and 2,3-butanediol[73] synthesis in cyanobacteria. In these cases, a soluble transhydrogenase was introduced to convert excess NADPH to NADH, which increased production of both products. Site-directed mutagenesis of an NADH-specific lactate dehydrogenase increased the affinity for NADPH and improved lactic acid productivity even further[74]. NADH-specific enzymes in pathways for ethanol, *n*-butanol, and 1,2-propanediol synthesis have also been replaced with NADPH-specific alternatives with higher activity[57,75,76] as a means to utilize the high NADPH availability in cyanobacteria. In paper II, we explored two different enzymatic reactions to convert excess NADPH to NADH, with the aim to increase butanol production. The first strategy was based on overexpression of the soluble transhydrogenase previously used for lactic acid and

2,3-butanediol production. The transhydrogenase acts by transferring a hydride ion from NADPH to NAD^+, generating one NADH and one $NADP^+$ molecule. In contrast, our second strategy utilized a phosphatase, which converts NADPH to NADH by removing the phosphate group through hydrolysis (Figure 10). Both strategies can theoretically increase the total NADH pool, but the impact on NADH/NAD^+ and NADPH/$NADP^+$ ratios will differ between the two.

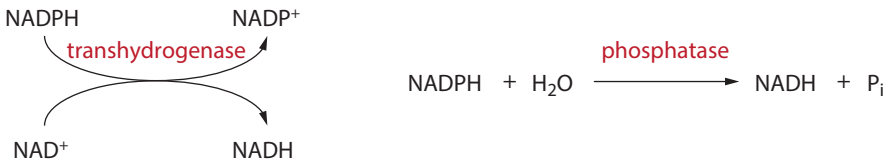

Figure 10. Catalytic reactions converting NADPH to NADH. Transhydrogenases mediate NADH formation by transferring a hydride ion from NADPH to NAD^+, while NADPH phosphatases hydrolyze NADPH into NADH.

Co-localization of pathway enzymes

Spatial co-localization of enzymes is commonly occurring throughout nature as a means to preserve pathway fidelity and avoid diffusion of toxic or volatile intermediates. Co-localization can be established either through compartmentalization of enzymes, or through complex formation of enzymes or enzyme subunits. For example, cyanobacteria contain organelle-like carboxysomes that encapsulate carbonic anhydrase and ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This provides a high local concentration of CO_2 and subsequently increases the CO_2-fixation rate of RuBisCo due to the limited presence of O_2 as a competing substrate[77] (Figure 11). In contrast, multi-enzyme complexes can enable *channeling* of intermediates between the active sites of the complex, which minimizes intermediate diffusion, increases the reaction rate, and can also improve the thermodynamics of the reaction[78]. The two-step conversion of indole-3-glycerol

phosphate into tryptophan, catalyzed by tryptophan synthase, is a well-studied example of direct substrate channeling. The separate subunits of the enzyme complex form a hydrophobic tunnel through which the intermediate indole is transferred to the second active site[79]. In the pyruvate dehydrogenase complex, consisting of three enzymes that catalyze the conversion of pyruvate into acetyl-CoA, channeling is instead mediated by flexible arms that bind and transfer intermediates between the active sites[80]. An additional co-localization mechanism is the docking of enzymes onto a non-catalytic scaffold (Figure 12A). Signaling cascade proteins have been found to co-localize onto scaffolds to facilitate efficient signal propagation[81]. Another example is the extracellular formation of cellulosomes, found on the cell-surface of some anaerobic microorganisms, which contain cellulose-degrading enzymes tethered to surface-anchored scaffolds[82]. The benefits gained from spatial organization of enzymes have inspired synthetic biologists to apply co-localization strategies also on heterologous pathways.

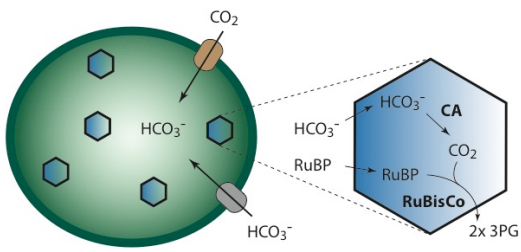

Figure 11. Co-localization of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase (CA) within a carboxysome increases CO_2 fixation rate by limiting the presence of O_2 as a competing substrate.

Fusion proteins

A simple technique for bringing enzymes into close proximity relies on the translation of a fusion of the proteins, often separated by a short linker. Fusion of glycerol-3-P dehydrogenase and glycerol-3-P phosphatase resulted in close to 100% increased glycerol titer in *E. coli*, as compared to expression of the free enzymes[83].

Another successful example was demonstrated for terpene synthesis, where fusion of two of the pathway enzymes resulted in a 2-fold increased production of patchoulol in *S. cerevisiae*[84]. However, the concept of enzyme fusions as a means for co-localization suffers from several limitations. The number of enzymes that can be fused is typically limited to two or three due to inefficient folding or poor solubility of large complexes. This in turn hinders enzyme balancing through the introduction of more than one copy of each enzyme[85]. In addition, several enzyme fusions have resulted in *reduced* activities and product titers[86]. Oligomeric proteins are potentially more vulnerable in this sense, as the fusion may sterically hinder proper subunit interaction. An attempt to improve PHB production in *Arabidopsis* by fusing the two homotetramers PhaA and PhaB, converting acetyl-CoA to 3-hydroxybutyryl-CoA, resulted in a 50% decrease in PHB accumulation[87]. Some of these limitations could be overcome through post-translational co-localization of pathway enzymes onto a scaffold.

Synthetic scaffolds

During the last few years, synthetic DNA-, RNA-, and protein-based scaffolds have been developed and increased product titers from several pathways, including glucaric acid, hydrogen and 1,2-propanediol synthesis routes[88–90]. Although the mechanisms behind the increased productivities are not fully known, the oligomeric nature of some of the tested pathway enzymes could potentially allow interactions between multiple scaffold complexes (Figure 12B), resulting in the formation of large enzyme agglomerates[85]. Thus, the improved metabolite processing might not be an effect of efficient metabolite channeling between the enzymes of one particular scaffold, but rather the increased likelihood of processing by any of the enzymes within the agglomerate[91]. Dueber et al. constructed a synthetic scaffold by linking three protein-protein interaction domains from the metazoan signaling system and fusing their corresponding ligands to enzymes in the mevalonate pathway, which resulted in a 77-fold increase in mevalonate titer[90]. All three enzymes are oligomeric, and the formation of larger enzyme clusters is hence likely.

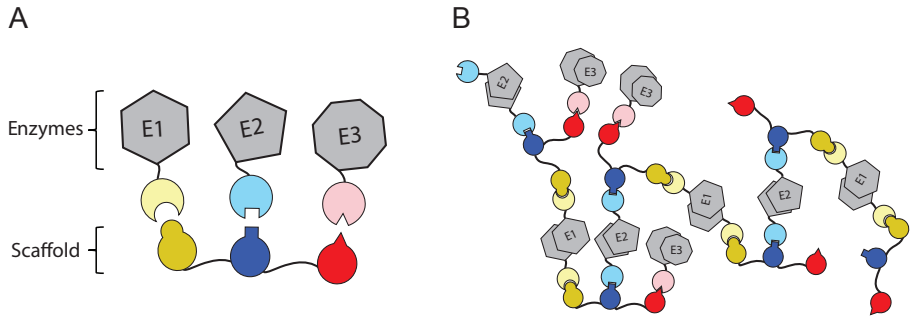

Figure 12. Co-localization of enzymes onto a non-catalytic scaffold. A) Pathway enzymes fused to recognition domains with specific affinity to the scaffold components. B) Oligomeric enzymes (here exemplified as homo- and heterodimers) can bind more than one scaffold, enabling the formation of large enzyme agglomerates. For homotetramers, each enzyme can bind up to four scaffolds, increasing the complexity of the clusters.

Depending on the ligand of choice, the affinity handle may not only enable binding to the scaffold, but could potentially also increase enzyme solubility. This has been shown for several tags, including the IgG-binding Z-domain derived from staphylococcal protein A[92]. The Z-domain is a highly stable and soluble three helical bundle consisting of 58 amino acids. Combinatorial randomization of 13 predefined positions of the two first helices has enabled construction of large libraries of new Z-variants, called affibodies, from which high affinity binders to theoretically any target of interest can be selected. This has generated strong binders to various molecules, including antibodies and the breast cancer-associated HER2 receptor, with affinities (K_D) in the μM to pM range[93]. The libraries have also been used to select anti-idiotypic affibodies, i.e. Z-variants with high and specific affinity to other Z-variants[94,95]. Due to their physical properties as well as the relative ease of developing and selecting novel strong binders, affibodies could be suitable as affinity handles for co-localization purposes. In paper III, we constructed affibody-scaffolds with the aim to co-localize two or three enzymes from a farnesene or PHB pathway, respectively, and increase product titers. Pathway enzymes were fused to different Z-variants, and their respective anti-idiotypic partners were linked to form a separately expressed scaffold. Since the same enzymes catalyze the first two reaction steps of

the PHB and *n*-butanol pathways, increased accumulation of PHB resulting from co-localization of pathway enzymes would suggest that also the butanol pathway could benefit from co-localization strategies.

Execution of genetic modifications

Homologous recombination

Gene deletions and insertions in cyanobacteria are traditionally conducted through double homologous recombination. In this method, a target region of the host genome is replaced with a gene cassette that is flanked by guiding homology regions and introduced on a non-replicating plasmid. The gene cassette typically contains an antibiotic resistance combined with any additional genes of interest. This limits the possible number of transformation rounds due to the restricted availability of suitable resistance cassettes. Colonies typically appear within 1-2 weeks, but may need restreaking in order to reach full segregation into all chromosome copies. Counter-selection methods based on the combined introduction of an antibiotic resistance and a sensitivity gene, which are replaced with the genes of interest in a second transformation step, have been developed to circumvent the problem of resistance build-up. The antibiotic resistance is used as selection marker after the first transformation, whereas sensitivity to e.g. sucrose or nickel is used for the second selection[96,97]. The second recombination event required for marker-less gene modification makes the transformation procedure even more time consuming, where a minimum of four weeks is anticipated for a marker-less mutant to be obtained. There is hence a great need for the development of fast, multiplex gene modification methods in cyanobacteria.

CRISPR/Cas9 and CRISPRi

Since 2012, a new genome editing method has gained enormous popularity and has been applied in a wide range of cell types, including mammalian cells, yeasts and bacteria[98]. This technique is based on CRISPR/Cas systems (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) – a type of immune defense naturally present in many bacteria and archea, with CRISPR/Cas9 from *Streptococcus pyogenes*[99] so far being the most well-characterized and broadly applied CRISPR system. These bacteria incorporate short fragments (protospacers) of invading viral or plasmid DNA between CRISPR repeat sequences into their genome. The transcript from the repeat/spacer array is cleaved into crRNA (CRISPR RNA), which hybridize with tracrRNA (trans-activating crRNA), followed by binding to the Cas9 nuclease. The Cas9 is guided to its target by the crRNA, which contains a complementary sequence to part of the invading DNA. Wild-type Cas9 inactivates the foreign DNA through the introduction of a double-strand break (Figure 13). Importantly, a protospacer adjacent motif (PAM) must be present next to the homologous target DNA to allow Cas9 processing, hence avoiding cleavage of the CRISPR array. For biotechnological purposes, synthetic CRISPR arrays can be introduced and co-expressed with Cas9, or variants thereof, for targeted genomic editing. Similar to PCR primers, the 20 bp protospacers can be designed to target in principal any host genes containing suitably located PAM sequences. The induced double-strand break can either be repaired through error-prone non-homologous end joining, often resulting in a frame shift caused by insertion(s) or deletion(s), or through high-fidelity homology-directed repair, which can be utilized to insert exogenous DNA flanked by homology regions that enables its use as a repair template[98].

The endonuclease activity of Cas9 can be abolished through the introduction of two point mutations, without affecting the RNA-binding capability[99]. Nuclease-deficient Cas9 (dCas9) has been used for CRISPR interference (CRISPRi), where the dCas9 represses gene expression simply by physically blocking transcription[100]. In a similar fashion, CRISPR can also be used for activating purposes (CRISPRa) by fusing dCas9 to a transcription activator[101]. Three native CRISPR/Cas systems have been

identified on one of the plasmids of *Synechocystis* sp. PCC 6803[102], but heterologous CRISPR/Cas-mediated genome editing is yet to be demonstrated in cyanobacteria. However, in paper IV we show the first implementation of CRISPRi in cyanobacteria, which enables time-efficient, reversible and controlled repression of (at least) four genes simultaneously.

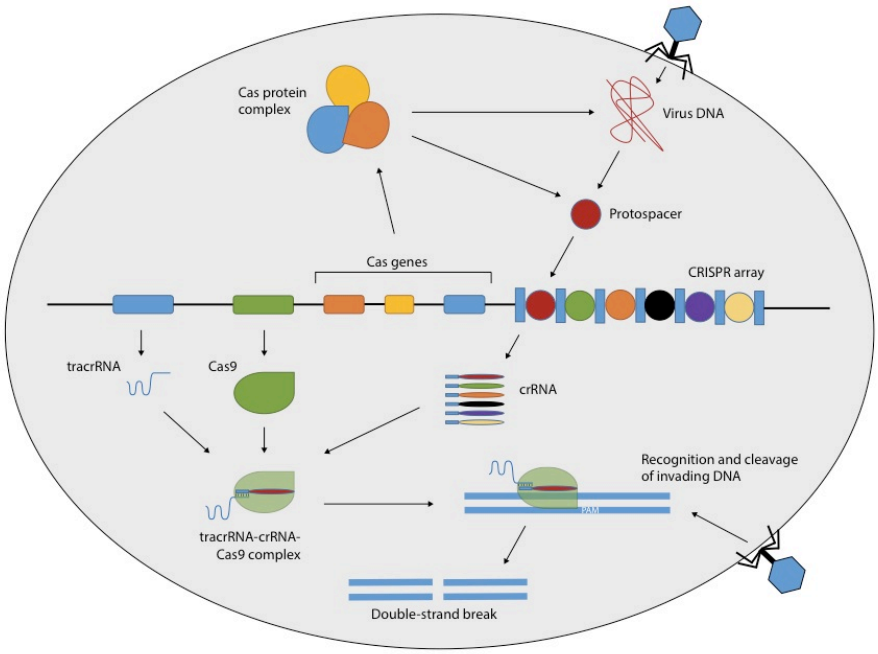

Figure 13. Overview of the CRISPR/Cas9 system. Short fragments (protospacers) of foreign DNA are incorporated into a CRISPR array in the bacterial genome. crRNA from the transcribed CRISPR array hybridize to tracrRNA and associate with Cas9. The crRNA contains a complementary sequence to the foreign DNA, which guides the tracrRNA-crRNA-Cas9 complex to its target upon invasion. Cas9 inactivates the invading DNA by introducing a double strand break.

Applications of pathway optimization strategies in present investigation

Modulation of acetyl-CoA levels for increased *n*-butanol production in *Synechocystis* (paper I)

In paper I, we aimed to identify and utilize driving forces for *n*-butanol production in cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). *Synechocystis* has, in contrast to other model cyanobacteria strains, a native PHB-pathway that can be rerouted to *n*-butanol synthesis through the introduction of only three heterologous enzymes. A chimeric version of the *Clostridia* butanol pathway was assembled by introducing an enoyl-CoA hydratase (PhaJ), trans-enoyl-CoA reductase (Ter), and bifunctional aldehyde/alcohol dehydrogenase (AdhE2). PHB can accumulate to 10% of DCW as a carbon reservoir and redox sink when culturing *Synechocystis* under nitrogen deplete conditions for one week[103]. We thus hypothesized that the changed carbon and cofactor distribution induced by nitrogen starvation could also increase flux through the *n*-butanol pathway. The starting-strains, JA01 and JA02 (Table 2) expressed PhaJ, Ter and AdhE2 under the moderately strong *psbA2* promoter and produced 6 mg/L and 12 mg/L of butanol respectively after 14 days at nitrogen-replete conditions. Acetyl-CoA was only rerouted to butanol at nitrogen-deplete conditions when PHB synthesis was abolished (JA02, Figure 14). Nitrogen starvation increased the *specific* butanol production (i.e. butanol per DCW) up to 3-fold, confirming a higher flux through the butanol pathway, but the overall titer was reduced to 7 mg/L due to ceased growth at this condition.

Table 2. *Synechocystis* strains used in this study.

	Plasmid	Genome modification
JA01	pJA2-P_{psbA2} *phaJ ter adhE2*	None
JA02	pJA2-P_{psbA2} *phaJ ter adhE2*	Δ*phaEC*::SpR
JA03	pJA2-P_{psbA2} *phaJ ter adhE2*	Δ*phaEC*::SpR, ΔNSI::P_{trc}*phaAB* CmR
JA04	pJA8-P_{trc} *phaJ ter adhE2*	Δ*phaEC*::SpR
JA05	pJA8-P_{trc} *phaJ ter adhE2*	Δ*phaEC*::SpR, ΔNSI::P_{trc} *phaAB* CmR
JA06	None	ΔNSI::P_{trc} *xfpk* CmR
JA07	pJA8-P_{trc} *phaJ ter adhE2*	Δ*phaEC*::SpR, ΔNSI::P_{trc} *xfpk* CmR

The increased specific productivity was not simply an effect of the known upregulation of PhaA and PhaB (catalyzing the two shared steps of the PHB and butanol pathways) during starvation, since overexpression of these (JA03 and JA05) at nitrogen-replete conditions increased the secretion of acetate and not butanol (Figure 14 and Figure 15). Titers at both growth conditions were increased 3-fold by overexpressing the three heterologous genes under the strong promoter P_{trc}, indicating a limiting activity from one or more of the three enzymes in the parent strain. In order to identify driving forces behind increased specific butanol productivity at nitrogen-deplete conditions, we quantified cofactors and metabolites with potential influence on butanol accumulation such as acetyl-CoA, acetate and NADH (Figure 15). This revealed a 2-fold increase of acetyl-CoA in wild-type during starvation, which may be a key driving force for increased flux towards PHB or butanol.

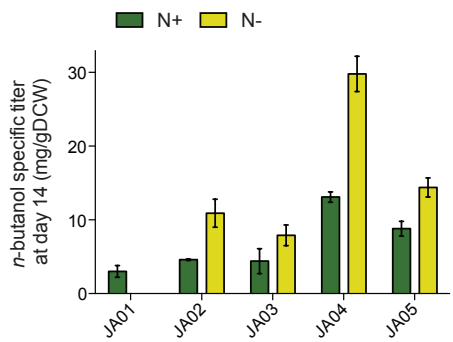

Figure 14. Specific production of *n*-butanol after 14 days of cultivation at nitrogen replete (N+) or deplete (N-) conditions. No butanol was detected at nitrogen deplete conditions from strain JA01, which had an intact PHB synthesis pathway.

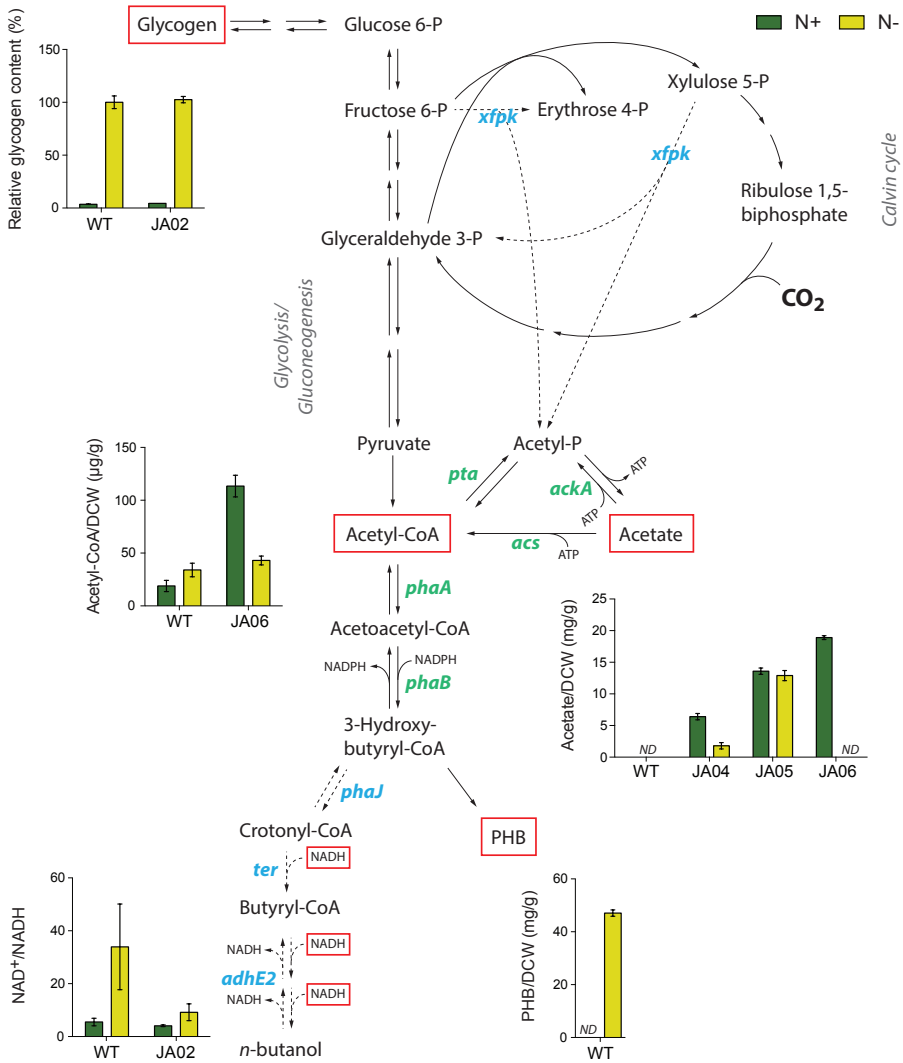

Figure 15. Overview of metabolic effects on *Synechocystis* 6803 during nitrogen starvation. Metabolites with potential influence on butanol synthesis were extracted from cultures grown at nitrogen replete (N+) and deplete (N-) conditions. Glycogen content is relative to WT levels at N-, defined as 100%. Native genes (in green): *phaA* (beta-ketothiolase), *phaB* (acetoacetyl-CoA reductase), *pta* (phosphotransacetylase), *ackA* (acetate kinase), *acs* (acetyl-CoA synthetase). Heterologous genes (in blue): *xfpk* (phosphoketolase, *B. breve*), *phaJ* (enoyl-CoA hydratase, *A. caviae*), *ter* (trans-enoyl-CoA reductase, *T. denticola*) and *adhE2* (bifunctional aldehyde/alcohol dehydrogenase, *C. acetobutylicum*). *ND* not detectable.

Since starvation hampers growth and CO_2-fixation, we sought a way to increase the acetyl-CoA pool also at nitrogen-replete conditions, with the aim to increase specific productivity and titer of butanol. Phosphoketolases are enzymes catalyzing one or both of the two reactions below:

Xylulose 5-P + P_i → Acetyl-P + H_2O + Glyceraldehyde 3-P

Fructose 6-P + P_i → Acetyl-P + H_2O + Erythrose 4-P

Introduction of a phosphoketolase in cyanobacteria could form a shortcut between the Calvin-Benson-Bassham (CBB) cycle and acetyl-CoA (Figure 15), allowing formation of acetyl-CoA from only two units of CO_2 instead of the three required in the path through lower glycolysis. As a consequence, less ATP per formed acetyl-CoA will be consumed in the CBB cycle, which more than compensates for the absence of ATP formation in lower glycolysis. This theoretically results in a more ATP-efficient route to acetyl-CoA, and hence also to *n*-butanol. The potential effect from incorporation of phosphoketolase reactions in *Synechocystis* was evaluated *in silico* by introducing the butanol pathway reactions together with the two phosphoketolase reactions into a genome-scale model[46]. Flux balance analysis (FBA) predicted that the phosphoketolase reactions would account for 40% and 100% of all generated acetyl-CoA during light-limited conditions when optimizing for maximal biomass formation and butanol production, respectively. The model also predicted an increased growth rate (from 0.026 to 0.029 h^{-1}), increased theoretical butanol productivity (Figure 16A) and a 45% decrease in total ATP cost (associated with CO_2 fixation) for butanol synthesis in the presence of a phosphoketolase. Introduction of a phosphoketolase from *B. breve* (Xfpk) into wild-type (JA06) resulted in a 6-fold increased acetyl-CoA pool at nitrogen-replete conditions (Figure 15). Combined expression of Xfpk and the butanol pathway enzymes in strain JA07 increased butanol titer by 1.7-fold (to 37 mg/L at day 8) and specific titer by 2-fold (Figure 16B) under nitrogen-replete conditions. The limited final titers and the secretion of acetate indicate that bottlenecks, such as NADH shortage or low enzyme activity due to oxygen sensitivity, are present in the downstream steps of the butanol pathway. Addressing these, as well as deleting or downregulating additional enzymes from competing pathways (e.g. acetate kinase) will be necessary to increase titers further.

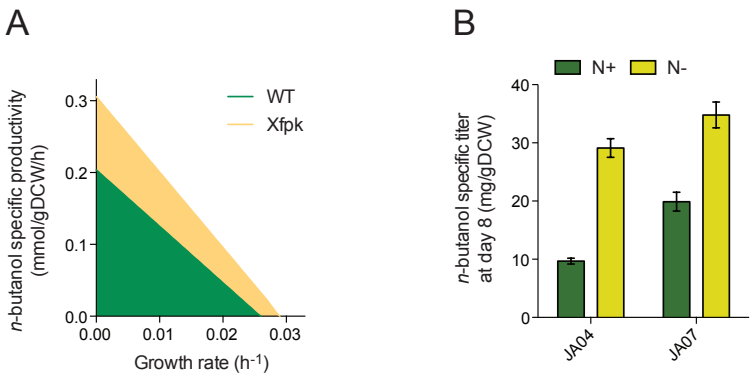

Figure 16. Theoretical and experimental effect of phosphoketolase (Xfpk) overexpression on butanol production. A) Phenotypic phase plane for biomass and butanol during autotrophic growth (photon uptake set to 18.7 mmol/gDCW/h). Xfpk increases theoretical butanol productivity. B) Butanol specific titers at day 8 from strains with (JA07) and without (JA04) Xfpk overexpressed during nitrogen replete (N+) and deplete (N-) conditions.

In conclusion, this study demonstrated that nitrate-depletion or phosphoketolase overexpression can increase the acetyl-CoA pool in *Synechocystis*, creating a driving force for increased *n*-butanol production. Introduction of a phosphoketolase for increased acetyl-CoA levels may also serve as a valuable engineering strategy to increase titers of other acetyl-CoA-derived products in cyanobacteria.

Complementary effects of ATP, acetyl-CoA and NADH driving forces increase butanol production in *Synechocystis* (paper II)

In this study, we built upon the findings in paper I in order to further improve *n*-butanol production from *Synechocystis* sp. PCC 6803. The weak thermodynamic driving force for the condensation of two acetyl-CoA into acetoacetyl-CoA can, as shown in paper I, be improved by increasing the acetyl-CoA pool. An alternative strategy, recently applied in *Synechococcus* for butanol production[104], relies on the ATP-driven carboxylation of acetyl-CoA into malonyl-CoA. Malonyl-CoA is further condensed with acetyl-CoA in a decarboxylation reaction, generating acetoacetyl-CoA (Figure 17). Here, we investigated whether the two strategies could have an additive effect on butanol production in cyanobacteria. Additionally, we attempted to increase the NADH availability for the NADH-dependent butanol synthesis reactions by overexpressing a soluble trans-hydrogenase or a NADP(H) phosphatase. Both enzymes could theoretically catalyze the formation of NADH from NADPH generated from the light reactions, although with different mechanisms (Figure 17).

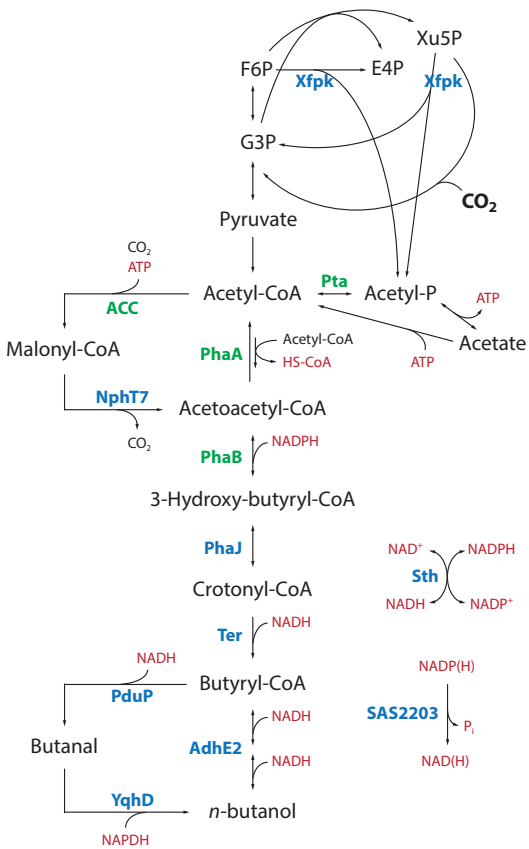

Figure 17. Overiew of the routes to *n*-butanol evaluated in this study. Heterologous enzymes are in blue, and native enzymes in green. A soluble transhydrogenase (Sth, *P. aeruginosa*) and a NADP(H) phosphatase (SAS2203, *S. aureus* strain MSSA476) were introduced in an attempt to increase the NADH supply. NphT7 (acetoacetyl-CoA synthase, *Streptomyces* sp. strain CL190), ACC (acetyl-CoA synthetase), PduP (CoA-acylating propionaldehyde dehydrogenase, *S. enterica*), YqhD (alcohol dehydrogenase, *E. coli*).

A base strain was constructed by chromosomal integration of *phaJ* and *ter*, along with an aldehyde dehydrogenase (*pduP*) and alcohol dehydrogenase (*yqhD*) replacing the oxygen-sensitive AdhE2, at the *phaEC* site, thus disrupting PHB synthesis. Additionally, an acetoacetyl-CoA synthase (NphT7) was introduced individually (JA32) or together with a phosphoketolase (Xfpk, JA33). An increased acetyl-CoA pool, resulting from Xfpk expression or nitrogen starvation, did not increase butanol production in these strains (Figure 18A). The presence of a β-ketothiolase (PhaA) – although downregulated at nitrogen replete conditions – could allow the thiolysis of acetoacetyl-CoA back to acetyl-CoA, which subsequently could be lost to competing pathways such as acetate production. This might mask the potential benefit of Xfpk expression on butanol accumulation. Deletion of *phaA* increased specific titers of butanol by 3.5-fold and 7-fold in the absence (JA41) and presence (JA42) of Xfpk, respectively, with a 70% increase in butanol production from JA42 compared to JA41.

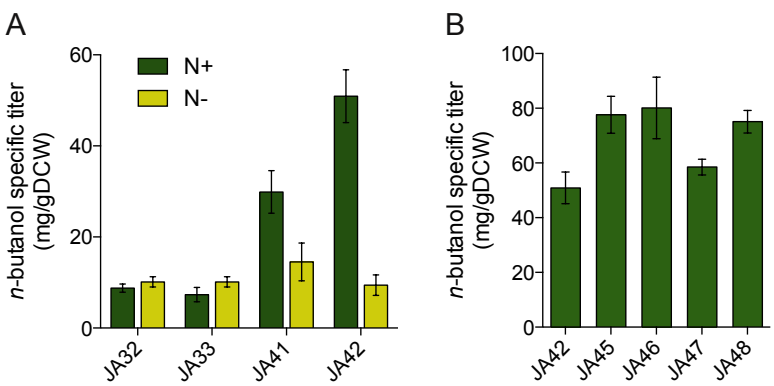

Figure 18. Specific titers of butanol four days after induction. A) Strains expressing NphT7 in the absence (JA32 and JA41) or presence (JA33 and JA42) of Xfpk. *phaA* was deleted in strains JA41 and JA42. Xfpk expression increased butanol production only in the absence of PhaA. N+, nitrogen replete conditions; N-, nitrogen deplete conditions. B) NADP(H) phosphatase (JA45) and soluble transhydrogenase (JA46) expression increased specific titers by 50%. No additive effect was observed from co-expression of the two (JA48). PhaB overexpression (JA47) had no effect on butanol production.

With the aim to identify factors limiting butanol production in strain JA42, we overexpressed an inositol monophosphatase/NADP(H) phosphatase (JA45), a soluble transhydrogenase (JA46), and an acetoacetyl-CoA reductase (PhaB, JA47). The transhydrogenase and phosphatase strains produced 50% more butanol than JA42, resulting in titers of 100 mg/L in four days. The increased production resulting from phosphatase expression was in line with predictions made through genome-scale modeling. PhaB overexpression did not increase production significantly (Figure 18B). Co-expression of the phosphatase and transhydrogenase (JA48) had no additive effect on butanol synthesis. Acetate quantification from stain JA41-47 revealed acetate accumulation in the order of 100-300 mg/L, indicating an insufficient ability to pull excess acetyl-CoA and/or acetyl-P into the butanol synthesis pathway.

In conclusion, we found that increasing the acetyl-CoA pool through Xfpk overexpression could increase butanol production by 70% even in the presence of a strong driving force – consisting of two irreversible reactions – for the conversion of acetyl-CoA into acetoacetyl-CoA. This further strengthens the hypothesis that Xfpk overexpression could be used as a general strategy to increase titers of acetyl-CoA derived products, such as fatty acids and alkanes. Expression of a soluble transhydrogenase or a NADP(H) phosphatase, in an attempt to increase the NADH availability for the reductions of crotonyl-CoA and butyryl-CoA, increased specific titers by an additional 50%. Future efforts to improve butanol production should focus on increasing the flux from acetyl-P and acetyl-CoA into the butanol synthesis pathway, e.g. by upregulating acetyl-CoA carboxylase and phosphotransacetylase, and blocking acetate formation. Quantification of intermediates and cofactors in the butanol pathway will aid the identification of other bottlenecks within the pathway, while also revealing differences in $NADH/NAD^+$ and $NADPH/NADP^+$ ratios resulting from expression of the soluble transhydrogenase compared to the NADP(H) phosphatase.

Co-localization of pathway enzymes using an affibody scaffold improves production of sesquiterpenes in *Saccharomyces cerevisiae* (paper III)

In paper III, we explored the utility of an affibody-based enzyme co-localization platform for increased product titers from two heterologous pathways in two different hosts. The scaffold strategy was initially applied on a farnesene synthesis pathway in *S. cerevisiae* and the *Synechocystis* PHB pathway expressed in *E. coli* to test the generality of the approach, with the long-term aim to extend the scaffold system to other pathways and hosts, such as *n*-butanol synthesis in cyanobacteria. The sesquiterpene farnesene can be used as surfactant, emollient, or diesel substitute, and is produced via the mevalonate pathway (Figure 19A). In order to aid channeling of the intermediate farnesyl diphosphate (FPP) into the farnesene branch of the pathway, thus reducing diffusion to competing reactions, we aimed to bring the two enzymes farnesyl diphosphate synthase (FPPSyn) and farnesene synthase (FarnSyn) into close proximity using a protein scaffold.

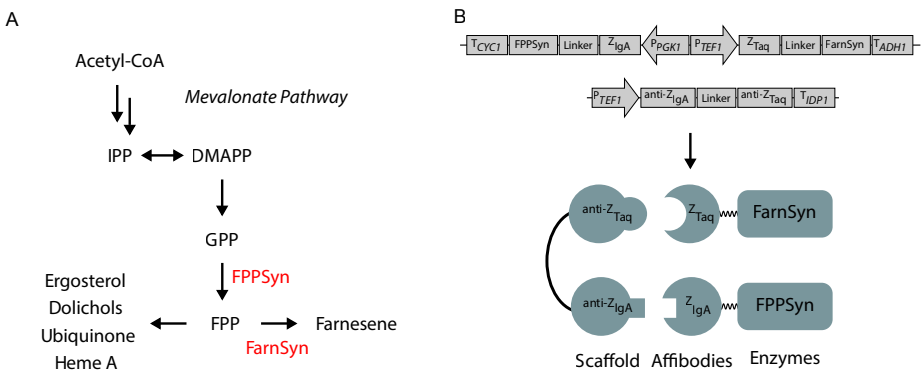

Figure 19. Co-localization of farnesene pathway enzymes using an affibody scaffold. A) Farnesene was produced recombinantly in *S. cerevisiae* via the native mevalonate pathway. B) Farnesyl diphosphate synthase (FPPSyn) and farnesene synthase (FarnSyn) were fused to affibodies Z_{IgA} and Z_{Taq} and co-expressed with a scaffold containing the corresponding anti-idiotypic affibodies to mediate enzyme co-localization and channeling of FPP into the farnesene synthesis branch of the pathway.

Two small affinity proteins (affibodies), Z_{IgA} and Z_{Taq}, were fused to FPPSyn and FarnSyn and co-expressed with a scaffold consisting of two anti-idiotypic affibodies (anti-Z_{IgA} and anti-Z_{Taq}) with specific affinity to Z_{IgA} and Z_{Taq}, respectively. The enzyme-fused affinity tags enable co-localization through the interaction with the scaffold (Figure 19 B).

Simultaneous binding of Z_{IgA} and Z_{Taq} to the scaffold was verified in a Förster resonance energy transfer (FRET) assay, where the idiotypic affibodies were fused to YFP and CFP (Figure 20). Presence of scaffold enabled co-localization, indicated by CFP quenching and YFP fluorescence, but careful tuning of the relative scaffold concentration was found to be crucial for the co-localization strategy to be efficient.

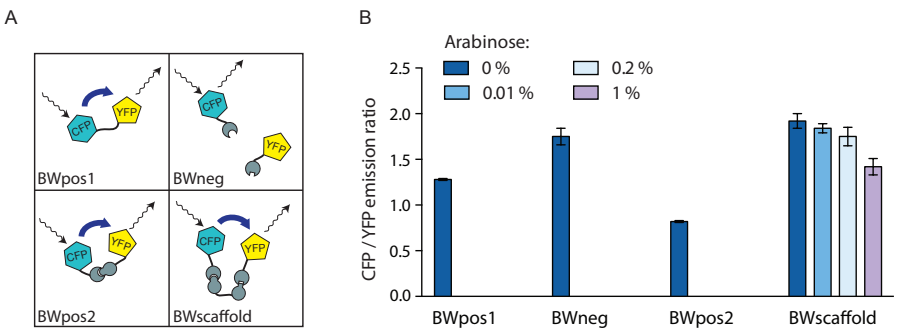

Figure 20. FRET assay verifies interaction between scaffold and affibody-fused proteins. A) Schematic representation of the constructs used for the FRET assay, including one negative and two positive controls. The negative control (BWneg) expressed affibody-tagged fluorophores in the absence of scaffold. Blue arrows indicate energy transfer. B) FRET analysis of control samples (no arabinose added) and affibody-fused fluorophores co-expressed with varying concentration of scaffold. CFP was excited at 415 nm and emission from CFP and YFP was detected at 475 and 530 nm, respectively. A reduced 475 nm/530 nm signal ratio indicates FRET.

Modulation of enzyme and scaffold expression levels in *S. cerevisiae* for farnesene production was accomplished by varying promoter strengths and expression sites; different combinations of chromosomal integration and expression from plasmid were evaluated for the enzyme and scaffold cassettes. Scaffold expression from a weak promoter had no effect on farnesene titers. In contrast, chromosomal integration of the affibody-fused enzymes combined with scaffold expression from a moderately strong promoter on a low-copy plasmid increased farnesene titers up to 120%, and yield 135%. Additionally, a smaller increase in farnesene titer was observed when expressing affibody-fused enzymes in the absence of scaffold (Figure 21). This may be an effect of improved folding or solubility, as previously seen *in vitro* for affibody-fused proteins[92].

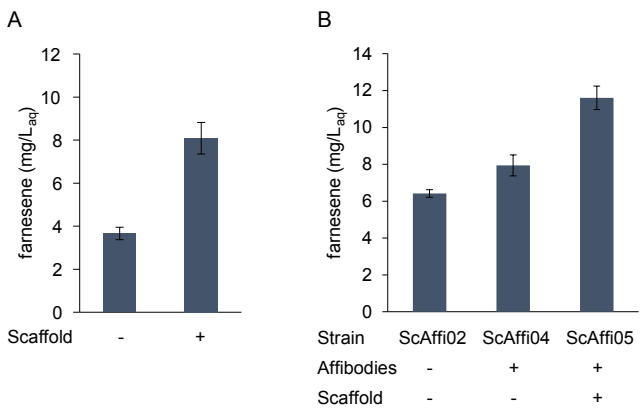

Figure 21. Effect of affibody fusions and scaffold expression on farnesene production in *S. cerevisiae*. A) Farnesene production in fed-batch cultivations in the presence and absence of scaffold. Titers were quantified 6 h after initiation of the feed phase. B) Farnesene titers after 72 h cultivations in shake flasks. Farnesene enzymes without (ScAffi02) or with (ScAffi04) affibody-tags, in the presence or absence of scaffold.

Finally, the scaffold strategy was tested on a PHB pathway heterologously expressed in *E. coli*. The PHB pathway consists of three oligomeric enzymes, catalyzing the three-step conversion of acetyl-CoA into polyhydroxybutyrate. The first step – condensation of two acetyl-CoA into acetoacetyl-CoA – is, as previously discussed in this thesis, thermodynamically unfavorable unless the acetyl-CoA:acetoacetyl-CoA ratio is very high. Efficient metabolite channeling between the pathway enzymes could potentially reduce the concentrations of intermediates, which are further sequestered in the insoluble PHB polymer. Decreased acetoacetyl-CoA availability would improve the thermodynamics of the condensation reaction, thus pulling more substrate into the pathway. As the PHB and *n*-butanol pathways share the two initial reaction steps, a similar strategy could be applied for butanol synthesis, where the fourth reaction step (conversion of crotonyl-CoA to butyryl-CoA by Ter) could serve as the irreversible, product-sequestering step. To allow scaffolding of all three PHB pathway enzymes, a third anti-idiotypic affibody pair, Z_{Her2} and anti-Z_{Her2} [95,105], was introduced. The idiotypic affibodies were fused N-terminally to the enzymes, and the three anti-idiotypic counterparts were assembled into a scaffold. A 5-fold increase in PHB was observed for the Z-fused enzymes in the absence of scaffold, compared to untagged enzymes, after five hours of cultivation (Figure 22). Strains carrying two plasmids (one for enzyme expression and one for the scaffold) accumulated less PHB than strains carrying only one of the plasmids, but Z-fusions resulted in higher PHB titers also here. However, scaffold expression did not contribute to the increased accumulation of polymer, since equal levels were detected from a strain where the scaffold was replaced with an affibody (Z_{WT}) lacking affinity to the other Z-variants. Similar results were observed after extended cultivation time (from 5 to 24 h), as well as at lower enzyme expression levels. Potential reasons for the lack of scaffolding could include non-optimal enzyme stoichiometry and/or enzyme:scaffold ratio.

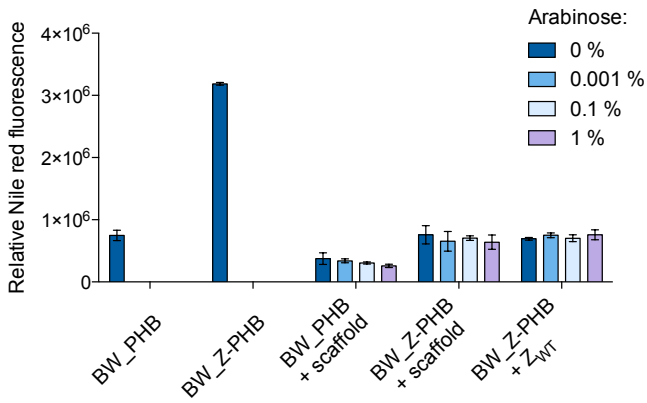

Figure 22. Effect of affibody fusions and scaffold expression on PHB accumulation in *E. coli*. BW_PHB: enzymes without Z-tag, BW_Z-PHB: Z-fused enzymes. Z_{WT} has no known affinity to the other affibodies and was used in replacement of scaffold as a negative control. PHB was quantified through Nile red staining after 5 hours of cultivation.

In summary, we show that affibodies can aid increased bioproduct formation either when assembled into a scaffold for enzyme co-localization purposes, or when fused to pathway enzymes to improve folding or solubility. The scaffold effect on farnesene production may result from agglomerate channeling (i.e. clustering of multiple enzymes), since at least one of the two enzymes is oligomeric and thus capable of binding more than one scaffold. The potential benefits from scaffolding of PHB pathway enzymes could be further investigated by fine-tuning enzyme:scaffold expression ratios, or by introducing multiple repeats of affibodies in the scaffold, allowing changed enzyme stoichiometry. The increased PHB accumulation observed in strains expressing affibody-fused enzymes suggest that Z-tags could potentially increase also butanol titers, since the pathways share the two initial reaction steps converting acetyl-CoA to 3-hydroxybutyryl-CoA.

Multiple gene repression in cyanobacteria using CRISPRi (paper IV)

In this study, we implemented a CRISPR interference (CRISPRi) platform in *Synechocystis* sp. PCC 6803 to enable fast, multiplex, reversible gene repression. A simplified variant of the crRNA:tracrRNA complex, where vital parts of the two are combined and transcribed as a single guide RNA unit (sgRNA)[99], was co-expressed with a nuclease-deficient Cas9 (dCas9) for targeted blocking of transcription (Figure 23). Several constitutive and anhydrotetracycline (aTc) inducible promoters of varying strengths, controlling dCas9 or sgRNA transcription, were evaluated based on their ability to allow continuous or tightly regulated repression. dCas9 and the sgRNA were introduced at two different regions of the genome. P_{trc} GFP was introduced at a third position and used as a reporter for transcription repression. We found that GFP expression could be efficiently repressed by over 99% using constitutive promoter P_{psbA2} and/or TetR-repressed promoters P_{L31} and P_{L03}, even in the absence of the inducer aTc. A significantly weaker but less leaky promoter, P_{L22}, allowed inducible repression of GFP expression by 94%, while only repressing expression by 10% at the non-induced state (Figure 24).

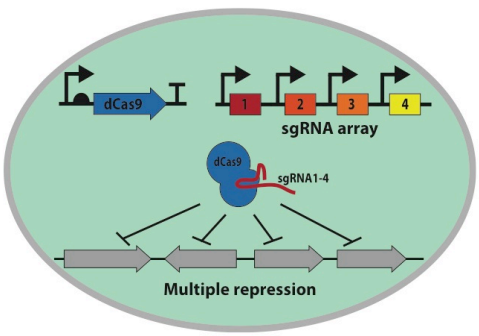

Figure 23. Schematic representation of the CRISPRi strategy used in this study. Transcribed single guide RNA (sgRNA) binds and guides nuclease-deficient Cas9 (dCas9) to a target gene with complementary DNA sequence. Binding of the sgRNA-dCas9 complex physically blocks gene transcription.

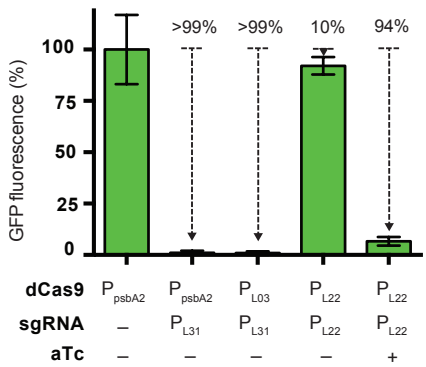

Figure 24. GFP repression by dCas9-sgRNA. Strong repression was observed when dCas9 was expressed from constitutive P_{psbA2}, or P_{L03} even in the absence of inducer aTc. Inducible repression was achieved with the tightly regulated P_{L22} promoter.

Next, CRISPRi was used to knock down native genes *phaE* (encoding one of the two PHB synthase subunits) and *glgC* (encoding ADP-glucose pyrophosphorylase), required for PHB and glycogen accumulation, respectively. Both carbon storage polymers accumulate to high levels upon nitrogen depletion. Knockdown of *phaE* using constitutively expressed dCas9 abolished PHB accumulation completely after two days of nitrogen starvation, demonstrating the high performance of CRISPRi even at conditions associated with protein degradation and reduced protein synthesis[106]. Previous attempts to knock out *glgC* have required multiple rounds of restreaking to reach full segregation[107]. Using CRISPRi, transcripts of *glgC* were reduced to 10% of wild-type levels upon induction of P_{L22} dCas9 and P_{L31} sgRNA, resulting in the characteristic non-bleached phenotype and a 75% decrease in glycogen during nitrogen starvation.

One of the most attractive features of CRISPRi is the possibility to repress multiple genes simultaneously, with potential to save weeks or months of work in relation to traditional knockout through homologous recombination. We explored this feature by targeting three to four putative aldehyde reductases and dehydrogenases for parallel repression. Three out of four targets were successfully repressed 10-20 fold in the 4-sgRNA mutant, correlating well with expression levels from individual knockdown strains (Figure 25). These three had a transcription start site (TSS) directly upstream of their respective open reading frames in the genome. Transcription from the fourth

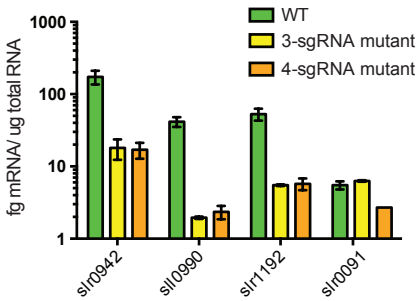

Figure 25. Multiplex gene repression of three (yellow) or four (orange) aldehyde reductase/-dehydrogenase genes. Over 10-fold repression was observed for all but one gene (*slr0091*), which is the 3rd gene of an operon. Transcripts were quantified with RT-qPCR. dCas9 and sgRNAs were constitutively expressed from P_{psbA2} and P_{L31} (no TetR cassette), respectively.

target was repressed 2.5-fold, which may be related to the lack of a TSS adjacent to the gene, since this appears to be the third gene of an operon.

In conclusion, this study demonstrates the use of CRISPRi for multiplex, reversible repression of gene transcription in *Synechocystis* 6803. CRISPRi can serve as a time-efficient alternative to cumbersome recombination-based gene knockout, as well as simplify downregulation of genes essential for growth. A multiplex gene regulation method will be a necessity for the realization of growth-coupled bioproducts (e.g *n*-butanol) formation at photoautotrophic conditions, since this will likely require simultaneous repression of a large number of reactions[108]. The CRISPRi platform could potentially be modified, e.g. by fusing dCas9 with a transcription activator, to also allow controlled *upregulation* of genes. This study is the first demonstration of a heterologous CRISPR-based gene regulation technology in cyanobacteria.

III. BIOFUEL TOXICITY AND TOLERANCE

The improved understanding of microbial metabolism and the expanding toolbox for metabolic engineering has enabled a continuous increase in production yields of various advanced biofuels. A major challenge of microbial biofuel synthesis is the toxicity effects these compounds impose on the production host, resulting in reduced growth and limited titers. The tolerance limit depends on the host, but also on the class of biofuel. Toxicity effects typically increase with hydrophobicity, as can be realized by comparing tolerance to alcohols of varying lengths[109]. Extending the carbon chain of mildly toxic ethanol by only two carbon units reduces the tolerance in wild-type strains of *E. coli* and *S. cerevisiae* dramatically (Table 3). An increased tolerance to alcohols and other harmful biofuels is required for the production processes to become economically feasible. However, an increased tolerance does not necessarily equal increased titers or yields, but can in fact result in unchanged or even reduced production[110]. This chapter will describe the underlying mechanisms of biofuel toxicity as well as strategies to reduce toxicity effects and/or increase tolerance without negatively affecting production.

Table 3. Tolerance limits of commonly used microorganisms to C2-C6 alcohols.

Carbons	Solvent	Microorganism	Tolerance[a] (g/L)	Reference
C2	Ethanol	*E. coli*	32	111
		S. cerevisiae	60	112
		Synechocystis 6803	9	113
C4	1-Butanol	*E. coli*	5	114
		S. cerevisiae	10	114
		Synechocystis 6803	2	115
		C. acetobutylicum	7	20
C6	1-Hexanol	*E. coli*	0.8	116

[a]Tolerance is defined as the concentration reducing growth by approximately 50 %.

Toxicity mechanisms

Solvent toxicity responses are complex and involve altered expression of multiple chromosomally dispersed genes of various functions. Exposure to toxic levels of chemicals damages DNA, RNA, proteins and lipids, which affect basic cellular processes such as DNA synthesis and repair, as well as membrane integrity[117]. Solvents accumulate in the cell membrane, resulting in increased membrane fluidity and permeability, which in turn may cause the release of ATP, ions, phospholipids and proteins, disrupt the proton gradient and affect nutrient transport[109,118]. The dissipated proton motive force can perturb the respiratory balance and induce oxidative stress[119]. The cellular response to solvent stress involves induction of mechanisms that counteract the negative effects from solvent exposure. These include stabilizing membrane alterations, and upregulation of heat shock proteins and molecular pumps (Figure 26). Increasing the proportion of *trans*- to *cis*-unsaturated fatty acids can decrease membrane fluidity, which has been found to be the immediate response in gram-negative *Pseudomonas putida* upon exposure to solvents such as butanol, hexanol and toluene[120]. Membrane fluidity can also be reduced by altering the saturated-to-unsaturated fatty acid ratio, as a long-term response to solvent stress[121]. Heat shock proteins commonly serve as chaperons and assist in synthesis, transport, folding and degradation of proteins under normal growth conditions. A broad range of stress conditions, including solvent exposure, induces the upregulation of heat shock proteins to aid refolding and prevent aggregation of proteins. The expression of efflux pumps of broad specificity enables export of solvent from the cytoplasm to the extracellular milieu[118], which can alleviate the toxicity effects further.

Knowledge of microbial solvent responses is highly valuable as guidance for engineering efforts aiming to increase tolerance. These mechanisms are commonly explored on a global scale using proteomics, transcriptomics and metabolomics technologies, and complemented with physiological and molecular analysis tools[118]. Solvent toxicity in model organisms – native producers in particular – has been extensively studied during the past few decades. Reports on solvent toxicity responses in cyanobacteria are however scarce, since these hosts have not been

engineered for biofuel production until recently. During the last five years, systems biology approaches have been undertaken to study the stress responses to ethanol[122,123], *n*-butanol[21,124,125] and hexane[126] in *Synechocystis* 6803. One of these studies is presented in paper V, where the transcriptomic response to two different *n*-butanol concentrations was explored and utilized to increase tolerance.

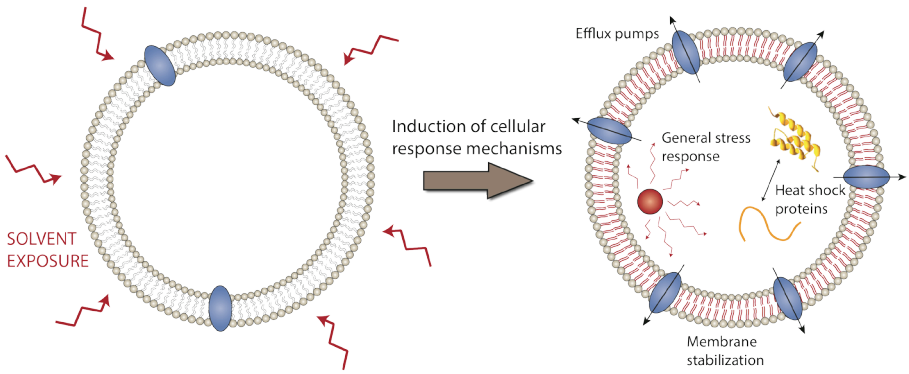

Figure 26. Solvent tolerance mechanisms. Solvent exposure induces cellular response mechanisms, including activation of general stress response genes, upregulation of efflux pumps and heat shock proteins, and stabilizing modifications to the membrane composition.

Alleviating toxicity and increasing tolerance

Implementation of known tolerance mechanisms

As previously described, native tolerance mechanisms toward solvent stress include upregulation of heat shock proteins, transporters and stabilizing modifications to cell membranes. Implementation of these strategies through genetic engineering has been demonstrated as a means to increase tolerance. Overexpression of *groESL* heat shock genes in *C. acetobutylicum* reduced butanol-dependent growth inhibition by 85% while increasing solvent titers by 33%[127]. GroESL overexpression in *E. coli* had a similar effect, increasing tolerance to all five short-chain alcohols tested, including *n*-

butanol[128]. In paper V, we show that overexpression of a heat shock protein can also increase tolerance to butanol in cyanobacteria. Membrane modifying mechanisms introduced to improve ethanol tolerance in *E. coli* include upregulation of cell wall biosynthesis genes[129,130], and increased saturated-to-unsaturated fatty acid ratio through overexpression of fatty acid biosynthesis gene *fabA*[131]. However, strategies to decrease membrane permeability could potentially reduce biofuel titers in the absence of solvent pumps due to impeded secretion out of the cell[109].

Biofuel tolerance can be indirectly increased through the use of efflux pumps. These are membrane transporters that reduce the intracellular toxin concentration by actively pumping out toxic compounds from the cytoplasm and periplasm to the extracellular space – a process typically fueled by ATP or a proton gradient. This not only reduces toxicity effects, but can also increase titers from biofuel cell factories by releasing trapped solvent to the exterior, and by thermodynamically favoring the forward synthesis reaction through continuous product removal[110]. Efflux pumps have mainly been studied for their ability to export antibiotics, but several transporters have also been shown to act on a variety of solvents[109]. Most studied solvent pumps, found in e.g. *E. coli* and *P. putida*, can export hydrophobic compounds such as toluene, hexane, heptane and nonane[132,133], but not hydrophilic short-chain alcohols. Several examples of increased solvent tolerance and/or production resulting from heterologous expression of efflux pumps have been demonstrated, e.g. for the terpene limonene[134] and isoprenoid zeaxanthin[135]. Attempts to identify natural efflux pumps capable of exporting short-chain alcohols have met with limited success[109,110]. However, efflux pump engineering recently enabled active export of *n*-butanol in *E. coli*, resulting in 25% increased growth rate and 40% increased titer[136,137]. Implementation of solvent-pumps could potentially be beneficial also in biofuel-producing cyanobacteria. However, overexpression of membrane proteins can impede growth dramatically, and expression thus requires careful balancing[110].

Adaptive evolution and transcription machinery engineering

Solvent tolerance can also be increased through global modification processes based on random mutagenesis and selection, or modulation of regulatory networks. Native tolerance development rests upon the complex interplay between multiple protective mechanisms that are still not well understood[138]. Techniques allowing broad diversification of the genomic or transcriptomic landscape of the host, followed by a selection process, are thus valuable for improving tolerance and identifying unforeseen resistance mechanisms. Adaptive laboratory evolution (ALE) is a simple and commonly used method to improve fitness of microbes in a certain environment. ALE is based on serial passage where cultures are diluted into media containing gradually increasing selection pressure, e.g. in the form of elevated solvent concentration. Spontaneous mutations resulting in improved tolerance will accumulate in the culture over time as less fit mutations are outcompeted. Typically, ALE experiments are performed for 100-2000 generations, with a higher adaptation rate during the first 100-500 generations. UV radiation or mutagenic chemicals can be applied to speed up the mutagenesis process[139]. Examples where ALE has been used for increased solvent tolerance include adaptation to ethanol and butanol by *E. coli*[129], *S. cerevisiae*[140] and *Synechocystis* 6803[141]. The latter was performed during 395 days and resulted in a 150% increase in tolerance to *n*-butanol. Based on metabolome analysis of the evolved strains, butanol tolerance was suggested to be related to differential regulation of glycerol, amino acids, fatty acids, and intermediates of glycolysis and the Calvin cycle[141].

Adaptive laboratory evolution often results in mutagenesis of regulatory genes[142]. Thus, an alternative and more time-efficient method for tolerance development is based on the direct targeting of regulatory hubs, either through modulated expression of the native regulatory genes or through selections from a library of mutated regulators[143]. In paper VI, we increased the resistance of *Synechocystis* 6803 to *n*-butanol and elevated temperatures by modulating the expression of a selection of regulatory genes involved in temperature-, oxidative-, and osmotic stress responses, including a sigma factor and members of two-component regulatory systems. Random mutagenesis of a housekeeping sigma factor in *E. coli* has also been applied

to increase tolerance to several solvents, such as ethanol, butanol, hexane and toluene[144,145]. Here, upregulation of ATP-generating reactions were found to be important for solvent tolerance, possibly as a means to compensate for reduced ATP-levels during solvent stress and to supply energy for active solvent export[145].

In situ product recovery

Toxicity effects from biofuel production can be alleviated or avoided through continuous removal of product from the culture before the solvent accumulates to toxic concentrations. Various *in situ* solvent removal techniques have been developed for the recovery of butanol from *Clostridia* fermentations, including liquid-liquid extraction, perstraction, gas stripping and pervaporation. Methods for liquid-liquid extraction and perstraction are based on the extraction of solvent from the aqueous culture to an organic phase, either through direct mixing of the two phases (liquid-liquid extraction), or by separating the aqueous and organic phase with a membrane (perstraction)[146]. Pervaporation also makes use of a membrane that is placed in contact with the culture broth and allows transfer of volatile solvents in their gas form through the membrane, while gas stripping is performed by bubbling e.g. nitrogen or fermentation gas through the culture to extract the solvent to the gas phase. The solvent is recovered from the gas phase through condensation[147]. These techniques are valuable complements to genetic modulation for decreasing toxicity effects and improving productivity.

Present investigation of butanol toxicity and tolerance mechanisms in cyanobacteria

Using transcriptomics to improve butanol tolerance (paper V)

n-Butanol titers from engineered cyanobacteria are currently below concentrations impeding growth[148]. However, the anticipated future increase in productivity will eventually be limited by the inherently low tolerance to butanol. To address this problem, we aimed to take advantages of key native response mechanisms to *n*-butanol in *Synechocystis* 6803, with the underlying hypothesis that boosting natural response mechanisms could increase tolerance. We assessed the transcriptomic effect of butanol exposure by performing RNA sequencing (RNA-Seq) on wild-type *Synechocystis* cultivated in the presence of 40 mg/L (B+) and 1 g/L (B++) exogenously added butanol, where the latter corresponds to the highest concentration that does not reduce the growth rate. The selected concentrations allowed us to investigate whether low levels of butanol, with no affect on phenotype, are harmful enough to induce a stress response. Indeed, we found that both 40 mg/L and 1 g/L of butanol had a significant impact on the transcriptomic landscape, but with an elevated effect at the higher concentration. 276 genes were differentially expressed at the B++ condition, while the B+ condition resulted in differential expression of 78 genes – most of them affected at both conditions. The expression fold change was however more dramatic at the higher butanol concentration (Figure 27).

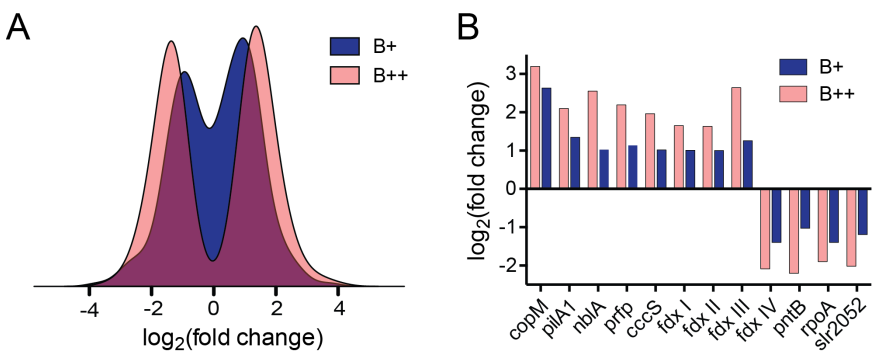

Figure 27. A) Expression fold change of differentially expressed genes at B+ and B++ conditions. B) Fold change of differentially expressed genes showing a strong butanol dosage-dependent response.

Despite this, only a small group of genes showed dosage-dependent expression patterns. The transcriptomic effect of butanol stress was found to be diverse, causing both up- and downregulation of genes from a wide range of functional categories such as two-component systems, transporters and amino acid metabolism (Figure 28). The largest functional group among upregulated genes was related to photosynthesis. To get a clearer view of the effect of butanol on the photosystems and electron transport, we complemented the transcriptomics data with measurements of reactive oxygen species (ROS) and fluorescence from phycocyanin and chlorophyll pigments at different butanol concentrations. A butanol-dependent increase was observed for both ROS accumulation and pigment fluorescence (Figure 29). The chlorophyll content, determined through chlorophyll extraction and subsequent quantification, was however unchanged, indicating that the increased fluorescence was an effect of reduced photochemical quenching due to damages to the photosystems, rather than an effect of increased pigment content. The upregulation of photosynthesis-related genes may thus be a means to repair damaged photosystem components.

Butanol stress also induced the expression of transporters and two-component systems involved in metal homeostasis. Iron is highly abundant in electron transport proteins and readily attacked by ROS[149]. The strong upregulation of iron transporters

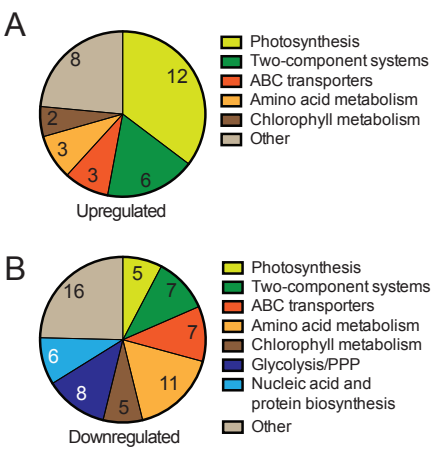

Figure 28. Differentially expressed genes categorized into KEGG orthology groups. A) Upregulated genes. B) Downregulated genes. Hypothetical proteins are not included.

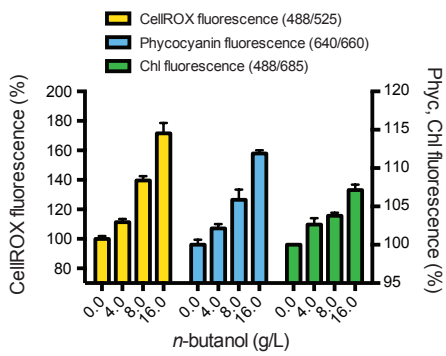

Figure 29. Intracellular ROS levels, determined with CellROX Green, and fluorescence from phycocyanin and chlorophyll (Chl) after exposure to different concentrations of *n*-butanol for 1 hour.

indicates a perturbed metal homeostasis, possibly caused by membrane leakage and degradation of iron-containing proteins. In addition, butanol exposure upregulated a heat shock protein (HspA) known to both aid protein folding and reduce membrane fluidity[150].

Based on the hypothesis that upregulated genes with potential to restore membrane integrity or reduce oxidative stress could be important for tolerance, we selected candidate genes for overexpression. Several of these were found to improve tolerance to butanol. In particular, overexpression of HspA increased growth rate both in the presence of *n*-butanol and isobutanol. It also improved viability after a short-term butanol shock (Figure 30).

Our results suggest that butanol exposure induces oxidative stress, compromises cell membranes, and impairs photosynthetic electron transport. Overexpression of HspA likely improved tolerance by stabilizing membranes and/or folding proteins, and could be combined with other toxicity-neutralizing mechanisms. Approximately 65% of all genes significantly affected by butanol had unknown function. Thus, further increased understanding of the butanol toxicity mechanisms in *Synechocystis* could be gained from this study in the future as more genes are characterized and annotated.

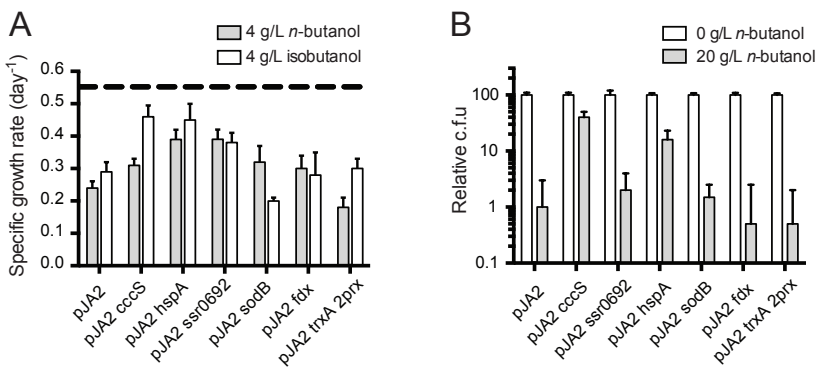

Figure 30. Butanol tolerance of strains overexpressing selected cyanobacteria genes on replicating vector pJA2. A) Growth rates in the presence of 4 g/L butanol. Dashed line corresponds to the growth rate of wild-type in the absence of butanol. B) Viability of strains incubated with 20 g/L butanol for 1 h, followed by growth on BG-11 agar plates. CFUs of butanol-shocked samples are relative to non-shocked control samples for each strain.

Increased temperature and butanol tolerance of *Synechocystis* 6803 through overexpression of SigB (paper VI)

Stress tolerance are complex phenotypes that typically rely on differential regulation of multiple cellular functions. Tolerance engineering can thus profit from simultaneous targeting of several genes. For this reason, we aimed to improve butanol and heat tolerance by modulating the regulatory machinery of *Synechocystis* 6803. The response mechanisms towards solvent exposure overlap with those of heat and oxidative stress, e.g. through the uprgeulation of heat shock proteins and ROS degrading enzymes[21,151,152]. Hence, increasing tolerance to one type of stress could potentially improve tolerance also to other stress conditions. To this end, we selected six stress-related regulatory genes for overexpression or deletion, and evaluated the resulting mutant strains with respect to heat and butanol tolerance.

Heat tolerance can be a valuable phenotype for industrial strains, since temperature may fluctuate with sun light intensity. The regulatory genes were selected based on their reported involvement in temperature-, oxidative- and osmotic stress responses. Two thirds of the mutants showed a substantially increased tolerance to a two hour heat shock treatment at 45 °C compared to wild-type (Figure 31). At 46 °C, the SigB overexpressing

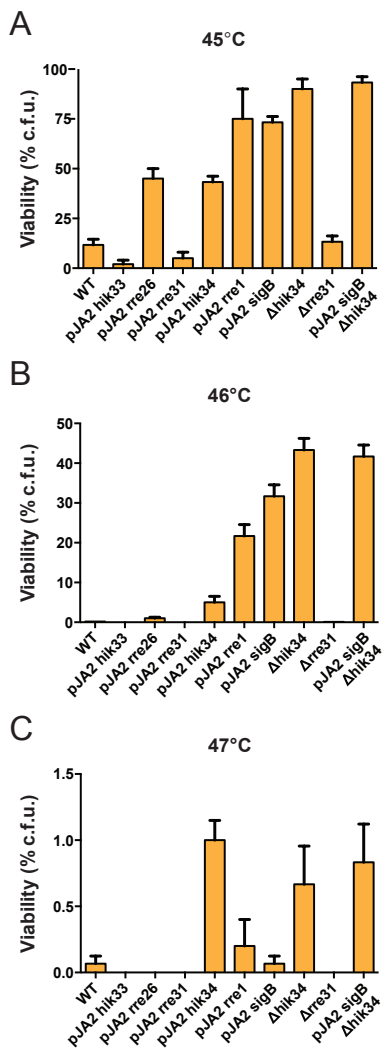

Figure 31. Viability of *Synechocystis* strains after 2 h heat treatment at A) 45 °C, B) 46 °C, and C) 47 °C. Viability is defined as percentage of colony forming units relative to non-treated cells. Note scale-bar differences.

mutant and the Hik34 knock-out showed a viability of 32-43% (determined as the percent of colony forming units), while wild-type viability was less than 1%. Combining *sigB* and Δ*hik34* did however not result in an additive tolerance effect. None of the mutant strains showed a viability above 1% at 47 °C, but the thermotolerance of *hik34*, Δ*hik34*, and *sigB* Δ*hik34* mutants was increased 10-15 fold in relation to wild-type.

Next, we evaluated viability of the mutant strains after one hour exposure to 2.5% (v/v) *n*-butanol (20 g/L). This concentration was extremly toxic to most strains. However, the mutant overexpressing SigB showed significant tolerance also at this condition, with a viability of 15% (Figure 32).

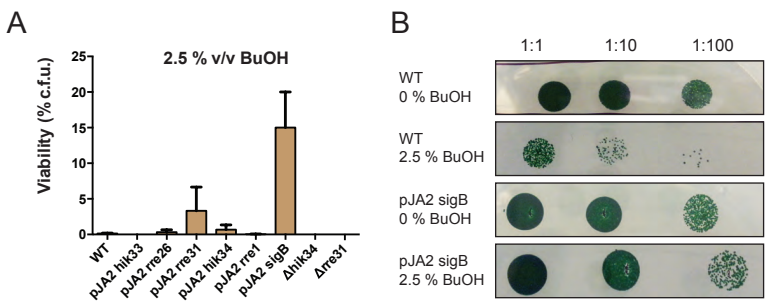

Figure 32. Effects of butanol shock. A) Viability of *Synechocystis* mutants after 1 h incubation in 2.5% (v/v) *n*-butanol. B) Growth of wild-type and SigB strains 8 days after butanol shock.

Butanol and heat stress are both associated with accumulation of reactive oxygen species[21,151]. We hypothesized that the increased thermo- and/or butanol tolerance in some of our studied mutant strains could be an effect of a decrease in intracellular ROS. Accumulation of reactive oxygen species, measured as fluorescence from a ROS-sensitive reagent, was found to be significantly reduced at both stress conditions in the *sigB* strain. However, the thermotolerant Δ*hik34* mutant had elevated levels of ROS in relation to wild-type, indicating the induction of other response mechanisms to heat stress in this strain (Figure 33).

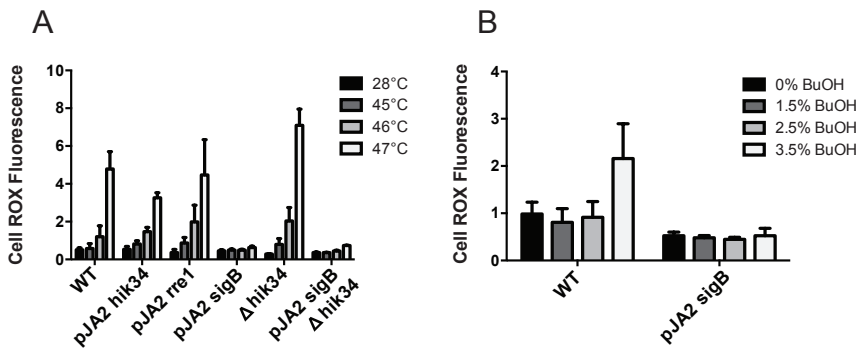

Figure 33. Intracellular ROS levels in mutant strains subjected to A) heat stress, or B) butanol stress. ROS was determined from CellROX Green fluorescence.

In summary, we have shown that modulation of the regulatory network in cyanobacteria can be used to increase tolerance to different stress conditions. In particular, upregulation of SigB was found to increase tolerance to both heat and butanol stress. The decreased levels of ROS resulting from SigB overexpression further strengthen the hypothesis that ROS accumulation is an important contributor to solvent toxicity.

CONCLUSIONS AND FUTURE OUTLOOK

Using photosynthetic organisms such as cyanobacteria for the direct conversion of carbon dioxide and light into biofuels and other compounds is a promising, green alternative to fossil-dependent chemical production.

In this thesis, several metabolic engineering strategies to increase butanol production in cyanobacteria were demonstrated. Genome-scale modeling was used to predict the metabolic effects of different gene additions, and promising candidates were selected for experimental evaluation. Introduction of a shortcut from the Calvin-Benson-Bassham cycle to acetyl-CoA through the overexpression of a phosphoketolase (Papers I-II) was found to be an effective way to increase the acetyl-CoA pool, and subsequently butanol titers, in *Synechocystis*. Phosphoketolase expression is anticipated to allow increased production also of other fuels and fuel precursors stemming from the acetyl-CoA node of central metabolism, including fatty acids, alkanes, isopropanol and propane. Attempts to increase NADH levels trough the introduction of a transhydrogenase or a NADP(H) phosphatase further improved butanol production, and final titers of 100 mg/L were reached within four days. Quantification of cofactors and intermediates relevant for butanol synthesis could in the future aid the identification of bottlenecks in our best-producing *Synechocystis* strains. The scaffolding strategy demonstrated in Paper III, where it increased farnesene titers by 120% in *S. cerevisiae,* could be used to co-localize butanol pathway enzymes around a rate-limiting step to potentially improve metabolite channeling, thus alleviating intermediate build-up and increasing butanol production.

In Paper IV, CRISPRi was implemented in cyanobacteria for multiplex gene repression. We demonstrated simultaneous repression of three genes by >90%, but the technology can likely be applied for strong, parallel repression of even more genes and will be a valuable addition to the toolbox for metabolic engineering of cyanobacteria. The multiplexity makes CRISPRi a time-efficient method with potential to save weeks or even months of work compared to traditional genome engineering techniques in cyanobacteria. CRISPRi can be used to further optimize

butanol-producing *Synechocystis* strains by simplifying downregulation of competing pathways. Fusion of dCas9 to a transcription activator could potentially also enable multiplex, inducible gene activation (CRISPRa) in the future. Ultimately, genome-scale modeling will be used to select multiple gene targets for simultaneous up- and downregulation, with the aim to divert all fixed CO_2 into biofuel at a certain growth stage. A multiplex gene regulation or gene-editing platform, such as CRISPR-based technologies, will be crucial for the implementation of the predicted modifications.

In Paper V-VI, butanol toxicity mechanisms in *Synechocystis* were identified and means to increase tolerance were explored. Boosting the cell's natural response mechanisms by overexpressing a heat shock protein or a stress-related sigma factor increased butanol tolerance in the wild-type. Future experiments will reveal whether these mechanisms could improve also butanol *production*, if implemented in a butanol-producing strain. This would be of particular importance in strains where butanol production reaches titers that impede growth.

Taken together, the work presented in this thesis could contribute to the development of photosynthetic cell factories for large-scale production of biofuels at high titers.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Rådande globala klimatförändringar tros till stor del bero på koldioxidutsläpp orsakade av mänsklig aktivitet. En betydande del av utsläppen kommer från transportsektorn, där huvudsakligen fossila bränslen såsom bensin och diesel används som drivmedel. För att minska utsläppen behöver fossila bränslen ersättas med *förnybara* alternativ. Både fossila och förnybara bränslen bildar koldioxid vid förbränning i motorer. Den avgörande skillnaden är ursprunget av koldioxiden. Fossila bränslen innehåller kol som sedan länge lämnat det naturliga kolkretsloppet. Förbränning av dessa ökar den totala mängden kol i omlopp, vilket successivt leder till högre koldioxidhalter än vad jordens växtlighet kan ta upp. Förnybara bränslen produceras istället från diverse grödor och växter, d.v.s. från källor som inte lämnat kolets kretslopp, och förbränning av dessa bidrar därmed inte till en nettoökning av koldioxid i atmosfären.

Etanol är än så länge det främsta förnybara substitutet för bensin och framställs vanligtvis genom jäsning. Mikroorganismer matas med grödor såsom sockerrör och majs, och omvandlar sockret till alkohol. Bränsleproduktion från ätbara grödor står i konkurrens med matindustrin och har därför mötts av kritik. Socker för jäsning kan istället utvinnas från restprodukter från skogs- och jordbruksindustrin, men detta kräver en kostsam förbehandling av materialet. Ett annat alternativ är att producera bränslen *direkt* från koldioxid och solljus. Fotosyntetiska organismer, såsom cyanobakterier, använder solljus som energikälla och koldioxid som föda, och behöver därmed inget socker för att kunna växa. Genom genmodifiering kan cyanobakterierna omprogrammeras för att omvandla koldioxiden till biobränsle. I den här avhandlingen har cyanobakterier genmodifierats för att producera butanol. Butanol är liksom etanol en alkohol, men är ett betydligt bättre bränsle eftersom det är lättare att hantera och transportera och har ett högre energiinnehåll, vilket tillåter längre körsträcka per liter bränsle. Cyanobakterier kan inte producera butanol naturligt. Därför introducerades nödvändiga gener, handplockade från diverse bakterier, i cyanobakteriernas kromosom på konstgjord väg.

Våra initiala genmodifierade cyanobakteriestammar producerade blygsamma mängder butanol. För att förbättra processen genmodifierades stammarna ytterligare.

I artikel I och II introducerade vi flera nya gener, samtidigt som andra gener togs bort, för att få mer koldioxid att omvandlas till butanol istället för oönskade biprodukter. Koldioxidens vandring genom cyanobakteriecellen kan liknas vid en ström bilar på en motorväg. För att så många bilar som möjligt ska ta sig från punkt A till punkt B, eller från koldioxid till butanol, på kortast möjliga tid behöver vi bredda vägen och tillåta höga hastigheter (introduktion av nya, snabba enzymer), samt blockera avfarter (ta bort gener) så att ingen bil råkar köra fel. Med hjälp av dessa strategier lyckades vi öka butanolproduktionen från drygt 1 mg/L på sju dagar till över 100 mg/L på fyra dagar.

Att genmodifiera cyanobakterier är en tidsödande process. Borttagning av en gen tar ofta 2-4 veckor, och generellt kan man bara ta bort en gen i taget. Vill man blockera många avfarter kan detta alltså ta månader att åstadkomma. I artikel IV implementerade vi en ny sorts genregleringsteknik i cyanobakterier, för att snabba på modifieringsprocessen. Tekniken, kallad CRISPRi, möjliggör stark nedreglering av flera gener samtidigt. Vi demonstrerade metodens potential genom att simultant nedreglera tre gener med över 90% effektivitet. Chanserna är goda att CRISPRi kan användas för att nedreglera betydligt fler gener parallellt, och metoden är ett viktigt tillskott i verktygslådan för genmodifiering av cyanobakterier.

En utmaning med butanolproduktion är bränslets toxiska egenskaper. Vid höga koncentrationer blir butanolen giftig för cellerna, och produktionen avstannar innan tillräckliga nivåer har nåtts. I artikel V och VI undersökte vi de bakomliggande mekanismerna för butanoltoxicitet i cyanobakterier. Genom att upreglera cellernas egna försvarsmekanismer kunde vi få cyanobakterierna att överleva i höga butanolkoncentrationer i betydligt större utsträckning än tidigare.

I den här avhandlingen demonstreras olika strategier för att öka produktionen av butanol i cyanobakterier. Flera av dessa strategier kan även implementeras för att öka produktionen av andra bränslen och kemikalier i cyanobakterier.

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