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Metabolic Engineering of *Synechocystis* PCC 6803 for Butanol Production

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Abstract
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There is an urgent demand for renewable alternatives to fossil fuels since the extraction and utilization cause a series of environmental problems in the world. Thus, the utilization of solar energy has attracted much attention in the last decades since there is excess amount of light on Earth. Photosynthetic microorganisms, such as cyanobacteria, can be a good biological chassis to convert solar energy directly to chemical energy. It has been demonstrated that cyanobacteria can produce various compounds which can be used asfourth-generation biofuels. This thesis focuses on the photo-autotrophic production of two biofuel compounds, isobutanol and 1-butanol, in the unicellular cyanobacterial strain *Synechocystis* PCC 6803. In the studies of isobutanol production, the endogenous alcohol dehydrogenase of *Synechocystis* encoded by *slr1192* showed impressive activity in isobutanol formation. In addition, a-ketoisovalerate decarboxylase (Kivd) was identified as the only heterologous enzyme needed to be introduced for isobutanol production in *Synechocystis*. Kivd was further recognized as a bottleneck in the isobutanol production pathway. Therefore, Kivd was engineered via rational design to shift the preferential activity towards the production of isobutanol instead of the by-product 3-methyl-1-butanol. The best strain pEEK2-ST expressing $Kivd^{S286T}$ showed dramatically increased productivity, and the activity of Kivd was successfully shifted further towards isobutanol production. A cumulative isobutanol titer of 911 mg L^{-1} was observed from this strain after 46 days growth under 50 μmol photons m^{-2} s^{-1} with pH adjusted to between 7 and 8. A maximum production rate of nearly 44 mg $L^{-1}d^{-1}$was reached between days 4 and 6. Similar metabolic engineering strategies were employed to generate 1-butanol producing *Synechocystis* strains and then to stepwise enhance the production. By selecting the best enzymes and promotors, 836 mg L^{-1} in-flask 1-butanol was produced. By optimizing the cultivation condition, an in-flask titer of 2.1 g L^{-1} and a maximal cumulative titer of 4.7 g L^{-1} were observed in the long-term cultivation. This thesis demonstrates different metabolic engineering strategies for producing valuable compounds in *Synechocystis,* exemplified with butanol, and how to enhance production systematically.

Keywords: Synechocystis PCC 6803, biofuel, isobutanol, 1-butanol, metabolic engineering, protein engineering

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Be the change that you wish to see in the world.

-Mahatma Gandhi

梦想, 可以天花乱坠，理想，是我们一步一个脚印踩出来的坎坷道路。

-三毛

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Englund, E., Andersen-Ranberg, J., **Miao, R.,** Hamberger, B., Lindberg, P. (2015) Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. *ACS Synthetic Biology*, *4*: 1270-1278

II **Miao, R.**, Liu, X., Englund, E., Lindberg, P., Lindblad, P. (2017) Isobutanol production in *Synechocystis* PCC 6803 using heterologous and endogenous alcohol dehydrogenases. *Metabolic Engineering Communications*, *5*: 45-53.

III **Miao, R.,** Xie, H., Ho, F. M., Lindblad, P. (2018) Protein engineering of α-ketoisovalerate decarboxylase for improved isobutanol production in *Synechocystis* PCC 6803. *Metabolic Engineering*, *47*: 42-48.

IV **Miao, R.**, Xie, H., Lindblad, P. (2018) Enhancement of photosynthetic isobutanol production in engineered cells of *Synechocystis* PCC 6803. *Manuscript*

V Liu, XF.*, **Miao, R.***, Lindberg, P., Lindblad, P. (2018) Engineering *Synechocystis* PCC 6803 for 1-butanol production. *Manuscript*

VI **Miao, R.,** Wegelius, A., Dural, C., Liang, F., Khanna, N., Lindblad, P. (2017) Engineering cyanobacteria for biofuel production. In: Hallenbeck, P. (Ed.), Modern Topics in the Phototrophic Prokaryotes, Environmental and Applied Aspects. Chapter 11: 351-393. Springer International Publishing, Switzerland. ISBN: 978-3-319-46259-2

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Contents

Abbreviation

OCDE	Organization for Economic Cooperation and Development
GOE	Great Oxygenation Event
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
CBB cycle	Calvin-Benson-Bassham cycle
PEPc	Phosphoenolpyruvate carboxylase
TCA-cycle	Tricarboxylic acid cycle
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
RON	Research octane number
MON	Motor octane number
NADPH	Nicotinamide adenine dinucleotide phosphate
ThDP	Thiamine diphosphate
RBS	Ribosome binding site
BCD	Bicistronic design
5'UTR	5' untranslated region
PHB	Polyhydroxybutyrate
ADH	Alcohol dehydrogenase
AlsS	Acetolactate synthase
IlvC	Acetohydroxy acid isomeroreductase
IlvD	Dihydroxy acid dehydratase
PK	Phosphoketolase
NphT7	Acetoacetyl-CoA synthase
PhaB	(R)-3-hydroxybutyryl-CoA dehydrogenase
PhaJ	(R)-specific enoyl-CoA hydratase
Ccr	Crotonyl-CoA reductase
Pta	Phosphotransacetylase
PduP	CoA-acylating aldehyde dehydrogenase
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
Synechocystis	*Synechocystis* PCC 6803
S. elongatus	*Synechococcus elongatus* PCC 7942

L. lactis	*Lactococcus lactis*
A. caviae	*Aeromonas caviae*
S. collinus	*Streptomyces collinus*
S. enterica	*Selmonela enterica*
B. subtilis	*Bacillus subtilis*
P. aeruginosa ATCC 15442	*Pseudomonas aeruginosa* ATCC 15442

Introduction

Motivation of the study

Global energy consumption keeps rising at a significantly high speed, especially in the countries outside of the Organization for Economic Cooperation and Development (OECD). The estimated global population in 2040 is more than 9 billion, which means the energy demand at that time will be at least 30% higher than today [1]. Among all different kinds of fuel types, fossil fuels (*e.g.* petroleum, coal and natural gas) are still the major energy sources even though the consumption of renewable energy and nuclear energy has already increased dramatically [1].

The fossil fuel geographical distribution together with the oil industry have shaped the international economy map and caused international disputes to a great extent [2]. From 1973 till 2012, approximately 25% to 50% of the interstate wars were linked to one or more oil-related reasons [3]. Furthermore, the food industry heavily depends on oil for machine-based production, which leads to an increase in food prices worldwide and famine in some poor regions [4].

Therefore, the development of a decentralized and democratized global energy system is an urgent need in the modern age [5]. Renewable energy (*e.g.* solar fuel, biofuel, wind power, hydropower, etc.) is a potential solution that leads to an end of the oil-dominated society, thereby reduces the pollution of harmful particles from fossil fuel waste and the conflicts caused by the geographical restriction of traditional energy resource.

The sun delivers gigantic amount of energy to our Earth every day, and the solar energy reaching the atmosphere in one hour approximately equals the entire global energy consumption in one year [6]. Thus, if there are more efficient ways to harvest and store solar energy at any place on the earth, many of the problems discussed above will be solved or at least attenuated. In this case, photosynthetic microorganisms (*e.g.* microalgae and cyanobacteria) are good candidates to be utilized as converters of solar energy and CO_2 to chemical energy. Compared to higher photosynthetic plants, microalgae and cyanobacteria grow faster and their genetic characteristics are simpler and better studied. All these advantages allow us to do modifications on a molecular level to improve their capacity to produce valuable chemical compounds. This thesis is my contribution towards establishing a better cyanobacterial cell factory for producing biofuels from sunlight and atmosphere CO_2.

Different generations of biofuels

A biofuel is defined as the fuel which has a minimum of 80% content by volume of materials that are derived from living organisms harvested within the 10 years proceeding its manufacture [7]. Nowadays, biofuels are classified into four generations. The first-generation biofuels are made from sugars and vegetable oils in food crops. The sourcing of feedstocks for the first generation biofuels has a high risk to cause land usage competition and negative impacts on biodiversity [8]. Therefore, some renewable and conventional biofuel alternatives, such as second generation biofuels have gained extensive attention. The feedstock for second generation biofuels should not be food crops (*i.e.* the part of plant to fulfill food purpose), nor based on the plants that occupy arable lands. Specifically speaking, most of the second generation biofuels are made from lingocellulosic biomass feedstock using advanced technological process [9]. However, the liquid fuel extraction from lignocellulosic biomass requires a series of complex chemical and physical treatments, which makes it challenging to produce the second generation biofuels economically at large scale. The third-generation biofuels refer to the algae biomass-based biofuels such as biodiesel and biogas. Algae are one of the oldest life-forms on Earth, they may have an oil content as high as 20-50% dry weight of biomass [10]. Unlike the production of feedstock for first and second generation biofuels, algae cultivation can be performed all year round with less freshwater demand than terrestrial crops [11]. Moreover, since algae are more robust than most higher plants, wastewater and industrial dust waste can be used to provide nutrients for algal cultivation [12]. Even though the third generation biofuels have so many advantages, more investigations and knowledge are nevertheless needed to overcome the negative energy balance [10] and to increase the product diversity. Thus, intensive research on developing the fourth-generation biofuels are being carried out worldwide. This class of biofuels refer to the ones produced by algae or other microorganisms via synthetic biology and metabolic engineering approaches [13]. Generally, these approaches are applied to generate engineered strains which can produce specific non-natural compounds or have better metabolic fluxes towards the production of target compounds. Moreover, when utilizing photosynthetic organisms, genetic engineering is also being done to improve both the CO_2 fixation and the photosynthetic efficiency in order to increase the product yield [14][15].

Biofuel production in cyanobacteria

The production of various valuable compounds has been demonstrated in different microorganisms, especially in the famous heterotrophic model microorganisms *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). However, cyanobacteria have recently become another favorite platform for the production of the fourth generation biofuel due to their attractive ability to grow photo-autotrophically using sunlight, CO_2 and water. The biomass and/or biofuel generated is produced directly from photosynthesis and independent of any carbohydrate feedstock.

Cyanobacteria

Cyanobacteria are a group of Gram-negative prokaryotes that can be found in varied environments across all latitudes, such as fresh water, sea water, soil, rocks, and even deserts. Before 1960, cyanobacteria were called 'blue-green algae' due to their color and were classified along with green, red and brown algae. In 1960s, many biochemical characters of cyanobacteria were discovered, such as their bacteria-like sensitivities to antibiotics and their lack of organelles (*e.g.* mitochondria and chloroplasts) [16]. Therefore, these blue-green microbes were finally classified as bacteria and the new name 'cyanobacteria' appeared in the microbiology world since then.

Oceanic cyanobacteria are believed to be the first cause of the Great Oxygenation Event (GOE) on the earth 2.45 billion years ago [17]. How cyanobacteria evolved the ability to split water remained unclear for a long time since there were no evolutionary precursors identified until the discovery of two classes of non-photosynthetic cyanobacteria [18, 19]. These two sister groups of non-photosynthetic cyanobacteria have the same non-photosynthetic ancestor as the photosynthetic cyanobacteria, and the divergence of cyanobacteria to have the ability to produce O_2 happened approximately 100 million years before the GOE [20] via horizontal gene transfer [21].

Cyanobacteria comprise diverse morphological groups of filamentous, unicellular, and colonial strains. Some filamentous strains have the ability to differentiate their cells into various cell types when the environmental condition changes. For instance, when *Nostoc punctiforme* need to fix atmospheric nitrogen, while maintaining the vegetative cells for normal photosynthesis, the filaments will differentiate a new type of cells called heterocysts, which have an anaerobic intercellular environment [22]. This differentiation is mainly for the sake of the nitrogen fixing enzyme, nitrogenase, which is highly oxygen sensitive.

Cyanobacteria possess many other advantages to be a promising chassis for biofuel production. For example, they provide more than three times higher solar energy capturing efficiency than plants [11]. In addition, their CO_2/HCO_3^- uptake and concentrating systems can accelerate the

photosynthesis process by minimizing the effects from photorespiration [23][24]. Genetically, cyanobacteria have relatively simple and transformable genomes which provide an opportunity to engineer the cells for diverse biotechnological applications. In addition, some cyanobacteria highly tolerant to the changes of certain environmental factors, e.g. salinity, pH, temperature, or light intensity, which makes it feasible to cultivate them in sea water, industrial wasted water and dust, or in some other harsh environmental conditions on a large scale.

The model cyanobacterial strain used in this thesis is *Synechocystis,* a well-studied unicellular strain which was isolated from a freshwater pond in Oakland (California) and it was the first phototrophic organism to be fully sequenced. *Synechocystis* is a non-nitrogen fixing cyanobacterium and the substrain we used in the study of this thesis can tolerate glucose, therefore, it can grow photoautotrophically, mixtrophically, and heterotrophically [25]. Genetically, *Synechocystis* is transformable and exogenous DNA can be transferred into the cell via natural transformation, electroporation, or conjugation [26, 27]. Furthermore, the function of more than half of the genes in *Synechocystis* have been identified or deduced and all the related information is collected in

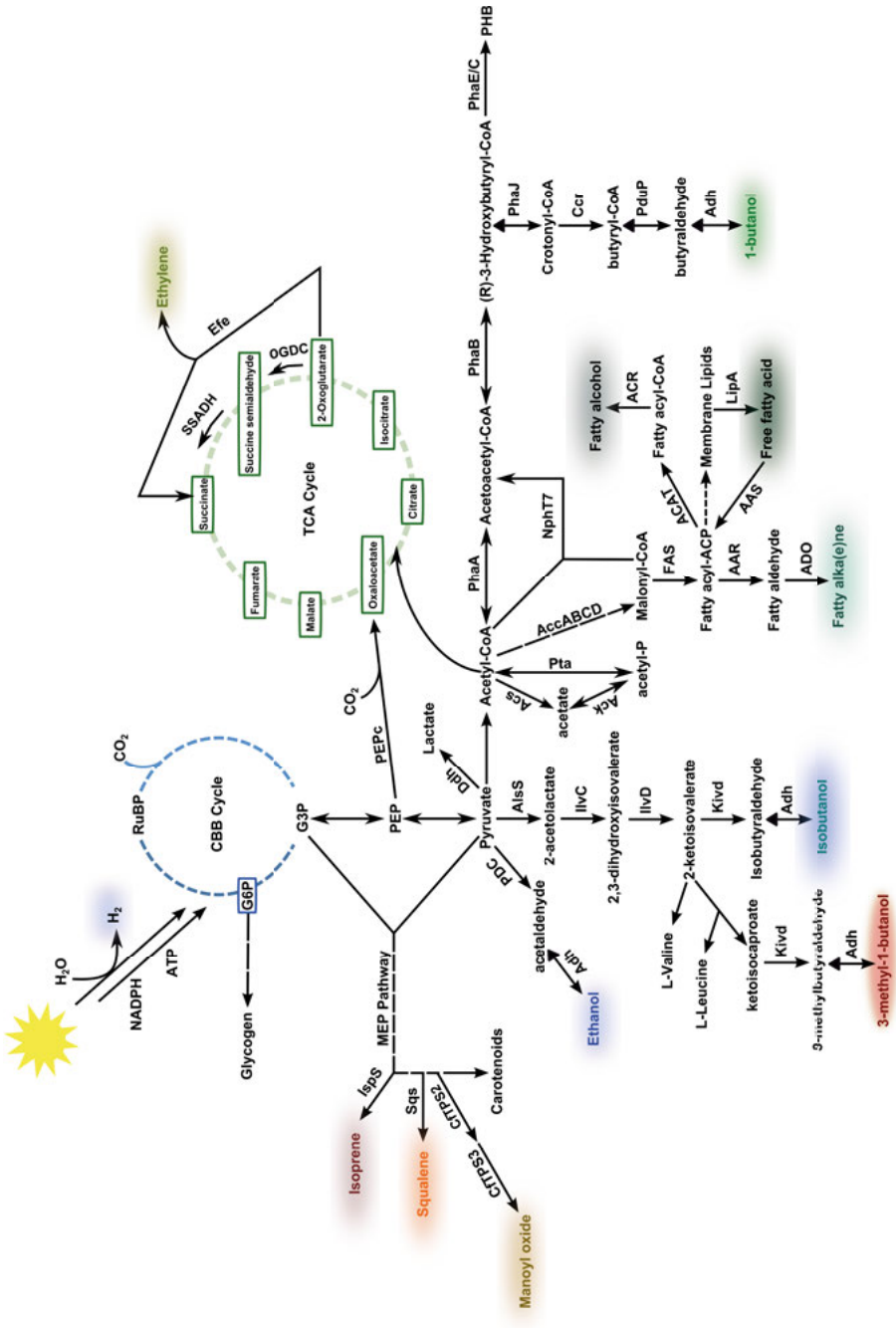

H2O
H2
NADPH
ATP
CO2
RuBP
CBB Cycle
G6P
Glycogen
G3P
MEP Pathway
IspS
Isoprene
Sqs
Squalene
CrtPSZ
CrtOYB
Carotenoids
Manoyl oxide
PEP
PEPc
CO2
Oxaloacetate
Citrate
Isocitrate
2-Oxoglutarate
OGDC
Succine semialdehyde
SSADH
Succinate
Fumarate
Malate
TCA Cycle
Ethylene
Efe
Pyruvate
Ldh
Lactate
PDC
acetaldehyde
Adh
Ethanol
AlsS
2-acetolactate
IlvC
2,3-dihydroxyisovalerate
IlvD
2-ketoisovalerate
L-Valine
Kivd
Isobutyraldehyde
Adh
Isobutanol
L-Leucine
ketoisocaproate
Kivd
3-methylbutyraldehyde
Adh
3-methyl-1-butanol
Acetyl-CoA
Acs
acetate
Ack
acetyl-P
Pta
AccABCD
Malonyl-CoA
FAS
Fatty acyl-ACP
AAR
Fatty aldehyde
ADO
Fatty alka(e)ne
PhaA
Acetoacetyl-CoA
PhaB
(R)-3-Hydroxybutyryl-CoA
PhaE/C
PHB
NphT7
PhaJ
Crotonyl-CoA
Ccr
butyryl-CoA
PduP
butyraldehyde
Adh
1-butanol
Fatty alcohol
ACR
Fatty acyl-CoA
AcAT
Membrane Lipids
LipA
Free fatty acid
AAS

Figure 1. Examples of photosynthetic production of different valuable compounds in cyanobacteria.

CyanoBase, a genome database for cyanobacteria. In light of this genetic knowledge and all the recent developments on metabolic engineering technology and synthetic biology, *Synechocystis* has been established as a model system for the investigations of cyanobacterial genetic regulation, photosynthesis, carbon fixation, and metabolic flux regulation. Moreover, it has also been engineered as a cell factory for the production of different valuable compounds, such as biofuel and pharmaceutical compounds [28].

Metabolic engineering of cyanobacteria

In cyanobacteria, some of the natural metabolites are already suitable to be used as biofuels, such as lipids, some carbohydrates, and hydrogen. In addition, cyanobacteria have also been engineered to produce some non-natural biofuel products, for example, long chain alcohols, fatty alcohols (Fig. 1). No matter which kind of products are wanted from cyanobacteria, metabolic engineering is an unreplaceable approach for introducing and navigating the metabolic flux towards the aimed products [29]. Metabolic engineering refers to the directed improvements of cellular properties made by the introduction of new biochemical reactions or the modification of specific existing ones [30]. Metabolic engineering has various applications, such as improving the yield of native metabolites, extending the range of substrates, and producing novel products in different hosts [31].

In order to engineer the metabolism of cyanobacteria, the development of synthetic biology tools, such as vectors, promotors, transcription factors, ribosome binding sites, and post-transcription and translation regulators are needed (Fig. 2) [32]. Below is a summary of the development of some synthetic biology tools for *Synechocystis*, the model strain used in this thesis.

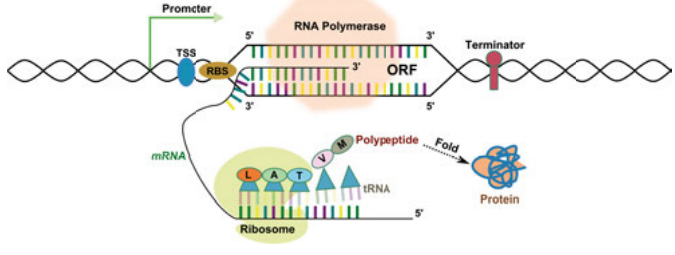


Figure 2. Simple schema of transcription and translation. TSS: Transcription starting site; RBS: Ribosome binding site; ORF: Open reading frame; mRNA: Messenger RNA.

Vectors

When genes are introduced into *Synechocystis*, they are either on a self-replicating vector or integrated on the chromosome. The broad-host-range self-replicating vector pPMQAK1 contains RSF1010-derived replicon which allows the vector to be replicated and maintained in different host strains, such as *E. coli*, *Synechocystis*, *Anabaena* 7120 and *Nostoc punctiforme*, and this vector can be transformed quickly into *Synechocystis* via conjugation [33]. Different integrated vectors have also been generated for introducing genes into neutral sites or any other specific loci on the genome [34][35]. Since several neutral sites on the genome and also on the endogenous plasmids have been identified in *Synechocystis,* EYFP expression levels from these loci have been compared to the expression level on the self-replicating vector pPMQAK1 [36] [33]. Due to the difference in copy number, EYFP expression level from the neutral site on the endogenous plasmid was 3-5 times higher than that from pPMQAK1 and 8-14 times higher than that from neutral sites on the genome [36]. Moreover, the neutral site on the endogenous plasmid was not able to be fully segregated after transformation, which indicated that genetic stability may be an issue when we integrate DNA there.

Promoters

In *Synechocystis*, there are several strong native constitutive promoters, including the *psbA2* promoter that is responsible for the expression of D1 protein in photosystem II, the *rbcL* promoter for the expression of RuBisCO large subunit, and the *cpc560* promoter for the expression of phycocyanin beta subunit. They can be employed when continues high expression of operons are desired [37][38][39]. However, an inducible and tunable promoter is required when the product of the expressed enzyme(s) is toxic to the host cell or when the transcription of the target DNA (*e.g.* in CRISPRi system) is required at specific time points. Unfortunately, there are not many choices of inducible promoters for *Synechocystis* so far. The commonly used isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter P*trc*, which works well in *E. coli*, *S. cerevisiae*, and even in *S. elongatus*, can only be used as a strong constitutive promoter in *Synechocystis* since the repressor LacI cannot release the operator on the promoter completely after induction. Therefore, the promoter does not have an induction level that is comparable to its original strength without repression [33]. However, a set of P*tac* derived promotors varing -10 and -35 regions showed tunable IPTG-induction [40]. In addition, several *Synechocystis*-native inducible promoters have been characterized and the Ni^{2+} and Co^{2+} induced promoter P*nrsB* that drives the transcription of a set of Ni^{2+} stress protection transporters have shown a high induction rate with low leakage [41][42][37]. Furthermore, the Tet-promoters performed well in *Synechocystis* with wide dynamic ranges, but the light sensitivity of the inducer

anhydrotetracycline makes them difficult to work with in photosynthetic organisms [43].

5' untranslated region (5' UTR)

The ribosome binding site is another important component that effects gene expression strongly. Thus, a library of different native and artificial RBSs have been tested in *Synechocystis* [44][45]. The results indicated that the strength of different RBSs can be effected significantly by the DNA sequences nearby and the variation is consistence in both *Synechocystis* and *E. coli* since these two organisms share the same core anti-Shine-Dalgarno sequence in their ribosomes [46][47][48]. However, using strong promoters and strong RBSs does not always lead to high expression since the mRNA of the expressed gene together with the upstream 5' untranslated region (5' UTR) may form unpredictable secondary structure which can interfere the initiation of translation [49][50]. Therefore, some reliable regulation/modification methods such as bicistronic design (BCD) and self-cleaving ribozyme (RiboJ) are needed to overcome this potential problem and provide precise and reliable gene expression [51][52]. BCD is a special construct with a short coding sequence at the junction between 5' UTR and the gene of interest (GOI), which makes the translation more reliable via 'melting' the secondary structures that insulate the RBS. RiboJ is an effective insulator part which can cleave the 5' UTR from mRNA in order to leave a stable translatable structure. These two elements were tested in *E. coli* but only few studies have been carried out in *Synechocystis* [53].

Strategies to enhance biofuel production in cyanobacteria

So far, the production for all of the biofuel compounds in cyanobacteria is far less than what is needed to commercialize the products (Table. 1). Extensive efforts are therefore being made to enhance the productivity of target products (Paper VI). In this section, several strategies for improving the yield of the aimed compounds in cyanobacteria are discussed.

Table 1. *Titer or productivity of selected compounds that have been produced in cyanobacteria. (Results from this thesis are not included in this table)*

Product	Strain	Titer /productivity	Cultivation condition	Time (Days)	Reference
Ethanol	*Synechocystis* PCC6803	2.3 g L^{-1}	100 µmol photon m^{-2} s^{-1} BG11 with 50 mM $NaHCO_3$	9	[54]
Isopropanol	*S. elongatus* PCC7942	146 mg L^{-1}	Dark anaerobic 5 days and light (150 µmol photon m^{-2} s^{-1}) aerobic 10 days, BG11	15	[55]
Isobutanol	*S. elongatus* PCC7942	~ 500 mg L^{-1}	150 µmol photon m^{-2} s^{-1}, BG11 with 50 mM $NaHCO_3$ and 10 mg L^{-1} thiamine per Day	8	[56]
1-Butanol	*S. elongatus* PCC7942	~ 317 mg L^{-1}	50 µmol photon m^{-2} s^{-1}, BG11 with 50 mM $NaHCO_3$	12	[57]
Ethylene	*Synechocystis* PCC6803	9.7 ml L^{-1} h^{-1}	200 µmol photon m^{-2} s^{-1}, BG11 with 5% CO_2, semi continues	16	[58]
Isoprene	*S. elongatus* PCC7942	1.26 g L^{-1}	100 µmol photon m^{-2} s^{-1}, BG11 with 5% CO_2 in photobioreactor	21	[59]
Squalene	*S. elongatus* PCC7942	~ 50 mg L^{-1}	100 µmol photon m^{-2} s^{-1}, BG11 with 5% CO_2 and 10 mM MOPS	14	[60]
Free Fatty Acid	*S. elongatus* PCC7942	640 mg L^{-1}	180 µmol photon m^{-2} s^{-1}, BG11 with 2% CO_2, two-phase cultivation	10	[61]
Alkanes (Heptadecane)	*Nostoc punctiforme*	12.9 % of Dry cell weight	135 - 160 µmol photon m^{-2} s^{-1}	8	[62]

Optimizing light harvesting capacity

Cyanobacteria only utilize the narrow absorbable spectrum range, light between 400 to 700 nm, like most of the other oxygen-evolving photosynthetic organisms [63]. Thus, investigations on the special chlorophylls that can absorb light above 700nm have gained much attention in recent years and some genetic engineering efforts were done to introduce these special chlorophylls into the model cyanobacterial strains in order to expand their light harvesting capacities [64][65][66]. Moreover, cyanobacteria have a large light harvesting complex which allows the organism to harvest enough light energy in low light conditions. However, if consider the large-scale cultivation in photo-bioreactors, the large antenna size increases the risk of photodamage for the cells on the top layer of a mass culture while decreases the light source for the cells on the bottom. Therefore, optimizing the antenna size is a potential strategy for increasing photosynthetic rates under high light [67][68].

Enhancing carbon fixation

Carbon fixation efficiency is another important characteristic of cyanobacteria that determines whether the strain is an optimal platform for biochemical production, especially for those carbon-based products. RuBisCO is the essential CO_2-fixing enzyme in the Calvin-Benson-Bassham (CBB) cycle that has been investigated intensively in the last decades. A number of studies have been carried out to either overexpress or engineer RuBisCO to enhance CO_2 fixation capacity in cyanobacteria, resulted in improved growth of the strain and the production of the target carbon-based products [15][69][70]. Moreover, phosphoenolpyruvate carboxylase (PEPc), which catalyzes the conversion of phosphoenolpyruvate to oxaloacetate, also plays an important role in carbon utilization in cyanobacteria. Since the catalysis of PEPc is irreversible towards the TCA cycle, overexpressing PEPc may be helpful for the production of some TCA cycle-linked products, such as ethylene and 3-hydroxypropionic acid [71], though not for pyruvate-based products, like lactate, isobutanol, or ethanol.

Redirecting metabolic fluxes

Besides optimizing the fundamental capacities of the host strain, redirecting metabolic flux towards the target products and enhancing the catalytic efficiency of the target metabolic pathways are other important strategies to increase the production of specific products. Sometimes, heterologously expressing new functional enzymes or overexpressing endogenous enzymes are not enough to gain a reasonable productivity, thus, knocking out (or down) potential competing pathways may also be needed to reroute the metabolic flux further. Homologous recombination is a matured traditional method for knocking out the essential gene or operon of the competing pathway [72]. However, many other methods, for example, riboswitch, antisense RNA, and CRIPSRi system have also been developed and demonstrated in cyanobacteria for knocking out or down target gene(s) [73][74][75][76][77]. These newer methods can achieve the goal to regulate the expression of multiple genes at the same time which overcome the obstacle of limited choice of antibiotics in homologous recombination. In addition, the design of metabolic engineering is more and more meticulous nowadays, which means what we would like to do with the metabolic pathways is not only ON-OFF switching but also tuning. With the help of these tunable tools, some essential pathways, *e.g.* TCA cycle and glycogen synthesis pathway, can be redesigned to benefit the production of target products without sacrificing cells survival and growth.

Enhancing the turnover rate of pathways

To optimize the catalytic efficiency of a metabolic pathway, the enzymes within the pathway become the main roles since some enzymes have slow turnover rates and some enzymes catalyze reversible reactions, or they can

utilize different substrates to generate different products at the same time (Fig. 1). Enzyme engineering is an efficient way to change the characteristics of enzymes even though it often requires the crystal structure of the enzyme and huge amount of efforts on structure remodeling and mutation testing [78][79][80]. Directed evolution is a method of enzyme engineering, which is based on generating random mutations in the enzyme, thus, normally a larger mutant library and intensive screening are required [81][82]. The other method is rational design and it is a knowledge-based modification, usually starting with the 3D structure of the target enzyme or some similar enzymes. Using this method, the more structural information we know, the more precise design we can make, and thereby, the smaller mutant library we need to generate [82]. Unfortunately, most of the enzyme engineering studies have been performed in *E. coli* since it is the most mature biological model system to provide enough amount of protein for *in vitro* assay. However, if we want to use cyanobacteria as a chassis, more effort should be put in examining the performance of engineered enzymes in cyanobacteria because the significant genetic and metabolic differences between *E. coli* and cyanobacteria may result in totally different performances of the engineered enzymes.

Isobutanol and 1-butanol production in cyanobacteria

Currently, ethanol is the main gasoline additive in the fuel market and is normally made from biomass fermentation using sugarcane and corn as feedstocks [83]. The largest ethanol fuel producer is the U.S. which had a production of nearly 6 billion liters in 2017 and in Europe, E85 (85% ethanol) becomes one of the main fuel resources for vehicles. However, ethanol has a relatively low energy density, only 66% of that of gasoline, which leads to a lower mileage volume compared to gasoline and the users need to refuel their vehicles more frequently [84]. Moreover, due to many other negative properties, such as its hygroscopic behavior, ethanol is not the most optimal supplement for gasoline [85]. Therefore, more alternatives are needed to be investigated and also to be produced via a more environmental friendly strategy.

One of the potential candidates is butanol, a four-carbon alcohol which has four isomers, 1-butanol, isobutanol, sec-butanol, and tert-butanol. Butanol can be used as a paint solvent, an ink ingredient, an artificial flavorant, a solvent in chemical industry, and a base for perfumes, etc. Nowadays, isobutanol and 1-butanol have been recognized as attractive gasoline additives since they have significantly higher energy density, lower vapor pressure, lower water solubility and also lower hygroscopicity compared to ethanol [86]. Furthermore, 1-butanol has a research octane number (RON) of 96 and a motor octane number (MON) of 78 while isobutanol has an even more favorable RON of 113 and a MON of 94 [87]. These qualities make them good 'drop in' fuels for the petroleum infrastructure without any risk of corrosion of engines and pipelines [88]. In addition, isobutanol and 1-butanol can be used as precursors for the production of a number of other valuable compounds, such as isobutene and butyl acrylate, respectively [89][90].

Examples of isobutanol production in cyanobacteria

Isobutanol production in cyanobacteria was first demonstrated in *Synechococcus elongatus* PCC 7942 (*S. elongatus*) with the heterologous expression of the keto-acid isobutanol synthesis pathway which starts from pyruvate and consists five steps (Fig. 3) catalyzed by enzymes from different organisms [37]. All of these five genes were integrated into the neutral sites on the chromosome and the transcribed by the IPTG induced promoters P*trc* and $P_{LlacO1.}$ As result, approximately 450 mg L^{-1} isobutanol was produced in 6 days when using BG11 media with the addition of 50 mM $NaHCO_3$ and the pH of the culture was adjusted to 7.5 using 10 N HCl every day. One strategy to further increase isobutanol production in *S. elongatus* is to redirecting more carbon into the isobutanol synthesis pathway. In a follow up study, Li *et al.* tried to knock out the glycogen synthesis pathway since glycogen is a major carbon storage in cyanobacterial cells [56]. The hypothesis was the isobutanol

synthesis pathway could be an alternative metabolic sink in the glucose-1-phosphate adenylyltransferase (*glgC*) deficient *S. elongatus* strain to rescue the growth retardation caused by the absence of glycogen synthesis [56]. Unfortunately, the existence of isobutanol synthesis pathway was not able to completely rescue the phenotype caused by *glgC* deficient. This may due to either the isobutanol produced from the culture was high enough to cause the growth inhibition, or because the ATP and NADPH requirement in isobutanol synthesis was less than glycogen synthesis, so isobutanol synthesis pathway cannot recycle cofactors as efficiently as the glycogen synthesis pathway. However, the deletion of glycogen synthesis pathway successfully redirected the carbon flux towards isbutanol production and resulted in more carbon distribution in isobutanol formation than in biomass formation, which led to higher productivity per cell.

Autotrophic, heterotrophic and mixtrophic productions of isobutanol in *Synechocystis* were examined using the strain heterogeneously expressing α-ketoisovalerate decarboxylase (Kivd) and an alcohol dehydrogenase (AdhA) from *Lactococus lactis* (*L. lactis*) that catalyze the last two steps of the isobutanol synthesis pathway [91] (Fig. 3). Most isobutanol titer was detected in the mixtrophic culture where 50 mM $NaHCO_3$ and 0.5% glucose were supplemented into the BG11 media. Moreover, an *in situ* oleyl alcohol trap was employed to reduce the negative feedback from the isobutanol produced, resulting in a more than doubled isobutanol titer was observed. However, the OD_{730} of the mixtrophic culture was above 10 which indicates that the high titer of isobutanol was contributed by a dramatic increase of biomass.

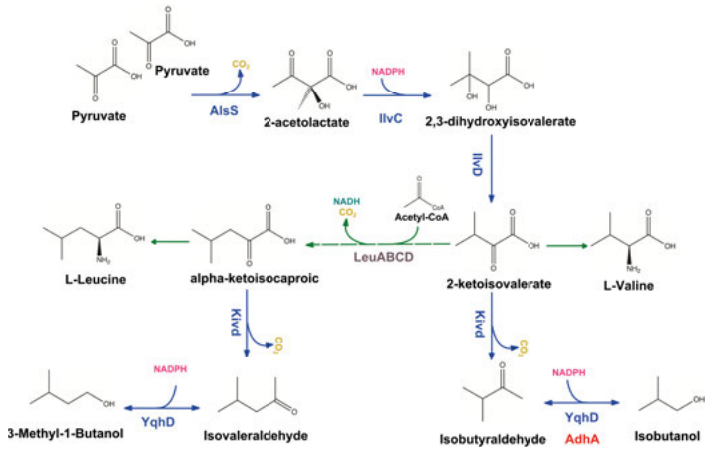


Figure 3. Isobutanol biosynthesis pathways have been demonstrated in different cyanobacterial strains [15][56][91]. All the heterogenous enzymes that have been employed are in blue and red: **AlsS**: acetolactate synthase from *B. subtilis*, **IlvC**: acetohydroxy acid isomeroreductase from *E. coli*, **IlvD**: dihydroxy acid dehydratase from *E. coli*, and **Kivd**: α-ketoisovalerate decarboxylase from *L. lactis,* **YqhD**: alcohol dehydrogenase from *E. coli,* **AdhA**: alcohol dehydrogenase from *L. lactis.*

Examples of 1-butanol production in cyanobacteria

1-butanol photoautotrophic production was first demonstrated in *S. elongatus* with the expression of the modified CoA-dependent 1-butanol synthesis pathway (Fig. 4). In this modified pathway, the oxygen sensitive complex Bcd/EtfAB from *C. acetobutylicum* was replaced by a NADH-dependent trans-enoyl-CoA reductase, Ter, from *Treponema denticola* (*T. denticola*) and the thiolase, Thl, was replaced by AtoB from *E. coli* because of the interest of gaining higher activity. These changes were based on the experience from former study in *E. coli* and all these genes were integrated into the neutral sites on the chromosome of *S. elongatus*. Unfortunately, the engineered strain showed barely detectable 1-butanol production (near 1 mg L^{-1}) under light aerobic condition, and trace amount (2.3 mg L^{-1}) 1-butanol was detected when the cells were grown under light in 5% CO_2 and 95% N_2 mixed gas. Surprisingly, when darkness and anoxic were applied together to the cells, they produced approximately 14.5 mg L^{-1} 1-butanol which indicated that some other factors in the pathway such as the cofactors and substrate concentration may need the anoxic condition to fulfill the requirement of 1-butanol production.

Further modifications have been done in the CoA-dependent pathway to increase the driving force of 1-butanol production in *S. elongatus* (Fig. 4). An acetoacetyl-CoA synthase, NphT7, from *Streptomyces* sp. CL190 was introduced into the pathway to catalyze the reaction from malonyl-CoA to acetoacetyl-CoA and resulted in detectable (6.5 mg L^{-1}) 1-butanol production in phototrophic condition [92]. Moreover, in the same study, all the NADH-dependent enzymes in the original CoA-dependent pathway were changed to NADPH- utilizing ones since NADPH is the reduction power produced during photosynthesis. By doing so, 1-butanol phototrophic production was increased to 29.9 mg L^{-1} which strengthened the importance of cofactor preference in metabolic engineering. However, the second last enzyme in the 1-butanol pathway, CoA-acylating butyraldehyde dehydrogenase Bldh from *Clostridium saccharoperbutylacetonicum N1-4* (*C. saccharoperbutylacetonicum N1-4*), is an oxygen sensitive enzyme which might be an obstacle for 1-butanol phototrophic production [93]. Thus, Lan *et al.* replaced this enzyme with the CoA-acylating propionaldehyde dehydrogenase, PduP, from the 1,2-propanediol degradation pathway in *Salmonella enterica* (*S. enterica*) and 1-butanol in-flask titer was increased to 317 mg L^{-1} after 12 days cultivation.

Besides all the studies in *S. elongatus*, *Synechocystis* PCC 6803 (hereafter *Synechocystis*) has also been used as a platform for 1-butanol production [94]. Both genetic engineering and cultivation condition modification were performed in the study to enhance acetyl-CoA pool for increased 1-butanol production. Three essential enzymes in the 1-butanol synthesis pathway were expressed on a self-replicating vector under the control of the strong P_{trc} promoter while the carbon storage PHB synthesis pathway was blocked by eliminating *phaE* and *phaC*. This engineered strain produced around 22 mg L^{-1} 1-

butanol under both nitrogen-starvation and nitrogen-repletion conditions. However, the specific titer observed during nitrogen-starvation was three times higher than during nitrogen-repletion. A further expressing of the phosphoketolase, Xfpk from *Bifidobacterium breve* (*B. breve*), resulted in a 2-fold increase of 1-butanol titer which was 37 mg L^{-1} in nitrogen-replete condition, but showed only minor effects during nitrogen starvation.

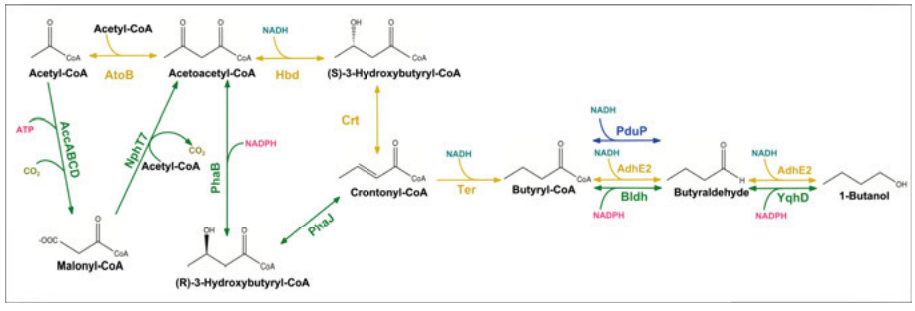


Figure 4. 1-Butanol biosynthesis pathways demonstrated in different cyanobacterial strains. The pathway in yellow was the first 1-butanol pathway examined in *S. elongatus*, then the green pathway was utilized to enhance the production via ATP driving force and NADPH preference, and further improvement was done by using PduP instead of Bldh to overcome the oxygen sensitivity barrier. AtoB: acetyl-CoA acetyltransferase from *E. coli*, Hbd: 3-hydroxybutyryl-CoA dehydrogenase from *C. acetobutylicum*, Crt: crotonase from *C. acetobutylicum*, Ter: trans-2-enoyl-CoA reductase from *T. denticola*, AhdE2: bifunctional aldehyde/alcohol dehydrogenase from *C. acetobutylicum*, NphT7: acetoacetyl-CoA synthase from *Streptomyces* strain CL190, PhaB: acetoacetyl-CoA reductase from *R. eutropha*, PhaJ: (R)-specific enoyl-CoA hydratase from *Aeromonas. caviae*, Bldh: butyraldehyde dehydrogenase from *C. saccharoperbutylacetonicum* N1-4, PduP: CoA-acylating propionaldehyde dehydrogenase from *S. enterica.* (Paper V)

Aim

The aim of this PhD thesis can be summarized into three points:

I To generate and characterize isobutanol and 1-butanol producing *Synechocystis* PCC 6803 strains.

II To improve isobutanol production in *Synechocystis* PCC 6803 by engineering a key bottleneck enzyme.

III To further improve isobutanol and 1-butanol production in *Synechocystis* PCC 6803 by optimizing cultivation conditions.

Results and Discussion

Construction of vectors for genetic engineering in *Synechocystis* (Papers I, II, and V)

Integrative vectors

In order to integrate a gene of interest (GOI) into different loci on the chromosome of *Synechocystis*, and to simplify the cloning process, a series of integrative vectors, pEERM, containing all the genetic parts for gene expression and integration were constructed. pEERM vectors have a high copy number in *E. coli* since it has the pMB1 replicon used in pUC vectors. Different versions of pEERM contain different promoters, antibiotic cassettes, and homologous recombination regions (Paper I). The insertion of DNA fragments can be done using a BioBrick-like standardized cloning strategy [95]. In each pEERM, a promoter is located between EcoRI and XbaI restriction enzyme cutting sites, followed by the BioBrick suffix sequence containing SpeI and PstI sites. All the insertions are required to have XbaI site on its 5' end and BioBrick suffix sequence on its 3' end for keeping the same cloning sites after each ligation (Fig. 5A). pDDH is a variant of the pEERM vectors made for integrating isobutanol synthesis genes, and it has the homologous region for replacing *ddh* (*slr1556*) in *Synechocystis*' chromosome (Paper II). Besides, other versions of pEERM vectors were generated for 1-butanol production in *Synechocystis* (Paper V).

Self-replicating vectors

A series of self-replicating vectors, pEEK, were generated to express genes of interest (GOI) independently from the chromosome of *Synechocystis* (Fig. 5B). pEEK vectors carry the broad-host-range RSF1010 replicon, which allows the GOI on these vectors to be transferred from *E. coli* to *Synechocystis* directly by conjugation (Paper II). Moreover, this replicon has been shown to have a copy number rang between 10 to 30 in *Synechocystis*, which is similar or slightly higher than the average copy number of the *Synechocystis* chromosome, depends on the cultivation condition and selection pressure [96]. pEEK2 is the pEEK variant used in this thesis, which contains a strong promoter P*trc* to drive the transcription of the operon and a BCD sequence on the 5' UTR for more reliable and stable gene expression. GOI can be ligated into

the vector via BglBrick [97], and the scar made from BglII - BamHI ligation becomes a part of the linker sequence between the Strep-tag and gene of interest. *ccdB* sensitive *E. coli* strains are required for the cloning process when use pEEK2, since this vector contains *ccdB* encoded toxic selection marker protein which can kill the *E. coli* cell by causing DNA cleavage [98].

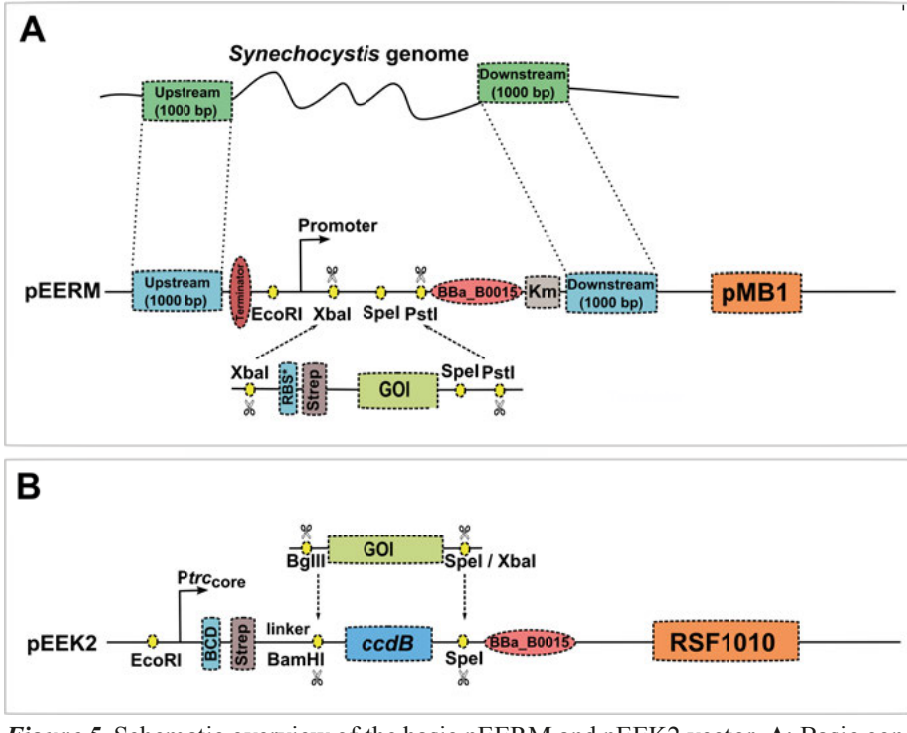


Figure 5. Schematic overview of the basic pEERM and pEEK2 vector. **A**: Basic construct of pEERM series vectors. Upstream and Downstream are the homologous recombination regions for integration. BglII - EcoRI and BamHI - SalI are the cloning sites for changing the upstream and downstream regions, respectively. pMB1 is the high copy number replicon. **B**: Construct of pEEK2 vector. RSF1010 is the broad-host-range replicon. *ccdB* is employed in the construct for enhanced colony selection efficiency. GOI:Gene of interest. BBa_B0015: BioBrick terminator. BCD: Bicistronic design.

Photosynthetic isobutanol production in *Synechocystis* (Papers II, III, and IV)

Isobutanol production in *Synechocystis* using different alcohol dehydrogenases (Paper II)

During cultivations in 30 °C under 50 - 100 μmol photons m^{-2} s^{-1} light intensity, there is a high risk of isobutanol evaporation through air exchange in the E-flasks with cotton cap. Thus, plug-sealed tissue culture flasks were chosen for cultivating the engineered *Synechocystis* strains, and an evaporation examination was performed before the cultivation.

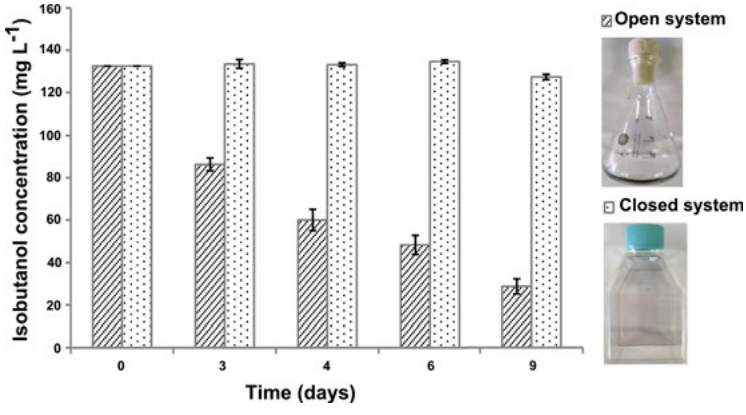


Figure 6. Comparison of isobutanol evaporation in the cotton capped E-flask (open system) and in the plug-sealed tissue culture flask (closed system). All the flasks were shaken for 9 days in the 30 °C growth room, under 50 μmol photons m^{-2} s^{-1} light intensity. (Paper II)

As expected, isobutanol evaporation could barely be observed in the closed system, while in the open system, only 36% and 22% isobutanol remained in the flask on day 6 and day 9, respectively (Fig. 6). This result indicates that the plug-sealed tissue culture flask can efficiently prevent product evaporation. In addition, the tissue culture flask provides better culture mixture and light supply since it has larger horizontal surface while shaking compared to E-flasks. Therefore, the closed system was employed in this thesis to perform all the cultivation experiments for isobutanol and 1-butanol production in *Synechocystis*.

The precursor for isobutanol production, α-ketoisovalerate (KIV), is produced via the L-Valine and L-leucine biosynthesis pathways which naturally exist in *Synehcocystis* (Fig. 7). Thus, to heterologously produce isobutanol in *Synechocystis*, the α-ketoisovalerate decarboxylase (Kivd) from *L. lactis* was employed since the *E. coli* strain expressing Kivd showed higher isobutanol production than the strains expressing Pdc6 or Aro10 from *S. cerevisiae* and

Thi3 from *C. acetobutylicum* [99]. Furthermore, it was shown that the engineered *S. elongatus* strain could not produce detectable amount of isobutanol without expressing heterologous alcohol dehydrogenases (ADH) [15], which indicates the activity of *S. elongatus* endogenous ADH is low towards isobutanol synthesis. In *Synechocystis*, the endogenous ADHs showed high *in vivo* activity towards ethanol production, but had not been examined for isobutanol production [100]. Therefore, in this study, two *Synechocystis* endogenous ADHs, encoded by *slr1192* and s*lr0942*, were examined and compared with two ADHs from *E. coli,* YqhD and YjgB, that had been shown to have high activity towards isobutanol production (Fig. 7).

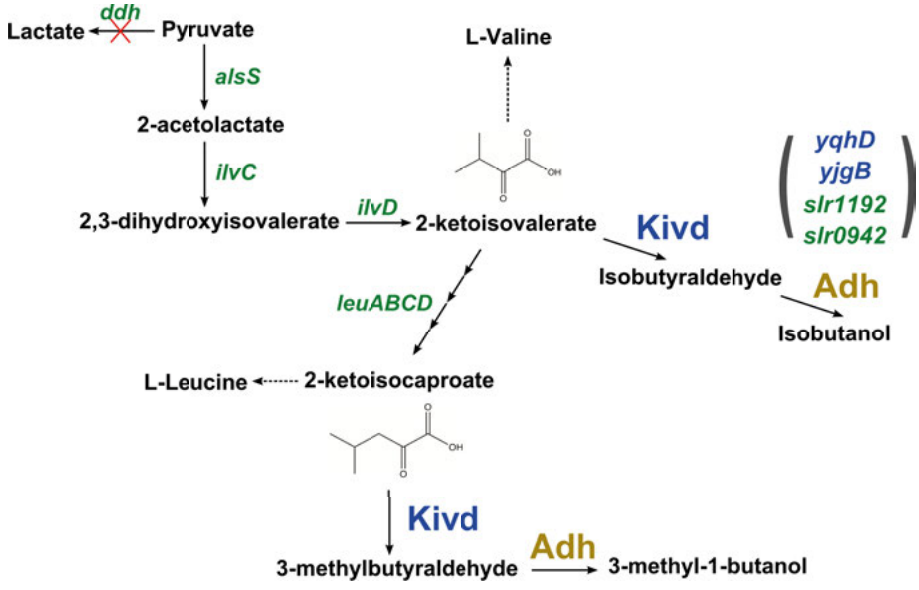


Figure 7. Isobutanol and 3-methyl-1-butanol biosynthesis pathway in *Synechocystis*. *Synechocysits* endogenous genes are marked in green, heterologous genes are marked in blue. The knock-out locus is marked with a red cross.

Initially, each of the ADH encoding genes was co-expressed with Kivd in the same operon driven by P*psbA2* promoter [101] on pDDH vector, which resulted in strains Syn-IB-1, -2, -3, and -4, respectively (Table 2). After 6 days cultivation, only trace amount of isobutanol was produced from these 4 strains and no significant difference among the strains was observed. The low production may due to the low expression levels of Kivd and AHDs in all the strains (Fig. 8). Therefore, in order to get higher expression levels of the genes, strain Syn-IB-5 (Table 2) was generated by changing the P*psbA2* promoter in Syn-IB-4 into a stronger Ptrc_{core} promoter followed by a BCD sequence. In addition, strain Syn-IB-6, which has the identical operon as strain Syn-IB-5

on the self-replicating vector pEEK2 instead of the chromosome (Table 2), was also generated, since it has been reported that genes expressed on RSF1010 replicon based self-replicating vector showed higher expression level than the ones expressed on the chromosome in *Synechocystis* [36]. As expected, the expression levels of the genes and isobutanol titer were found to be step-wise increased comparing strains Syn-IB-4, -5, and -6 (Fig. 8B). Notably, the isobutanol production per OD_{750} from strain Syn-IB-6 was more than 4 times higher than that from Syn-IB-5, which indicated that compared to using a stronger promoter on the chromosome of *Synechocystis*, overexpressing genes on a high copy number self-replicating vector gave a more significant increase on protein expression and isobutanol production (Fig. 8B). Therefore, strains Syn-IB-7, -8, and -9 were generated for the further examination of different ADHs together with strain Syn-IB-6. In addition, strain pEEK2-Kivd, was also generated as a better control strain to show the isobutanol production from *Synechocystis* native ADHs (Table 2). Surprisingly, the isobutanol titer observed from strain pEEK2-Kivd at day 6 was more than doubled compared to that from all the other strains (Fig. 8B).

Table 2. *Genetic information of the engineered Synechocystis strains.*

Strain Name	Expressed gene(s)	Promoter	Location	Additional ADH source
Syn-IB-1	*kivd, yqhD*	P*psbA2*	*slr1556*	*E. coli*
Syn-IB-2	*kivd, yjgB*	P*psbA2*	*slr1556*	*E. coli*
Syn-IB-3	*kivd, slr0942*	P*psbA2*	*slr1556*	*Synechocystis*
Syn-IB-4	*kivd, slr1192*	P*psbA2*	*slr1556*	*Synechocystis*
Syn-IB-5	*kivd, slr1192*	Ptrc_{core}BCD	*slr1556*	*Synechocystis*
Syn-IB-6	*kivd, slr1192*	Ptrc_{core}BCD	pEEK2	*Synechocystis*
Syn-IB-7	*kivd, yqhD*	Ptrc_{core}BCD	pEEK2	*E. coli*
Syn-IB-8	*kivd, yjgB*	Ptrc_{core}BCD	pEEK2	*E. coli*
Syn-IB-9	*kivd, slr0942*	Ptrc_{core}BCD	pEEK2	*Synechocystis*
Syn-pEEK2	Empty vector control strain			
pEEK2-Kivd	*kivd*	Ptrc_{core}BCD	pEEK2	-

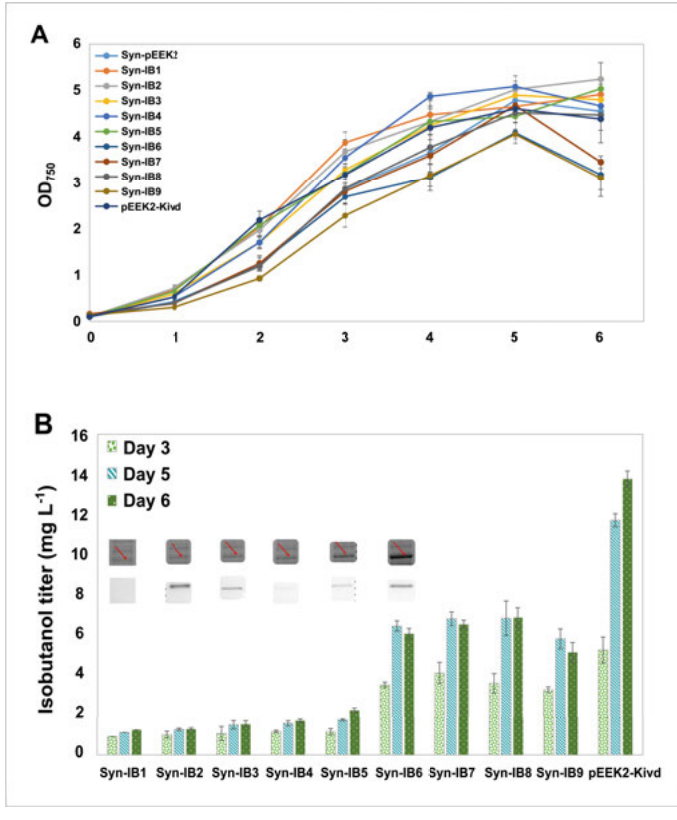


Figure 8. Growth, isobutanol titer and protein expression level of the engineered *Synechocystis* strains. **A**: Growth of all the engineered Synechocystis strains during 6 days cultivation. **B**: Isobutanol titer observed on day 3, 5, and 6 in all the isobutanol producing strains. The SDS-PAGE boxes with red arrows indicate the expression levels of Kivd in strains Syn-IB-1 to Syn-IB-6, while the Western-immunoblot boxes below indicate the expression levels of ADHs in strains Syn-IB-1 to Syn-IB-6.

However, there was no significant difference on isobutanol production per cell among all the strains (Fig. 9A), which indicates that there might not be enough substrates for the additionally expressed ADHs to show their catalytical differences. Herein, to overcome this barrier, external substrate for ADH, isobutyraldehyde, was added into the cultures and isobutanol titer was detected after 24 hours. As expected, isobutanol titer from all the other strains were higher than that from strain pEEK2-Kivd, meaning the amount of external substrate could saturate the native ADHs in *Synechocystis* and the additionally expressed ADHs were able to produce more isobutanol. Moreover, significantly different isobutanol titers were observed from strains Syn-IB-6, -7, -8, and -9, and the ADHs encoded by *yqhD* and *slr1192* seem to have better catalytic efficiency on catalyzing isobutyraldehyde into isobutanol in *Synechocystis* (Fig. 9B).

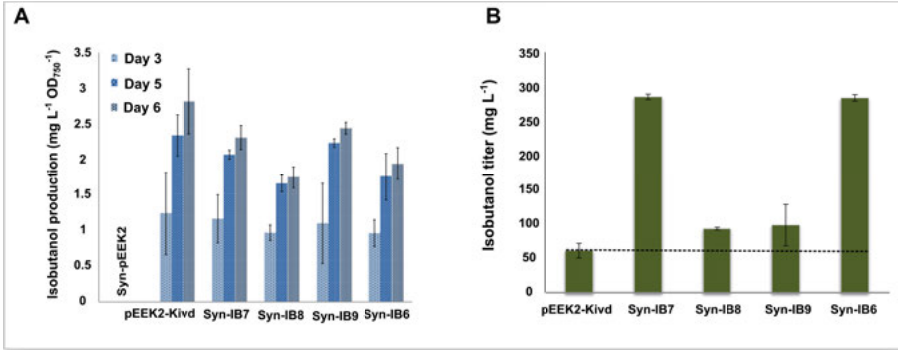


Figure 9. **A**: Isobutanol production observed on day 3, 5, and 6 in the engineered *Synechocystis* strains containing pEEK2-based plasmids, Syn-pEEK2 is the empty vector control strain. **B**: Isobutanol titer observed in strains pEEK2-Kivd, Syn-IB-6, -7, -8, and -9, after 24 hours cultivation in the presence of external isobutyraldehyde. (Modified from Paper II)

Interestingly, strain Syn-IB-6 showed better growth and less bleaching than strain Syn-IB-7 during the 24 hours cultivation with the presence of external isobutyraldehyde. This may due to the additional expression of *slr1192* played a role in enhancing the stress tolerance of *Synechocystis* [102][103], or (and) due to a higher catalytic efficiency of *slr1192* encoded ADH which converted the more toxic isobutyraldehyde into the less toxic isobutanol faster than YqhD.

In summary, this was the first study showed that α-ketoisovalerate decarboxylase is the only heterologous enzyme that needs to be introduced into *Synechocystis* for isobutanol production, and it is a potential bottleneck in the pathway. Moreover, the ADH encoded by *slr1192* has a high catalytic efficiency on isobutanol production in *Synechocystis*. In addition, this study indicated that using the self-replicating vector pEEK2 to express genes could result in increased transcription, translation and product formation in *Synechocystis*.

Protein engineer for improved isobutanol production in *Synechocystis* (Paper III)

Kivd was identified as a bottleneck in the isobutanol synthesis pathway in Paper II, since isobutanol production levels in the engineered *Synechocystis* strains were positively correlated to the expression levels of Kivd. Moreover, the Kivd employed here was from *L. lactis* IFPL730, which is a ThDP-dependent 2-keto acid decarboxylase that can utilize different substrates *e.g.* α-ketoisovalerate, α-ketoisocaproate, and α-phenylpyruvate. This is the reason why two products, isobutanol and 3-methyl-1-butanol (3M1B) were observed when Kivd was introduced into *Synechocystis* (Fig. 7) (Paper II). The substrate for isobutanol production contains one carbon less than the substrate for 3M1B production. Therefore, minimizing the substrate binding pocket of Kivd is a promising strategy to enhance the activity and preferential shift towards substrate α-ketoisovalerate (KIV), and thereby enhance isobutanol production.

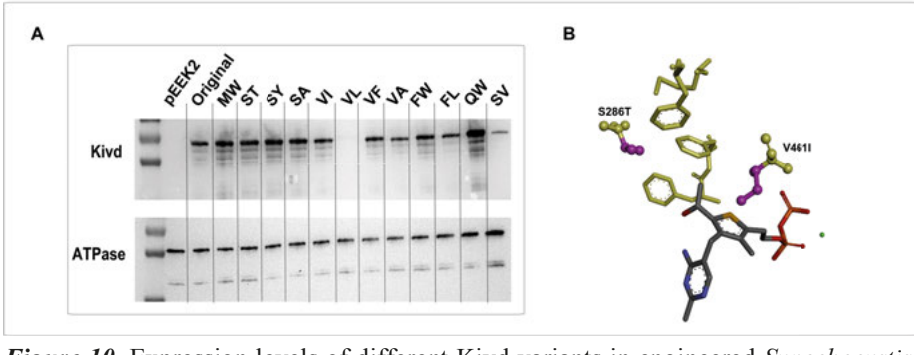


Figure 10. Expression levels of different Kivd variants in engineered *Synechocystis* strains and the structure of the predicted active site of Kivd. **A**: Anti-Strep tag Western-immunoblot to detect expression levels of different Kivd variants, anti-ATPase Western-immunoblot to detect expression levels of ATPase in the engineered strains as loading control. pEEK2-empty vector control strains, Original-Kivd without mutagenesis, MW-M538W, ST-S286T, SY-S286Y, SA-S286A, VI-V461I, VL-V461L, VF-V461F, VA-V461A, FW-F542W, FL-F542L, QW-Q377W, and SV-S286T & V461I. **B**: Mutations of the residues in the predicted active site of the best Kivd variants ST, VI, and SV, with ThDP (large multi-color molecule on the bottom) as cofactor. The green dot is Mg^{2+}. The yellow residues are the unchanged ones and the violet parts are the differences between the original residues and the mutated residues. Strains VI and ST have the corresponding change and the combined strain SV has both. (Paper III)

Twelve new engineered *Synechocystis* strains with different Kivd variants were generated using site mutagenesis based on rational design. Nine of the variants (MW, ST, SY, VI, VL, VF, FW, QW, and SV) are with minimized substrate binding pockets and the other three variants (SA, VA, and FL) are with enlarged substrate binding pockets for examining the opposite situation.

The expression levels of Kivd (Fig. 10A) and isobutanol titers (detailed in Paper III) observed in all these engineered strains varied significantly. Among all the strains producing isobutanol, strains VI, ST, and SV were selected for further investigations since they either showed the highest isobutanol production or the highest isobutanol to 3M1B titer ratio. All these selected strains and the control strain were cultivated for 8 days in seal-plugged tissue culture flasks under 50 μmol photons m^{-2} s^{-1}. Strains VI and ST showed 51.5 mg L^{-1} and 59.6 mg L^{-1} isobutanol in-flask titer on day 8, respectively, which were more than 3 times higher than the control strain (Fig. 11B). In contrast, strain SV showed only 6 mg L^{-1} isobutanol in-flask titer on day 8 (Fig. 11B), which may due to the extremely low expression level of this double mutated Kivd variant (Fig. 10A).

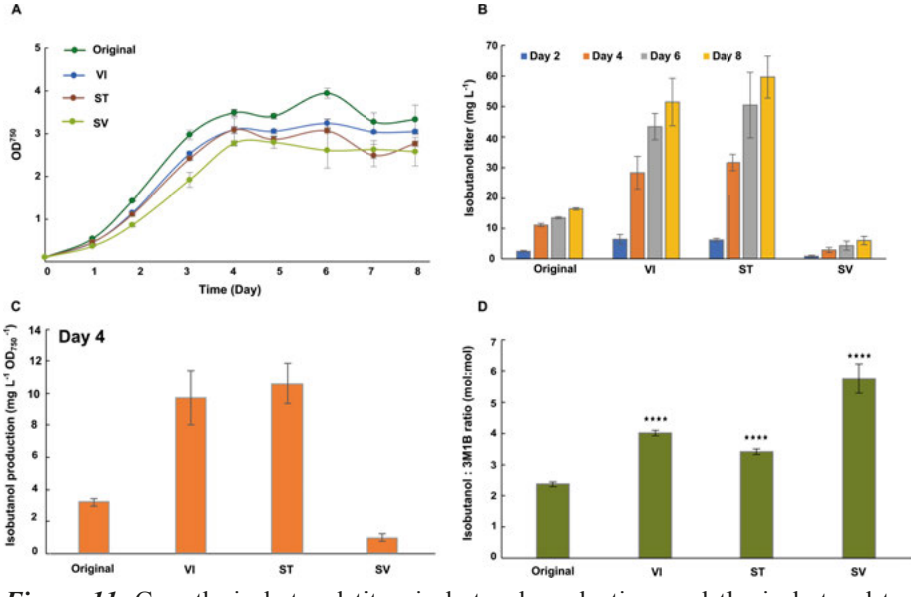


Figure 11. Growth, isobutanol titer, isobutanol production, and the isobutanol-to-3M1B molar ratio from the selected engineered *Synechocystis* strains. **A**: Growth of the selected strains during 8 days cultivation. **B**: Isobutanol in-flask titer on day 2, day 4, day 6, and day 8. **C**: Isobutanol production from different stains on day 4. **D**: Molar ratio of isobutanol and 3M1B in different engineered *Synechocystis* strains. The calculation was done based on the in-flask titer on day 8. Asterisks represent significant differences between the corresponding strain and the control strain (Original), **** = $p < 0.0001$ in t-test. All the results represent the mean of two technical replicates with three biological replicates, error bars represent the standard deviation. (Paper III)

Moreover, the isobutanol-to-3M1B molar ratio was quantified in the selected strains on day 8 (Fig. 11D). Strains VI, ST, and SV showed statistically significant increase of isobutanol-to-3M1B molar ratio. Notably, strain SV showed the highest ratio, which was 2.4-fold higher than that of the control strain. This result quantitatively shows that the double mutation

S286T/Val461I affects the preferential shift of Kivd towards isobutanol production more significantly compared to the different single mutations.

In order to investigate if the difference on isobutanol in-flask titer from the selected strains was only due to different Kivd expression level or (and) due to different catalytic activity, the *in vivo* activities of Kivd variants towards substrates KIV and KIC were estimated using the calculation of isobutanol / 3M1B production per specific unit of Kivd protein. In addition, the relative expression levels of different Kivd variants were quantified via Western-immunoblot (Fig. 12). The *in vivo* Kivd activity towards isobutanol (Fig. 13A) and 3M1B

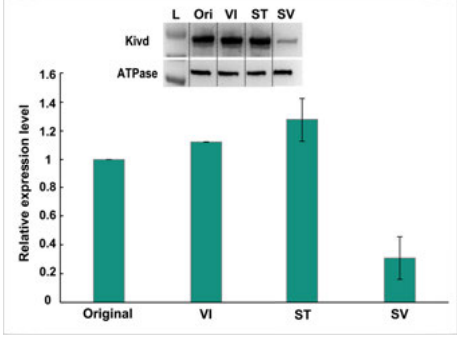


Figure 12. Relative expression levels of the Kivd variants in the selected engineered *Synechocystis* strains. They were quantified based on band intensity on the Western-immunoblot images using QuantityOne software. Kivd expression was detected via anti-Strep tag Western-immunoblot and anti-ATPase Western-immunoblot was employed as loading control. Results represent the mean of three technical replicates with two biological replicates, error bars represent standard deviation. (Modified from Paper III)

(Fig. 13B) production in strains VI and ST were significantly increased compared to in the control strain. However, the increase of *in vivo* activity towards the substrate KIV was higher than the increase of *in vivo* activity towards the substrate KIC, which resulted in the higher isobutanol-to-3M1B molar ratio in these two strains (Fig. 11D). Furthermore, strain SV showed similar Kivd *in vivo* activity towards substrate KIV and significantly lower activity towards the substrate KIC compared to in the control strain (Fig. 13), which resulted in the highest isobutanol-to-3M1B molar ratio among all examined strains (Fig. 11D).

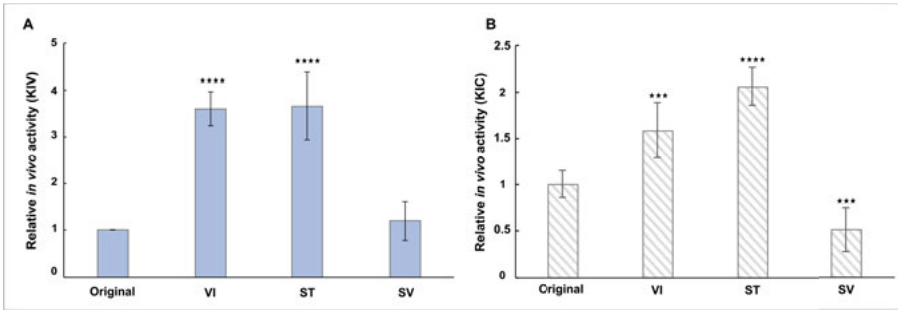


Figure 13. Relative *in vivo* (in *Synechocystis*) activity from different Kivd using substrates KIV and KIC. **A**: Relative *in vivo* activity using KIV as substrate. Relative activity in *Synechocystis* engineered strain equals to isobutanol production (mg L^{-1} OD^{-1}) divided by the relative protein expression level. **B**: Relative *in vivo* activity using KIC as substrate. Relative activity in *Synechocystis* engineered strain equals to 3M1B production (mg L^{-1} OD^{-1}) divided by the relative protein expression level. Asterisks represent significant differences between the corresponding strain and the original strain, *** = $0.0001 < p < 0.001$ and **** = $p < 0.0001$ in t-test. All the results represent the mean of two technical replicates per two biological replicates. The error bars represent the standard deviation of these calculated values. (Figure from Paper III)

In addition, *in vitro* activity assays using KIV as substrate was also performed on crude extracted protein from the selected strains, and the activities were normalized according to the relative expression level of the corresponding Kivd variant (Fig. 12). Similar to the *in vivo* results, strains VI, ST, and SV showed significant increase on Kivd *in vitro* activity towards the substrate KIV (Fig. 14).

Both the *in vivo* and *in vitro* activity results indicate that the enlargement of Val461 to isoleucine might help the substrate to anchor in the pocket in an appropriate position, making the interaction between the substrate and the cofactor easier. Moreover, S286, which was not suggested as a part of the active site of Kivd, is nevertheless an important amino acid for both the overall activity of Kivd and the preferential shift towards isobutanol production.

In summary, this study is the first demonstration on engineering protein for increasing production of specific compound in *Synechocystis* and the evaluation of the engineered proteins based on deep analyses of their *in vivo* performance. It clearly demonstrates that a single amino acid substitution can significantly affect the expression level and the function of a protein in *Synechocystis*. Therefore, protein engineering is a powerful tool to modify the output of a cyanobacterial metabolic pathway.

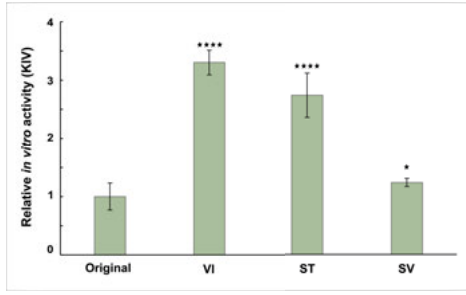


Figure 14. *In vitro* activity of different Kivd variants using substrate KIV. Crude extracted protein from each strain was used in the *in vitro* assay and the concentrations were normalized to the same level for all reactions. Then, the relative activity was calculated based on the relative Kivd expression level in the different strains. It represents the relative NADH consumption in a certain time per same amount of Kivd protein. *= $p < 0.05$ and **** = $p < 0.0001$ in t-test. All the results represent the mean of two technical replicates per two biological replicates. The error bars represent the standard deviation of these calculated values. (Figure from Paper III)

Further enhancement of isobutanol production in *Synechocystis* (Paper IV)

In Paper II and Paper III, isobutanol production in *Synechocystis* has been improved step by step from below 0.5 mg L^{-1} OD^{-1} to 18.6 mg L^{-1} OD^{-1} via genetic engineering and protein engineering approaches. Nevertheless, it was still much lower than the isobutanol production observed in heterotrophic organisms. This contrast makes the concept of photosynthetic isobutanol production less competitive. Therefore, the study in Paper IV focused on further enhancement of isobutanol production in *Synechocystis* via modifying the cultivation condition and addressing other potential bottleneck enzyme(s) than Kivd.

Firstly, the best isobutanol producing strain ST (pEEK2-ST) generated in Paper III was utilized here for examining three different light intensities and two different pH adjustments. Generally, the *Synechocystis* cells are grown under relatively low light condition (30 - 50 µmol photons m^{-2} s^{-1}) in the laboratorium thus 15 µmol photons m^{-2} s^{-1} and 100 µmol photons m^{-2} s^{-1} were applied in this study for low light condition and high light condition, respectively. 50 µmol photons m^{-2} s^{-1} was used as medium light condition. Cultures growing under 50 µmol photons m^{-2} s^{-1} showed better overall performance, faster growth and higher isobutanol titer, compared to the cultures growing under the other two light intensities. The cultures growing under 100 µmol photons m^{-2} s^{-1} showed the fastest growth till day 3 but a quick bleaching phenotype after day 4, which resulted in a low isobutanol in-flask titer in the end (Fig. 15A). Hence, 50 µmol photons m^{-2} s^{-1} was further employed to the cultures for investigating different pH adjustments.

HEPES buffering was one of the pH adjustment methods examined since HEPES is a widely used organic chemical buffering agent in cell culture. A significant amount of HEPES was added to the cultures every second day in this study since the initial $NaHCO_3$ concentration was as high as 50 mM and 2 ml BG11 media with 500 mM $NaHCO_3$ was supplemented to each culture every second day after sampling, which resulted in an even higher $NaHCO_3$ concentration in the cultures. The growth and isobutanol in-flask titer of the HEPES buffered cultures were significantly higher than that of the cultures without any pH adjustment though HEPES could not buffer the cultures back to pH between 7 and 8, and the final pH observed in the cultures was around 10 (Fig. 15B).

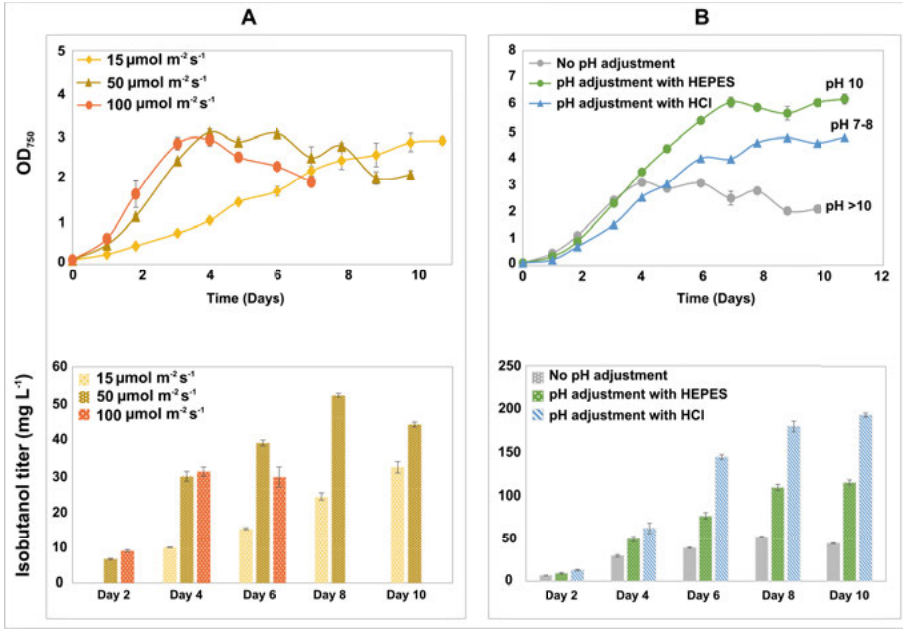


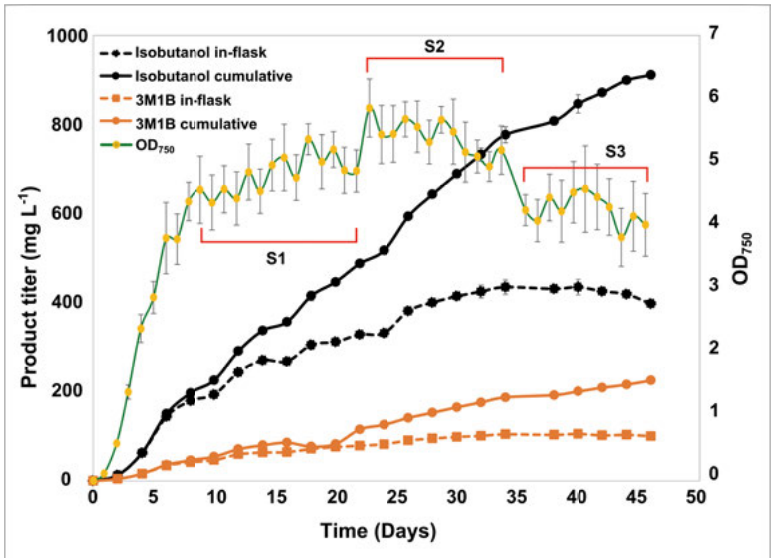
Figure 15. Growth and isobutanol titer of strain pEEK2-ST growing in different cultivation conditions. **A**: Growth and isobutanol titer of the cells growing under low (15 µmol photons m^{-2} s^{-1}), medium (50 µmol photons m^{-2} s^{-1}), and high (100 µmol photons m^{-2} s^{-1}) light intensities without any pH adjustment. **B**: Growth and isobutanol titer of the cells growing under 50 µmol photons m^{-2} s^{-1} with different pH adjustments, HEPES buffering and HCl titration. Cells without any pH adjustment were the control. The aimed pH range is between 7 and 8. The pH labeled on the growth curves are the actual pH observed in the end of the cultivation. (Paper IV)

In order to have a more efficient pH adjustment, certain amount of 37% HCl was added every day to titrate the cultures to pH between 7 and 8. Surprisingly, the titration contributed to a more than 40% increase of isobutanol in-flask titer compared to that from the HEPES buffered cultures though the growth of the titrated cultures was not as fast as the HEPES buffered ones (Fig. 15B). Moreover, isobutanol produced per cell in the titrated cultures was 2.4 times higher than in the HEPES buffered cultures on day 10.

The notable difference on growth between the cultures with and without pH adjustment may indicate that the closed system together with the culture pH affected the carbon uptake in the cells. When the culture has alkaline pH above 10, the carbon form in the media towards more to CO_3^{2-}, which cannot be used by the cells. Thus, by adjusting the pH to a range between 7 and 8, the carbon equilibrium shifted towards HCO_3^- and CO_2 formation, and these HCO_3^- and CO_2 molecules could get into the cells and provide additional carbon, which resulted in better growth and higher isobutanol production.

Surprisingly, the HCl titrated cultures maintained in stationary phase (s) for more than 30 days without losing the ability of producing isobutanol and

3M1B. The cultivation was kept till day 46 when the isobutanol in-flask titer had dropped for 3 continues measurements (Fig. 16). A maximal isobutanol in-flask titer of 435 mg L^{-1} was observed on day 40. However, the in-flask titer could not fully represent the exact amount of product produced by the cells since there was a dilution in each culture every second day from the exchange of 2 ml withdrawn culture for isobutanol extraction and 2 ml fresh media for fulfilling the original volume. Therefore, the cumulative isobutanol titer was calculated to 847 mg L^{-1} on day 40 when the cultures showed the highest in-flask titer with a final cumulative titer of 911 mg L^{-1} in the end of the cultivation on day 46. The cumulative titer for 3M1B was also calculated in the same way resulting in a final cumulative titer of 225 mg L^{-1} was gained on day 46. Furthermore, a highest isobutanol production rate of 43.6 mg L^{-1} was observed between days 4 and 6 coinciding with cells in maximal growth rate (Fig. 16). This indicates that maintaining the culture in the growth phase using chemostate may be an efficient way to further improve isobutanol total production in *Synechocystis*.



Isobutanol production rate

	Time	In-Flask (mg L^{-1} Day^{-1})	Cumulative (mg L^{-1} Day^{-1})
Growth Phase	Days 2-10	22.6 ± 0.4	26.6 ± 0.3
Steady state Phase (total)	Days 10-46	5.7 ± 0.2	19.0 ± 0.3
Steady state Phase (S1)	Days 10-22	11.2 ± 0.5	21.9 ± 0.4
Steady state Phase (S2)	Days 23-35	10.3 ± 1.5	25.9 ± 1.8
Steady state Phase (S3)	Days 36-46	-4.2 ± 0.7	12.9 ± 0.8
Maximal rate	Days 4-6	41.1 ± 3.5	43.6 ± 3.3

Figure 16. Growth, in-flask titer, cumulative titer of both products, and isobutanol production rate from the long term cultivated engineered *Synechocystis* strain ST. S1: Steady state phase I, S2: Steady state phase II, S3: Steady state phase III. The culture was cultivated under 50 µmol photons m^{-2} s^{-1} light intensity with HCl titrated pH between 7 and 8. Results represent the mean of three biological replicates, error bars represent standard deviation. (Modified from Paper IV)

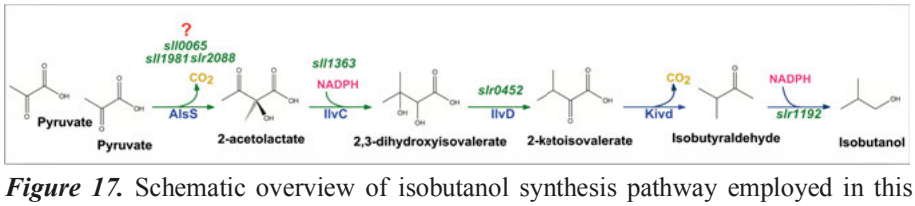


Figure 17. Schematic overview of isobutanol synthesis pathway employed in this study. The *Synechocysits* endogenous genes are in green while the heterogenous enzymes are in blue. AlsS: acetolactate synthase from *B. subtilis*, IlvC: acetohydroxy acid isomeroreductase from *E. coli*, IlvD: dihydroxy acid dehydratase from *E. coli*, and Kivd: α-ketoisovalerate decarboxylase from *L. lactis*. (Paper IV)

After the investigation on cultivation conditions, additional engineered *Synechocystis* strains containing one operon or two convergent orientated operons expressing Kivd^{S286T} and other genes in the isobutanol synthesis pathway were generated to potentially identify any other metabolic bottlenecks (Fig. 17). All the engineered strains were cultivated for 8 days, and expression level of all the enzymes were detected on crude extracted protein on day 2 while isobutanol titer was detected on day 2, 4, and 8.

Since there was no clear clue on the genes encoding *Synechocysits* endogenous acetolactate synthase (AlsS), three related genes, *sll0065*, *sll1981*, and *slr2088* were examined in this study together with the heterogenous AlsS from *B. subtilis* (Fig. 17). Unfortunately, the construct containing Kivd^{S286T} and AlsS (*B. subtilis*) could not be conjugated into *Synechocystis* successfully, which may due to the high activity of the enzyme [104]. Thus, strain pEEK2-ST1192OP-AlsS, supposed to have low expression level of AlsS was generated. Nevertheless, isobutanol titer observed from three biological replicates of this strain showed high variation and no AlsS expression could be detected via the anti-Flag tag Western-immunoblot. Furthermore, not as expected, the control strain pEEK2-ST still showed the highest isobutanol production among all the strains containing one operon, the expression level of Kivd^{S286T} in different strains varied significantly, and the expression of some co-expressed enzymes could not be detected via the anti-Flag tag Western-immunoblot (Fig. 18A). Interestingly, strain pEEK2-ST1981 showed the lowest isobutanol titer, which was 5 times less than the control strain pEEK2-ST on day 8. This may be another evidence for the α-ketoglutarate decarboxylase function of *sll1981* encoded enzyme in TCA cycle [58]. In this study, the expression of *sll1981* might lead more carbon flux towards TCA cycle instead of L-Leucine or L-Valine synthesis pathways (Fig. 18A).

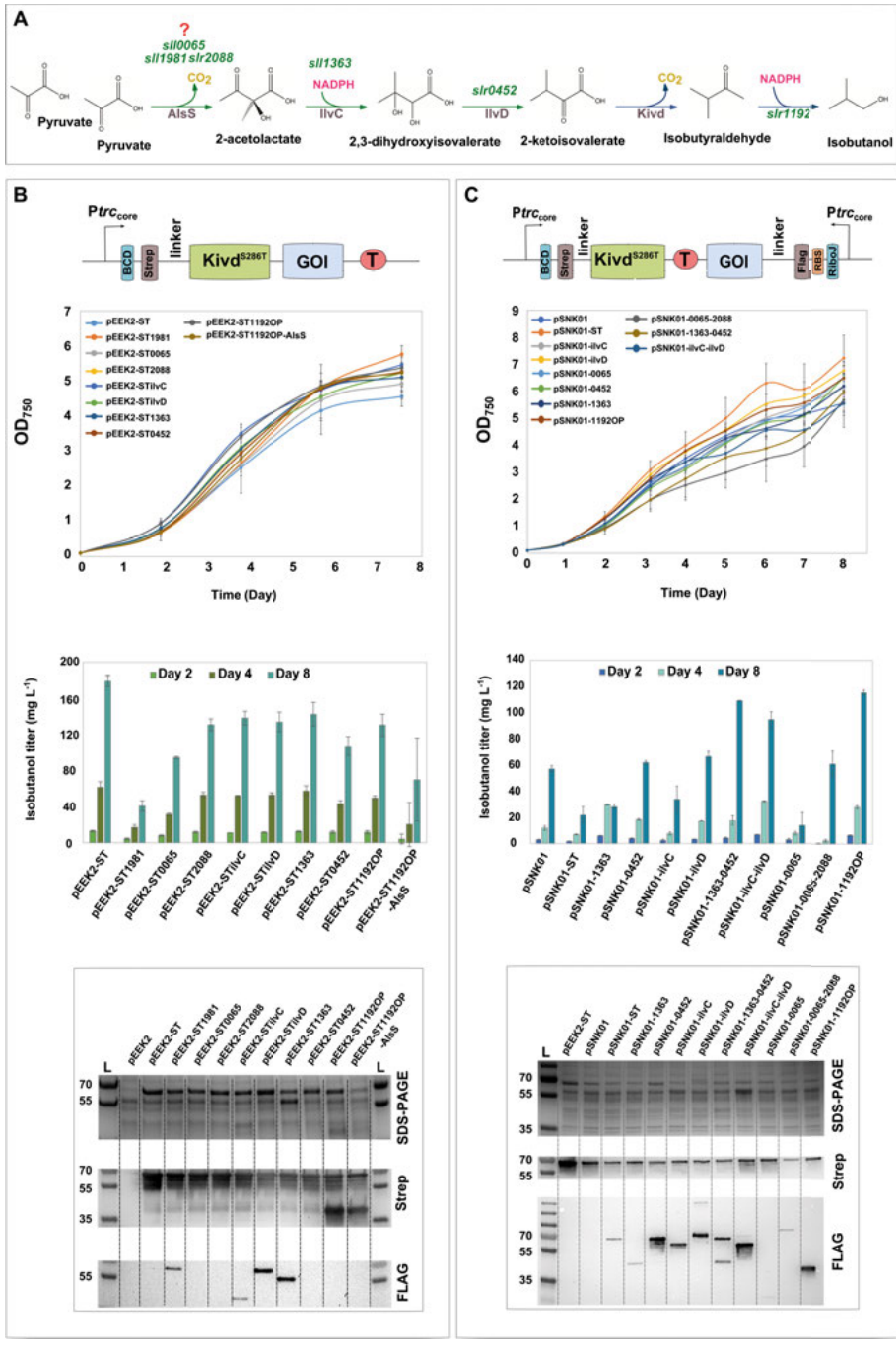

A
Pyruvate
Pyruvate
?
sll0065
sll1981 slr2088
CO2
AlsS
2-acetolactate
sll1363
NADPH
IlvC
2,3-dihydroxyisovalerate
slr0452
IlvD
2-ketoisovalerate
CO2
Kivd
Isobutyraldehyde
NADPH
slr1192
Isobutanol
B
Ptrc core
BCD
Strep
linker
Kivd S286T
GOI
T
OD750
Time (Day)
pEEK2-ST
pEEK2-ST1981
pEEK2-ST0065
pEEK2-ST2088
pEEK2-STilvC
pEEK2-STilvD
pEEK2-ST1363
pEEK2-ST0452
pEEK2-ST1192OP
pEEK2-ST1192OP-AlsS
Isobutanol titer (mg L-1)
Day 2
Day 4
Day 8
SDS-PAGE
Strep
FLAG
C
Ptrc core
BCD
Strep
linker
Kivd S286T
T
GOI
linker
Flag
RBS
RiboJ
Ptrc core
OD750
Time (Day)
pSNK01
pSNK01-ST
pSNK01-ilvC
pSNK01-ilvD
pSNK01-0065
pSNK01-0452
pSNK01-1363
pSNK01-1192OP
pSNK01-0065-2088
pSNK01-1363-0452
pSNK01-ilvC-ilvD
Isobutanol titer (mg L-1)
Day 2
Day 4
Day 8
SDS-PAGE
Strep
FLAG

Figure 18. Genetic constructs, protein expression, and isobutanol titer from the engineered *Synechocystis* strains. **A**: Basic construct, SDS-PAGE, Western-immunoblot, and isobutanol titer from the strains containing a single operon for expressing Kivd^{S286T} and other gene(s) in the isobutanol synthesis pathway. Anti-Strep tag Western-immunoblot was used to detect the expression of Kivd^{S286T} and ADH (codon optimized *slr1192*) while anti-Flag tag Western-immunoblot was used to detect the expression of all the other genes **B**: Basic construct, SDS-PAGE, Western-immunoblot, and isobutanol titer from the strains containing two convergent orientated operons for expressing Kivd^{S286T} and other gene(s) in the isobutanol synthesis pathway. Anti-Strep tag Western-immunoblot was used to detect the expression of Kivd^{S286T} while anti-Flag tag Western-immunoblot was used to detect the expression of all the other genes in the convergent operon. Protein size: AlsS: 62 kDa, IlvC: 54 kDa, IlvD: 65 kDa, Kivd (ST): 61 kDa, Sll1981: 60 kDa, Sll1363: 40 kDa, Slr0452: 59 kDa, Slr1192: 36 kDa, Sll0065: 21 kDa, Slr2088: 68 kDa. (Modified from Paper IV)

In order to have identical expression level for Kivd (ST) and higher expression level for the other genes, strains containing two convergent orientated operons were generated, and the control strain was pSNK01, which contains Kivd^{S286T} in the P*trc*$_{core}$-BCD driven operon and CcdB in the P*trc*$_{core}$-RiboJ driven operon. pEEK2-ST was used as an additional control strain while examining the expression levels of Kivd (ST) in different strains. Surprisingly, this control strain showed significantly higher Kivd (ST) expression level than all the strains containing two operons (Fig. 18B). The low Kivd^{S286T} expression in the strains with two operons may due to the interference between the two operons which was caused by supercoiling [105]. However, the expression levels for all the other genes could be detected clearly by employing the additional convergent orientated operon, which was an improvement compared to the one operon strains. The strains expressing Kivd^{S286T} with either *ilvC-ilvD* or *sll1363-slr0452* showed significantly higher isobutanol production than the control strain pSNK01 while the strains expressing each of these genes solely with Kivd^{S286T} did not show any improvement on isobutanol production. Unfortunately, the isobutanol titers from all the strains with two operons were lower than that from strain pEEK2-ST, which due to the significantly lower Kivd^{S286T} expression level in those strains, and the contribution from expressing the other genes in the pathway could not overcome the negative effect from the low Kivd^{S286T} expression.

In summary, this study clearly showed the importance of applying suitable cultivation conditions for enhancing the production of isobutanol in *Synechocystis*. Moreover, the observations in this study indicate that the expression level of Kivd^{S286T} can be significantly affected by the co-expression of other genes either in the same operon or in a convergent orientated operon. Therefore, choosing suitable (optimal) location and strategy for gene expression in *Synechocystis* plays an important role in improving the production.

Photosynthetic production of 1-butanol in *Synechocystis* (Paper V)

Photosynthetic 1-butanol biosynthesis via an acetyl-CoA dependent pathway has been studied for nearly a decade in two model cyanobacterial strains, *Synechocossus elongatus* and *Synechocystis*. Many steps in this pathway have been improved by using oxygen tolerant enzymes, NADPH dependent enzymes and ATP driving force [106][92][57]. Herein, the enzymes that showed good performance in all the previous investigations, were selected for better isobutanol production (details in Paper V). After massive amount of comparison of different enzymes in the first stage of this study, the best 1-butanol producing strain, SynBuOH-8, was generated and nearly 140 mg L^{-1} in-flask 1-butanol titer (38 mg L^{-1} OD_{750}^{-1}) was observed after 8 days cultivation in the closed system using $NaHCO_3$ supplemented BG11 media without pH adjustment (Fig. 19). In strain SynBuOH-8, five optimal enzymes for catalyzing malonyl-CoA to butyraldehyde were integrated into *Synechocysits* genome together with an additional copy of *Synechocystis* endogenous alcohol dehydrogenase, encoded by *slr1192*. These six genes were expressed in three different loci on the genome and the transcription of all three operons were driven by the short version of *Synechocystis* native P*psbA2* promotor (Fig. 20 blue) [37].

In order to further improve 1-butanol production, a mutated NADPH-dependent acetoacetyl-CoA reductase, PhaB (T173S), was employed since it has more than 3 times higher K_{cat} than the wild-type PhaB from *R. eutropha* and it showed enhanced *in vivo* activity when expressed in *Corynebacterium glutamicum* (*C. glutamicum*) (Fig. 20 purple) [107]. As expected, the utilization of this highly active PhaB enhanced 1-butanol in-flask titer to 180 mg L^{-1}. Furthermore, the *Synechocystis* cells did not easily segregated when the operon with an additional copy of *slr1192* was integrated into the genome. Thus, *slr1192* was replaced by the codon re-optimized version (*slr1192*OP) and resulted in more than one third increase of 1-butanol production (Fig. 21 purple).

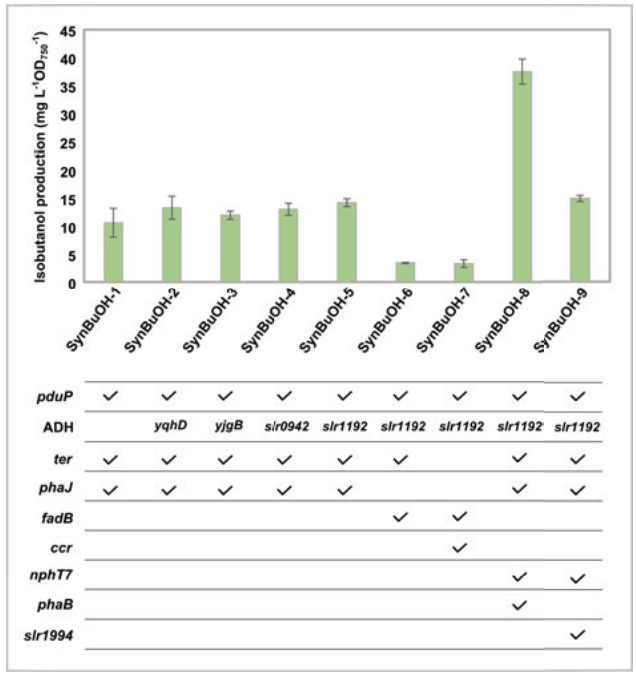


Figure 19. Selection of enzymes in 1-butanol synthesis pathway in *Synechocystis*. All the enzymes that were claimed to have high activity were combined and examined for 1-butanol synthesis in this study. (Paper V)

The third improvement stage mainly focused on selecting stronger promotors and suitable 5'UTR sequences to enhance the expression level of the butanol synthesis pathway in *Synechocystis*. Different combinations of P*psbA2*, Pcpc_{560}, P*trc*2O, Ptrc_{core}BCD, and Ptrc_{core}RiboJ for the three operons were examined (details in Paper V). In addition, a NADPH dependent crotonyl-CoA reductase (Ccr) from *S. collimus* was used to replace the NADH dependent crotonyl-CoA reductase (Ter) from *T. denticola* (Fig. 20 red) since there are more NADPH than NADH in cyanobacteria and the NADPH/NADH ratio in *Synechocystis* is much higher than in *E. coli* [108][109][110][111]. In the screening, strain SynbuOH-29 utilizing Ptrc_{core}RiboJ, Ptrc_{core}BCD, and Ptrc_{core} promotors showed the highest in-flask 1-butanol titer of 572 mg L^{-1} after 11 days cultivation.

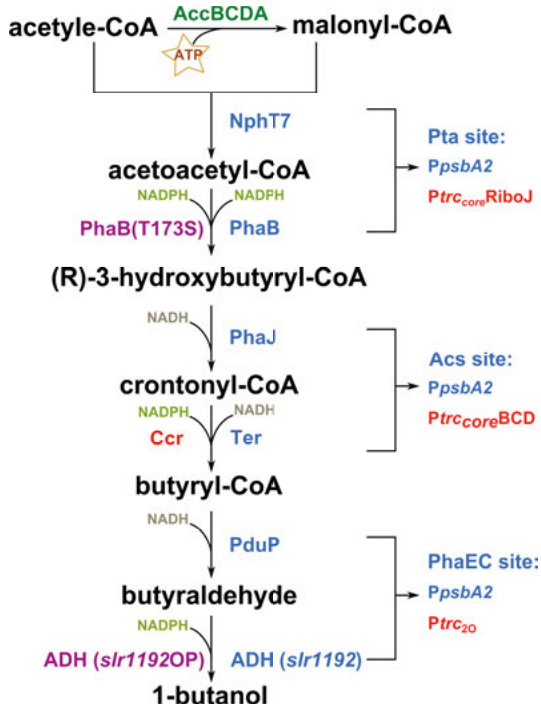


Figure 20. Strategic improvements on the 1-butanol biosynthesis pathway in *Synechocystis*. **Stage 1 (in blue)**: Basic 1-butanol synthesis pathway driven by P*psbA2* promotor, (in blue) was integrated into three *loci* (Pta, Acs, and PhaEC sites) in the genome of *Synechocystis*. **Stage 2 (in purple)**: Two enzymes were replaced for higher activity. **Stage 3 (in red)**: Ter was replaced by Ccr and promotors were changed to three strong P*trc*-based variants: Ptrc_{core}RiboJ, Ptrc_{core}BCD, and Ptrc_{core}. NphT7: acetoacetyl-CoA synthase from *Streptomyces* strain CL190, PhaB: acetoacetyl-CoA reductase from *R. eutropha*, PhaB (T173S): mutated version of PhaB, PhaJ: (R)-specific enoyl-CoA hydratase from *A. caviae*, Ter: crotonyl-CoA reductase from *T. denticola*, Ccr: crotonyl-CoA reductase from *S. collimus*, PduP: CoA-acylating propionaldehyde dehydrogenase from *S. enterica.* ADH (*slr1192*): alcohol dehydrogenase from *Synechocystis*, ADH (*slr1192*OP): codon re-optimized version of ADH (*slr1192*).

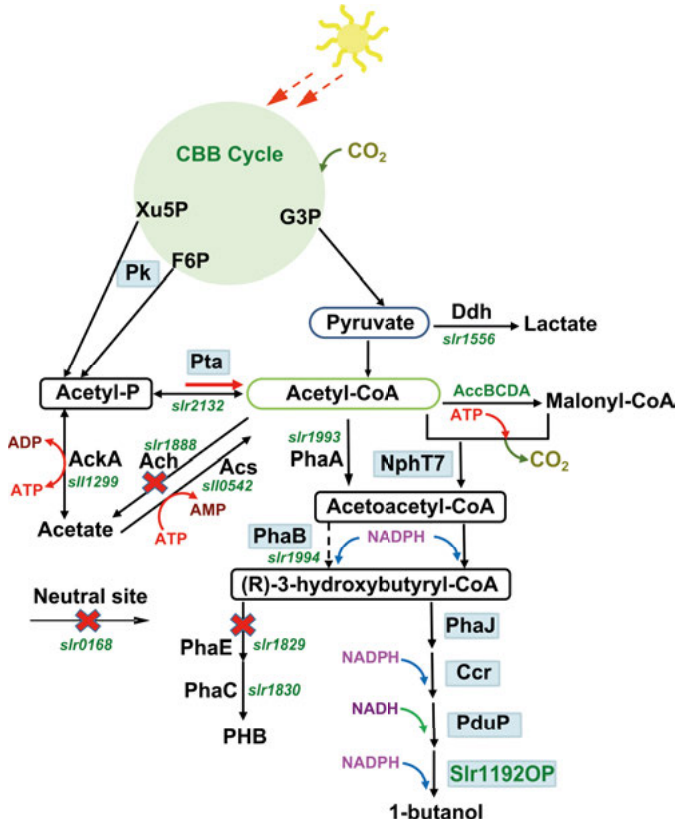


Figure 21. Schematic overview of the final optimized 1-butanol biosynthesis pathway introduced into *Synechocystis*. The endogenous genes are in green and the introduced genes are in blue boxes. The red crosses indicate the knockout *loci* in *Synechocystis* genome. NphT7: acetoacetyl-CoA synthase from *Streptomyces* strain CL190, PhaB (T173S): mutated version of acetoacetyl-CoA reductase from *R. eutropha*, PhaJ: (R)-specific enoyl-CoA hydratase from *A. caviae*, Ccr: crotonyl-CoA reductase from *S. collimus*, PduP: CoA-acylating propionaldehyde dehydrogenase from *S. enterica.* Slr1192OP, codon re-optimized version of Slr1192 (ADH) from *Synechocystis*, Pk: phosphoketolase from *P. aeruginosa*, Pta: phosphate acetyltransferase from *B. subtilis,* Xu5P: xylulose 5-phosphate, F6P: fructose 6-phosphate, G3P: glyceraldehyde 3-phosphate.

After optimizing every single step of the pathway from acetyl-CoA to 1-butanol, additional metabolic modification was done to increase the acetyl-CoA pool in *Synechocystis* for even higher 1-butanol production (Fig. 21). Nine different phosphoketolase (PK) from different organisms were examined in this study and the one from *P. aeruginosa* showed the best performance (details in Paper V). Moreover, in order to enhance the flux from acetyl-P to acetyl-CoA, an additional Pta from *B. subtilis* was introduced into *Synechocystis* in the same operon as NphT7 and PhaB^{T173S}, resulting in strain SynBuOH-44,

which showed 836 mg L^{-1} in-flask and 1 g L^{-1} cumulative 1-butanol titer on day 11.

In light of the results from Paper IV, 50 µmol photons m^{-2} s^{-1} light and HCl titrated pH were utilized for long-term cultivation of the best 1-butanol producing strain SynBuOH-44 in this study. As expected, the 1-butanol production from this engineered strain dramatically increase when grown under 50 µmol photons m^{-2} s^{-1} with HCl titrated pH between 7 and 8. Due to the impressively high 1-butanol production, product feedback inhibition and carbon supply might become obstacles in the culture. Thus, instead of taking samples and supplementing new BG11 with $NaHCO_3$ every second day, this procedure was done every day. By doing so, the strain managed to grow and produce 1-butanol for more than 20 days with the highest in-flask titer observed being 2.13 g L^{-1} and a cumulative titer of 4.71 g L^{-1} on day 23 and 27, respectively (Fig. 22).

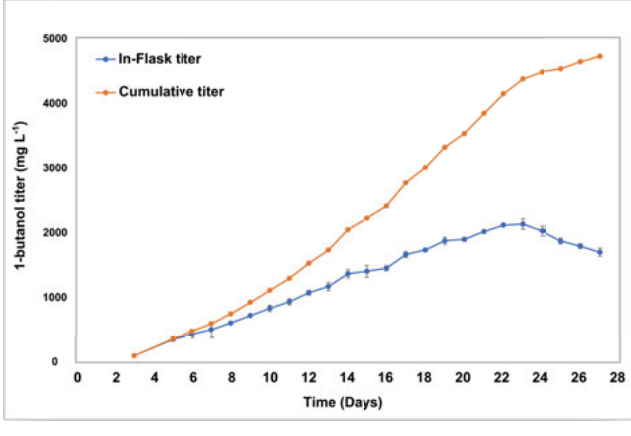


Figure 22. 1-butanol in-flask and cumulative titer during a long-term cultivation of the best 1-butanol producing *Synechocystis* strain. Cultivation condition: Initial OD_{750} = 0.5, BG11 media supplemented with 25 mM $NaHCO_3$, 50 µmol photons m^{-2} s^{-1}, sampled every second day for 1-butanol detection before OD_{750} reached 2 and afterwards sampled every day. Same volume of fresh BG11 media with 500 mM $NaHCO_3$ were added to each culture after each sampling. pH was adjusted to the range between 7 and 8 every day with HCl. (Paper V)

In summary, this study demonstrates a well-designed systematic strategy on engineering *Synechocystis* for producing a valuable chemical, 1-butanol. The investigations include engineering on genetic level, transcription/translation level, and over all metabolic flux level. In addition, the 1-butanol titer was finally boosted when a more suitable cultivation condition was employed.

Conclusions and outlook

This thesis demonstrates step-wise engineering strategies for producing valuable compounds in the cyanobacterial stiran *Synechocystis* and how to enhance the production systematically. Take the development of isobutanol production in *Synechocystis* as an example, the improvement from gene level to protein level and finally to the cultivation system level successfully enhanced photosynthetic isobutanol production titer hundreds of times (Fig. 23). Through these impressive results for both isobutanol and 1-butanol production, we can clearly see the potential future of cyanobacteria as green cell factories which can compete with the heterotrophic organisms.

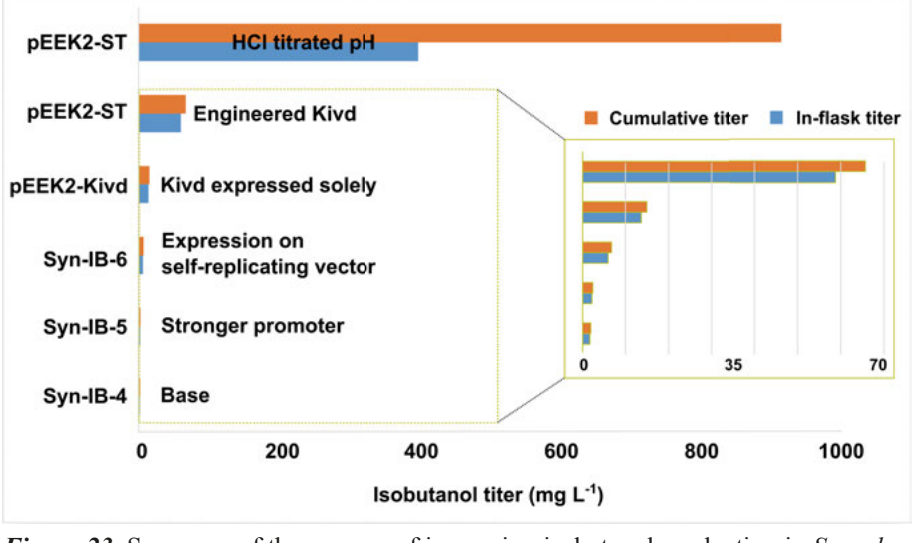


Figure 23. Summary of the process of improving isobutanol production in *Synechocystis* via different approaches. **Syn-IB-4**: P*psbA2* promoter - (*kivd, slr1192*) expressed on *slr1556* locus. Strains **Syn-IB-5**: P*trc*$_{core}$ promoter with bicistronic design – (*kivd, slr1192*) expressed on *slr1556* locus. **Syn-IB-6**: P*trc*$_{core}$ promoter with bicistronic design – (*kivd, slr1192*) expressed on pEEK2 vector. **pEEK2-Kivd**: P*trc*$_{core}$ promoter with bicistronic design – (*kivd*) expressed on pEEK2 vector. **pEEK2-ST**: P*trc*$_{core}$ promoter with bicistronic design – (best *mutated kivd*) expressed on pEEK2 vector.

Besides the modifications within the aimed biosynthesis pathways, additional changes in the entire metabolic net should be considered as well in order to

further improve the productivity of the engineered strains to a commercially viable level. For instance, it was determined that the largest portion of carbon flux in *Synechocystis* is funneled towards sugar and fatty acid synthesis pathways [101]. Therefore, alter this carbon flux partitioning in the cell would be useful to elevate the production of compounds link to other carbon consuming pathways, though it was claimed that under photo-autotrophic conditions, the re-routing of carbon flux in *Synechocystis* is significantly harder than under mixotrophic or heterotrophic conditions [112]. Moreover, re-direct carbon flux from growth to production in the cell using CRISPRi system could be another efficient strategy to optimize the carbon usage [113].

In addition, selected cultivation system is another important factor that directly affects the yield of aimed product. For example, when the aimed product is a volatile, suitable collecting system in (organic trap) or outside (condenser) the photo-bioreactor is preferred to avoid the loss of products from evaporation [91][114][115]. Some organic solvents, such as dodecane, are not toxic for the cells, thus they can be added directly on the top of the culture as a trap layer for removing specific products from the culture [116][117].

In summary, this thesis enriches the knowledge about synthetic biology in cyanobacteria, highlights the power of protein engineering, and overall helps the development of cyanobacteria as a chassis for high-flux production of valuable compounds.

Svensk sammanfattning

Världens energibehov ökar i en otroligt hög takt på grund av den alltmer tilltagande folkmängden samt den moderna livsstilen. Inom 20 år kommer det finnas mer än 9 miljarder människor i världen. Detta betyder att energibehovet kommer att öka med mer än 30 % jämfört med dagens energiförbrukning. Dessutom är fossila bränslen såsom olja, kol och naturgas fortfarande de största energikällorna. Den geografiska fördelningen av fossila bränslen kan orsaka en rad problem, exempelvis internationella konflikter och stigande matpriser. Förutom detta kan framställningen och förbrukningen av fossila bränslen leda till att toxiska ämnen uppkommer. Dessa ämnen kan både vara livsfarliga och skada miljön. Därför är vi i omedelbart behov av att framställa renare och förnybara energikällor som ej är centraliserade till ett fåtal ställen i världen. Solenergi kan bli den utmärkta förnybara energikällan, eftersom solljus finns överallt och mängden ljus som når atmosfären varje timme är tillräcklig för att tillfredsställa människans energibehov för ett år. Att hitta ett effektivare sätt att utnyttja och lagra solenergi skulle därför kunna lösa flera av de ovanstående problemen. Solceller är ett effektivt och direkt sätt att överföra solenergi, medan mikroorganismer med fotosyntetisk förmåga (ex. mikroalger och cyanobakterier) kan vara effektiva i detta hänseende genom att omvandla solenergi och koldioxid till kemisk energi.

Cyanobakterier är en grupp fotosyntetiska gramnegativa prokaryoter som har funnits på jorden i nästan 3 miljarder år. De är ursprunget till att det finns syre i atmosfären. *Synechocystis* är en encellig cyanobakteriestam som kan överleva i många olika miljöer. Bakterien upptäcktes och isolerades för första gången från en sjö i Kalifornien. DNA-sekvensen för *Synechocystis* hela genom är känd och det finns väl utvecklade metoder för att ändra i genomet.

Synechocystis används vid metabolisk genmodifiering i syfte att producera olika värdefulla föreningar såsom etylen, alkoholer, terpenoider, vätgas och fettsyror (artikel VI). I denna avhandling visas hur *Synechocystiskan* modifieras till att producera kemikalierna och biobränslena isobutanol och 1-butanol. Båda dessa ämnen är värdefulla ämnen som direkt kan användas som bränsle i dagens förbränningsmotorer, eftersom de har liknande energiinnehåll som bensin samt är stabilare och säkrare än etanol (ett av de huvudsakliga alternativa bränslena idag).

Varken isobutanol eller 1-butanol produceras naturligt av *Synechocystis*. Detta betyder att det inte finns några syntesvägar i bakteriens metaboliska system som leder till att dessa föreningar skapas. Därför har gener som kodar för de enzymer som skapar dessa metaboliska system valts ut från andra organismer. Dessa gener har sedan lagts in i *Synechocystis*, antingen i genomet eller som separata plasmider utanför genomet. För att introducera gener till olika ställen i en cell har speciella DNA-molekyler som kallas för vektorer (används som bärare för att på artificiell väg få in främmande genetisk material in till en cell) skapats (artikel I och II). Ett väldigt viktigt enzym, Kivd (a-ketoisovalerat dekarboxylas från *Lacotcocus lactis*) som är ett mellansteg i syntesvägen för isobutanol, kan använda olika substrat och producera olika ämnen. I artikel II producerades isobutanol och 3-metyl-1-butanol (3M1B) samtidigt när Kivd fördes in i *Synechocystis* och enzymet identifierades som en flaskhals i syntesvägen för isobutanol. Fyra separata snarlika enzymer som katalyserar sista steget i syntesvägen för isobutanol undersöktes också och det enzymet som finns i *Synechocystis* naturligt visade bäst resultat. I artikel III modifierades strukturen för Kivd-substratets bindningsplats för att förbättra produktionen av isobutanol. Detta genom att anpassa bindningsfickan till substratet för isobutanolproduktionen, som är betydligt mindre än sunbstratet för 3M1B-produktionen. Fler än 10 olika nya Kivd-enzymer designades och skapades. Några av stammarna med dessa nya Kivd-enzymer producerade en markant större mängd av både isobutanol och 3M1B än ursprungsstammen samt hade en signifikant ökad andel isobutanol jämfört med 3M1B. Det bäst modifierade Kivd-enzymet producerade mer än 3 gånger så mycket isobutanol jämfört med ursprungsenzymet. Eftersom produktionen av isobutanol fortfarande var låg, trots den bäst modifierade stammen, så kunde det finnas andra hinder för produktionen förutom Kivd. I artikel IV utfördes därför fler genetiska modifieringar och olika odlingsmiljöer för *Synechocystis* undersöktes. Resultatet visar att proteinnivån för Kivd påverkas avsevärt av den genetiska miljön i nära anslutning till den proteinkodande genen. En optimal odlingsmiljö identifierades och genom att applicera detta odlingsförhållande, artikel IV, producerades 911 mg L^{-1} kumulativ isobutanoltiter av den stam som producerade knappt 60 mg L^{-1} isobutanol artikel III. Liknande tillvägagångssätt för att påverka produktionen av isobutanol, genetisk modifiera samt optimera odlingsmiljön användes för produktionen av 1-butanol i *Synechocystis* och en kumulativ titer på 4.7 g L^{-1} uppmättes från den bäst modifierade stammen (artikel V).

Alla artiklarna i denna avhandling visar hur man kan använda genetisk modifiering, syntetisk biologi, proteinmodifiering och metabolisk optimering för att skapa nya modifierade stammar av *Synechocystis* och för att förbättra produktionen av värdefulla föreningar. Mer specifikt undersöker denna avhandling i detalj de enskillda enzymens funktion och bidrag i den introducerade syntesvägen. I avhandlingen visas också att produktionen av isobutanol och 1-butanol stegvis kan förbättras genom att använda av flera olika strategier.

Slutresultatet av dessa undersökningar, de kumulativa produktioner av isobutanol och 1-butanol i cynaobakterier som presenteras i denna avhandling, är de högsta uppmätta i litteraturen.

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