

Improved 1-octanol synthesis for biofuel production in *Synechocystis* sp. PCC 6803

Ian Sofian Yunus¹, Zhixuan Wang², Pachara Sattayawat¹, Jonathan Muller¹, Fessehay W. Zemichael², Klaus Hellgardt², and Patrik R. Jones^{1,*}

¹Department of Life Sciences, Imperial College London, SW7 2AZ, London, United Kingdom

²Department of Chemical Engineering, Imperial College London, SW7 2AZ, London, United Kingdom

*Correspondence to: p.jones@imperial.ac.uk

Abstract

1-Octanol has gained interest as a biofuel candidate to supplement or even substitute diesel fuel. By harnessing the power from sunlight and CO₂ as the carbon source, cyanobacteria has recently been engineered for renewable production of 1-octanol. The productivity, however, remained low. In the present work, we report efforts to further improve the 1-octanol productivity. By screening multiple thioesterases from different plant species, we successfully obtained several candidate thioesterases with improved activity and selectivity towards octanoyl-ACP. Through structure/function trials we learned that the current knowledge and/or state-of-the art computational tools to determine the most appropriate cleavage site for the chloroplast transit peptide of a thioesterases are insufficient to generate a functionally expressed thioesterase in *Synechocystis*. Additionally, by systematically tuning the inducer concentration and light intensity, we further improved the 1-octanol titer, reaching up to 35% carbon partitioning (AB% of the theoretical limit), a carbon accumulation rate of ED% (relative to biomass) and a titer of 526 ± 5.1 mg/L 1-octanol in 12 days. Long-term cultivation experiments demonstrated that the strain can be stably maintained for at least 30 days and/or over ten times serial dilution. Surprisingly, the optimized strain was genetically stable in contrast to earlier strains having lower productivity (and hence reduced chance of reaching toxic product concentrations). Altogether, optimized choice of enzymes and environmental conditions (e.g., inducer concentration and light intensity) substantially improved 1-octanol production. When cultured under continuous turbidostat conditions, the bioproduction system reached an accumulative titer of >3.5 g/L 1-octanol over close to 180 days.

1. Introduction

In scenarios to limit global warming to 1.5 degrees C, the world will need to reach net-zero CO₂ emissions on average by 2050 (The United Nation Intergovernmental Panel on Climate Change (IPCC), 2018). Many mitigation strategies are needed to meet this target. A key goal has to be the replacement of fossil fuels with alternative low-carbon fuels or alternative energy systems. Intensive research efforts have aimed to develop renewable and more sustainable fuel substitutes in the last few decades. Particularly attractive is the concept to utilise photosynthetic organisms, such as cyanobacteria, for the direct conversion of CO₂ into precursors or ready-to-use biofuels (Choi et al., 2020; Gao et al., 2016; Khan and Fu, 2020; Lu, 2010; Miao et al., 2020; Nozzi et al., 2013; Oliver et al., 2016; Quintana et al., 2011; Savakis and Hellingwerf, 2015; Sitther et al., 2020; Wang et al., 2020). Many of these engineered strains have achieved biocatalysis, i.e. channeling more than 50% of fixed carbon into product (e.g., 2,3-butanediol (60%; (Oliver et al., 2013)), 1-butanol (60%; (Liu et al., 2019))), demonstrating the potential of cyanobacteria as an engineerable chemicals production platform. To achieve financially sustainable fuel production using photosynthetic microorganisms still remains an unmet goal due to the present high cost of production (Tredici2016), and unfair subsidised competition from the fossils fuel industry (Perin2019). Many of these biofuel molecules, and derivatives thereof (Sattayawat2020) also have other uses that merit higher prices, hence providing an opportunity for a financially feasible start to commercialization and the beginnings of a cyanobacteria-based chemicals manufacturing industry.

Production of biofuel compounds, however, faces several challenges. Although compatible with current fuel infrastructure, ethanol serves as a rather poor gasoline substitute due to its hygroscopicity and low energy density. For these reasons, efforts have shifted toward longer carbon chain fuels, such as 1-butanol. 1-Butanol has higher energy density compared to ethanol. However, like ethanol, 1-butanol requires energy intensive separation steps due to its high solubility in water. To lower the product separation cost, synthetic pathways for bioproduction of insoluble biofuel compounds such as free fatty acids (Liu et al., 2011; Ruffing, 2014), hydrocarbons (Tan et al., 2011; Wang et al., 2013; Yunus et al., 2018), and 1-octanol (Yunus and Jones, 2018) have also been developed. Hydrocarbons and 1-octanol are attractive biofuel candidates as they do not require further chemical modification, unlike fatty acids which require esterification or hydrotreatment. However, attempts to produce hydrocarbons ($\geq C_{15}$) in engineered cyanobacteria have all resulted in intracellular accumulation of products (Yunus et al., 2018). In contrast, 1-octanol is spontaneously excreted from cyanobacteria into the liquid medium (Yunus and Jones, 2018), and combustion tests show that it can be used as a direct replacement of diesel fuel (Kremer et al., 2015).

There are at least four routes for 1-octanol biosynthesis (Akhtar et al., 2015; Dellomonaco et al., 2011; Machado et al., 2012; Marcheschi et al., 2012). Fig. 1A shows the schematic diagram of the 1-octanol synthetic pathway that relies on carboxylic acid reductase. In our previous work, we first implemented this pathway in *Synechocystis* and demonstrated that cyanobacteria can be genetically engineered to produce 1-octanol (Yunus and Jones, 2018). However, the productivity and carbon partitioning were relatively low, reaching 7 mg/L/day and 50 mg/g cell dry weight respectively. Additionally, the chosen thioesterase (Tes3) displayed broad specificity, resulting in the production of longer-chain

fatty alcohols (C10-OH to C18-OH). Protein quantification also indicated that the thioesterase was the limiting enzyme in the engineered pathway.

Herein, efforts to further improve the 1-octanol titer are described (Fig. 1B). These involved screening for superior thioesterases, introduction of native porins/transporters, and optimization of the cultivation system (e.g., light intensity and inducer concentration). Additionally, we also tested if the engineered strain could be stably maintained and cultured continuously.

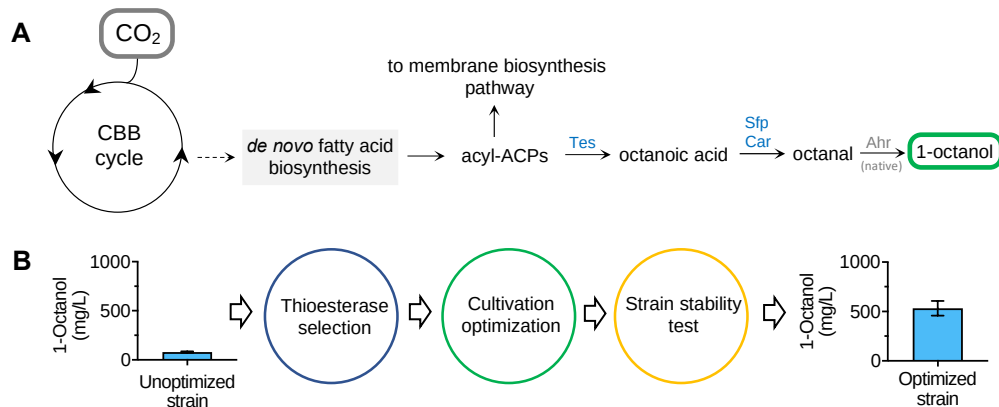


Figure 1. Strategies to improve 1-octanol production in cyanobacteria. (A) Schematic diagram of 1-octanol biosynthesis pathway implemented in this work. This pathway begins with *de novo* fatty acid biosynthesis which elongates acyl-ACPs, the building block of membrane lipids. A thioesterase (Tes) is required to terminate the elongation of acyl-ACPs. Consequently, the substrate specificity of the thioesterase is important to determine the carbon chain length of the final products (i.e., fatty alcohols). A thioesterase that is highly specific toward octanoyl-ACP may result in the high production of octanoic acid. A carboxylic acid reductase (Car), with its maturation factor (Sfp; phosphopantetheinyl transferase), then converts octanoic acid into octanal. Octanal is further reduced into 1-octanol by native aldehyde reductase(s) (Ahr). (B) Summary of efforts to optimize the production of 1-octanol.

2. Results and Discussion

2.1 Screening of C8-preferring acyl-acyl carrier protein thioesterases

Acyl-acyl carrier protein (acyl-ACP) thioesterase is an enzyme responsible for catalyzing the hydrolysis of a thioester bond that links an acyl chain to the sulfhydryl group of the phosphopantetheine prosthetic group of ACP. The acyl-ACP thioesterase terminates the acyl-ACP elongation process and plays an important role in determining the chain length of free fatty acid-derived products. For example, *E. coli* thioesterase 'tesA has broad specificity for C12-C18 acyl-ACP and has been used for fatty acid, fatty alcohol, fatty acid ethyl ester, and fatty alkane production in several microorganisms (Akhtar et al., 2013; Cho and Cronan, 1995; Liu et al., 2011; Xu et al., 2013; Zhou et al., 2016). FatB1 thioesterase from *Umbellularia californica* has preferential activity on lauryl-ACP (Voelker et al., 1992) and has been used for production of lauric acid (Work et al., 2015) and methyl laurate (Yunus et al., 2020) in cyanobacteria. Similarly, FatB3 from coconut plant *Cocos nucifera* L. which has a rather broad specificity of C12-C16 acyl-ACP has been expressed in *Arabidopsis* and altered the content of C12:0-C16:0 fatty acids (Yuan et al., 2017). Although a number of plant acyl-ACP thioesterases which are highly specific to C8-ACP have also been identified using *E. coli* (Jing et al., 2011), the outcome of plant-based thioesterase expression in a microbial host, however, has not always resulted in the desired metabolic phenotype. For example, attempts to produce C8:0

and C10:0 fatty acids in *Synechocystis* sp. PCC 6803 by expressing FatB2 thioesterase from *Cuphea hookeriana* resulted in a substantial increase in the accumulation of C12:0 fatty acid instead (Liu et al., 2011). Similarly, expressing a C12-preferring thioesterase from *Umbellularia californica* or a C14-preferring thioesterase from *Cinnamomum camphorum* in *Synechocystis* sp. PCC 6803 did not drastically change the free fatty acid composition (Liu et al., 2011). As many plant thioesterases are localized in the chloroplast, we speculated that the plant-based thioesterase might have not been functionally expressed in cyanobacteria when the chloroplast transit peptide was not truncated properly.

Three plant thioesterases which are known to show activities toward octanoyl-ACP, namely CaFatB3 from *Cuphea avigera* var. *pulcherrima*, ChoFatB2 from *Cuphea hookeriana*, and CpFatB1 from *Cuphea palustris* were selected to evaluate this hypothesis in *Synechocystis* sp. PCC 6803. All thioesterases encoding genes contain a putative chloroplast transit peptide in the N-terminal amino acid sequence. Previous studies have shown that the cleavage site of the chloroplast transit peptide might vary between plant thioesterases (Dehesh et al., 1996a, 1996b; Voelker et al., 1992). This complicates metabolic engineering dependent on thioesterases. To find a desirable method that can be used to determine the cleavage site of plant thioesterases, we evaluated different methods for prediction of truncation locations based on: (1) chloroplast transit peptide prediction method (ChloroP, <http://www.cbs.dtu.dk/services/ChloroP/>) (Emanuelsson et al., 1999), (2) identification of the putative plastid-targeting peptide cleavage site located at LPDW amino acid sequence (Jha et al., 2006; Kim et al., 2015; Moreno-Pérez et al., 2011; Sánchez-García et al., 2010), and (3) established knowledge from previously published articles (Dehesh et al., 1996a, 1996b; Jing et al., 2011; Voelker et al., 1992). In some cases, we also performed site-directed mutagenesis to modify the amino acid sequence according to Lozada et al., 2018. The list of all tested modifications are shown in Table 6.1. The resulting thioesterases were cloned in a broad-host range RSF1010 plasmid under a cobalt-inducible promoter PcoA (Peca et al., 2008) (Fig. 2A (i)) and expressed in *Synechocystis* sp. PCC 6803 strain lacking the acyl-ACP synthetase encoding gene (hereafter Δaas) (Kaczmarzyk and Fulda, 2010) for free fatty acid (FFA) production. For fatty alcohol production, the plasmids were transformed into the Δaas strain harboring integrated *sfp* and *car* in the DNA chromosome that had been constructed in a previous study (Sattayawat et al., 2020) (Fig. 2A (ii)).

Table 1. Details of thioesterase modification

Organism	Thioesterase	Modification		Truncation Reference
		The first amino acid residue after truncation [¶]	Changes in amino acid sequence	
<i>Cuphea palustris</i>	CpFatB1	-	-	Wild-type sequence
	'CpFatB1.1	A58	-	ChloroP [§]
	'CpFatB1.2	L90	-	LPDW cleavage site
	'CpFatB1.3	R120	-	Dehesh et al., 1996b
	'CpFatB1.4	F114	N122S, I169M	Lozada et al., 2018
<i>Cuphea avigera</i> var. <i>pulcherrima</i>	CaFatB3	-	-	Wild-type sequence
	'CaFatB3.0	K21	-	This work
	'CaFatB3.1	A58	-	ChloroP [§]
	'CaFatB3.2	L89	-	LPDW cleavage site
	'CaFatB3.3	R118	-	Following Dehesh et al., 1996b
	'CaFatB3.4	V102	-	ChloroP [§]
	'CaFatB3.5	H112	D120S, I157M	This work
<i>Cuphea hookeriana</i>	ChoFatB2	-	-	Wild-type sequence
	'ChoFatB2.1	A58	-	ChloroP [§]
	'ChoFatB2.2	L89	-	LPDW cleavage site
	'ChoFatB2.3	R118	-	Following Dehesh et al., 1996b

[¶] The first amino acid residue as compared to the wild-type sequence

[§] <http://www.cbs.dtu.dk/services/ChloroP/>

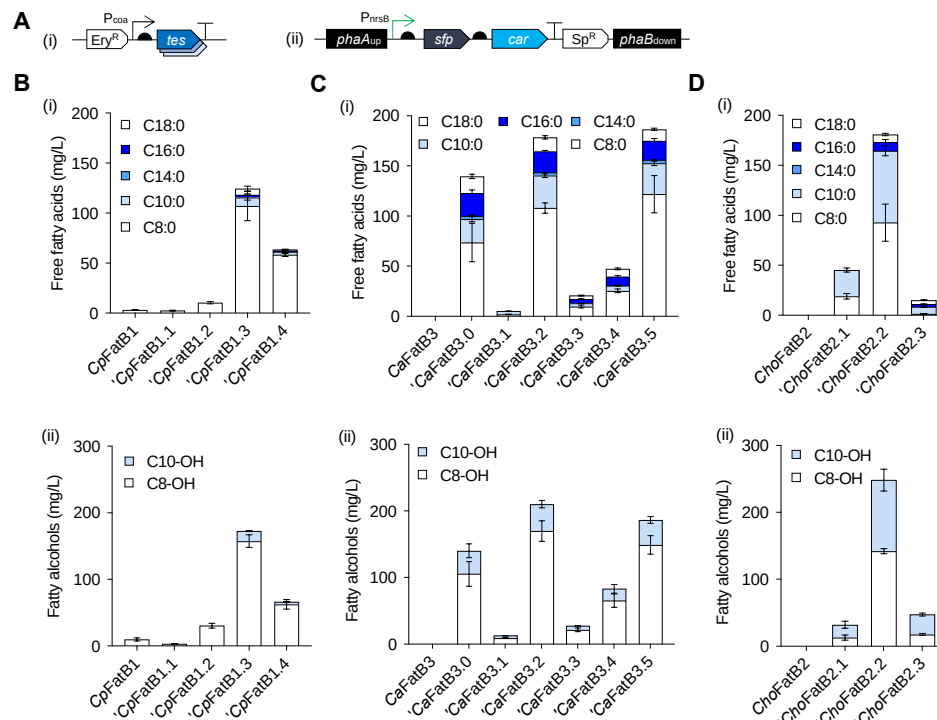


Figure 2. Expression of different variants of thioesterases for production of free fatty acids and fatty alcohols in *Synechocystis* sp. PCC 6803. (A) The thioesterase (*tes*) was cloned into a RSF1010 plasmid under a *P_{coa}* promoter (i) whilst a phosphopantetheinyl transferase (*sfp*) and a carboxylic acid reductase (*car*) were integrated into the *Synechocystis* genome (ii). (B) *CpFatB1* from *Cuphea palustris* (UniProt: Q39554). (C) *CaFatB3* from *Cuphea avigera* var. *pulcherrima* (UniProt: R4J2L6). (D) *ChoFatB2* from *Cuphea hookeriana* (UniProt: Q39514). For fatty acid analysis (Fig. 2B-D (i)), all samples were induced on day 2 with 5 μ M cobalt and free fatty acids were extracted on day 10 from the whole liquid culture by a hexane extraction method. For fatty alcohol analysis (Fig. 2B-D (ii)), all liquid cultures were overlaid with 30% isopropyl myristate and samples from the overlay were taken for analysis on day 10. Liquid cultures were induced on day 2 with 5 μ M cobalt to induce the expression of the thioesterase and/or 15 μ M nickel to induce the expression of *Sfp* and *Car*. Error bars represent standard deviations of three biological replicates

FatB1 from *Cuphea palustris* (*CpFatB1*) is arguably one of the most well-studied C8-preferring thioesterases, especially when compared to the other two thioesterases. We, therefore, began the study by truncating the chloroplast transit peptide of *CpFatB1*. *'CpFatB1.3*, truncated according to Dehesh et al. (1996), showed the highest free fatty acid titer, reaching 106 ± 13 mg/L octanoic acid 8 days after induction (Fig. 2B (ii)). This was almost a two-fold increase in the octanoic acid titer compared to *Tes3*, the thioesterase we used previously (Yunus and Jones, 2018). Additionally, octanoic acid accounted for more than 90% of the total FFAs with *'CpFatB1.3*, whereas the C8 chain-length represented only approximately 40% of the total FFAs synthesised by *Tes3*-expressing cells. The truncation of the predicted cleavage site given by the ChloroP prediction tool, however, failed to improve the titer (*'CpFatB1.1*; Fig. 2B (i,ii)). Similarly, cleaving the thioesterase at the LPDW site did not significantly improve the titer (*'CpFatB1.2*; Fig. 2B(i,ii)). It must be pointed out, however, that we did not confirm whether any of the truncations resulted in variation or a loss of protein synthesis. *'CpFatB1.4*, which was truncated by following a study by Lozada and colleagues (Lozada et al., 2018), yielded the highest C8-specificity (>95%), albeit with lower

titer compared to 'CpFatB1.3. It was noticeable that under 5 μ M cobalt induction, the strain carrying 'CpFatB1.4 grew poorly compared to the other strains (Supplementary Fig. 1B). This was in agreement with what had been observed in previous studies when excessive inducer concentration was used (Sattayawat et al., 2020) or when the thioesterase was expressed in a higher copy number plasmid (Lozada et al., 2018).

Another plant known to accumulate medium chain free fatty acids is *Cuphea avigera* var. *pulcherrima*. The seeds from *Cuphea avigera* var. *pulcherrima* plant accumulate >90% of C8:0 and C10:0 fatty acids (Graham and Kleiman, 1992). It has also been revealed that *CaFatB3* cDNA is the predominantly expressed thioesterase in the *Cuphea avigera* var. *pulcherrima* seeds (Kim et al., 2015). Additionally, heterologous expression of *CaFatB3* in *Camelina sativa* and *Arabidopsis thaliana* resulted in the accumulation of C8:0 and C10:0 fatty acids in *Camelina* oil and in *Arabidopsis* seeds (Kim et al., 2015; Tjellström et al., 2013). Heterologous expression of *CaFatB3* in *E. coli* has been demonstrated (Sattayawat et al., 2020), however the composition of C8:0 in comparison to other FFAs was unclear. Similarly to 'CpFatB1.1, which was truncated according to ChloroP prediction tool, expressing 'CaFatB3.1 and 'CaFatB3.4 (Supplementary Fig. 2A) in *Synechocystis* resulted in a low titer of free fatty acids and fatty alcohols (Fig. 2C (i,ii)). In contrast with 'CpFatB1.2 (Fig. 2B (i,ii)), truncating *CaFatB3* thioesterase at the LDPW site ('CaFatB3.2) resulted in a higher titer of free fatty acids and fatty alcohols, reaching 106 mg/L (Fig. 2C (i)) and 163 mg/L (Fig. 2C (ii)) in 10 days, respectively. Surprisingly, truncating *CaFatB3* at the same cleavage site as 'CpFatB1.3 drastically reduced the fatty acid and fatty alcohol titer (compare 'CpFatB1.3; Fig. 2B and 'CaFatB3.3; Fig. 2C). To understand whether a similar modification of *CaFatB3* like that of 'CpFatB1.4 (Fig. 2B) would result in a similar outcome (i.e., a growth defect upon 5 μ M cobalt induction), we also created 'CaFatB3.5. Interestingly, the strain did not show any growth defect when induced with 5 μ M cobalt (Supplementary Fig. 2B) and the free fatty acid (Fig. 2C (i)) and fatty alcohol (Fig. 2C (ii)) titer were relatively comparable with the other thioesterases. These results suggest that the optimum truncation of one thioesterase might not be applicable to other plant thioesterases.

Whilst *Cuphea hookeriana* seeds accumulate 50% of C8:0 and 20% of C10:0 (Graham and Kleiman, 1992), the expression of its *FatB2* thioesterase (designated as *ChoFatB2*) in several genetically engineered plants or microbes resulted in various outcomes. For example, *Brassica napus* plant transformed with *ChoFatB2* yielded 11% of C8:0 and 27% of C10:0 fatty acids in seeds (Dehesh et al., 1996b). Expression of *ChoFatB2* in *E. coli* resulted in 50% C16, 25% C18, 15% C8, and 10% C10 fatty acid ethyl esters (Steen et al., 2010). Cyanobacteria expressing *ChoFatB2* unexpectedly produced 20.5% C12:0 and 2.5% C10:0 fatty acids. (Liu et al., 2011). Recently, *ChoFatB2* was also expressed in engineered *E. coli* and the strain produced 85% C8:0 fatty acid (Feng et al., 2018). A single residue mutation at amino acid residue number 198, from isoleucine to glutamic acid (I198E), shifted the enzyme substrate preference to C10:0, resulting in the production of 57.6% C10:0 fatty acid (Feng et al., 2018). Here, similar to the previous two thioesterases, *ChoFatB2* was truncated at different locations (Supplementary Fig. 3A) and tested for the production of free fatty acids and fatty alcohols. The expression of 'ChoFatB2.1 increased the titer of both C8 and C10 fatty acids (Fig. 2D (i)) and fatty alcohols (Fig. 2D (ii)) compared to the wild-type thioesterase (*ChoFatB2*), with 46% C8-OH and 54% C10-OH products. Truncating the thioesterase at the LPDW motif not only resulted in the highest fatty acid titer (181 mg/L) and fatty alcohol titer so far (250 mg/L) but also shifted the product chain length to 63% C8-OH and 37% C10-OH ('ChoFatB2.2; Fig. 2D (ii)). This came at expense of biomass accumulation, however (Supplementary Fig. 3B). Lastly,

the truncation of chloroplast transit peptide like the truncation of 'CpFatB1.3, forming 'ChoFatB2.3 thioesterase, did not further improve the product titer (Fig. 2D).

The expression of thioesterases in Δaas and Δaas -P_{nrsB}-*sfp-car* resulted in the production of different compositions of free fatty acids and fatty alcohols. Truncation of the thioesterase at the LPDW motif was successful in 'CaFatB3.2 (Fig. 2C) and 'ChoFatB2.2 (Fig. 2D), however a similar approach failed to yield a desired result in 'CpFatB1.2 (Fig. 2B). Similarly, truncation of 'CpFatB1.3 at the RPNM sequence according to Dehesh et al., 1996b was successful in 'CpFatB1.3 (Fig. 2B), however it was not successful in 'CaFatB3.3 (Fig. 2C) and 'ChoFatB3.3 (Fig. 2D). Finally, additional amino acid mutations on the 'CaFatB3.5 (Fig. 2D) did not further improve its performance as well as 'CpFatB1.4 (Fig. 2B). Altogether, it could be concluded that the truncation of plant thioesterases is crucial for their functionality in *Synechocystis*. The only question is where to make the truncation? Although ChloroP has been commonly used to predict the chloroplast transit peptides and their cleavage sites of proteins in rice, barley, Arabidopsis, and several eukaryotic algae such as *Chlamydomonas* (Lee et al., 2019; Lupette et al., 2019; Wanniarachchi et al., 2018), this prediction tool failed to create a functionally expressed thioesterase, at least in three different tested thioesterases. This study highlights that improvements in the algorithms underlying truncation site predictions would be desirable to more accurately inform where optimal cleavage sites of proteins should be made.

2.2 Tuning the 'CpFatB1.4 protein expression level improves 1-octanol titer

On the basis of the previous results shown in Figure 2, we identified that 'CpFatB1.3 and 'CpFatB1.4 were the two thioesterases showing the highest specificity for 1-octanol production (Fig. 2B (ii)). We also noticed the strain carrying 'CpFatB1.4 grew poorly compared to the other strains, both in fatty acid- and fatty alcohol-producing strains (Supplementary Fig. 1B). The observed growth defect might be the reason for the low product titer achieved from this strain and 5 μ M of cobalt was probably not the optimum inducer concentration for the strain carrying 'CpFatB1.4. To understand this better, the strain carrying 'CpFatB1.4, were subsequently induced with different concentrations of cobalt inducer (0 – 5000 nM). Induction of 'CpFatB1.4 strain with lower cobalt concentration resulted in increased production of 1-octanol (Fig. 3A). The highest 1-octanol titer (241 ± 13.7 mg/L) and yield (201 ± 8.3 mg/g biomass) were achieved from the strain expressing 'CpFatB1.4 induced with 625 nM of cobalt (Fig. 3A and B). The titer, yield, and cell density, however, decreased whenever the strain was induced with a higher cobalt concentration (Fig. 3C). Many metabolic bottlenecks are still likely to persist but several potential physiological issues may also hinder further improvement. Some of the latter include: (1) product toxicity caused by poor 1-octanol transport from cells to the liquid medium, (2) metabolic burden caused by excessive carbon partitioning to 1-octanol, and (3) insufficient acyl-ACP supply for biosynthesis of carbon skeletons required for building a well-functioning cell membrane. To test these hypotheses, two further experiments were carried out. In the first experiment, the potential native *Synechocystis* porins/transporters with the highest homology to the well-established *E. coli* AcrA, AcrB, and TolC proteins were identified and co-expressed with the 1-octanol synthetic pathway to promote product excretion and reduce product toxicity. In the second experiment, the 1-octanol pathway was introduced into the *Synechocystis* wild-type strain to avoid excessive carbon distribution to 1-octanol product as a result of cells inability to recycle free fatty acids back to acyl-ACPs in the Δaas strain.

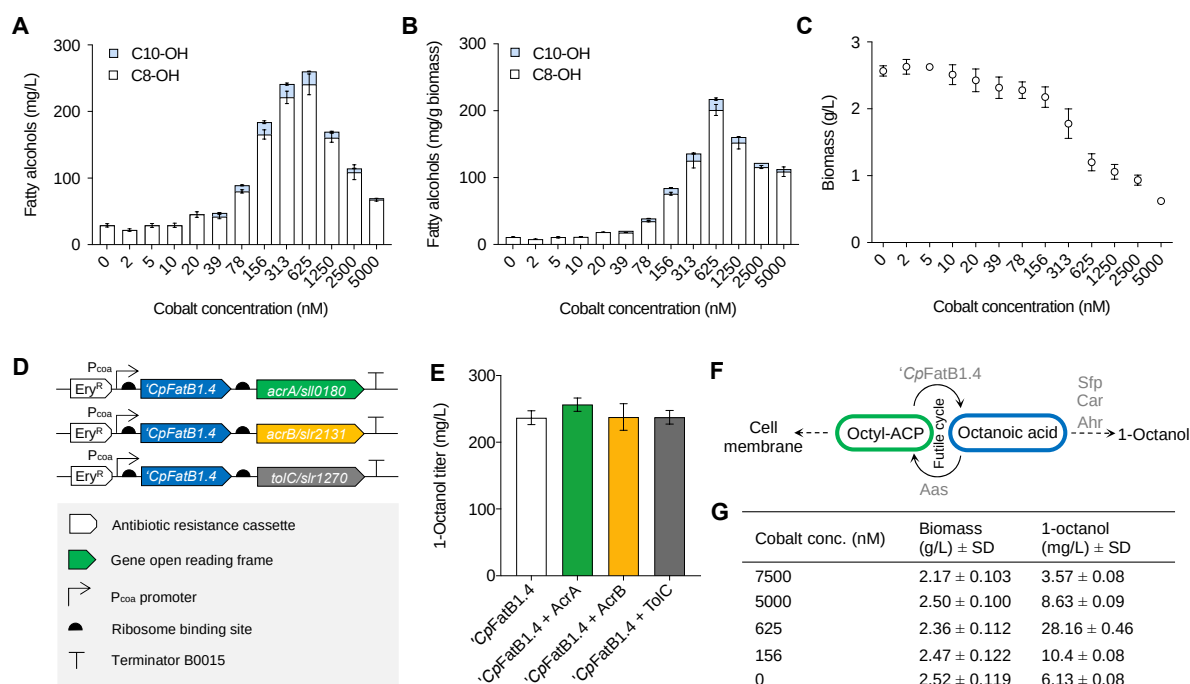


Figure 3. Tuning the expression level of 'CpFatB1.4 to improve the 1-octanol production. (A) Fatty alcohol titer, (B) yield, and (C) growth profile of the strain expressing 'CpFatb1.4 under different concentrations of cobalt (0 – 5000 nM). (D) Schematic diagram of plasmids expressing native *Synechocystis* transporters. (E) 1-Octanol titer obtained from strains expressing plasmids shown in Fig. 3D in comparison with a control experiment ('CpFatB1.4 only). (F) Futile cycle conversion of octanoyl-ACP to and from octanoic acid by 'CpFatB1.4 and Aas. (G) Biomass accumulation (g/L) and 1-octanol titer obtained from expression of 'CpFatB1.4 in the wild-type strain carrying Sfp and Car under different concentrations of cobalt. All liquid cultures were overlaid with 30% isopropyl myristate and samples from the overlay were taken for analysis using GC-MS on day 10. Liquid cultures were induced on day 2 with 5 μ M cobalt (Fig. 3E) or different concentration of cobalt (Fig. 3F) to induce the expression of the thioesterase, and 15 μ M nickel to induce the expression of Sfp and Car. Error bars represent standard deviations of two to three biological replicates.

Expression of resistance-nodulation-cell (RND) efflux pumps such as AcrAB-TolC and MdlB has been shown to improve *E. coli* tolerance toward limonene and 3-methyl-2-buten-1-ol (Dunlop et al., 2011; Foo et al., 2014). In addition, alleviating growth inhibition in some cases also results in increased production of n-butanol, limonene, and isopentenol (Mukhopadhyay, 2015). To identify the acting transporters of 1-octanol in cyanobacteria, the disruption of several putative porins/transporters was attempted in *Synechocystis* sp. PCC 6803 lacking an acyl-ACP synthetase gene (Δaas strain). Several genes such as *slr0180*, *slr2131*, and *slr1270* were identified as the closest homologous proteins to *E. coli* AcrA, AcrB, and TolC (Kato et al., 2015) which have been identified as the transporters responsible for 1-octanol efflux in this heterotroph (Akhtar et al., 2015; unpublished data). Suicide vectors to disrupt the genes with a gentamicin or spectinomycin selection marker cassette were created. Transformation with the plasmids resulted in several viable colonies. Upon increasing the antibiotic concentration to 20 mg/L, however, the colonies did not survive and after several rounds of restreaking on BG11-Co agar plates containing 10 mg/L antibiotic, it was not possible to isolate a fully segregated mutant. It has been reported in a previous study that knocking out the *tolC* gene in *Synechocystis* increases cells susceptibility to selected antibiotics (Oliveira et al., 2016). A possible explanation for the failure to knock out *tolC* in this study is that the Δaas mutation results in the accumulation of free fatty acids which also likely are exported by the same transporter complex and that this was lethal to the cells.

313 Recently, Bellefleur and co-workers also reported the characterization of a free fatty acid
314 active transportation mechanism in *Synechocystis*. Curiously, they were able to isolate and
315 characterize a null mutant of Δaas lacking *acrA* or *acrB* gene (Bellefleur et al., 2019).

316 Given the failure in obtaining a fully segregated and viable mutant of the transporter
317 encoding genes mentioned above, a reverse strategy was instead tried. Three RSF1010
318 plasmids encoding either *AcrA*, *AcrB*, or *TolC* (from *Synechocystis*), in all cases combined with
319 '*CpFatB1.4*, were used to transform the Δaas -Sfp-CAR strain (Fig. 3D). The resulting strains
320 were induced on day 2 with 625 nM cobalt and 15 μ M nickel, and cultured with an isopropyl
321 myristate solvent overlay for 8 days and the overlay was sampled for GC-MS analysis on day
322 10. Fig. 3E shows the 1-octanol titer obtained from the samples. If *Synechocystis* *AcrAB-TolC*
323 was involved in 1-octanol efflux, the over-expression of only one of its members could be
324 expected to influence this process either positively or negatively. None of the strains differed
325 in the titer of 1-octanol compared to the control strain ('*CpFatB1.4*) (Fig. 3E) suggesting that
326 native *AcrA*, *AcrB*, and *TolC* possibly have no role to play in fatty alcohol excretion in
327 *Synechocystis* sp. PCC 6803, or that expression of the protein was not sufficient. It is also
328 possible that 1-octanol excretion to the liquid medium in *Synechocystis* occurs by passive
329 diffusion rather than active transport (i.e., does not requires transporters). In contrast,
330 Bellefleur and co-workers reported that expression of *E. coli* *AcrB* in their free fatty acid-
331 producing strain significantly increased the titer of extracellular free fatty acid and rescued
332 the sensitivity of the strain to chloramphenicol (Bellefleur et al., 2019), suggesting that efflux
333 of both fatty acids and chloramphenicol in cyanobacteria could be promoted by a membrane
334 transporter.

335 Previous studies show that acyl-ACP synthetase (*aas*) is active on 8-bromooctanoic
336 acid or 8-phenyloctanoic acid (Beld et al., 2014). Therefore, it is also possible that *aas* is able
337 to convert octanoic acid produced by '*CpFatB1.4* back into octanoyl-ACP as a building block
338 for cell membrane biosynthesis. Although this would result in a futile cycle (Fig. 3F), it may
339 help to alleviate some of the stress caused by excessive carbon re-distribution of octanoyl-
340 ACP into 1-octanol. To test this hypothesis, we also introduced the 1-octanol synthetic
341 pathway into the *Synechocystis* sp. PCC 6803 wild-type strain and compared it with the
342 equivalent strain in the Δaas background. The *sfp* and *car* genes were integrated into the
343 chromosome as described previously (Fig. 2A (ii)) (Sattayawat et al., 2020), whilst '*CpFatB1.4*
344 was introduced in the RSF1010 plasmid with a *Pcoa* promoter (Fig. 2A (i)). Fig. 2G shows that
345 induction of the liquid cultures above 5000 nM had a slight effect on growth, whereas
346 induction below 5000 nM did not cause a growth defect. Interestingly, no 1-decanol was
347 detected from the overlay, although it was observed in the Δaas strain. Six hundred and
348 twenty-five nM cobalt still gave the highest 1-octanol titer (28.16 mg/L), consistent with the
349 previous results obtained in the Δaas deletion strain (Fig. 2A). However, the highest titer was
350 almost 10 times lower than the 1-octanol titer achieved from the Δaas -Sfp-Car-'*CpFatB1.4*
351 strain. This indicated that a futile cycle had most likely formed with both acyl-ACP synthetase
352 and the carboxylic acid reductase competing for octanoic acid. Since only <30 mg/L 1-octanol
353 was achieved from the wild-type strain expressing '*CpFatB1.4*, *sfp*, and *car*, implementing the
354 1-octanol pathway in the wild-type strain was not a satisfactory option to explore further.
355 However, evidence supporting the initial hypothesis (i.e., the reason for the negative growth
356 caused by high induction of '*CpFatB1.4* was mainly due to excessive removal of octanoyl-ACP
357 from native FAS required for growth) was obtained.

2.3 Optimization of cultivation condition improves cell growth, titer, and carbon partitioning of 1-octanol accordingly

As the induction of the wild-type strain carrying the 1-octanol pathway above the optimum inducer concentration (e.g., 625 – 5000 nM) did not cause metabolic stress (i.e., growth defect) and co-expression of the native porins/transporters did not improve cell growth or product titer, this suggested that the growth defect shown in Fig. 3C was caused by insufficient supply of acyl-ACP for cell membrane biosynthesis rather than product toxicity. At a certain induction level, the introduction of the improved 1-octanol synthetic pathway likely resulted in a limitation in available cellular resources (primarily carbon source) for growth, thus imposing a cellular metabolic burden. To find a remedy for this issue, one possibility is to provide a greater carbon flux to fatty acid metabolism overall or to better optimize the balance of resources between the introduced pathway and metabolism required for growth. Some form of dynamic sensor as reported for *E. coli* (Xu et al., 2014) could be useful if such a system would work in cyanobacteria. An alternative to this concept is to identify conditions that would allow metabolism to continue with no or minimal growth, i.e., a quiescent state of growth. Unfortunately, to date, there has been no study that we are aware of that examine these possibilities in cyanobacteria.

Light also plays an important role in controlling cyanobacterial growth (Cuellar-Bermudez et al., 2015; lasimone et al., 2018; Jahn et al., 2018). As cyanobacteria harvest energy from light (e.g., sunlight) to convert carbon dioxide into organic carbon molecules (e.g. acetyl-CoA) thereafter used as precursor for the fatty acid biosynthesis pathway, one mechanism to boost the carbon flux to fatty acid metabolism may be to enhance the input of energy and force the cell to dissipate it into other end-products other than biomass (i.e., excreted carbon products) by increasing the inducer concentration. To increase carbon flux to the fatty acid metabolism pathway, the improved 1-octanol strain was cultivated at a higher light intensity. At first, the 1-octanol titer was compared between liquid cultures grown in two different light conditions (Fig. 4A, Condition 1 and 2). Both liquid cultures were grown at 60 $\mu\text{mol photons/m}^2\cdot\text{s}$ for 48 hours, following induction with 625 nM cobalt. After 48 hours, one of the liquid cultures was cultivated at 120 $\mu\text{mol photons/m}^2\cdot\text{s}$, whilst the other remained at 60 $\mu\text{mol photons/m}^2\cdot\text{s}$. The liquid cultures grown under higher light condition (120 $\mu\text{mol photons/m}^2\cdot\text{s}$) produced a significantly higher 1-octanol titer (Fig. 4B). However, the strain also accumulated higher biomass (Fig. 4C), leading to a relatively lower carbon partitioning of 1-octanol per g cell dry weight (Fig. 4D). Compared to the strain grown under Condition 1, the biomass of strains grown under Condition 2 increased by nearly 320 mg/L, whilst the 1-octanol titer only increased by 71 mg/L (compare Condition 1 and Condition 2 in Fig. 4B and C). This suggests that more carbon flux was diverted to cell membrane biosynthesis at increased light intensities rather than to 1-octanol synthesis and an adjustment of cobalt inducer concentration was needed.

To re-balance the carbon partitioning of 1-octanol per g CDW of biomass, the concentration of cobalt inducer was therefore re-evaluated. Under the revised environmental conditions, the highest titer (526 ± 5.1 mg/L 1-octanol and 54 ± 2.7 mg/L 1-decanol) and carbon partitioning (336 ± 9.2 mg/g CDW or 33.6% carbon partitioning) of 1-octanol was achieved when the culture was induced with 625 nM cobalt repetitively on day 2, 3, and 4 (Condition 4; Fig. 4D). When induced with additional 625 nM on day 5 and/or day 6, however, the strains accumulated lower overall fatty alcohols titer (Condition 5 and 6; Fig. 4B). Ten days after induction, these strains (i.e., strain grown under Condition 5 and 6) turned pale blue,

indicating toxicity from 1-octanol. A similar phenotype had also been observed when cyanobacterial strains were fed with 1-octanol in the absence of overlay (Yunus and Jones, 2018). Nevertheless, these experiments illustrated that 1-octanol production could be improved by optimizing the cultivation condition (e.g., higher light intensity and higher inducer concentration). However, a more elaborate cultivation system and metabolic engineering strategy would be needed in order to achieve strains with greater culture stability and consequently higher 1-octanol titer and carbon partitioning. Overall, these simple but powerful experiments highlight the complexity of the systems involved and that exploitation of the full potential may not be reached without combinatorial optimization of many parameters. It does, however, illustrate the flexibility of the system with promise of substantial improvement as a reward.

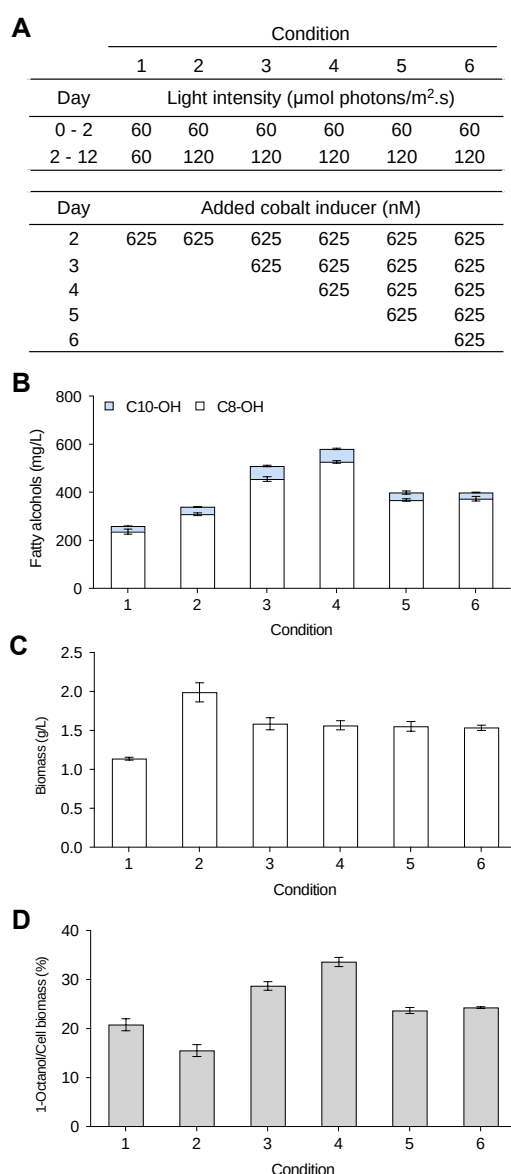


Figure 4. Re-evaluation of inducer concentration together with optimizing light intensity for 1-octanol production of strain $\Delta\text{aas-Sfp-Car-Pcoa-CpFatB1.4}$. (A) Different conditions used to cultivate the strain. The fatty alcohol titer (B), biomass accumulation (C), and carbon partitioning (D) achieved from the strains grown under different conditions. Overlay was sampled on day 12 and analyzed by GC-MS. Error bars represent standard deviations of three biological replicates.

2.4 1-Octanol producing strain displays stability and robustness upon environmental perturbations in long-term cultivation

Our previous study indicated that a serial dilution of the Δaas -Pcoa-4-TPC3 (*tes3*, *sfp*, and *car*) strain displayed reproducible genetic instability characterized by complete loss of metabolic phenotype and random mutations in the *tes3* gene (Yunus and Jones, 2018). Further evaluation of the strains developed in this study (Fig. 5A) showed that the ability to produce 1-octanol was not always lost. For example, strain Δaas -Sfp-Car-*'CpFatB1.4* could be stably maintained for at least ten serial dilutions. In comparison to the first sample, the 1-octanol produced by the subsequent samples ranged from 73% to 111% of 1-octanol titer of the first sample (Fig. 5B). The fluctuation in the 1-octanol titer might be attributed to the variations in the growth profile (Fig. 5B). To understand if the 1-octanol strains developed in this study could also be stably maintained in a semi fed-batch experiment, we cultivated several strains over 30 days with medium replenished twice, on day 10 and 20 (Fig. 5C). Fig. 5D shows that the Δaas -Sfp-Car strain expressing *'CaFatB1.0*, *'CpFatB1.3*, and *'CpFatB1.4*, could still maintain the production of 1-octanol over at least two rounds of serial dilution during a 30-day cultivation period although there was a slight decrease in productivity following the second dilution (Fig. 5D). The highest titer was 743.08 ± 53.82 mg/L 1-octanol and 56.75 ± 2.67 mg/L 1-decanol in 30 days from the strain expressing *'CpFatB1.4* (Fig. 5B). Altogether, these experiments demonstrated that the potential of the 1-octanol producing strains had not yet reached its limit. However, upon long term cultivation, the productivity decreased, presumably due to the long contact of 1-octanol with cyanobacterial cells. This issue could be partially solved by converting 1-octanol into its respective ester, octyl acetate, which has been shown to be less toxic towards cyanobacteria than 1-octanol as described in our previous study (Sattayawat et al., 2020). HERE SOMEWHERE WE NEED TO COMMENT ON THE GENETIC STABILITY - I think this is one of the more interesting observations in this paper - i.e. why are these strains more stable than the first ones? We need to at least speculate on this.

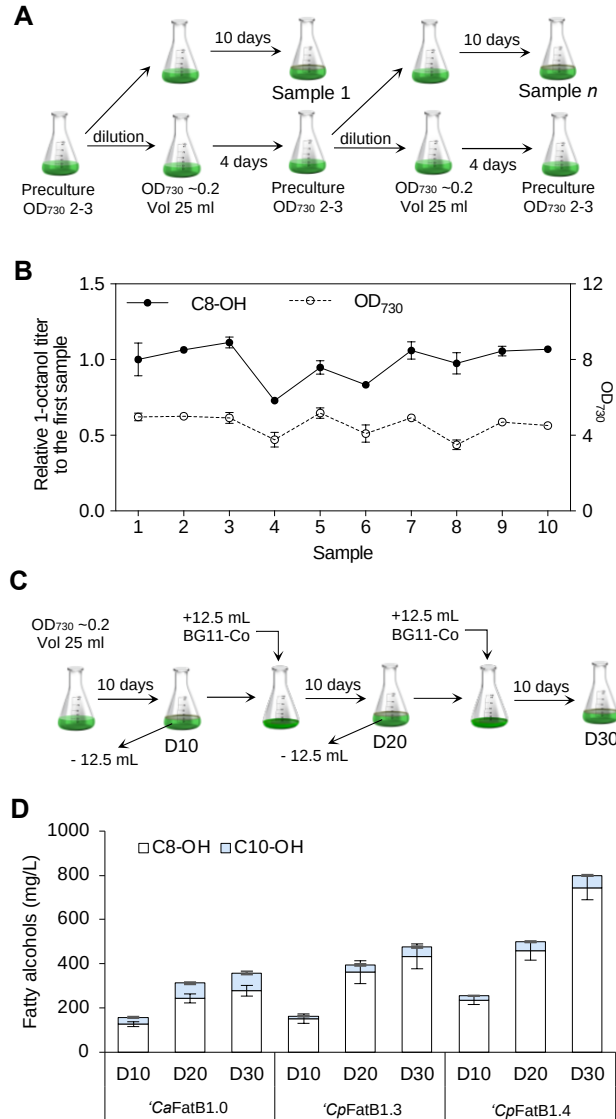


Figure 5. Evaluation of strain stability over long cultivation period. (A) Schematic cultivation diagram of Δaas -Sfp-Car-'CpFatB1.4 strain over ten serial dilutions. Preculture with OD₇₃₀ 2-3 was prepared to inoculate liquid culture with a starting OD₇₃₀ ~0.2 (25 ml). One set of liquid cultures was incubated for ten days (induced on day two) for 1-octanol production experiment, whilst the other was incubated for four days and used for the preculture of production experiment of sample *n*. The experiment was repeated until Sample No. 10 was obtained. (B) Cell density (OD₇₃₀) and relative 1-octanol titer of sample *n* compared to the first sample measured on day ten. (C) Schematic cultivation diagram of strains shown in Figure 5D. Liquid culture (25 ml) with initial OD₇₃₀ 0.2 was prepared. On day two, the cultures were induced and incubated for eight days. On day 10, solvent overlay was sampled for GC-MS analysis and 12.5 ml of liquid culture was replaced with fresh BG11-Co medium followed by induction with half concentration of inducers added on day two. This experiment was continued until day 30 (D30). (D) Fatty alcohols profile obtained from day 10 (D10), day 20 (D20), and day 30 (D30) from different strains. For the strains carrying 'CaFatB1.0 or 'CpFatB1.3, the liquid cultures were re-induced with 2.5 μ M cobalt and 7.5 μ M nickel. For the strains carrying 'CpFatB1.4, liquid cultures were re-induced with 312.5 nM cobalt and 7.5 μ M nickel. Samples from the overlay were taken on day 10, day 20, and day 30 (D30). Error bars represent standard deviations of three biological replicates.

To investigate the robustness of the 1-octanol producing strain upon environmental perturbations (e.g., pH, light intensity, and feed flow rate), we also cultivated the Δaas -Sfp-Car-'CpFatB1.4 strain in a flat plate photobioreactor under batch (Fig. 6A), chemostat (Fig. 6B), and turbidostat (Fig. 6C) mode. Figure 6D and 6E show the growth profile and 1-octanol

productivity of the 1-octanol producing strain, respectively, upon cultivation in the photobioreactor for 165 days. Initially, the liquid culture (700 ml) was maintained in a batch mode and bubbled with air under continuous and constant light intensity (50 μ E). On day two, liquid culture was induced with 625 nM cobalt and 15 μ M nickel and overlaid with 200 ml hexadecane. After the strain could be stably maintained for XXX~14 days[Athena please confirm the number of days here] reaching 1-octanol productivity of approximately 20 mg/L/day, the batch mode was switched to a chemostat mode where 0.2 ml/min fresh medium was pumped in to the photobioreactor whilst other parameters were kept constant (Fig. 6B). To maintain the same volume of liquid culture in the photobioreactor, 0.2 ml/min liquid culture was also removed from the photobioreactor to the effluent collection container. The cell biomass was kept constant until day 66. To understand the effect of pH on 1-octanol production, we perturbed the pH value by pumping in 1-5% CO₂/Ar. The pH was altered from 10 to 9.6-9.8, which resulted in an increase of both biomass and 1-octanol production. However, when the pH reached 9.4, we observed a reduction in the 1-octanol productivity as well as in the cell biomass accumulation. The productivity was recovered when the pH was adjusted back to 9.6. To understand the effect of feed/effluent flow rate and light intensity on 1-octanol productivity, we varied the flowrate (0.24 – 0.27 ml/min) and also light intensity (35 – 45 μ E). Upon applying a higher flow rate (0.27 ml/min; day 113), both cell biomass and 1-octanol productivity were significantly reduced. Likewise, we also observed a lower 1-octanol productivity as well as cell biomass when the light intensity was lowered from 50 μ E to 35 μ E (day 125 – 130). This implies that the 1-octanol productivity was largely influenced by the amount of cell biomass in the liquid culture. Nevertheless, 1-octanol productivity could be recovered when the flow rate or light intensity was re-adjusted.

To achieve higher biomass accumulation in the liquid culture, we then operated the photobioreactor in a turbidostat mode. An additional recirculation loop was added to the photobioreactor setup, involving pumping the culture through a cross flow filtration membrane (hollow fiber membrane). This membrane retained the cells within the photobioreactor, with only filtered medium going to the effluent reservoir. Since the biomass was retained in the PBR, we observed an increase in the biomass accumulation (1.5 g/L; Fig. 6D) and 1-octanol productivity (~70 mg/L/day; Fig. 6E). To supply more nutrient to the photobioreactor, we subsequently increased the feed flow rate (0.27 – 0.3 ml/min). This turbidostat operation lasted for 10 days and it demonstrated almost three times improvement in 1-octanol productivity compared to the chemostat mode. It is likely that applying a higher flow rate and light intensity to the turbidostat system might further enhance the 1-octanol productivity.

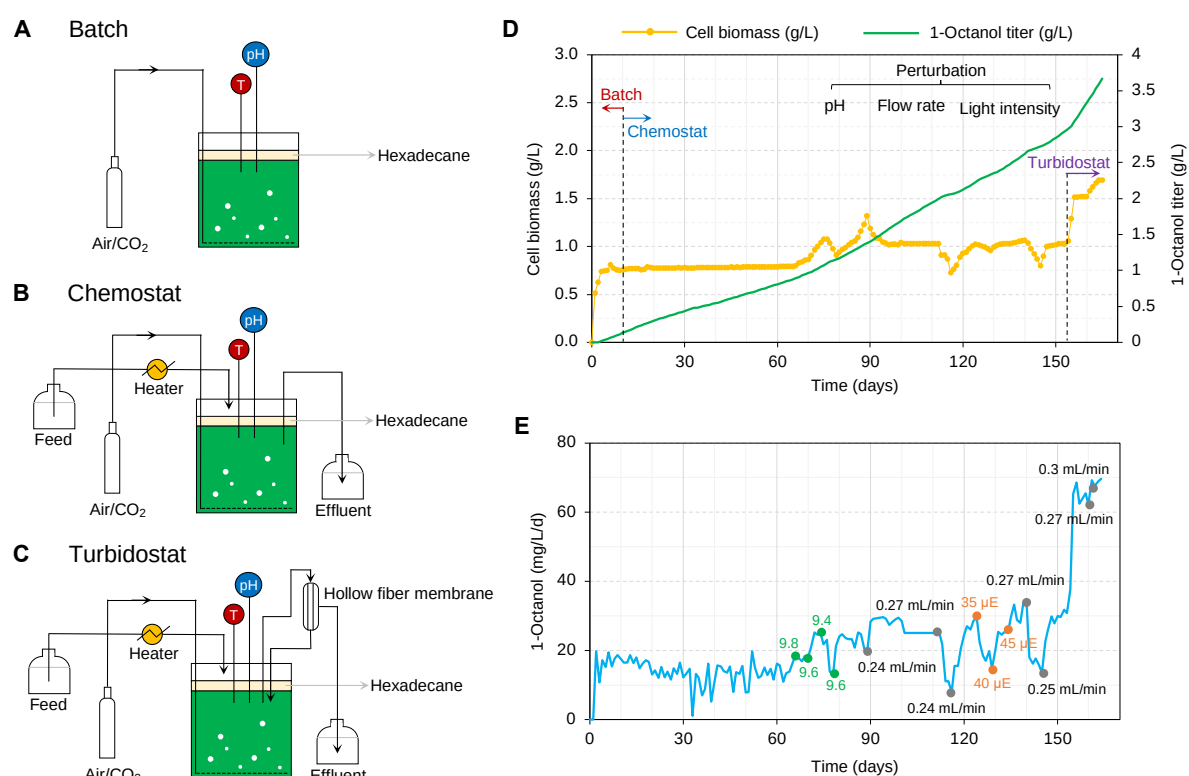


Figure 6. Evaluation of Δ as-Sfp-Car-'CpFatB1.4 strain in a flat plate photobioreactor under (A) batch, (B) chemostat, (C) turbidostat mode. (D) Effect of different mode of operation, pH, flow rate, and light intensity on the accumulation of biomass and 1-octanol. (E) 1-octanol productivity profile of the engineered strain for over 165 days.

3. Conclusion

In this work, three plant thioesterases which are known to show activities toward octanoyl-ACP, namely *CaFatB3* from *Cuphea avigera* var. *pulcherrima*, *ChoFatB2* from *Cuphea hookeriana*, and *CpFatB1* from *Cuphea palustris* were screened and modified to improve the 1-octanol production in the engineered *Synechocystis* sp. PCC 6803 strain. It was demonstrated that the truncation of the thioesterases is crucial for their functionality. Approximately 10-fold improvement in the 1-octanol titer was achieved by expressing 'CpFatB1.4 from *Cuphea palustris*, the best-performing thioesterase. By adjustment of the light intensity and inducer concentration, up to 520 mg/L 1-octanol was produced in 10 days after induction. Additionally, adding additional copies of 'CpFatB1.4 further improved the 1-octanol production. Up to 743.08 ± 53.82 mg/L 1-octanol was produced in 30 days. Long-term experiments also indicated that the strain could be stably maintained over at least 10 serial dilutions. Chemostat operation of 1-octanol strain could be stably maintained for over 165 days under the tested conditions. The 1-octanol concentration reached to about 3750 mg/L at an average rate of 20-25 mg/L/day. In turbidostat mode, the productivity was further improved to well above 60 mg/L/d. Further optimization on light intensity might result in even higher productivity.

4. Materials and Methods

4.1 Plasmid construction

E. coli DH5 α (Thermo Fisher Scientific) was used for propagation of all the plasmids used in this study. The strain was cultivated in lysogeny broth (LB) medium (LB Broth, Sigma Aldrich), 37°C, 180 rpm, supplemented with appropriate antibiotic(s) (final concentration: spectinomycin 50 μ g/mL, carbenicillin 100 μ g/mL, chloramphenicol 37 μ g/mL, and erythromycin 200 μ g/mL). All plasmids used in this study were constructed using Biopart Assembly Standard Idempotent Cloning (BASIC) as described previously (Storch et al., 2015; Yunus et al., 2018; Yunus and Jones, 2018). Primers used to modify the thioesterases are listed in Supplementary Table 1 and 2. The amino acid sequences of all thioesterases used in this study are listed in Supplementary Table 3. 'CaFatB3.0 to 'CaFatB3.5 thioesterases have a single point mutation at amino acid number 261 from serine to cysteine.

4.2 Cultivation condition for *Synechocystis* sp. PCC 6803

In this study, *Synechocystis* sp. PCC 6803 glucose-tolerant wild-type strain (obtained from Prof. Klaas Hellingwerf, University of Amsterdam, The Netherlands), *Synechocystis* sp. PCC 6803 strain lacking acyl-ACP synthetase (Δaas) (previously constructed, Yunus and Jones, 2018) and Δaas strain expressing a phosphopantetheinyl transferase (Sfp) and carboxylic acid reductase (Car) (Δaas -Sfp-Car) (previously constructed, Sattayawat et al., 2020) were used. All strains were cultivated in BG11 medium without cobalt (BG11-Co) and supplemented with appropriate antibiotic(s) (final concentration: kanamycin 50 μ g/mL, spectinomycin 50 μ g/mL, chloramphenicol 37 μ g/mL, and erythromycin 20 μ g/mL). The liquid cultures were grown in 100-ml flasks in an AlgaeTron AG 230 (Photon Systems Instruments (PSI), Czech Republic) at 30 °C with continuous warm-white LED illumination at 60 μ mol photons $m^{-2} s^{-1}$ and continuous shaking at 180 rpm (Unimax 1010 shaker, Heidolph Instruments). The AlgaeTron AG 230 was equipped with a CO₂ sensor and controller (Ecotechnics, UK) to control and monitor the concentration of CO₂ (1% (v/v)) inside the growth chamber. Liquid cultures were induced with 5 μ M cobalt (stock solution: 20 mM Co(NO₃)₂·6H₂O) and/or 15 μ M nickel (stock solution: 20 mM NiSO₄·6H₂O) unless stated otherwise. To capture the fatty alcohols (e.g., 1-octanol and 1-decanol), an isopropyl myristate solvent overlay (30% v/v) was added to the liquid cultures during cultivation.

4.3 Transformation methods for *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 was transformed by triparental conjugation. Firstly, RSF1010 plasmids were transformed into *E. coli* HB101, a cargo strain carrying the pRL623 plasmid (Elhai et al., 1997) and plated on LB agar containing chloramphenicol (37 μ g/mL) and erythromycin (200 μ g/mL) to isolate colonies carrying the pRL623 and RSF1010 plasmids, respectively. Individual colonies were picked and inoculated overnight. A conjugal strain, *E. coli* ED8654 (Elhai et al., 1997), was grown in LB medium containing carbenicillin (100 μ g/mL). Overnight grown cargo strain and conjugal strain, were mixed with the *Synechocystis* strain (OD₇₃₀ 1) and incubated for two hrs (30°C, 60 μ mol photons $m^{-2} s^{-1}$). Prior to mixing, all three strains were washed with fresh LB medium and BG11-Co medium, as appropriate, to remove the antibiotics. After 2 hrs of incubation, the mixed cultures were plated onto BG11 agar plates without antibiotics and incubated for two days (30°C, 60 μ mol photons $m^{-2} s^{-1}$). After

this incubation, cells were scraped from the agar plate, resuspended in 500 μ L of BG11-Co medium, and transferred onto a new agar plate containing 20 μ g/mL erythromycin. Cells were grown for one week until colonies appeared. Individual colonies were restreaked onto a new plate containing 20 μ g/mL erythromycin and used for subsequent experiments. All plasmids and strains used in this study are listed in Supplementary Table 4.

4.4 *Free fatty acid and fatty alcohol analysis*

Free fatty acid analysis was performed as described previously (Yunus et al., 2020). In brief, 100 μ L of 10 M H_3PO_4 was added to acidify 1 ml of cyanobacteria liquid culture. The mixed solution was vortexed briefly to homogenize the sample. To extract the free fatty acids from the liquid culture, 2 ml of n-hexane (VWR Chemicals) was added. Solutions were vortexed vigorously and the hexane layer was separated from the liquid culture by centrifugation at 3,500 $\times$ g for 2 min. The upper hexane layer was then collected and transferred into a fresh PyrexTM round bottom threaded culture glass tube (Corning 99449-13, Thermo Fisher Scientific) and closed with a CorningTM phenolic threaded screw cap (Corning 9998-13, Thermo Fisher Scientific). To maximize the extraction of free fatty acids, the liquid culture was extracted with 2 ml hexane one more time. After centrifugation, the upper hexane layer was transferred and combined with the previously collected hexane layer. Pentadecanoic acid (100 μ g) was added as internal standard. The hexane solvent was allowed to evaporate completely under a stream of N_2 . The dried samples were esterified using 500 μ L of 1.25 M HCl in methanolic solution (Sigma Aldrich) and incubated at 85°C for 2 hr. After methyl esterification, the samples were cooled down to room temperature and 500 μ L of n-hexane was added to extract the FAMES. The FAMES were then analyzed using gas chromatography-mass spectrometer (GC-MS). One microliter of sample was injected into using Hewlett Packard (HP) 6890 Series Gas Chromatograph (GC) equipped with a 5973 Mass Selective Detector and a DB-WAXetr column (Agilent, 122-7332). The injector temperature was 250 °C and helium was used as the carrier gas. The oven temperature was initially held at 100°C for 30 s, followed by a ramp at 30°C.min⁻¹ to 250°C and hold for 3 min. The free fatty acids were then quantified by comparing the peak area of FAMES in the samples with serially diluted external FAME standard.

For fatty alcohol analysis, 10 μ L of isopropyl myristate solvent overlay was sampled, mixed with 10 μ L of 1-nonanol (1 g/L dissolved in isopropyl myristate), and derivatized with 90 μ L N, O-Bistrifluoroacetamide (BSTFA) at 65 °C for 2 hr for derivatization. A serial dilution of 1-octanol and 1-decanol standard was also prepared to quantify the concentration of 1-octanol and 1-decanol in the samples. Samples were analyzed using GC-MS. One microliter of sample was injected into Hewlett Packard (HP) 6890 Series Gas Chromatograph (GC) equipped with a 5973 Mass Selective Detector and a DB-WAXetr column (Agilent, 122-7332). The injector temperature was set to 250 °C and helium was used as the carrier gas. The oven temperature was initially held at 70°C for 30 s, followed by a ramp at 30°C.min⁻¹ to 250°C and hold for 1 min.

4.5 *Measurement of cell dry weight*

Cell biomass (cell dry weight (CDW)) was calculated from OD₇₃₀ using the conversion factor of 0.36 gCDW/L per OD₇₃₀ (Yunus et al., 2020).

4.6 Photobioreactor experiment

The 1 L flat plate PBR used in these experiments was previously described in detail Tamburic et al., (2011). The PBR consists of two compartments. The first compartment was used to culture liquid cyanobacteria and was equipped with a pH probe (Consort, Belgium), a dissolved oxygen probe (Consort, Belgium), a photodiode for optical density measurements and a K-type thermocouple (Omega, United Kingdom). The mixing was achieved through a gas lift system (air/CO₂) which was connected to a gas diaphragm pump (KNF Neuberger, United Kingdom) and a stainless steel sparger at the bottom of the first compartment. The second compartment was connected to a water bath unit (Grant Instruments, United Kingdom) where the temperature was pre-set and water was continuously recycled using a liquid diaphragm pump (KNF Neuberger, United Kingdom). The PBR system was also equipped with 24 V DC Brightest Day Light White LED array (LED Wholesalers). The PBR could be modified to operate as chemostat to investigate the best separation strategy, taking into account the physical properties of the targeted products. For 1-octanol extraction, hexadecane overlay was used and samples were taken periodically.

Acknowledgement

This study received funding from IY is supported by a PhD scholarship from Indonesia Endowment Fund for Education (LPDP). XXX

References

- Akhtar, M.K., Dandapani, H., Thiel, K., Jones, P.R., 2015. Microbial production of 1-octanol : A naturally excreted biofuel with diesel-like properties. *Metab. Eng. Commun.* 2, 1–5. <https://doi.org/10.1016/j.meteno.2014.11.001>
- Akhtar, M.K., Turner, N.J., Jones, P.R., 2013. Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. *Proc. Natl. Acad. Sci. U. S. A.* 110, 87–92. <https://doi.org/10.1073/pnas.1216516110>
- Beld, J., Finzel, K., Burkart, M.D., 2014. Versatility of acyl-acyl carrier protein synthetases. *Chem. Biol.* 21, 1293–1299. <https://doi.org/10.1016/j.chembiol.2014.08.015>
- Bellefleur, M.P.A., Wanda, S.-Y., Curtiss, R., 2019. Characterizing active transportation mechanisms for free fatty acids and antibiotics in *Synechocystis* sp. PCC 6803. *BMC Biotechnol.* 19, 5. <https://doi.org/10.1186/s12896-019-0500-3>
- Cho, H., Cronan, J.E., 1995. Defective Export of a Periplasmic Enzyme Disrupts Regulation of Fatty Acid Synthesis. *J. Biol. Chem.* 270, 4216–4219. <https://doi.org/10.1074/jbc.270.9.4216>
- Choi, Y.-N., Lee, J.W., Kim, J.W., Park, J.M., 2020. Acetyl-CoA-derived biofuel and biochemical production in cyanobacteria: a mini review. *J. Appl. Phycol.* 32, 1643–1653. <https://doi.org/10.1007/s10811-020-02128-x>
- Cuellar-Bermudez, S.P., Romero-Ogawa, M.A., Vannela, R., Lai, Y.S., Rittmann, B.E., Parra-Saldivar, R., 2015. Effects of light intensity and carbon dioxide on lipids and fatty acids produced by *Synechocystis* sp. PCC6803 during continuous flow. *Algal Res.* 12, 10–16. <https://doi.org/https://doi.org/10.1016/j.algal.2015.07.018>
- Dehesh, K., Edwards, P., Hayes, T., Cranmer, A.M., Fillatti, J., 1996a. Two Nove1 Thioesterases Are Key Determinants of the Bimodal Distribution of Acyl Chain Length of Cuphea

palustvis Seed Oil. *Plant Physiol.* 110, 203–10.

Dehesh, K., Jones, A., Knutzon, D.S., Voelker, T.A., 1996b. Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of Ch FatB2, a thioesterase cDNA from *Cuphea hookeriana*. *Plant J.* 9, 167–172. <https://doi.org/10.1046/j.1365-313X.1996.09020167.x>

Dellomonaco, C., Clomburg, J.M., Miller, E.N., Gonzalez, R., 2011. Engineered reversal of the β -oxidation cycle for the synthesis of fuels and chemicals. *Nature* 476, 355–359. <https://doi.org/10.1038/nature10333>

Dunlop, M.J., Dossani, Z.Y., Szmidt, H.L., Chu, H.C., Lee, T.S., Keasling, J.D., Hadi, M.Z., Mukhopadhyay, A., 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol. Syst. Biol.* 7, 487. <https://doi.org/10.1038/msb.2011.21>

Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flores, E., Wolk, C.P., 1997. Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp strain PCC 7120. *J Bacteriol* 179. <https://doi.org/10.1128/jb.179.6.1998-2005.1997>

Emanuelsson, O., Nielsen, H., Heijne, G. Von, 1999. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* 8, 978–984. <https://doi.org/10.1110/ps.8.5.978>

Feng, Y., Zhang, Y., Wang, Y., Liu, J., Liu, Y., Cao, X., Xue, S., 2018. Tuning of acyl-ACP thioesterase activity directed for tailored fatty acid synthesis. *Appl. Microbiol. Biotechnol.* 102, 3173–3182. <https://doi.org/10.1007/s00253-018-8770-6>

Foo, J.L., Jensen, H.M., Dahl, R.H., George, K., Keasling, J.D., Lee, T.S., Leong, S., Mukhopadhyay, A., 2014. Improving Microbial Biogasoline Production in *Escherichia coli* Using. *t* 5, 1–9. <https://doi.org/10.1128/mBio.01932-14>.Invited

Gao, X., Sun, T., Pei, G., Chen, L., Zhang, W., 2016. Cyanobacterial chassis engineering for enhancing production of biofuels and chemicals. *Appl. Microbiol. Biotechnol.* 100, 3401–3413. <https://doi.org/10.1007/s00253-016-7374-2>

Gao, Z., Zhao, H., Li, Z., Tan, X., Lu, X., 2012. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energy Env. Sci* 5. <https://doi.org/10.1039/C2EE22675H>

Graham, S.A., Kleiman, R., 1992. Composition of seed oils in some Latin American *Cuphea* (Lythraceae). *Ind. Crops Prod.* 1, 31–34. [https://doi.org/https://doi.org/10.1016/0926-6690\(92\)90042-T](https://doi.org/https://doi.org/10.1016/0926-6690(92)90042-T)

Iasimone, F., Panico, A., De Felice, V., Fantasma, F., Iorizzi, M., Pirozzi, F., 2018. Effect of light intensity and nutrients supply on microalgae cultivated in urban wastewater: Biomass production, lipids accumulation and settleability characteristics. *J. Environ. Manage.* 223, 1078–1085. <https://doi.org/https://doi.org/10.1016/j.jenvman.2018.07.024>

Jahn, M., Vialas, V., Karlsen, J., Maddalo, G., Edfors, F., Forsström, B., Uhlén, M., Käll, L., Hudson, E.P., 2018. Growth of Cyanobacteria Is Constrained by the Abundance of Light and Carbon Assimilation Proteins. *Cell Rep.* 25, 478–486.e8. <https://doi.org/https://doi.org/10.1016/j.celrep.2018.09.040>

Jha, J.K., Maiti, M.K., Bhattacharjee, A., Basu, A., Sen, P.C., Sen, S.K., 2006. Cloning and functional expression of an acyl-ACP thioesterase FatB type from *Diploknema* (Madhuca) butyracea seeds in *Escherichia coli*. *Plant Physiol. Biochem.* 44, 645–655. <https://doi.org/https://doi.org/10.1016/j.plaphy.2006.09.017>

Jing, F., Cantu, D.C., Tvaruzkova, J., Chipman, J.P., Nikolau, B.J., Yandeu-Nelson, M.D., Reilly, P.J., 2011. Phylogenetic and experimental characterization of an acyl-ACP thioesterase family reveals significant diversity in enzymatic specificity and activity. *BMC Biochem.*

12, 44. <https://doi.org/10.1186/1471-2091-12-44>

Kaczmarzyk, D., Fulda, M., 2010. Fatty Acid Activation in Cyanobacteria Mediated by Acyl-Acyl Carrier Protein Synthetase Enables Fatty Acid Recycling. *Plant Physiol.* 152, 1598–1610. <https://doi.org/10.1104/pp.109.148007>

Kato, A., Takatani, N., Use, K., Uesaka, K., Ikeda, K., Chang, Y., Kojima, K., Aichi, M., Ihara, K., Nakahigashi, K., Maeda, S.I., Omata, T., 2015. Identification of a Cyanobacterial RND-Type Efflux System Involved in Export of Free Fatty Acids. *Plant Cell Physiol.* 56, 2467–2477. <https://doi.org/10.1093/pcp/pcv150>

Khan, S., Fu, P., 2020. Biotechnological perspectives on algae: a viable option for next generation biofuels. *Curr. Opin. Biotechnol.* 62, 146–152. <https://doi.org/https://doi.org/10.1016/j.copbio.2019.09.020>

Kim, H.J., Silva, J.E., Vu, H.S., Mockaitis, K., Nam, J., Cahoon, E.B., 2015. Toward production of jet fuel functionality in oilseeds: identification of FatB acyl-acyl carrier protein thioesterases and evaluation of combinatorial expression strategies in *Camelina* seeds 66, 4251–4265. <https://doi.org/10.1093/jxb/erv225>

Kremer, F., Blank, L.M., Jones, P.R., Akhtar, M.K., 2015. A Comparison of the Microbial Production and Combustion Characteristics of Three Alcohol Biofuels: Ethanol, 1-Butanol, and 1-Octanol. *Front. Bioeng. Biotechnol.* 3, 112. <https://doi.org/10.3389/fbioe.2015.00112>

Lee, J.H., Kim, Y.-C., Jung, Y., Han, J.H., Zhang, C., Yun, C.-W., Lee, S., 2019. The overexpression of cucumber (*Cucumis sativus* L.) genes that encode the branched-chain amino acid transferase modulate flowering time in *Arabidopsis thaliana*. *Plant Cell Rep.* 38, 25–35. <https://doi.org/10.1007/s00299-018-2346-x>

Liu, X., Miao, R., Lindberg, P., Lindblad, P., 2019. Modular engineering for efficient photosynthetic biosynthesis of 1-butanol from CO₂ in cyanobacteria. *Energy Environ. Sci.* 12, 2765–2777. <https://doi.org/10.1039/C9EE01214A>

Liu, X., Sheng, J., Curtiss III, R., 2011. Fatty acid production in genetically modified cyanobacteria. *Proc. Natl. Acad. Sci.* 108, 6899–6904. <https://doi.org/10.1073/pnas.1103014108>

Lozada, N.J.H., Lai, R.Y., Simmons, T.R., Thomas, K.A., Chowdhury, R., Maranas, C.D., Pfleger, B.F., 2018. Highly Active C8-Acyl-ACP Thioesterase Variant Isolated by a Synthetic Selection Strategy. *ACS Synth. Biol.* 7, 2205–2215. <https://doi.org/10.1021/acssynbio.8b00215>

Lu, X., 2010. A perspective: Photosynthetic production of fatty acid-based biofuels in genetically engineered cyanobacteria. *Biotechnol. Adv.* 28, 742–746. <https://doi.org/10.1016/j.biotechadv.2010.05.021>

Lupette, J., Jaussaud, A., Seddiki, K., Morabito, C., Brugière, S., Schaller, H., Kuntz, M., Putaux, J.-L., Jouneau, P.-H., Rébeillé, F., Falconet, D., Couté, Y., Jouhet, J., Tardif, M., Salvaing, J., Maréchal, E., 2019. The architecture of lipid droplets in the diatom *Phaeodactylum tricornutum*. *Algal Res.* 38, 101415. <https://doi.org/https://doi.org/10.1016/j.algal.2019.101415>

Machado, H.B., Dekishima, Y., Luo, H., Lan, E.I., Liao, J.C., 2012. A selection platform for carbon chain elongation using the CoA-dependent pathway to produce linear higher alcohols. *Metab. Eng.* 14, 504–511. <https://doi.org/10.1016/j.ymben.2012.07.002>

Marcheschi, R.J., Li, H., Zhang, K., Noey, E.L., Kim, S., Chaubey, A., Houk, K.N., Liao, J.C., 2012. A Synthetic Recursive “+1” Pathway for Carbon Chain Elongation. *ACS Chem. Biol.* 7, 689–697. <https://doi.org/10.1021/cb200313e>

778 Miao, R., Xie, H., Liu, X., Lindberg, P., Lindblad, P., 2020. Current processes and future
 779 challenges of photoautotrophic production of acetyl-CoA-derived solar fuels and
 780 chemicals in cyanobacteria. *Curr. Opin. Chem. Biol.* 59, 69–76.
 781 <https://doi.org/https://doi.org/10.1016/j.cbpa.2020.04.013>
 782 Moreno-Pérez, A.J., Sánchez-García, A., Salas, J.J., Garcés, R., Martínez-Force, E., 2011. Acyl-
 783 ACP thioesterases from macadamia (*Macadamia tetraphylla*) nuts: Cloning,
 784 characterization and their impact on oil composition. *Plant Physiol. Biochem.* 49, 82–87.
 785 <https://doi.org/https://doi.org/10.1016/j.plaphy.2010.10.002>
 786 Mukhopadhyay, A., 2015. Tolerance engineering in bacteria for the production of advanced
 787 biofuels and chemicals. *Trends Microbiol.* 23, 498–508.
 788 <https://doi.org/https://doi.org/10.1016/j.tim.2015.04.008>
 789 Nozzi, N., Oliver, J., Atsumi, S., 2013. Cyanobacteria as a Platform for Biofuel Production .
 790 *Front. Bioeng. Biotechnol.* .
 791 Oliveira, P., Martins, N.M., Santos, M., Pinto, F., Büttel, Z., Couto, N.A.S., Wright, P.C.,
 792 Tamagnini, P., 2016. The versatile TolC-like Slr1270 in the cyanobacterium *Synechocystis*
 793 sp. PCC 6803. *Environ. Microbiol.* 18, 486–502. [https://doi.org/10.1111/1462-](https://doi.org/10.1111/1462-2920.13172)
 794 [2920.13172](https://doi.org/10.1111/1462-2920.13172)
 795 Oliver, J.W.K., Machado, I.M.P., Yoneda, H., Atsumi, S., 2013. Cyanobacterial conversion of
 796 carbon dioxide to 2,3-butanediol. *Proc. Natl. Acad. Sci. U. S. A.* 110, 1249–54.
 797 <https://doi.org/10.1073/pnas.1213024110>
 798 Oliver, N.J., Rabinovitch-Deere, C.A., Carroll, A.L., Nozzi, N.E., Case, A.E., Atsumi, S., 2016.
 799 Cyanobacterial metabolic engineering for biofuel and chemical production. *Curr. Opin.*
 800 *Chem. Biol.* 35, 43–50. <https://doi.org/10.1016/j.cbpa.2016.08.023>
 801 Peca, L., Kós, P.B., Máté, Z., Vass, I., Farsang, A., 2008. Construction of bioluminescent
 802 cyanobacterial reporter strains for detection of nickel, cobalt and zinc. *FEMS Microbiol.*
 803 *Lett.* 289, 258–264. <https://doi.org/10.1111/j.1574-6968.2008.01393.x>
 804 Quintana, N., Van der Kooy, F., Van de Rhee, M.D., Voshol, G.P., Verpoorte, R., 2011.
 805 Renewable energy from Cyanobacteria: energy production optimization by metabolic
 806 pathway engineering. *Appl. Microbiol. Biotechnol.* 91, 471–490.
 807 <https://doi.org/10.1007/s00253-011-3394-0>
 808 Ruffing, A.M., 2014. Improved free fatty acid production in cyanobacteria with *Synechococcus*
 809 sp. PCC 7002 as host. *Front. Bioeng. Biotechnol.* 2, 17.
 810 Sánchez-García, A., Moreno-Pérez, A.J., Muro-Pastor, A.M., Salas, J.J., Garcés, R., Martínez-
 811 Force, E., 2010. Acyl-ACP thioesterases from castor (*Ricinus communis* L.): An enzymatic
 812 system appropriate for high rates of oil synthesis and accumulation. *Phytochemistry* 71,
 813 860–869. <https://doi.org/https://doi.org/10.1016/j.phytochem.2010.03.015>
 814 Sattayawat, P., Yunus, I.S., Jones, P.R., 2020. Bioderivatization as a concept for renewable
 815 production of chemicals that are toxic or poorly soluble in the liquid phase. *Proc. Natl.*
 816 *Acad. Sci.* 117, 1404 LP – 1413. <https://doi.org/10.1073/pnas.1914069117>
 817 Savakis, P., Hellingwerf, K.J., 2015. Engineering cyanobacteria for direct biofuel production
 818 from CO₂. *Curr. Opin. Biotechnol.* 33, 8–14.
 819 <https://doi.org/10.1016/j.copbio.2014.09.007>
 820 Sittther, V., Tabatabai, B., Fathabad, S.G., Gichuki, S., Chen, H., Arumanayagam, A.C.S., 2020.
 821 Chapter 18 - Cyanobacteria as a biofuel source: advances and applications, in: Singh, P.K.,
 822 Kumar, A., Singh, V.K., Shrivastava, A.K.B.T.-A. in C.B. (Eds.), . Academic Press, pp. 269–
 823 289. <https://doi.org/https://doi.org/10.1016/B978-0-12-819311-2.00018-8>
 824 Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B., Keasling,

J.D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562. <https://doi.org/10.1038/nature08721>

Storch, M., Casini, A., Mackrow, B., Fleming, T., Trehwitt, H., Ellis, T., Baldwin, G.S., 2015. BASIC: A New Biopart Assembly Standard for Idempotent Cloning Provides Accurate, Single-Tier DNA Assembly for Synthetic Biology. *ACS Synth. Biol.* 4, 781–787. <https://doi.org/10.1021/sb500356d>

Tamburic, B., Zemichael, F.W., Crudge, P., Maitland, G.C., Hellgardt, K. (2011). Design of a Novel Flat-plate Photobioreactor System for Green Algal Hydrogen Production. *International Journal of Hydrogen Energy*, 36, 6578-6591.

Tan, X., Yao, L., Gao, Q., Wang, W., Qi, F., Lu, X., 2011. Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. *Metab. Eng.* 13, 169–176. <https://doi.org/10.1016/j.ymben.2011.01.001>

Tjellström, H., Strawsine, M., Silva, J., Cahoon, E.B., Ohlrogge, J.B., 2013. Disruption of plastid acyl:acyl carrier protein synthetases increases medium chain fatty acid accumulation in seeds of transgenic Arabidopsis. *FEBS Lett.* 587, 936–942. <https://doi.org/10.1016/j.febslet.2013.02.021>

The United Nations Intergovernmental Panel on Climate Change (IPCC), 2018 <https://www.un.org/en/sections/issues-depth/climate-change/>

Voelker, T.A., Worrell, A.C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E., Davies, H.M., 1992. Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science* (80-). 257, 72 LP – 74. <https://doi.org/10.1126/science.1621095>

Wang, L., Chen, L., Yang, S., Tan, X., 2020. Photosynthetic Conversion of Carbon Dioxide to Oleochemicals by Cyanobacteria: Recent Advances and Future Perspectives . *Front. Microbiol.* .

Wang, W., Liu, X., Lu, X., 2013. Engineering cyanobacteria to improve photosynthetic production of alka(e)nes. *Biotechnol. Biofuels* 6. <https://doi.org/10.1186/1754-6834-6-69>

Wanniarachchi, R.V., Dametto, L., Sweetman, C., Shavrukov, Y., Day, A.D., Jenkins, L.C., Soole, L.K., 2018. Alternative Respiratory Pathway Component Genes (AOX and ND) in Rice and Barley and Their Response to Stress. *Int. J. Mol. Sci.* . <https://doi.org/10.3390/ijms19030915>

Work, V.H., Melnicki, M.R., Hill, E.A., Davies, F.K., Kucek, L.A., Beliaev, A.S., Posewitz, M.C., 2015. Lauric Acid Production in a Glycogen-Less Strain of *Synechococcus* sp. PCC 7002. *Front. Bioeng. Biotechnol.* 3, 48. <https://doi.org/10.3389/fbioe.2015.00048>

Xu, P., Gu, Q., Wang, W., Wong, L., Bower, A.G.W., Collins, C.H., Koffas, M.A.G., 2013. Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. *Nat. Commun.* 4, 1409.

Xu, P., Li, L., Zhang, F., Stephanopoulos, G., Koffas, M., 2014. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proc. Natl. Acad. Sci.* 111, 11299 LP – 11304. <https://doi.org/10.1073/pnas.1406401111>

Yuan, Y., Gao, L., Sun, R., Yu, T., Liang, Y., Li, D., Zheng, Y., 2017. Seed-specific expression of an acyl-acyl carrier protein thioesterase CnFatB3 from coconut (*Cocos nucifera* L.) increases the accumulation of medium-chain fatty acids in transgenic Arabidopsis seeds. *Sci. Hortic. (Amsterdam)*. 223, 5–9. <https://doi.org/https://doi.org/10.1016/j.scienta.2017.05.029>

Yunus, I.S., Jones, P.R., 2018. Photosynthesis-dependent biosynthesis of medium chain-length fatty acids and alcohols. *Metab. Eng.* 49. <https://doi.org/10.1016/j.ymben.2018.07.015>

872 Yunus, I.S., Palma, A., Trudeau, D.L., Tawfik, D.S., Jones, P.R., 2020. Methanol-free
873 biosynthesis of fatty acid methyl ester (FAME) in *Synechocystis* sp. PCC 6803. *Metab.*
874 *Eng.* 57, 217–227. <https://doi.org/https://doi.org/10.1016/j.ymben.2019.12.001>
875 Yunus, I.S., Wichmann, J., Wördenweber, R., Lauersen, K.J., Kruse, O., Jones, P.R., 2018.
876 Synthetic metabolic pathways for photobiological conversion of CO₂ into hydrocarbon
877 fuel. *Metab. Eng.* 49, 201–211. <https://doi.org/10.1016/j.ymben.2018.08.008>
878 Zhou, Y.J., Buijs, N.A., Zhu, Z., Qin, J., Siewers, V., Nielsen, J., 2016. Production of fatty acid-
879 derived oleochemicals and biofuels by synthetic yeast cell factories. *Nat. Commun.* 7,
880 11709. <https://doi.org/10.1038/ncomms11709>
881
882
883