Bioderivatization as a concept for renewable production of toxic or poorly soluble chemicals

Pachara Sattayawat

Imperial College London

Department of Life Sciences

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

Bio-based production has increasingly gained attention as an alternative technology to complement or even substitute petroleum-based production of valuable chemicals. This technology, however, relies on the use of microbial cell factories and at times faces several challenges, including toxicity of the target products to microbial hosts. Many plants and microorganisms are naturally capable of biosynthesizing toxic molecules, but they often convert them into derivatives with reduced toxicity or enhanced solubility before the molecules are stored or excreted. Inspired by this principle, a novel strategy, bioderivatization, was proposed. Bioderivatization is here defined as a purposeful biochemical derivatization of intended target molecules by altering the functional groups to overcome such challenges. *O*-acetylation and *O*-glucosylation were proposed and investigated as two bioderivatization strategies. As a proof-of-principle, the effect of bioderivatization on biosynthesis of a relatively toxic and poorly soluble chemical, 1-octanol, was evaluated. The existing 1-octanol pathway was first optimized to enable the production of 1-octanol at higher titer. Novel synthetic pathways to derivatize 1-octanol into octyl acetate and octyl glucoside were then implemented in *Escherichia coli* and cyanobacteria. The evaluation of bioderivatization on growth and productivity showed that the implementation of bioderivatization contributed to improved growth and/or productivity in most cases. To understand if the bioderivatization strategy can be implemented in a broader scope (i.e., to derivatize other toxic chemicals), this strategy was also applied to derivatize several other attractive chemicals from different chemical classes. The preliminary results successfully identified several potential chemical candidates, including 1-dodecanol, menthol, and eugenol that showed a higher degree of toxicity compared to their corresponding derivatives. Finally, this study was concluded by identification of several active key enzymes to derivatize these toxic compounds. Altogether, this study showed that bioderivatization could be considered a strategy to improve the bioproduction of toxic or poorly soluble chemicals.

Thesis supervisor: Dr. Patrik R. Jones

Declaration of originality

I hereby confirm that the work contained within this thesis is my own and other sources of information used are properly cited.

Pachara Sattayawat

Copyright declaration

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Chapter 1
Introduction

1.1 General introduction

1.1.1 Motivation

With growing concern for the use of petroleum-derived chemicals and its associated negative problems to the environment including climate change, there has been an urge to develop a more sustainable technology as an alternative to petroleum-based production. Microbial biotechnology has increasingly attracted the attention for manufacturing of highly valuable chemicals from renewable resources (Na, Kim & Lee, 2010; Du, Shao & Zhao, 2011; Lee *et al.*, 2012). However, the use of living microorganisms as cell factories faces several challenges resulting in commercially inefficient systems for bioproduction of chemicals. Toxicity of the products toward microbial hosts is one of the underlying challenges that may prevent commercialization of some bio-based products (Dunlop *et al.*, 2011; Yunus & Jones, 2018). Often the titer of target products needs to exceed the native tolerance level of host microorganisms in order for cost-efficient production (Dunlop *et al.*, 2011). For example, in one case, when the tolerance level of the production host was improved, the productivity also improved (Alper *et al.*, 2006). When bioproducts are produced in a biorefinery, the cost of product separation and purification is another issue preventing cost-efficient production of bio-based chemicals. In general, such downstream processing can account for more than 60% of the whole bioproduction cost (Ragauskas *et al.*, 2006; Bechthold *et al.*, 2008; Kiss, Grievink & Rito-Palomares, 2015; Kiss *et al.*, 2016; Najmi *et al.*, 2018). In summary, bio-based production must achieve high yield, titer and productivity in order to be cost-competitive with petroleum-based production. Therefore, there is need to overcome aforementioned challenges.

1.1.2 A proposed novel biotechnological strategy “bioderivatization”

To tackle the challenges in bio-based production, a novel biotechnological process called “bioderivatization” is proposed in this study. Bioderivatization is here defined as the process of purposeful *in vivo* transformation of chemicals into their derivatives by modification of functional groups. This concept was inspired by natural mechanisms that organisms are using to cope with their naturally synthesized toxic chemicals. More specifically, plants and yeast cope with toxic metabolites by converting them into less toxic derivatives, esters or glycosides. In yeast, ester formation has arguably been proposed as a detoxification

mechanism that converts more toxic chemicals into those that are less harmful (Saerens *et al.*, 2010). In plants, toxic chemicals are often modified via glycosylation reaction, which converts the chemicals into an inactive form upon storage (Jones & Vogt, 2001; de Roode *et al.*, 2003; Sirikantaramas, Yamazaki & Saito, 2008). In this study, both *O*-acetylation and *O*-glucosylation of alcohols are investigated as bioderivatization reactions. It is proposed that modifications of functional groups happen inside the cell to alleviate the toxicity of alcohols. After the derivatives are excreted from the cell, they can be reverse-derivatized by chemical or enzymatic processes to recover the original target (Figure 1.1). Additionally, derivatives that are beneficial for downstream processes can be designed to further improve the bio-based production. To evaluate bioderivatization concept, proof-of-principle compounds and their derivatives were identified, their synthetic pathways were designed and optimized. Then, cell growth, metabolisms and final product productivity were assessed.

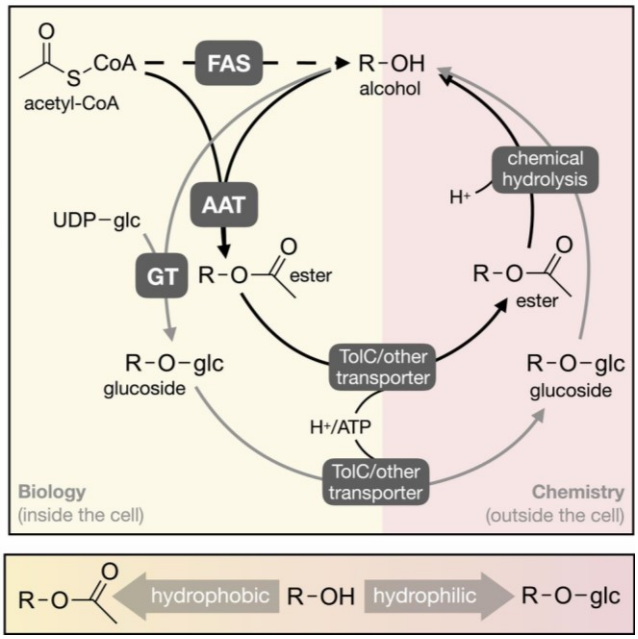

Figure 1.1 Nonstoichiometric overview of *O*-acetylation and *O*-glucosylation as bioderivatization reactions Target chemicals are enzymatically converted into ester or glucoside derivatives inside the cell and sequentially excreted from the cell. Implementation of *O*-acetylation or *O*-glucosylation alters the properties of target molecules differently; thus, open opportunities for more cost-efficient product:process separation. FAS, fatty acid biosynthesis; AAT, alcohol acetyltransferase; GT, glycosyltransferase. [Figure from Sattayawat, Yunus & Jones, 2020, permission not required, see Appendix L].

1.2 Objectives

1) To establish and investigate the concept of bioderivatization as a new biotechnological strategy via synthetic pathways
2) To investigate factors influencing the concept and the production of compounds of interest

3) To evaluate the utility of the bioderivatization concept for a broader range of other attractive compounds

1.3 Thesis organization

Chapter 2 presents a literature review on bio-based production, challenges in bio-based production, proposed strategy to overcome the challenges, metabolic engineering, synthetic biology and related fundamental knowledge to this study.

Chapter 3 describes all materials and methods used in this study.

Chapter 4 describes the optimization of an existing carboxylic acid reductase-dependent 1-octanol pathway.

Chapter 5 demonstrates the toxicity of 1-octanol compared with octyl acetate, the implementation of octyl acetate pathway in *E. coli* and the evaluation of bioderivatization via *O*-acetylation.

Chapter 6 demonstrates the toxicity of 1-octanol compared with octyl glucoside, the implementation of octyl glucoside pathway in *E. coli* and cyanobacteria and the evaluation of bioderivatization via *O*-glucosylation.

Chapter 7 demonstrates the toxicity of 1-dodecanol compared with lauryl acetate and the effort to implement 1-dodecanol and lauryl acetate pathway in *E. coli* and cyanobacteria.

Chapter 8 presents initial tests for feasibility of other attractive compounds from different chemical classes to further explore bioderivatization concept.

Chapter 9 concludes key findings and recommendations for future work from this study.

Chapter 2
Literature Reviews

2.1 Bio-based production of attractive chemicals

The renewable bio-based production of attractive and commercially valuable chemicals using microorganisms as production hosts has become a promising alternative to petroleum-based production. This biotechnology relies on microorganisms and their natural metabolisms to produce desirable compounds either by naturally existing pathways or the introduction of novel synthetic pathways. Metabolic engineering and synthetic biology are important tools for the production as they allow manipulation of host metabolism leading to enhanced production of natural and non-natural chemicals from host strains (Figure 2.1).

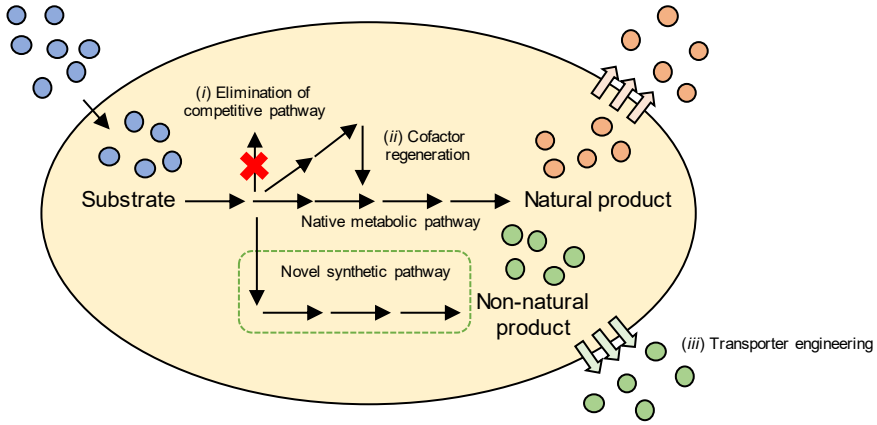

Figure 2.1 General scheme of microbial cell factories adapted from Lin and Tao, 2017
The diagram depicting natural and non-natural chemical production from renewable substrates and examples of engineering strategies to improve the production (*i*) elimination of competitive pathways (*ii*) cofactor regeneration (*iii*) transporter engineering.

2.1.1 Microbial cell factories

The use of microbial cell factories has been shown to be an efficient biotechnology for the production of desirable chemicals. The variation of microorganisms offers naturally existing choices for relatively optimal production. For example, the choice of renewable substrate can narrow down which group of microorganisms is suitable for the production. If the most basic carbon source, CO_2, is the substrate of interest, then the photosynthetic microorganisms would be an ideal choice as host strains. With recent advances in microbial technology, a number of chemicals have reached the ultimate goal of development by entering commercial phases such as succinic acid, polyhydroxyalkanoates (PHA), 1,3-propanediol,

1,4-butanediol (Gustavsson & Lee, 2016; Choi *et al.*, 2019). The advantages of this system have been shown through the actual production of several compounds (Lin & Tao, 2017).

Firstly, this biotechnology allows the production of desirable, value-added chemicals from non-complex, abundant and cheap substrates (Thakker, San & Bennett, 2013; Layton & Trinh, 2016b). Secondly, the use of whole-cell biocatalyst allows the implementation of pathways that comprise of cascades of reactions (Ladkau, Schmid & Bühler, 2014). Since the reactions are occurring inside of living cells, this should help the enzymes to stabilize under the intracellular conditions. Moreover, the addition of cofactors, which are commonly known as expensive compounds, can be omitted (Endo & Koizumi, 2001). The use of microorganisms as production hosts also bypasses the enzyme extraction and purification processes in enzymatic production of chemicals, which can be highly costly. In addition, the whole-cell fermentation is less expensive and the cells can be recycled in the new production batch (Mattam & Yazdani, 2013).

2.1.2 Role of metabolic engineering and synthetic biology

Metabolic engineering and synthetic biology are a useful tool in microbial technology. They can be employed at several levels from using basic efforts in manipulating native pathways inside host cells to constructing novel pathways and also using novel enzymatic mechanisms (Erb, Jones & Bar-Even, 2017). Metabolic engineering enables the manipulation and optimization of metabolic pathways in microorganisms that leads to desirable phenotypes such as enhanced production. Recent advances in synthetic biology accelerate the optimization of bio-based production and expand the list of bio-based products. Several approaches have been used in this manner. Metabolic engineering strategies include (*i*) engineering of carbon source utilization (*ii*) engineering of transporters to enhance product secretion (*iii*) elimination of competitive pathways and enhancement of precursor availability, (*iv*) engineering to increase cofactor supply and regeneration, etc (Lee *et al.*, 2012; Lin & Tao, 2017). The production of non-native compounds from microorganisms often involves an introduction of novel pathways into suitable hosts. This allows us to benefit from their attractive traits such as high growth and metabolism rates to produce desirable compounds. The general workflow for novel pathway construction is shown in Figure 2.2.

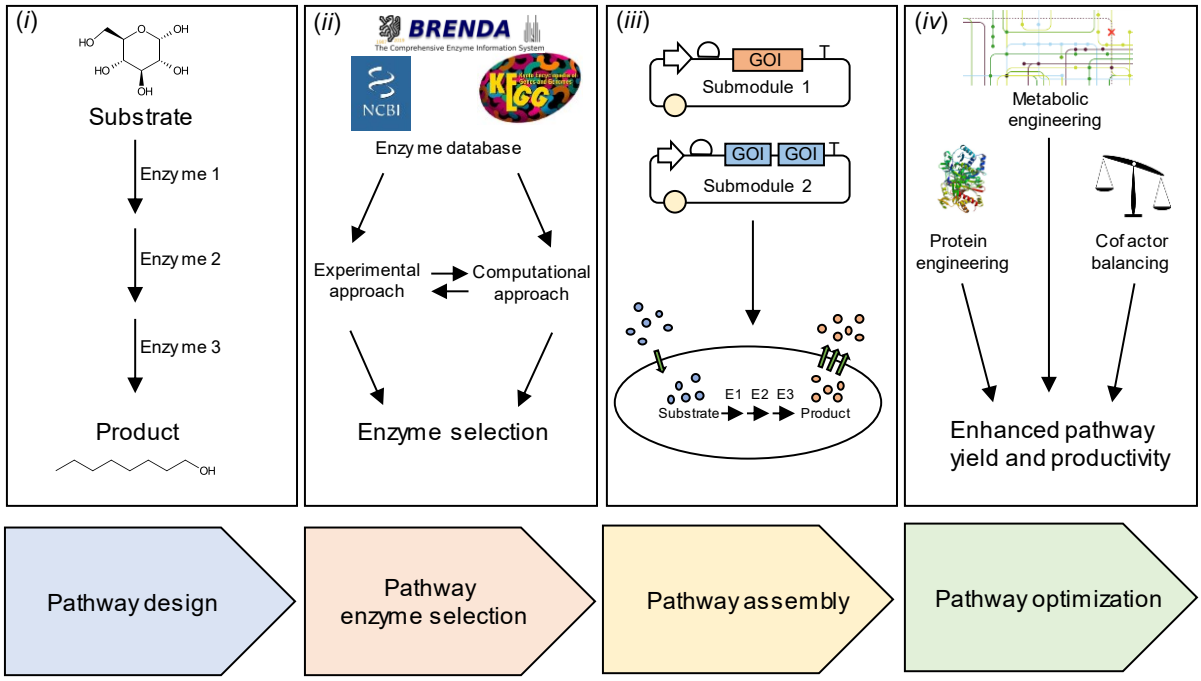

Figure 2.2 The simplified diagram depicting the novel pathway construction
Adapted from Liu *et al*., 2007; Martin *et al*., 2009; and Dhamankar and Prather, 2011. The overview of four key steps for novel pathway construction (*i*) Pathway design (*ii*) Pathway enzyme selection (*iii*) Pathway assembly (*iv*) Pathway optimization.

The pathway construction workflow is divided into four key steps as follows.

1) Pathway design

To design a new pathway, which has not been characterized, the native metabolic pathways of microorganisms are usually the starting point for novel pathway design. The use of naturally existing intermediates as precursors for the novel pathway helps simplify the process toward targets. For example, several synthetic pathways rely on native intermediates from *E. coli* fatty acid biosynthesis pathway or acetyl-CoA as precursors that are subsequently converted to desirable products via expression of heterologous enzymes (Guo *et al.*, 2014; Kallio *et al.*, 2014; Akhtar *et al.*, 2015). The final bio-chemicals of interest are usually selected based on their market demands (Choi, Kim & Lee, 2018). Metabolic pathways are usually composed of several reaction steps to transform substrates to intermediates and, finally, to final target products (Figure 2.2(*i*)). Each of the reaction step should be designed hypothetically that they are feasible and likely to happen when all essential components are available. With recent developments in bioinformatics, several software tools facilitate pathway prediction (Medema *et al.*, 2012). These tools predict possible metabolic pathways based on knowledge databases (Hou, Wackett & Ellis, 2003; McShan, Rao & Shah, 2003).

2) Pathway enzyme selection

Nature has variable collections of existing enzymes – this is one of the benefits for bioproduction. When each step of novel pathways has been designed, the enzyme suitable for each reaction are then identified. This step allows the production to be effective if appropriate enzymes are selected. With current advances in protein engineering, the chosen enzymes could be naturally existing or even the completely engineered ones (Martin *et al.*, 2009; Erb, Jones & Bar-Even, 2017). To search for suitable enzymes, enzyme databases (e.g., BRENDA, KEGG and NCBI) are valuable sources of knowledge. After the search to narrow down the number of potential enzymes, experimental or computational approaches can be used to validate or predict the overall performances of enzymes such as activity and specificity (Figure 2.2(*ii*)). Experimental approaches cover *in vitro* and *in vivo* studies of each enzyme toward substrates of interest. High throughput rapid screening assays for enzyme activity have been developed to facilitate this step. For instance, colorimetric screening assays for ester formation has been developed to rapidly verify the activity of alcohol acetyltransferases (AATs) (Lin, Zhu & Wheeldon, 2016; Löbs *et al.*, 2016). Computational approaches are used to predict the activity of enzymes. With increased knowledge in bioinformatics, computational tools could lessen the effort to conduct hand-on screening assays. For example, molecular docking and molecular dynamics simulation were used to investigate the molecular mechanism of AAT enzymes from *Cucumis melo var. cantalupensis* (Galaz *et al.*, 2013).

3) Pathway assembly

Once the enzymes are selected, the genes expressing each enzyme are assembled to form a pathway (Figure 2.2(*iii*)). In *E. coli*, a plasmid system is often used to express enzymes (Kallio *et al.*, 2014; Rodriguez, Tashiro & Atsumi, 2014; Akhtar *et al.*, 2015). Several DNA assembly techniques have been used to combine each genetic part together in a plasmid system for bioproduction e.g., traditional restriction/ligation cloning (Thanasomboon *et al.*, 2012), BASIC assembly (Yunus & Jones, 2018), Gibson assembly (Opgenorth *et al.*, 2019), etc.

4) Pathway optimization

Since novel synthetic pathways do not exist in heterologous production hosts; thus, the natural cellular environment may not be optimized for such synthetic pathways. Moreover, many factors could limit the production depending on each pathway of interest. In this section, protein engineering, cofactor regeneration and choice of promoter and ribosome binding site (RBS) as common strategies for pathway optimization are addressed (Figure 2.2(*iv*)).

To further improve the pathway efficiency, protein engineering has become a powerful tool to manipulate enzyme specificity and activity. Alteration of pathway enzyme performances allows each reaction step to efficiently function leading to the optimal production. A related example to this study is protein engineering of thioesterase enzyme to increase activity toward one of its substrates – C8 acyl-ACP, truncations and mutations of amino acid residues resulted in 15-fold increase in enzyme k_{cat} leading to the highest titer of end product (octanoic acid) reported (Lozada *et al.*, 2018).

As mentioned in Section 2.1, one of the advantages of microbial cell factories is the omission of cofactor supplementation; however, the supplementation of cofactors may still be needed depending on the pathway of interest and the choice of host microorganisms. In some cases, where host selection does not allow a sufficient supply of cofactors, cofactor regeneration is one of the common strategies for pathway optimization (Endo & Koizumi, 2001). Cofactors are non-protein compounds that are required for enzymes to function. For example, the overexpression of NADPH/ferredoxin/flavodoxin-oxidoreductase (Fpr) to facilitate cofactor regeneration of aldehyde deformylating oxygenase (ADO) enzyme in propane biosynthetic pathway resulted in higher propane production (Kallio *et al.*, 2014).

The choice of promoter and Ribosome Binding Site (RBS) influences the protein expression. Therefore, there have been studies on varying the choice of promoter and RBS. To give an example related to this study, varying of promoters and RBSs for 1-octanol production in cyanobacteria resulted in significantly different yields (Yunus & Jones, 2018). Promoter and RBS are known to be context-dependent, in other words, the same optimal promoters or RBSs may not apply to every pathway or host microorganism, optimization may be needed for each case separately (Carr, Beal & Densmore, 2017; Thiel *et al.*, 2018).

Altogether, metabolic engineering and synthetic biology facilitate the construction of novel synthetic pathways and development of high-performance strains.

2.1.3 Challenges in microbial technology

Although microbial technology has been developed for several decades, there are several unsolved challenges that affect the overall production and commercialization of bioproducts. Two challenges are outlined and focused in this study, (*i*) toxicity of products and (*ii*) cost of separation and purification processes.

Toxicity of the products to microbial hosts is one of the limitations in bio-based production. This often shows as noticeable defect phenotypes such as poor growth or low biomass accumulation, which results in low titer of target products. Non-native hosts without evolutionary adaptive traits to defend themselves from toxicity of non-natural molecules may be more susceptible. For example, a native 1-butanol producer could tolerate up to about 2%

(v/v) (Lin & Blaschek, 1983); however, non-native hosts such as *E. coli* could tolerate up to only 1.5 % (v/v) (Mukhopadhyay, 2015). To reach the ultimate goal for bioproduction as a commercialized product, the product titer of chemicals often needs to exceed the tolerance levels in order for the production to be cost-effective (Dunlop *et al.*, 2011). Enhancement of cellular product tolerance and implementation of *in situ* product separation from the cells to mitigate the toxicity have shown to result in improvement of productivity (Baez, Cho & Liao, 2011; Dunlop *et al.*, 2011; de Vrije *et al.*, 2013; Gong, Nielsen & Zhou, 2017).

The separation and purification processes are essential in bioproduction in order to achieve the required specifications of final bioproducts, these steps are known to cost ≥60% of the whole cost for bioproduction (Kiss *et al.*, 2016). In order for bio-based production to replace petroleum-based production, this challenge is considered as one of the bottlenecks for bioproduction. To my knowledge, separation and purification processes of 1-octanol, the first proof-of-principle chemical in this study, from fermentation broths have not been investigated. However, a well-known shorter-chain alcohol, 1-butanol, has been exploited thoroughly on this topic. Due to its high toxicity to microbial hosts, low final product concentration of 1-butanol has consequently caused the downstream separation and purification processes to be cost-ineffective (Huang, Ramaswamy & Liu, 2014; Patraşcu, Bîldea & Kiss, 2017). Several methods have been developed for biobutanol separation from cultivation media such as distillation, gas-stripping and liquid-liquid extraction (Huang, Ramaswamy & Liu, 2014; Kujawska *et al.*, 2015). Despite heavy research on this matter, the cost for these processes in biobutanol production is still considered high. The separation and purification techniques are often developed based on chemical properties of compounds of interest. For example, liquid-liquid extraction developed for biobutanol relies on hydrophobic property of biobutanol relatively compared to other contaminants in the system (e.g., ethanol) (Huang, Ramaswamy & Liu, 2014). Thus, the development of cost-efficient separation and purification of bioproducts is still an attractive field to study.

2.2 Bioderivatization

In nature, many organisms naturally synthesize toxic molecules, and, in many cases, these compounds accumulate as chemical derivatives. This mechanism is considered nature's ways to adapt through environmental changes. These detoxification mechanisms are highly effective in some cases. For example, cyanogenic glucosides that without derivatization would kill the plant itself can accumulate up to 30% of dry weight (Halkier & Møller, 1989). A similar detoxification mechanism has been proposed in yeast where native enzymes convert toxic metabolites to those that are less harmful (Saerens *et al.*, 2010).

Inspired by this principle, a novel biotechnology “Bioderivatization” is proposed to overcome aforementioned limitations in bio-based production (see Section 2.1.3). Bioderivatization is proposed as an *in vivo* transformation of the functional groups of the target compounds to make them more suitable for certain purposes. As mentioned in Section 1.2, *O*-acetylation (see Section 2.5) and *O*-glucosylation (see Section 2.6) were proposed as strategies for bioderivatization as they are known detoxification mechanisms in nature. In this study, the transformation aims to alleviate the toxicity of the products and potentially make them more suitable for downstream processes of separation and purification. As bioderivatization may also radically change the chemical properties (e.g., water solubility) of the target chemical and/or protect the molecule from further conversion (e.g., oxidation). This could open new opportunities for strategic product:process separation that is more cost-efficient. As mentioned in Section 2.1.3, separation and purification processes of bioproducts from fermentation broths are expensive. Therefore, with bioderivatization, the intentional alteration of chemical properties allows us to design final derivatives that facilitate these costly steps. After the derivative is excreted from the production host, it would need to be converted back to its original form, unless the particular derivative is also a desirable product. The original form of the derivatized products can be obtained through chemical or enzymatic reactions such as hydrogenation (Pritchard *et al.*, 2015) or hydrolysis following isolation from the bioreactor. The general bioderivatization concept is illustrated in Figure 2.3.

Biotransformation of bioproducts to their corresponding derivatives has been shown without a rationale or occurred unintentionally through interactions with native metabolism in the biotechnological host. For example, vanillin glucoside biosynthesis was studied as opposed to vanillin biosynthesis to investigate the activity of an enzyme that catalyzes the conversion of ferulic acid and its glucoside to vanillin and vanillin glucoside, respectively (Gallage *et al.*, 2014). Similarly, one study implemented the transformation of geraniol to geranyl glucoside in order to understand the enzyme function; however, they also observed enhanced tolerance from derivative-producing strains (Huang *et al.*, 2016). Moreover, geraniol synthesis in a study resulted in geranyl acetate formation through the action of native AATs (Liu *et al.*, 2016a). Furthermore, the effect of transformation of bioproducts to derivatives on separation and purification processes has also been investigated. A study on integrated *in situ* recovery of product via transformation of 1-butanol to butyl butyrate by catalyst feeding was conducted in order to alleviate 1-butanol toxicity and diminish downstream processing (van den Berg *et al.*, 2013). The systematic investigations of the concept have yet remained to be conducted and whether this strategy can be generally applied to other suitable chemicals still needs further investigation.

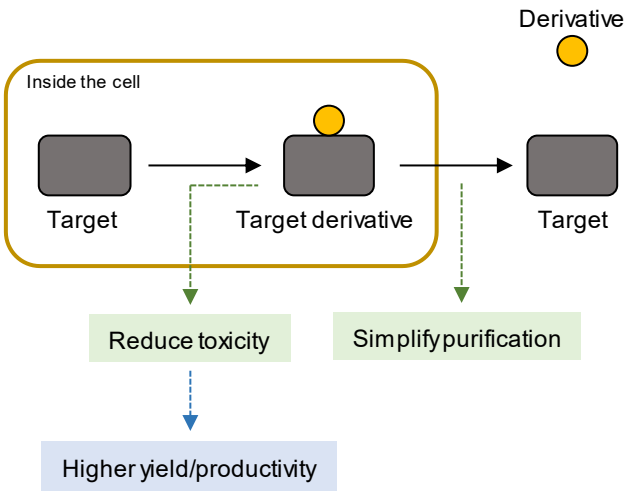

Figure 2.3 General schematic diagram for bioderivatization concept
The target molecule is enzymatically converted into a derivative (e.g., ester or glucoside) with potential consequences for both toxicity and water solubility inside of the cell. Once the product has been excreted, the original form of target molecule can be recovered by enzymatic or chemical processes that remove the conjugate group.

2.3 Model microorganisms

2.3.1 *Escherichia coli*

E. coli has been studied as a host microorganism in metabolic engineering for production of native and non-native desirable products. They have advantages over other bacteria as (*i*) they can utilize various carbon sources in both aerobic and anaerobic conditions (*ii*) they are fast-growing bacteria with high metabolic rates, which could facilitate the production rate (*iii*) genetic, metabolic and physiological traits are well-exploited including their full genome sequences reported (Blattner *et al.*, 1997), (*iv*) many genetic/molecular tools are available (Wang, Pfleger & Kim, 2017). Many attractive chemicals have been produced successfully in *E. coli* (Pontrelli *et al.*, 2018) including alcohols, fatty acids, terpenoids (Table 2.1).

Table 2.1 Examples of chemicals produced from engineered *E. coli* (Some data from Pontrelli *et al*., 2018)

Bioproduct	Titer	Reference
Vanillin	0.019 g/L	(Ni *et al.*, 2015)
Lauryl acetate	0.021 g/L	(Guo *et al.*, 2014)
Pentadecane	0.025 g/L	(Song, Yu & Zhu, 2016)
Propane	0.032 g/L	(Kallio *et al.*, 2014)
Ethyl butyrate	0.088 g/L	(Layton & Trinh, 2014)
1-Octanol	0.1 g/L	(Dellomonaco *et al.*, 2011)
Tetradecyl acetate	0.137 g/L	(Rodriguez, Tashiro & Atsumi, 2014)
Cinnamyl glucoside	0.259 g/L	(Zhou *et al.*, 2017)
Linalool	0.505 g/L	(Mendez-Perez *et al.*, 2017)
Butyraldehyde	0.630 g/L	(Ku, Simanjuntak & Lan, 2017)
1-Dodecanol	0.83 g/L	(Opgenorth *et al.*, 2019)
Octanoic acid	1.7 g/L	(Lozada *et al.*, 2018)
Geraniol	2 g/L	(Liu *et al.*, 2016a)
Succinic acid	22 g/L	(Thakker, San & Bennett, 2013)
1-Butanol	30 g/L	(Shen *et al.*, 2011)
Isobutyl acetate	36 g/L (in bioreactor)	(Tai, Xiong & Zhang, 2015)
Isobutanol	50 g/L (in bioreactor)	(Baez, Cho & Liao, 2011)
2,3-butanediol	88 g/L (fed-batch)	(Hwang, Lee & Lee, 2018)

*Titer is used to describe a concentration of compound in a solution (i.e., in g/L or mol/L (M))

Many of these bio-based chemicals including targeted chemicals in this study are produced via derived-native fatty acid synthetic pathways in *E. coli* (Yu *et al.*, 2014). The first step of fatty acid synthesis in *E. coli* is the formation of malonyl-CoA from acetyl-CoA by an acetyl-CoA carboxylase (AccABCD). Malonyl-CoA is then catalyzed by malonyl-CoA:ACP transacylase (FabD) to form malonyl-ACP. Then, fatty acid elongation cycle begins with generation of acetoacetyl-ACP via Claisen condensation of acetyl-CoA and malonyl-ACP by a β-ketoacyl-ACP synthase III (FabH). Next, 3-hydroxyacyl-ACP (or 3-hydroxybutyryl-ACP in the first cycle) is produced by a β-ketoacyl-ACP reductase (FabG) and then converted to enoyl-ACP (or 2-butenoyl-ACP in the first cycle) by a 3-hydroxyacyl-ACP (FabA and FabZ). Further, enoyl-ACP is reduced to acyl-ACP (or butyryl-ACP in the first cycle) by an enoyl-ACP reductase (FabI). Butyryl-ACP then enters the second round of elongation cycle by a condensation with malonyl-ACP catalyzed by β-ketoacyl-ACP synthase I or II (FabB/FabF) and the cyclic process of two carbon addition begins until acyl-ACPs of 16 and18 carbons. Acyl-ACPs are then converted to fatty acids via a thioesterase reaction of TesA. *E. coli* native fatty acid degradation pathway or β-oxidation pathway also plays an important role in renewable production of chemicals as, for example, fatty acid degradation pathway could compete for precursor intermediates of fatty acid-derived pathways for bio-based chemicals (Cao *et al.*, 2016). In fatty acid degradation pathway, fatty acids are activated by fatty acyl-CoA synthetase (FadD) to form acyl-CoA. Acyl-CoA is then converted to enoyl-CoA by an

acyl-CoA dehydrogenase (FadE). The following steps include hydration of enoyl-CoA to 3-hydroxyacyl-CoA by a enoyl-CoA hydratase/β-hydroxy acyl-CoA dehydrogenase (FadB/FadJ), dehydrogenation of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA by a enoyl-CoA hydratase/β-hydroxy acyl-CoA dehydrogenase (FadB/FadJ) and thiolation of 3-ketoacyl-CoA and CoA to form acyl-CoA and acetyl-CoA by a β-keto acyl-CoA thiolase (FadA/FadI), respectively. At the end of every degradation cycle, one molecule of acetyl-CoA is generated and further used in cellular metabolism. The overall fatty acid biosynthesis and degradation pathways (Lennen & Pfleger, 2013; Janßen & Steinbüchel, 2014; Tao *et al.*, 2016) are shown in Figure 2.4.

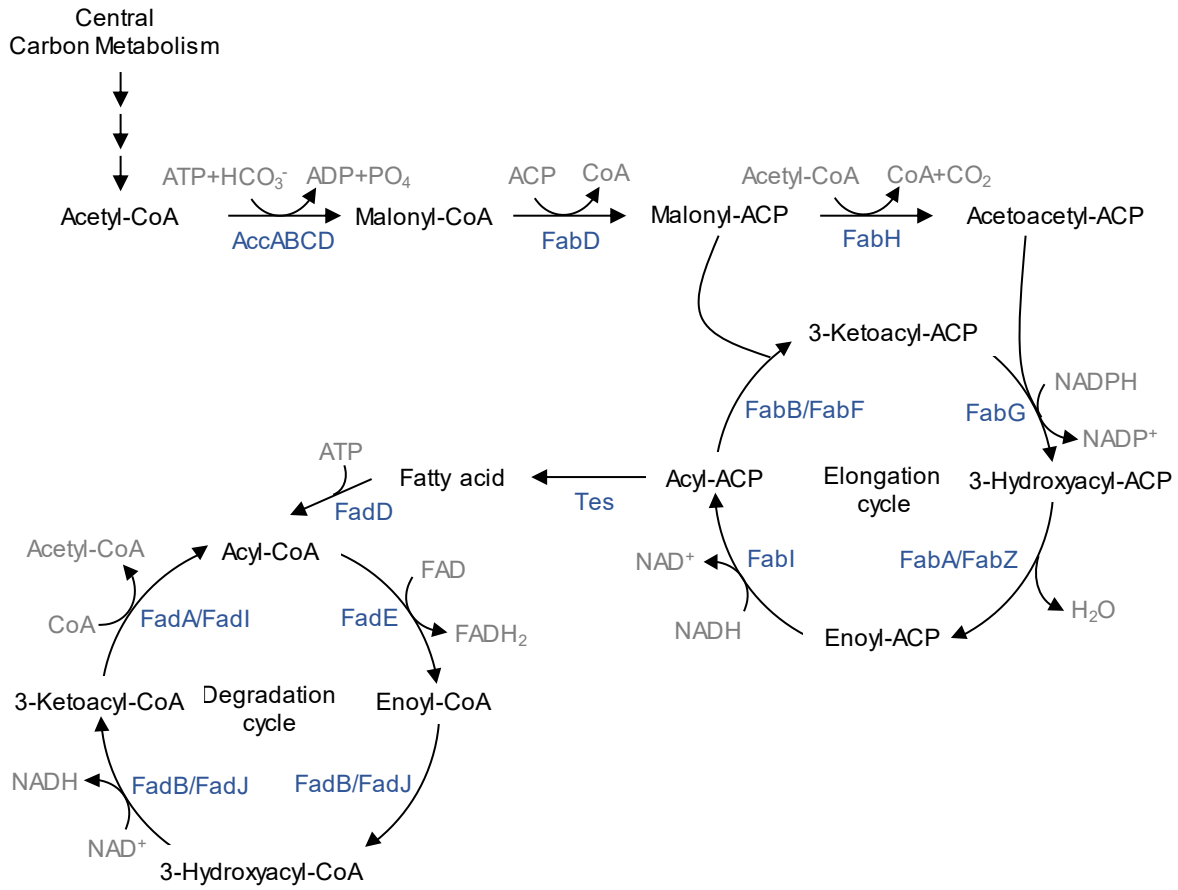

Figure 2.4 Native fatty acid biosynthesis and degradation cycles in *E. coli*
The diagram is adapted from Lennen and Pfleger, 2013; Janßen and Steinbüchel, 2014; and Tao *et al.*, 2016. AccABCD, Acetyl-coA carboxylase; FabD, Malonyl-CoA:ACP transacylase; FabH, β-Ketoacyl-ACP synthase III; FabB/FabF, β-Ketoacyl-ACP synthase I or II; FabG, β-Ketoacyl-ACP reductase; FabA/FabZ, β-hydroxyacylACP dehydratase/isomerase; FabI, enoyl acyl-ACP reductase; Tes, thioesterase; FadD, Fatty acyl-CoA synthetase; FadE, Acyl-CoA dehydrogenase; FadB/FadJ, Enoyl-CoA hydratase/β-hydroxy acyl-CoA dehydrogenase; FadA/FadI, β-Keto acyl-CoA thiolase. Pathway enzymes are indicated in blue.

2.3.2 Cyanobacteria

Cyanobacteria are another interesting host microorganism for bio-based production because of their ability to perform oxygenic photosynthesis. With this ability, cyanobacteria convert CO_2, H_2O, and sunlight through photosynthesis to chemicals of interest. A wide range of compounds have been produced in cyanobacteria via metabolic engineering (Savakis & Hellingwerf, 2015; Carroll *et al.*, 2018). This demonstrates that cyanobacteria can be genetically modified for production of attractive chemicals. The commonly used cyanobacterial strains in metabolic engineering are *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. PCC 7002, and *Synechocystis* sp. PCC 6803 (Ruffing, Jensen & Strickland, 2016), as they are unicellular cyanobacteria with well-exploited genome sequences (Chen & Widger, 1993; Kaneko *et al.*, 1995; Kaneko & Tabata, 1997; Holtman *et al.*, 2005; Nakao *et al.*, 2010). In this study, *Synechocystis* sp. PCC 6803 is used for metabolic engineering. This cyanobacterium was first isolated in 1968 and has been intensively studied (Yu *et al.*, 2013). Fatty acid biosynthesis in cyanobacteria is similar to in *E. coli*; however, it lacks degradation cycles (Figure 2.5). The ability to utilize fatty acid is catalyzed by acyl-acyl carrier protein synthetase (Aas) (Beld, Finzel & Burkart, 2014), which converts fatty acid back to acyl-ACP unlike in *E.coli* where fadD converts fatty acids to fatty acyl-CoAs before entering degradation cycles. The elongation cycles in cyanobacteria continue, until C16 and C18 acyl-ACPs are converted to phosphatidic acid – a building block for cell membrane biosynthesis – catalyzed by phosphate acetyltransferase (PlsX), acylglycerol-phosphate acyltransferase (PlsY), and lysophosphatidic acid acyltransferase (PlsC), respectively in three reaction steps (Wang *et al.*, 2020). This is another difference from *E. coli* cycles.

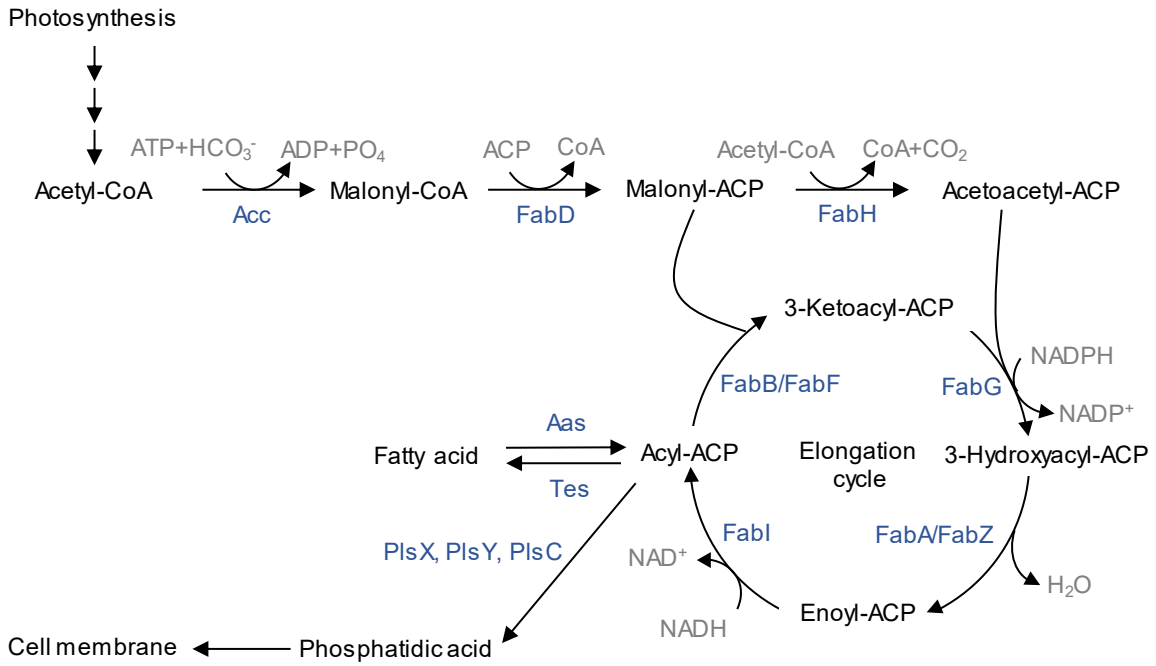

Figure 2.5 Native fatty acid biosynthesis in cyanobacteria
The diagram is adapted from Figure 2.4, Yunus, 2019; and Wang *et al.*, 2020. Acc, acetyl-coA carboxylase; FabD, malonyl-CoA:ACP transacylase; FabH, β-Ketoacyl-ACP synthase III; FabG, β-Ketoacyl-ACP reductase; FabA/FabZ, β-hydroxyacylACP dehydratase/isomerase; FabI, enoyl-ACP reductase I; FabB/FabF, β-Ketoacyl-ACP synthase I or II; Tes, thioesterase; Aas, acyl-ACP synthetase; PlsX, phosphate acetyltransferase; PlsY, acylglycerol-phosphate acyltransferase; PlsC, lysophosphatidic acid acyltransferase. Pathway enzymes are indicated in blue.

2.4 1-Octanol as the first proof-of-concept chemical

1-Octanol is a C8 fatty alcohol that can be used as a fuel because of its diesel-like properties (Kremer *et al.*, 2015) and has also been used as a precursor for ester synthesis in flavoring and fragrance industries. 1-Octanol has been reported to accumulate in some microorganisms and plants, however, at low levels (Hamilton-Kemp *et al.*, 2005; Song *et al.*, 2017). A comparison of well-known bio-based alcohols, ethanol and 1-butanol, with 1-octanol on microbial production and combustion characteristics has been comprehensively reviewed (Kremer *et al.*, 2015). In this review, 1-octanol was shown to be superior than other alcohols in some respects. One of the most beneficial characteristics is that 1-octanol has higher energy density compared with ethanol and 1-butanol. It is also shown to be more suitable for compression-ignition (CI) engines or also known as diesel engines.

2.4.1 1-Octanol synthetic pathways

1-Octanol has been produced by engineered *E. coli*, yeast and cyanobacteria (Dellomonaco *et al.*, 2011; Machado *et al.*, 2012; Marcheschi *et al.*, 2012; Akhtar *et al.*, 2015; Henritzi *et al.*, 2018; Yunus & Jones, 2018). To my knowledge, there are 4 existing synthetic

routes for 1-octanol production in *E. coli* (*i*) the reversal of β-oxidation (Dellomonaco *et al.*, 2011) (*ii*) redirecting branched-chain amino acid biosynthesis (Marcheschi *et al.*, 2012) (*iii*) extending the 1-butanol pathway (Machado *et al.*, 2012) and (*iv*) the carboxylic acid reductase-dependent pathway (Akhtar *et al.*, 2015; Henritzi *et al.*, 2018; Yunus & Jones, 2018).

The first synthetic route for 1-octanol is via the reversal of β-oxidation cycle. This pathway allows the production of linear *n*-alcohols (C≥4) and fatty acids. Acetyl-CoA is directly utilized with acyl-CoA by a thiolase (YqeF/FadA). This starts the reverse β-oxidation cycle (Figure 2.6) until the final step catalyzed by aldehyde-forming acyl-CoA reductases and alcohol dehydrogenases that convert acyl-CoAs and yield *n*-alcohols. 1-Octanol was produced at approximately 100 mg/L from this synthetic route (Dellomonaco *et al.*, 2011). However, this pathway yielded a mixture of various chain-length *n*-alcohols and required complex regulations and several strain modifications.

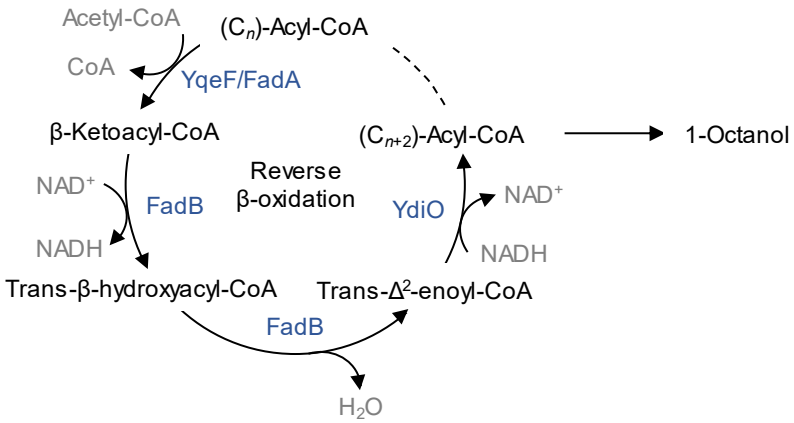

Figure 2.6 1-Octanol synthetic pathway via the reverse β-oxidation cycle adapted from Dellomonaco *et al*., 2011
YqeF/FadA, thiolase; FadB, hydroxyacyl-CoA dehydrogenase; FadB, enoyl-CoA hydratase; YdiO, enoyl-CoA reductase. The final step to generate 1-octanol is catalyzed by aldehyde-forming acyl-CoA reductases and alcohol dehydrogenases. Pathway enzymes are indicated in blue.

The second pathway is a synthetic "+1" recursive metabolic pathway, which relies on a cyclic process of one carbon addition. This pathway was derived from a branched-chain amino acid biosynthesis and protein engineering to alter enzyme activity was used to redirect the native branched-chain amino acid biosynthesis pathway toward targets of interest. The simplified pathway diagram is shown in Figure 2.7. Although 2-ketobutyrate is a natural metabolite, overexpression of ThrABC and IlvA was to drive the flux toward 2-ketobutyrate. LeuA was mutated (H97A, S139G, N167G, P169A, G462D) to catalyze 2-ketobutyrate (C4) and acetyl-CoA to form 2-ketopentanoate (C5) with a release of 1 molecule of CO_2 in the first cycle. 2-Ketononanoate was yielded after 5 repeating cycles and then converted to octanal and 1-octanol by decarboxylase (KivD) and dehydrogenase (Adh6), respectively. However,

the 1-octanol titer reported from this synthetic pathway was only 2 mg/L (Marcheschi *et al.*, 2012).

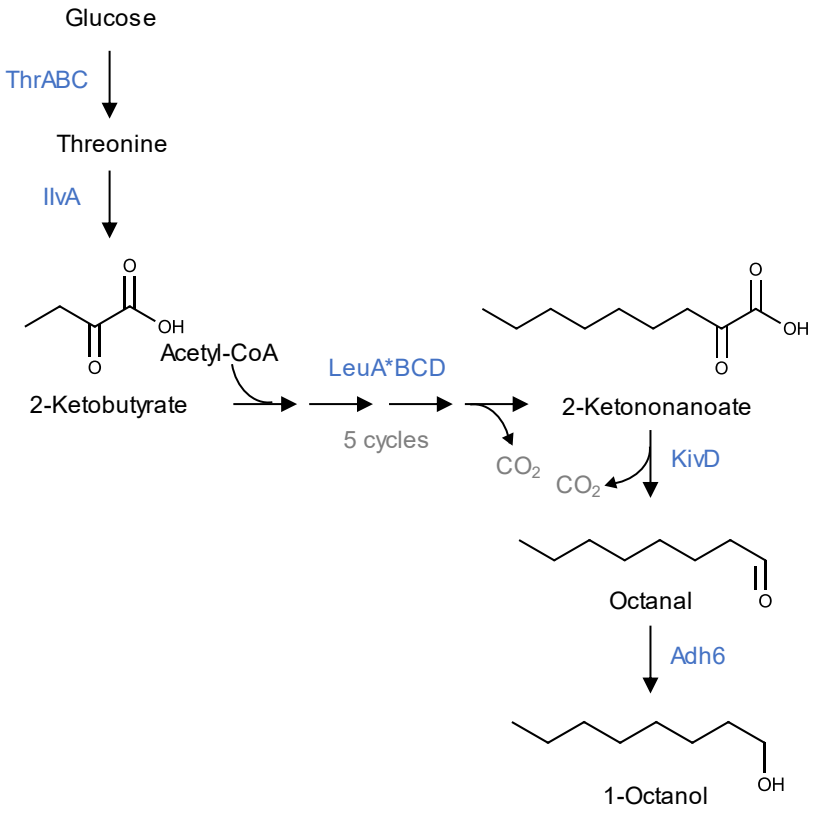

Figure 2.7 Simplified "+1" recursive metabolic pathway for 1-octanol production adapted from Marcheschi *et al.*, 2012
ThrABC, threonine operon; IlvA, *Bacillus subtilis* threonine dehydratase; LeuA*, mutated 2-isopropylmalate synthase; LeuCD, isopropylmalate isomerase complex; LeuB, isopropylmalate dehydrogenase; KivD, decarboxylase; Adh6, dehydrogenase. Pathway enzymes are indicated in blue.

The third pathway depends on a modified Clostridium CoA-dependent *n*-butanol synthesis pathway. The native elongation of acetyl-CoA to butyryl-CoA (chain elongation round 1) was extended by expression of acetyl-CoA acetyltransferase (BktB), which catalyzed the first step in chain elongation round 2. This enzyme converted acetyl-CoA and butyryl-CoA (intermediate from the chain elongation round 1) to 3-ketohexanoyl-CoA. The chain elongation continued for another round and octanoyl-CoA was produced prior to formation of octanal and 1-octanol by aldehyde/alcohol dehydrogenase. The simplified pathway diagram for the first and second chain elongation rounds is shown in Figure 2.8. 1-Octanol yielded from this pathway was approximately 70 mg/L (Machado *et al.*, 2012); however, this pathway requires the expression of several heterologous genes and yielded relatively low 1-octanol compared to other alcohols produced from the same pathway.

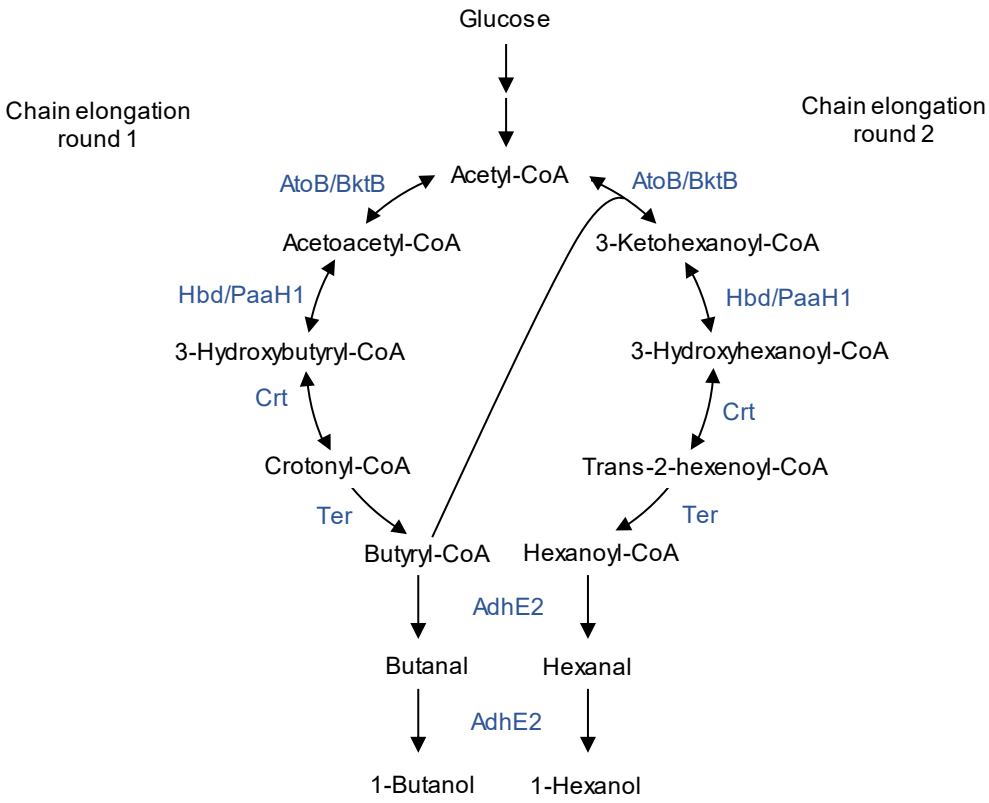

Figure 2.8 Simplified Clostridium CoA-dependent pathway for *n*-alcohol production adapted from Machado *et al.*, 2012

AtoB, acetyl-CoA acetyltransferase; BktB, *Ralstonia eutropha* β-ketothiolase; Hbd/PaaH1, *C. acetobutylicum* or *R. eutropha* 3-hydroxy-acyl-CoA dehydrogenase; Crt, *C. acetobutylicum* crotonase; Ter, *Treponema denticola* trans-enoyl-CoA reductase; AdhE2, *C. acetobutylicum* aldehyde/alcohol dehydrogenase. Pathway enzymes are indicated in blue.

The last synthetic route for 1-octanol biosynthesis is carboxylic acid reductase-dependent pathway. 1-Octanol was produced in *E. coli* at 62 mg/L from this synthetic route. In details, octanoyl-ACP from native fatty acid synthesis pathway is converted to octanoic acid and released free ACP by a thioesterase (Tes3). Octanoic acid is then converted to octanal by an activated carboxylic acid reductase (CAR). CAR is activated by a phosphopantetheinyl transferase (Sfp). Finally, octanal is catalyzed by an overexpressed aldehyde reductase (AHR) to form 1-octanol. The pathway diagram is shown in Figure 2.9. This pathway has advantages over the others as (*i*) the specificity toward 1-octanol is high at ~70% of total alcohol production depending mainly on the performance of the chosen thioesterase (Tes) (*ii*) the productivity is high at 4.4 mg/L/h compared to the others (*iii*) only 3-4 genes are expressed without major strain modifications needed (Table 2.2). With these advantages, however, the productivity of 1-octanol is insufficient for 1-octanol to be produced in a commercial system. As the maximum theoretical yield of 1-octanol from this pathway is 361 mg/g glucose and this work only reported 12 mg/g glucose. This emphasizes that there is room for improvements. Altogether, this pathway was selected in this study as the first proof-of-principle pathway for bioderivatization.

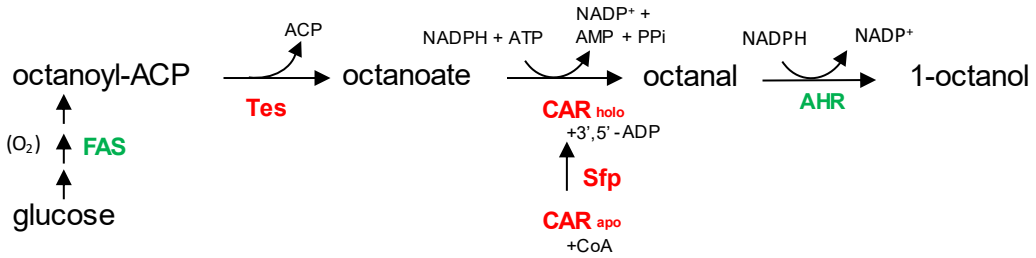

Figure 2.9 Carboxylic acid reductase dependent 1-octanol pathway adapted from Akhtar *et al.*, 2015
E. coli native fatty acid synthesis (FAS); Tes3, C8-preferring thioesterase; CAR, carboxylic acid reductase; Sfp, phosphopantetheinyl transferase; AHR, aldehyde reductase.

Table 2.2 summarizes current achievements on 1-octanol production using microbial cell factories and renewable substrates.

Table 2.2 1-Octanol production from *E. coli* via metabolic engineering (Data from Akhtar *et al.*, 2015)

Strain	Titers (mg/L)	Yield (mg/ g glucose)	Productivity (mg/L/h)	Cultivation condition	Reference
MG1655 *fadR, atoC*(con), *ΔarcA, Δcrp::crp*, ΔadhE, Δpta, ΔfrdA, ΔfucO, ΔyqhD, ΔfadD, fadBA, betA*	100	25	2.1	48 h, 37°C, shake-Flasks, M9-based medium with vitamins and metals	(Dellomonaco *et al.*, 2011)
ATCC98082 (threonine overproducing strain) *ΔrhtA, thrA, thrB, thrC, ilvA, leuA, leuB, leuC, leuD, kivD, adh6*	2	-	0.04	M9 medium with yeast extract	(Marcheschi *et al.*, 2012)
BW25113 *F^0 [traD36, proAB+, $lacI^q$ ZΔM15 (tetR)], ΔldhA, ΔadhE, ΔfrdBC, Δpta*	70	-	1.5	48 h, 30°C, culture tubes, terrific broth	(Machado *et al.*, 2012)
BL21(DE3) *tes, sfp, car, ahr*	62	12	4.4	14 h, 30°C, shake-flasks, M9-based medium with vitamins and metals	(Akhtar *et al.*, 2015)

* Modified gene

2.4.2 Enzymes in carboxylic acid reductase-dependent 1-octanol pathway

As mentioned, the carboxylic acid reductase-dependent pathway implemented by Akhtar *et al.*, 2015 for 1-octanol production depends on four enzymes, C8-preferring

thioesterase (Tes3), carboxylic acid reductase (CAR), phosphopantetheinyl transferase (Sfp), and aldehyde reductase (AHR). Thioesterase is an enzyme that catalyzes the formation of fatty acids by hydrolyzing the thioester bond between fatty acids and ACP or CoA. In *E. coli*, native thioesterases (Tes) terminate the elongation cycle and form fatty acids, the fatty acids then sequentially enter the degradation cycle (Figure 2.4). Thioesterase has been identified as a limiting enzyme in 1-octanol production in cyanobacteria via the carboxylic acid reductase-dependent pathway (Yunus, 2019). Lozada *et al*., 2018 recently reported a highly active C8 thioesterase variant originally from *Cuphea palustris* (CpFatB1) that could yield up to 1.7 g/L octanoic acid. The CpFatB1 thioesterase was modified and resulted in >90% specificity toward octanoyl-ACP. These modifications have also been employed in this study. The second enzyme, carboxylic acid reductase (CAR), is an enzyme that converts fatty acids and forms aldehydes. It was shown that CAR was active on a wide range of substrates (Akhtar, Turner & Jones, 2013) and was not limiting in 1-octanol production in cyanobacteria (Yunus, 2019). Phosphopantetheinyl transferase (Sfp) is an enzyme that activates CAR and maximizes the function of CAR (Venkitasubramanian, Daniels & Rosazza, 2007). Aldehyde reductases (AHR) are native enzymes in *E. coli* that catalyze the reduction of aldehydes into their corresponding alcohols. Although, overexpression of an AHR from *E. coli* has shown to improve the productivity of 1-butanol in carboxylic acid reductase-dependent pathway (Pásztor *et al.*, 2015) and removal of native AHRs has also been reported to result in higher production of aldehydes (Rodriguez & Atsumi, 2012). There are in total of 13 native AHRs reported in *E. coli* (Rodriguez & Atsumi, 2014); therefore, the overexpression of an extra AHR is optional.

2.4.3 The toxicity of alcohols

Alcohols have been shown to be toxic to microbial hosts (Ruffing & Trahan, 2014; Wilbanks & Trinh, 2017). The toxic effects often show as growth defects and low final product titers (Yunus & Jones, 2018). When a range of commonly found fermentation products (carboxylic acids, esters and alcohols) were tested for their toxicity, it showed that alcohols were more toxic than esters and carboxylic acids with the same total carbon number (≥C5) (Wilbanks & Trinh, 2017). Therefore, this is presumed to render from their hydroxyl group (-OH). The relatively small hydroxyl group of alcohols compared to carboxyl group (-COOH) of carboxylic acids, for example, increases the ability of alcohols to penetrate cell membranes (Wilbanks & Trinh, 2017). Since alcohols are in direct contact with microbial cell membranes and known to compromise the integrity of cell membranes. This sequentially affects membrane fluidity and destroys normal membrane structures. In response, cells radically change fatty acid compositions of cell membrane for adaptation (Ingram, 1976;

Ingram, Vreeland & Eaton, 1980). In a more severe instance, this adaptation is not enough to cope with the toxicity and results in cell membrane disruption and diminished growth (Huffer *et al.*, 2011), thereby observed as a growth defect. Moreover, protein misfolding and denaturation including key enzymes in central metabolism were observed in yeasts after exposure to high ethanol concentrations (Ding *et al.*, 2009). Intracellular production of chemical co-chaperones such as trehalose is another mechanism that microbial cells use to defend themselves as these co-chaperones could prevent the aggregation of misfolded proteins (Ding *et al.*, 2009). Other osmoprotectants such as glycine, betaine and glycerol also play an important role in alcohol stress responses. These molecules are produced to maintain proton gradients of cell membrane and, in turn, prevent cell lysis (Gonzalez *et al.*, 2003; Huffer *et al.*, 2011).

It is worth noting that each alcohol displays varying toxicity effects to cell growth, which may be due to differences in their alkyl-chains. 1-Octanol is more toxic than other shorter chain-length alcohols as it was shown that a 1-octanol concentration of 0.025% (v/v) is enough to reduce the growth of *E. coli* by 50% whilst 1-butanol and isobutanol, which are also produced by genetically modified *E. coli*, are less toxic only starting to show growth defects at 1.5% (v/v) and 0.8% (v/v), respectively (Mukhopadhyay, 2015). Similarly, 1-hexanol also showed high toxicity where 500 mg/L (~0.06% (v/v)) completely inhibited the growth of *E. coli* (Rodriguez, Tashiro & Atsumi, 2014). Overall, this may suggest that the toxicity is corelating with the number of carbon on alkyl-chains; however, 1-dodecanol, which is a C12 fatty alcohol, was not highly toxic to *E. coli* as the cells could still utilize all available carbon source within 19 h even in the presence of 100 g/L of 1-dodecanol (Liu *et al.*, 2016b). However, at this concentration, it is highly above the water solubility of 1-dodecanol at ~0.004 g/L (Institute for Occupational Safety and Health of the German Social Accident Insurance (IFA)., 2020) which may result in phase separation and limit the contact of 1-dodecanol to bacterial cell membrane.

2.5 Bioderivatization via *O*-acetylation

Esters are highly attractive compounds used in several industries. Comprehensive uses and economic aspects of esters have been reviewed by Riemenschneider and Bolt, 2005. They are most recognized for their pleasant odors as they contribute to scents in flowers and fruits (Aharoni *et al.*, 2000; Shalit *et al.*, 2003; Souleyre *et al.*, 2005). Thus, they are widely used in fragrance and flavoring industries. Moreover, they are considered as biofuels and solvents. For example, ethyl acetate is a common solvent used for extraction of plant-derived bioactive compounds (Lee *et al.*, 2014). To meet market demands, esters are produced via several chemical reactions including acylation with acyl halides and alkylation of metal carboxylates depending on particular esters in questions (Riemenschneider & Bolt, 2005).

Among these, the most common and simplest reaction is Fisher esterification of organic acid and alcohol substrates at high temperature in the presence of a strong acid as a catalyst (Riemenschneider & Bolt, 2005). In this study, esters are a group of derivatives investigated. This is inspired by a detoxification mechanism in yeasts (Saerens *et al.*, 2010) as mentioned in Section 2.2.

2.5.1 Biotechnological properties of esters

Esters are valuable compounds since they are used in several industries as mentioned. Additionally, they also have several valuable properties as a biotechnological product. Firstly, esters are generally less toxic than their corresponding alcohols or acids with the same carbon number (Wilbanks & Trinh, 2017). Secondly, it was hypothesized that the synthesis of esters regenerates free CoA without releasing high toxic concentrations of fatty acids under some conditions (Saerens *et al.*, 2010). Lastly, esters are known to be more volatile and less water-soluble than their corresponding alcohols with the same number of carbons which is likely to reduce the energetic cost of the separation and purification processes from the aqueous fermentation media via distillation (Oudshoorn, Van Der Wielen & Straathof, 2009). In addition, short chain length esters are generally excreted from the cell (Layton & Trinh, 2016b), thus bypasses the harvest process from intracellular fraction such as cell lysis and potentially reduces production costs.

2.5.2 Alcohol *O*-acetyltransferase (AAT)

Alcohol *O*-acetyltransferases (AATs) are enzymes in E.C. 2.3.1.84 category that catalyze the transfer of an acyl group (usually from an acetyl-CoA donor) to a primary alcohol acceptor in plant or yeast natural ester biosynthesis (Aharoni *et al.*, 2000; Mason & Dufour, 2000; Lin, Zhu & Wheeldon, 2016). This group of enzymes is generally recognized by 2 conserved regions 1) H-X-X-X-D for catalytic functions and 2) D-F-G-W-G for structural integrity (Ma *et al.*, 2005; Galaz *et al.*, 2013). As mentioned in Section 2.2, native AATs are found in yeasts arguably as a detoxification enzyme that converts toxic metabolites to esters (Saerens *et al.*, 2010). ATF1 and ATF2 are the most well-studied AATs from *S. cerevisiae* that contribute to fruity smells in wine by catalyzing ethyl acetate and isoamyl acetate formation (Lilly *et al.*, 2006). Moreover, AATs are responsible for fruity scents in many fruits including strawberry (Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004), apple (Souleyre *et al.*, 2005) and banana (Beekwilder *et al.*, 2004). Previous studies also suggested that AATs showed broad range specificity toward their substrates (Verstrepen *et al.*, 1988; Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004; Souleyre *et al.*, 2005; Rodriguez, Tashiro & Atsumi, 2014; Layton &

Trinh, 2016a). It has also been shown that similarity of AAT amino acid sequences could not be used to predict substrate preferences (Beekwilder *et al.*, 2004). To give an instance, SAAT and VAAT are AATs from two species of strawberry with 86% identical amino acid sequences; however, the substrate preference profiles of these enzymes are different as SAAT preferred C6-C10 aliphatic alcohols whereas VAAT preferred shorter ones (Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004). AAT enzymes used in this study and their reported activity are listed in Table 2.3.

Table 2.3 List of AAT enzymes used in this study and examples of their reported substrates

Enzyme	Source organism	Substrate		Reference
		Acyl-CoA	Alcohol	
CAT	*E. coli*	Acetyl-CoA	Chloramphenicol, Isobutanol	(Rottig & Steinbuchel, 2013;Rodriguez, Tashiro & Atsumi, 2014)
SAAT	*Fragaria ananassa cv. Elsanta*	Acetyl-CoA	1-Octanol, geraniol	(Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004)
ATF1	*Saccharomyces cerevisiae*	Acetyl-CoA	1-Octanol, 1-butanol	(Rodriguez, Tashiro & Atsumi, 2014; Layton & Trinh, 2016a)
VAAT	*Fragaria vesca*	Acetyl-CoA	Trans-2-hexen-1-ol	(Beekwilder *et al.*, 2004)
VpAAT1	*Vasconcellea pubescens*	Acetyl-CoA	Benzyl alcohol	(Balbontín *et al.*, 2010)

As mentioned, AATs have activity on a wide range of substrates, this table only summarizes related substrates in this study or substrates with high activity.

2.5.3 Ester production by genetically engineered *E. coli*

Esters have been produced by genetically engineered *E. coli* as the alternative for petroleum- and plant-derived compounds. The *O*-acetylation step often relies on the use of AATs to catalyze a wide range of alcohol and acyl-CoA substrates. The general schematic diagram of ester production in *E. coli* using AATs is illustrated in Figure 2.10. In 2014, a comprehensive study on ester biosynthesis from glucose was reported (Layton & Trinh, 2014). In this work, several synthetic pathways for alcohols (isobutanol, 1-butanol, isopropanol, ethanol and 1-propanol) were combined with the expression of SAAT. As a result, corresponding acetate and butyrate esters were formed. As mentioned, most AATs are active on a wide range of alcohols; therefore, a mixture of ester final products was observed from the same synthetic pathways that yield a mixture of alcohol intermediates. To give another instance, 2-keto acid-based alcohol pathway, which yields several branch-chained alcohols, was combined with an AAT in order to form a range of branch-chained esters. This allowed the production of several esters including isobutyl acetate and 2-phenethyl acetate

(Rodriguez, Tashiro & Atsumi, 2014). These achievements were marked and followed as several ester platforms were later developed in *E. coli* (Tai, Xiong & Zhang, 2015; Layton & Trinh, 2016b; Chacón, Kendrick & Leak, 2019; Lee & Trinh, 2019). Interestingly, there is no report on renewable production of octyl acetate, which is one of the targeted derivatives in this study, from *E. coli*. However, AATs that are capable of catalyzing 1-octanol and acetyl-CoA have been reported (Table 2.3) (Aharoni *et al.*, 2000; Rodriguez, Tashiro & Atsumi, 2014).

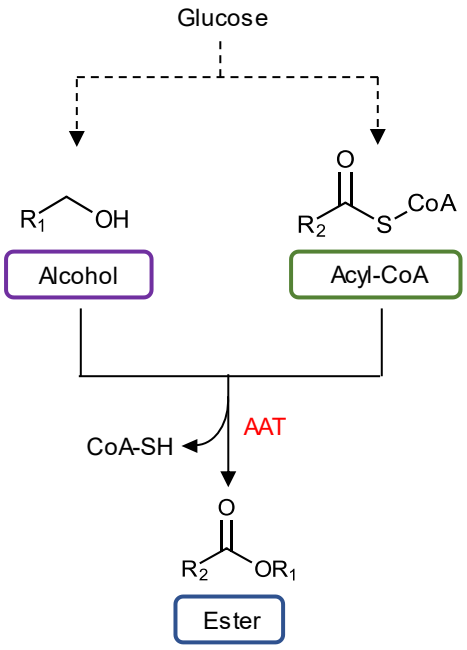

Figure 2.10 Simplified ester production pathway from glucose by AATs
AAT, alcohol acetyltransferase.

2.6 Bioderivatization via *O*-glucosylation

Glycosides are molecules in which sugar is bound to a non-sugar (aglycon) molecule through a glycosidic bond. The non-sugar moiety can vary from specialized metabolites to proteins and the sugar moiety can also range from diverse monosaccharides to polysaccharides (de Roode *et al.*, 2003). There are 4 types of glycosylation in nature, *O*- and *N*-glycosylation, which are commonly found, and *C*- and *S*-glycosylation, which are rarely found in nature. The type of glycosylation is classified based on the aglycon substrates, for example, *O*-glycosylation utilizes an alcohol or phenol where *N*-glycosylation utilizes nitrogen-containing group such as amine and amide (Lafite & Daniellou, 2012; Ati, Lafite & Daniellou, 2017). In this study, *O*-glucosylation is one of the strategical reactions of bioderivatization where the focus is on the transfer of glucose donor (mainly UDP-glucose) to alcohols. Glycosylation is catalyzed by two different types of enzymes; glycosyltransferases (E.C. 2.4.x.y), hereafter GTs (see Section 2.6.2), and glucosidases or glucoside hydrolase (E.C.

3.2.1.x), although the latter typically has evolved to hydrolyse glucosides to release the free aglycon (de Roode *et al.*, 2003; Yauk *et al.*, 2014). The use of glycosides depends on their aglycons. The industrial applications of glycosides are well-demonstrated by de Roode *et al*., 2003. For example, glycosides with long alkyl-chains or alkyl polyglucosides (APGs) are used in detergent and cosmetics industries as they possess surfactant and emulsifying properties (von Rybinski, 1996). On the other hand, glycoside with steroids aglycons are usually used for their antitumor properties (Schneider *et al.*, 2017). Currently, glycosides are synthesized by chemical or enzymatic reactions (de Roode *et al.*, 2003). In this study, glucosides are another group of derivatives investigated as mention in Section 2.2.

2.6.1 Glycoside in living organisms

Glycosides are synthesized naturally in many organisms, for example, they are presumed to play an important role in plant storage and transport of toxic/hydrophobic compounds (Jones & Vogt, 2001). Modification of natural metabolites via glycosylation in plants has three distinct effects outlined previously by Jones and Vogt in 2001 as follows: (*i*) increase solubilization, (*ii*) increase stabilization, and (*iii*) detoxification. The transfer of sugar, which is a highly polar molecule, onto an aglycon increases water solubility of the sugar-bound aglycon compared to free aglycon group (de Roode *et al.*, 2003). This property is considered to facilitate the mobilization and storage of metabolites. Moreover, glycosides are often more inert and non-reactive when compared to their corresponding aglycons (Calderon *et al.*, 1992). Therefore, the glycosylated molecules are less likely to participate in electron transfer, which lowers the reactivity of the molecules and improve their stability. Lastly, glycosylation appears to be a detoxification mechanism in higher plants, where it has been found that toxic metabolites and chemicals accumulate as glycosides. This includes both native specialized metabolites (Halkier & Møller, 1989) and externally applied xenobiotics, e.g., pollutants, pesticides, or even mycotoxin (Loutre *et al.*, 2003; Poppenberger *et al.*, 2003). To my knowledge, the specific impact of glycosylation on toxicity has not been well-studied, this could be due to the diversity within group of glycosides that may display different toxic effects on plants. In general, glycosides are known to be less toxic than their free aglycons as mentioned; therefore, accumulation of glycoside derivatives provides more benefits to plants. Furthermore, similar observations in genetically engineered microorganisms have been seen. For example, expression of enzymes responsible for glycosylation was found to enhance the tolerance of *E. coli* cells to externally supplied toxic molecule as the strain converted this molecule into a glucoside derivative and resulted in higher growth (Huang *et al.*, 2016).

2.6.2 Glycosyltransferase (GT)

Glycosyltransferases (GTs) are a group of enzymes that catalyze the transfer of saccharides from activated sugar donor onto saccharide or non-saccharide acceptors (Lairson *et al.*, 2008). To date, GTs are classified into 111 families under Carbohydrate-Active enZYmes classification (CAZy) (http://www.cazy.org/GlycosylTransferases.html) based on amino acid sequence similarities. Among these, family 1 (also known as UDP glycosyltransferase or UGTs) is the most well-studied family. The GTs from this family often catalyze UDP-glucose as the sugar donor (Wang & Hou, 2009). GTs have a great variation in the range of substrates and play a role in determining chemical diversity in plants (Wang & Hou, 2009). Many plant species mostly contain more than one GTs, for example, whole genome sequence of *Arabidopsis thaliana* revealed 120 GTs (Paquette, Møller & Bak, 2003). GTs used in this study are listed in Table 2.4.

Table 2.4 List of GT enzymes used in this study and examples of their reported substrates

Enzyme	Source organism	Substrate		Reference
		Sugar donor	Aglycon acceptor	
AdGT4	*Actinidia deliciosa*	UDP-glucose	Geraniol, hexanol, octanol and 3-octanol	(Yauk *et al.*, 2014; Schwab, Fischer & Wüst, 2015)
AtGT1	*Arabidopsis thaliana*	UDP-glucose	Hydroquinone	(Lim *et al.*, 2002)
MtG1	*Medicago truncatula*	UDP-glucose	Quercetin	(Shao *et al.*, 2005)
MtH2	*Medicago truncatula*	UDP-glucose	Kaempferol	(Li *et al.*, 2007)
VvGT1	*Vitis vinifera*	UDP-glucose	Cyanidin, geraniol	(Ford, Boss & Hæj, 1998; Schwab, Fischer & Wüst, 2015)

As mentioned, GTs have activity on a wide range of substrates, this table only summarizes related substrates in this study or substrates with high activity.

2.6.3 Glycoside production by genetically engineered *E. coli*

Similar to ester production in *E. coli*, glycosides can be produced by introducing GTs that catalyze alcohols and sugar donors to form corresponding glycosides. Several studies on production of glycosides in genetically engineered *E. coli* have been reported; however, not as many as ester production. Interestingly, the production often needed exogenous feeding of corresponding alcohols or key substrates; thus, not entirely produced from glucose or other cheap carbon sources. This may be due to the glycosides of interest in most cases were derived from complex alcohols or the studies mainly focused on the investigation of GT activity rather than the whole synthetic pathway. There are many reports on *in vivo* glycosylation of

quercetin in engineered *E. coli* due to high market demands of quercetin and its glycosides as bioactive compounds (Piao *et al.*, 2008; Davis, Murphy & Carmichael, 2009; Yin *et al.*, 2013). For instance, production of quercetin glucosides where quercetin was supplemented to *E. coli* expressing GTs (UGT73B3 or UGT84B1) in order to investigate activity and condition of the aforementioned GTs was reported (Xia & Eiteman, 2017). Moreover, novel quercetin glycosides that conjugated to dTDP-6-deoxytalose were also investigated and GTs that catalyzed such reaction were also identified (Yoon *et al.*, 2012). Other glucosides, geranyl glucoside and fisetin glucoside, were also reported in alcohol-feeding manner (Huang *et al.*, 2016; Pandey *et al.*, 2016). To my knowledge, only a few systems have been demonstrated to produce glucosides from cheap and abundant carbon sources. Cinnamyl alcohol glucoside or rosin was produced from engineered *E. coli* by co-expression of cinnamyl alcohol pathway with selected GT from *Rhodiola sachalinensis* (UGT73B6) and *A. thaliana* (UGT73C5) (Zhou *et al.*, 2017). Coculture of two *E. coli* strains, aglycone (AG) and glycoside (GD) strains, has also been used for a production of salidroside from glucose (Liu *et al.*, 2018). In some cases, the results from *in vitro* and *in vivo* glycoside production suggested that the activity of GTs may be limited due to (*i*) free UDP as a product inhibitor (Terasaka *et al.*, 2012) and (*ii*) insufficient UDP-glucose (Masada *et al.*, 2007; Huang *et al.*, 2016).

2.6.4 Sucrose synthase (SUS)

As mentioned in Section 2.6.3 that inhibitory effect of free UDP and the low availability of UDP-glucose showed to be the limiting factors for GT activity. UDP-glucose is a precursor for cell wall biosynthesis in all bacteria; thus, this metabolite is synthesized at a basal level. Therefore, cells maintain efficient regeneration of UDP-glucose for cellular essential needs (Ruffing & Chen, 2006). However, if UDP-glucose will be used as one of the substrates in synthetic pathways, this level of availability may not be enough for high production. Therefore, regeneration of this substrate is important given that the native UDP-glucose pool has a relatively low concentration. Sucrose synthase or SUS (EC 2.4.1.13) is an enzyme that catalyzes a reversible reaction to convert sucrose and UDP to a donor sugar, UDP-glucose, and one molecule of fructose (Geigenberger & Stitt, 1993). Even though SUS catalyzes a reversible reaction, a study suggested that SUS is primarily involved in sucrose breakdown. This reaction can be employed to regenerate UDP-glucose for glucoside production (Figure 2.11). In plant, SUS is an enzyme responsible for nucleotide sugar formation in carbohydrate metabolism (Salerno & Curatti, 2003; Baroja-Fernández *et al.*, 2012). UDP is the preferred substrate for this enzyme, even though it has also been shown to catalyze other nucleotide diphosphates such as ADP (Baroja-Fernández *et al.*, 2003, 2012). Moreover, as mentioned in Section 2.6.2, free UDP from glycosylation reaction could inhibit the function of GTs.

Therefore, by expressing SUS, not only should this effectively recycle the UPD-glucose pool, but also mitigate the inhibition of free UDP. The use of SUS has also been expressed and characterized in *E. coli* (Nakai *et al.*, 1997).

AtSUS1 is one of the sucrose synthases from *A. thaliana* (Zheng *et al.*, 2011) that has been well-studied including for UDP-glucose regeneration purposes (Masada *et al.*, 2007; Terasaka *et al.*, 2012). The use of SUS for enhancing glucoside production has been demonstrated (Masada *et al.*, 2007; Terasaka *et al.*, 2012; Huang *et al.*, 2016). Whether the effects were results from reducing inhibitory effects of free UDP or regeneration of UDP-glucose, the overall conclusion was that the expression of SUS could enhance the production of glucosides.

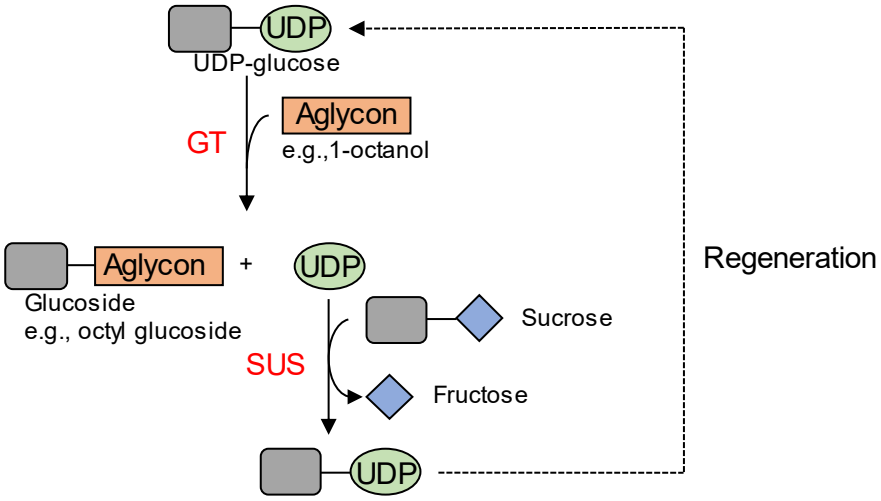

Figure 2.11 Simplified glucoside production and UDP-glucoside regeneration in *E. coli*
UDP-glucose and an aglycon (e.g., 1-octanol) are converted into glucoside (e.g., octyl glucoside) and free UDP by a glycosyltransferase (GT). To regenerate UDP-glucose, free UDP and sucrose are converted to UDP-glucose and fructose by a sucrose synthase (SUS).

Chapter 3
Materials and methods

3.1 *Esherichia coli*

E. coli DH5α (Thermo Fisher Scientific) was used for routine molecular cloning of all plasmids in this study. *E. coli* C43(DE3), BW25113 and JW1794-1 (Δ*fadD*) strains were used for protein expression and production. *E. coli* C43(DE3) is a strain derived from BL21(DE3), this strain is widely used for expression of toxic or membrane proteins under T7 promoter (Lucigen). *E. coli* BW25113 is a parental strain for the Keio Collection of single gene knockouts (Baba *et al.*, 2006) and is commonly used for recombinant protein expression and bioproduction (Yao *et al.*, 2013; Liu *et al.*, 2018). *E. coli* JW1794-1 was derived from BW25113 with only one distinct mutation to knockout *fadD* (Pech-Canul *et al.*, 2011).

3.1.1 LB (Lysogeny) medium

LB liquid medium was prepared by dissolving 6 g of LB powder (Sigma Aldrich) in 300 ml of ultrapure water (MilliQ, Merck). For LB agar, 1.5% (w/v) agar powder (Sigma Aldrich) was also added prior to sterilizing by autoclaving for 15 min at 121°C and 15 psi. Once sterilized, they were kept at room temperature. The LB agar was microwaved until fully melted and cooled down to 50-60°C before antibiotics were added, if needed, and poured onto 100-mm petri dishes (VWR).

3.1.2 M9 minimal medium

M9 minimal medium used in all production experiments were prepared from stock solutions as follows (Table 3.1).

Table 3.1 Stock solutions for M9 minimal medium

Composition	Stock concentration	Final concentration	Source
M9 salts	5x	1x	Sigma Aldrich
Glucose	20% (w/v)	2% (w/v)	Sigma Aldrich
$MgSO_4$	1 M	2 mM $MgSO_4$	Sigma Aldrich
$CaCl_2$	1 M	0.1 mM $CaCl_2$	Sigma Aldrich

The stock solutions were prepared and autoclaved separately. To prepare 1 L of M9 minimal medium with 2% (w/v) glucose, 200 ml of 5x M9 salts, 100 ml of 20% (w/v) glucose,

2 ml of 1M $MgSO_4$ and 100 µl of $CaCl_2$ were mixed before final volume was adjusted to 1 L by sterile ultrapure water. The antibiotics were then added to the solution, if needed.

3.1.3 Antibiotics preparation for *E. coli*

Table 3.2 is the list of antibiotic stock solutions and their final concentrations in culture media. The stock solutions were stored in -20°C freezer and thawed before use. To prepare an antibiotic stock solution, antibiotic powder was dissolved in appropriate solvent. Filter sterilization with a 0.2 µm syringe filter (PALL Corporation) was used to sterilize antibiotics that were dissolved in water. All stock solutions were then kept in -20°C freezer.

Table 3.2 Antibiotics preparation

Antibiotic	Stock concentration	Final concentration	Solvent	Source
Spectinomycin	50 mg/ml	50 mg/L	Ultrapure water	Sigma Aldrich
Carbenicillin	100 mg/ml	100 mg/L	Ultrapure water	Sigma Aldrich
Kanamycin	50 mg/ml	50 mg/L	Ultrapure water	Sigma Aldrich
Erythromycin	20 mg/ml	200 mg/L	Ethanol	Sigma Aldrich
Chloramphenicol	37 mg/ml	37 mg/L	Ethanol	Sigma Aldrich

3.1.4 Glycerol stocks for *E. coli*

All strains were cryopreserved in 16% (v/v) glycerol by mixing 800 µl of overnight cultures in LB liquid medium with 200 µl of sterile 80% (v/v) glycerol in a 2-ml cryogenic vial (VWR). The cryogenic vial was then stored in -80°C freezer. To revive *E. coli* strain from glycerol stocks, an inoculation loop was used to transfer the strains to a LB agar plate with suitable antibiotics or inoculate in LB broth in 50-ml centrifuge tube (Thermo Fisher Scientific or VWR).

3.1.5 Preparation of chemically competent cells

Chemically competent cells of *E. coli* strains were prepared according to Hanahan protocol (Hanahan, 1983). *E. coli* strains were cultivated overnight at 37°C, 180 rpm in 5 ml LB media with suitable antibiotics, if needed. The overnight culture (100 µl) was then used to inoculate in 100 ml fresh LB media with suitable antibiotics in a 500-ml Erlenmeyer flask and cultivated at 37°C, 180 rpm. After 2 h of incubation with OD_{600} around 0.3-0.5, the culture was cooled down on ice for 20 min before pouring to two 50-ml centrifuge tubes prior to centrifugation at 4,000 x*g* for 5 min at 4°C. Supernatant was discarded and the cell pellet was resuspended in 5 ml of ice-cold 100 mM $MgCl_2$ by slowly pipetting up and down. The cells were centrifuged at 4,000 x*g* for 5 min at 4°C and cell pellet was resuspended in 10 ml of ice-cold 100 mM $CaCl_2$. The centrifugation was repeated, and the cell pellet was resuspended in

5 ml of ice-cold 85 mM $CaCl_2$ with 15% glycerol. The cells were centrifuged again and finally resuspended in 2.5 ml of ice-cold 85 mM $CaCl_2$ with 15% glycerol before aliquoting 50 µl to pre-cooled microcentrifuge tubes. The tubes were stored at -80°C.

3.1.6 *E. coli* heat-shock transformation

Competent cells were taken out of -80°C freezer and thawed on ice for 20 min before 1 µl of plasmid(s) or 10 µl of ligation mixture was put in 50 µl of competent cells. The tubes were left on ice for another 30 min before heat shock at 42°C for 45 sec then put back on ice for 2 min. Fresh LB liquid medium (950 µl) was then added to each tube. The competent cells were then incubated at 37°C, 180 rpm for 1 h before centrifugation (8,000 x*g*, 2 min). Supernatant (900 µl) was discarded, the cells were resuspended and plated on LB agar with suitable antibiotics. LB agar plate was then incubated at 37°C in a benchtop incubator (Labnet international, Inc) for 1-2 days until colonies were observed.

3.1.7 *E. coli* cultivation condition

To prepare *E. coli* cultures for plasmid extraction or precultures for production, one single colony of *E. coli* from agar plate or from glycerol stock was inoculated in 5-10 ml of LB media with suitable antibiotics in 50-ml centrifuge tube then incubated overnight at 37°C, 180 rpm in a shaking incubator (New Brunswick™ Innova® 44/44R).

To cultivate *E. coli* for the production of compounds of interest, the overnight precultures were washed twice by centrifugation at 4,000 x*g* for 5 min and resuspending in fresh M9 minimal medium. The OD_{600} of resuspended cultures in M9 minimal medium was measured and calculated to adjust the volume for OD_{600} 0.1 in 25 ml M9 minimal medium in 100-ml Erlenmeyer flasks. All strains were incubated at 37°C, 180 rpm for 4 h before the induction and the incubation was continued at 30°C, 150 rpm for 48 or 72 h. A solvent overlay was applied to the culture at the time of induction to capture volatile products in some experiments as specified in the relevant section of results.

To cultivate *E. coli* in 96-well or 24-well plates, the precultures were prepared and washed in the same way with cultivation in flasks. Different volumes (200 µl for 96-well plate or 1 ml for 24-well plate) of culture media were used. The plate was incubated in Tecan Infinite M200 Pro Spectrophotometer (Tecan) at 37°C (unless mentioned otherwise) with continuous shaking at 432 rpm (amplitude 1, orbital shaking) and OD_{600} was measured every hour.

3.1.8 Cell density measurement

Cell optical density (OD) at 600 nm was measured in a 1.5-ml cuvette (Brandtech Scientific, Inc.) using a Tecan200P microplate reader as an indicator for growth. The fresh LB or ultrapure water was used as blank. If the cultures were dense, they were diluted prior to the

measurement. In this case, OD values were calculated accordingly to dilutions. To convert OD_{600} to biomass (g Cell Dry Weight/L (gCDW/L)), calibration curves correlating between biomass and OD_{600} for *E. coli* C43(DE3) and BW25113 were obtained (Appendix I).

3.1.9 *E. coli* toxicity assay

To investigate the toxic effects of different chemicals to cell growth, well-plates (24 or 96-well) were used and incubated in a Tecan microplate reader at 37°C, 432 rpm (see Section 3.1.7). A range of different concentrations of chemicals were added to *E. coli* culture with the same starting OD_{600} at the beginning of the cultivation. Where a fully soluble stock solution was not possible to prepare, each compound was spiked directly to each well. The specific growth rates were calculated for each treatment.

3.1.10 *E. coli* feeding experiment

To screen for activity of AAT enzymes on substrates of interest, feeding experiment in 100-ml Erlenmeyer flasks was conducted. The precultures were prepared similarly to the production assay (see Section 3.1.7). The strains were cultivated in M9 minimal medium with 2% (w/v) glucose and induced with 0.5 mM IPTG at the beginning of the incubation. The cultures were incubated at 30°C, 150 rpm. After 2 h, menthol (5 mM) or eugenol (2.5 mM) was spiked to the cultures and the incubation was continued at the same condition for 48 h.

3.2 Cyanobacteria

Synechocystis sp. PCC 6803 obtained from Prof. Klaas Helingwerf (University of Amsterdam) was used as a wild-type cyanobacterial strain in this study. Two modified *Synechocystis* sp. PCC 6803 strains, (*i*) *aas* gene knocked out strain and (*ii*) *aas* gene knocked out with *sfp-car* integrated in chromosomal DNA strain, were used in this study as described in Yunus, 2019.

3.2.1 BG11-Co

BG11 medium without cobalt (referred as BG11-Co) was used in this study as cobalt-inducible promoter was used. To prepare BG11-Co, stock solutions were prepared according to Table 3.3.

Table 3.3 BG11-Co preparation

Composition	Stock concentration	Stock preparation in ultrapure water (100 ml)	Final concentration
BG11-Co	100x	*	1x
Na_2CO_3	1000x	2 g	1x
K_2HPO_4	1000x	3.05 g	1x
Ferric ammonium citrate	1000x	0.6 g	1x

* Preparation of BG11-Co is mentioned below

One liter of BG11-Co stock solution (100x) was composed of $NaNO_3$ (149.6 g), $MgSO_4.7H_2O$ (7.49 g), $CaCl_2.2H_2O$ (3.6 g), Na-citrate.$2H_2O$ (0.89 g), 0.25 M NaEDTA (1.12 ml) pH 8.0, and 1000X trace mineral solution (100 ml). The trace element solution was composed of H_3BO_3 (2.86 g), $MnCl_2.4H_2O$ (1.81 g), $ZnSO_4.7H_2O$ (0.22 g), $Na_2MoO_4.2H_2O$ (0.39 g), and $CuSO_4.5H_2O$ (0.079 g) in 1 L. All solutions were stored at 4°C. To sterilize the medium, all components were mixed together according to Table 3.3 prior to autoclaving for 15 min at 121°C and 15 psi. To prepare BG11-Co agar, 1.5% (w/v) Difco Bacto agar (BD Biosciences) and 0.78% (w/v) of sodium thiosulfate pentahydrate (Sigma Aldrich) were added in the mixed solution before autoclaving. Once the media were sterilized, they were kept at room temperature.

3.2.2 Antibiotics preparation for cyanobacteria

Antibiotics for cyanobacteria cultivation were prepared and used as in Section 3.1.3, except that the final concentration of erythromycin was 20 mg/L for cyanobacteria instead of 200 mg/L. In the case where antibiotics were used to maintain integrated or knocked out genes, one fifth concentration of antibiotics was used.

3.2.3 Glycerol stocks for cyanobacteria

All strains were cryopreserved in 16% (v/v) glycerol by mixing 800 µl of 2-4 days cultures in BG11-Co liquid medium with 200 µl of sterile 80% (v/v) glycerol in a 2-ml cryogenic vial. The cryogenic vial was then stored in -80°C freezer. To revive cyanobacterial strains from glycerol stocks, an inoculating loop was used to transfer the strain from the cryogenic vial to freshly prepared BG11-Co agar plate with suitable antibiotics to maintain the plasmid, if needed. The plate was incubated in a Sanyo MIR-253 light incubator (Panasonic Biomedical) at 30°C for 2 days before transferring to an Algaetron AG 230 growth chamber (Photon System Instruments) (see Section 3.2.4).

3.2.4 Cyanobacteria cultivation condition

To cultivate *Synechocystis* sp. PCC 6803 strains for the production of compounds of interest, all strains were inoculated in 5 ml of BG11-Co with appropriate antibiotics in a 6-well plate and incubated in an Algaetron for 2-3 days. The cultivation condition in the Algaetron was set with continuous illumination (warm-white LED) at 60 µmol photons/m^2·s and 1% (v/v) CO_2 at 30°C. OD_{730} of precultures was then measured to calculate the volume for inoculation in 25 ml BG11-Co in 100-ml Erlenmeyer flask with the starting OD_{730} of 0.2. For liquid cultures, 6-well plates (5 ml of culture in each well) or 100-ml Erlenmeyer flasks (25 ml of culture) were incubated on a shaker rotating at 180 rpm. Cobalt- (Pcoa) and nickel-inducible (PnrsB) promoters were induced with 625 nM-6.25 µM Cobalt ($Co(NO_3)_2.6H_2O$) and 15 µM nickel ($NiSO_4.6H_2O$), respectively. When inducer and solvent overlay were used, they were added on day 2 after inoculation.

To maintain cyanobacterial strains, they were restreaked on BG11-Co agar plates with suitable antibiotics every 1-2 weeks and incubated in an Algaetron.

3.2.5 Triparental conjugation

Triparental conjugation (Wolk *et al.*, 1984; Elhai & Wolk, 1988) was used to transform plasmids into cyanobacterial host cells in this study. Two *E. coli* strains were used as (*i*) a cargo strain (HB101; a strain carrying a cyanobacterial helper plasmid with chloramphenicol selection marker) and (*ii*) a conjugal strain (IY135; a strain carrying a conjugal plasmid with carbenicillin selection marker). A plasmid of interest was transformed into *E. coli* HB101 by typical heat shock transformation (see Section 3.1.6) and plated on LB agar with chloramphenicol and selection marker for the plasmid of interest. The transformants (HB101 carrying plasmid of interest) and a conjugal strain (IY135) were inoculated and incubated overnight at 37°C prior to the triparental conjugation. *Synechocystis* sp. PCC 6803 was cultivated in 6-well plate in an Algaetron for 2 days to OD_{730} of ~1. *E. coli* and cyanobacteria were washed 2-3 times with fresh LB or BG11-Co in order to remove all antibiotics from the cultivation. Then, 100 µl of a conjugal strain, 100 µl of a cargo strain and 100 µl of cyanobacterial strain were mixed together in a 1.5 ml microcentrifuge tube and incubate in an Algaetron for 2 h. The mixture was then plated on a BG11-Co agar without antibiotics and incubated in a SANYO incubator at 30°C for 2 days. The cells were then transferred to a new BG11-Co agar plate with suitable antibiotics. The incubation continued until colonies were visible.

3.2.6 Cell density measurement

Optical density at 730 nm of cyanobacterial cultures was measured in a 1.5-ml cuvette using a Tecan microplate reader as an indicator for growth. In the case where the cultures

were dense, they were diluted in ultrapure water prior to the measurement. In this case, OD values were calculated accordingly to dilutions. To convert OD_{730} to biomass (g Cell Dry Weight/L (gCDW/L)), calibration curve correlating between biomass and OD_{730} for *Synechocystis* sp. PCC 6803 was obtained (Appendix I).

3.2.7 Cyanobacteria toxicity assay

Shake flasks were used to investigate the toxic effects of different compounds towards cyanobacterial growth. The same Algaetron condition was used to cultivate the cyanobacterial strains (see Section 3.2.4). The compounds of interest were added on day 2 after inoculation onwards, unless stated otherwise. In the presence of solvent overlay, 10% (v/v) solvent was used to overlay the culture.

3.3 Molecular biology methods

3.3.1 Plasmid extraction

Plasmid DNA extraction from *E. coli* was carried out using QIAGEN miniprep kit (QIAGEN). The extraction was conducted following the manufacturer's instructions.

3.3.2 DNA purification from agarose gel

DNA purification from agarose gel was carried out using QIAquick Gel Extraction Kit (QIAGEN).

3.3.3 Measurement of DNA quantity and purity

DNA quantity and purity were measured by absorbance measurements at 260 and/or 280 nm. Two µl of nuclease free water was put on a designated well on a Tecan Infinite 200Pro Nanoquant plate before absorbance at 260 and 280 nm were measured with a Tecan microplate reader to set as a blank. Then, the blank was wiped out with Kimwipes (Kimberly-Clark) before 2 µl plasmid DNA or PCR product was put on the same designated well. The absorbances were measured again. For DNA quantification, the absorbance at 260 nm was measured. For DNA purity, the ratio between 260/280 nm was calculated and the value of 1.8-2.0 was considered acceptable for further uses.

3.3.4 Primer and gBlocks

All primers and gBlocks were synthesized from Integrated DNA Technologies (IDT). All genes were codon optimized for bacteria. *Bsa*I prefix (5'-TCTGGTGGGTCTCTGTCC-3') and suffix (5'-TCTGGTGGGTCTCTGTCC-3') were added to the gene sequence before gene

start codon and after stop codon, respectively. All primers and gene sequences used in this study were listed in Appendix D and K.

3.3.5 Polymerase Chain Reaction (PCR)

PCR reaction with Q5 High-Fidelity DNA polymerase (New England BioLabs (NEB)) was used to amplify gene fragments that would be subsequently used in gene cloning in this study. The PCR reaction listed in Table 3.4 was mixed in a PCR tube (VWR). The PCR was then conducted in a Thermocycler (Biometra) with conditions listed in Table 3.5. Primers are listed in Appendix D.

Table 3.4 Q5 High-Fidelity DNA polymerase PCR reaction

Component	Volume (µl)
DNA template	1-3
10 mM dNTPs	0.5
10 µM forward primer	1.25
10 µM reverse primer	1.25
5x Q5 polymerase reaction buffer	5
Q5 Polymerase	0.25
Nuclease free water	to 25
Total	25

Table 3.5 Q5 High-Fidelity DNA polymerase PCR conditions

Step	Temperature	Time	No. of cycle
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	Primer dependent*	30 sec	
Extension	72°C	30 sec/kb	
Final extension	72°C	2 min	1
Hold	4°C	∞	-

* Annealing temperature was calculated for each set of primers using online NEB Tm Calculator (New England BioLabs)

3.3.6 Diagnostic colony PCR for *E. coli* and cyanobacteria

Colony PCR was used to primarily validate a positive insert in a plasmid from a transformant colony. For *E. coli*, a single colony from transformation plate was picked and resuspended in 10 µl autoclaved ultrapure water before incubating at 95°C for 10 min using a Thermocycler. For cyanobacteria, biomass was resuspended in 10 µl ultrapure water before incubating at 95°C for 10 min using a Thermocycler and at -80°C for 10 min. The freeze-thaw step was carried out 3 times. The suspension was then spun down and 3 µl of the supernatant was used as DNA template. DreamTaq green PCR Master Mix (2x) (Thermo Fisher Scientific)

was used to prepare PCR reaction as follows (Table 3.6). The PCR conditions are shown in Table 3.7. Primers are listed in Appendix D.

Table 3.6 DreamTaq green colony PCR reaction

Component	Volume (µl)
DNA template	3
10 µM Forward primer	1
10 µM Reverse primer	1
DreamTaq Master Mix (2x)	12.5
Nuclease free water	to 25
Total	25

Table 3.7 DreamTaq green PCR conditions

Step	Temperature	Time	No. of cycle
Initial denaturation	95°C	3 min	1
Denaturation	95°C	30 sec	30
Annealing	57°C	30 sec	
Extension	72°C	1 min/kb	
Final extension	72°C	15 min	1
Hold	4°C	∞	-

3.3.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used to validate the size of DNA. Agarose gel was prepared by adding 0.8 g of agarose into 100 ml 1xTAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA pH 8.3) and melted in a microwave. Once the gel was completely melted and cooled down at room temperature, SYBRsafe DNA stain (Thermo Fisher scientific) was added to the gel (5 µl of DNA stain for 100 ml of melted agarose). The gel was then poured onto a gel casting tray, and a comb with desirable size/well number was put on to create wells. Once the gel solidified, the comb was taken out, and the gel was moved to an electrophoresis tank (Bio-Rad Laboratories) containing 1xTAE buffer. DNA was mixed with gel loading dye (6x) (New England BioLabs (NEB)) before loading into the well. DNA ladder (New England BioLabs (NEB)) was also loaded into one of the wells in order to determine the size of the DNA samples. Gel electrophoresis was set at 100 V for 25-30 min. Once the run finished, the agarose gel was visualized on a blue-light transilluminator (Clare Chemical Research) or a UV gel imager (GelDoc-It, Ultra-Violet Products Ltd.).

3.3.8 pJET cloning

All gBlocks, genes and DNA parts for BASIC assembly (see Section 3.3.12) were stored in pJET storage plasmid (Yunus & Jones, 2018). CloneJET PCR cloning kit (Thermo Fisher Scientific) was used for cloning. With this commercial kit, pJET 1.2 blunt vector is a

backbone for each DNA part. The reaction was adapted from manufactures' protocol (Table 3.8). The ligation reaction was incubated at room temperature for 10-15 min before all was transformed to *E. coli* DH5α and plated on LB agar with 100 mg/L carbenicillin. The plate was incubated at 37°C overnight and diagnostic colony PCR was performed to validate the insertion using primers provided with the commercial kit (pJET-forward and pJET-reverse).

Table 3.8 pJET cloning reaction

Component	Volume (µl)
2x Reaction buffer	5
PCR product or gBlock	1-4
pJET 1.2 blunt vector	0.5
T4 DNA ligase	1
Nuclease free water	to 10
Total	10

3.3.9 Traditional cloning

Traditional restriction enzyme/DNA ligase cloning was used to clone all plasmids with T7 promoter in this study (Appendix C). All restriction enzymes were ordered from New England BioLabs (NEB) and used according to the manufacturer's protocol (see Section 3.3.10). T4 ligase from Promega was used for ligation reaction (see Section 3.3.11).

3.3.10 Plasmid restriction digestion

To digest plasmids or PCR products, restriction enzymes from New England BioLabs (NEB) were used and the reaction was prepared according to the manufactures' protocols (Table 3.9). The digestion reaction was incubated at 37°C for 1 h or overnight before further uses.

Table 3.9 Restriction digestion reaction

Component	Volume (µl)
PCR product	6-8
Restriction enzyme I	1
Restriction enzyme II	1
Cutsmart	3
Nuclease free water	to 30
Total	30

3.3.11 Plasmid ligation

To ligate plasmids, T4 ligase from Promega was used as a ligation enzyme. The reaction was prepared according to the manufactures' protocols (Table 3.10) and incubated

at room temperature for 30 min or overnight before transformation to *E. coli* DH5α (see Section 3.1.6).

Table 3.10 Ligation reaction

Component	Volume (µl)
T4 DNA ligase buffer	2
Fragment 1	from calculation*
Fragment 2	from calculation*
T4 DNA ligase	1
Nuclease free water	to 20
Total	20

* Added volume of DNA fragments was calculated accordingly to manufacturer's recommendation

3.3.12 BASIC assembly

Biopart Assembly Standard for Idempotent Cloning (hereafter BASIC) assembly (Storch *et al.*, 2015) was used to construct most of the plasmids in this study. This method for molecular cloning is efficient and time-effective when a number of plasmids need to be constructed. As mentioned, all DNA parts with restriction sites for *Bsa*I restriction enzyme (*Bsa*I prefix and suffix) were stored in pJET storage plasmid. The *Bsa*I sites were put before and after the DNA parts of interest. The simplified BASIC assembly workflow is illustrated in Figure 3.1.

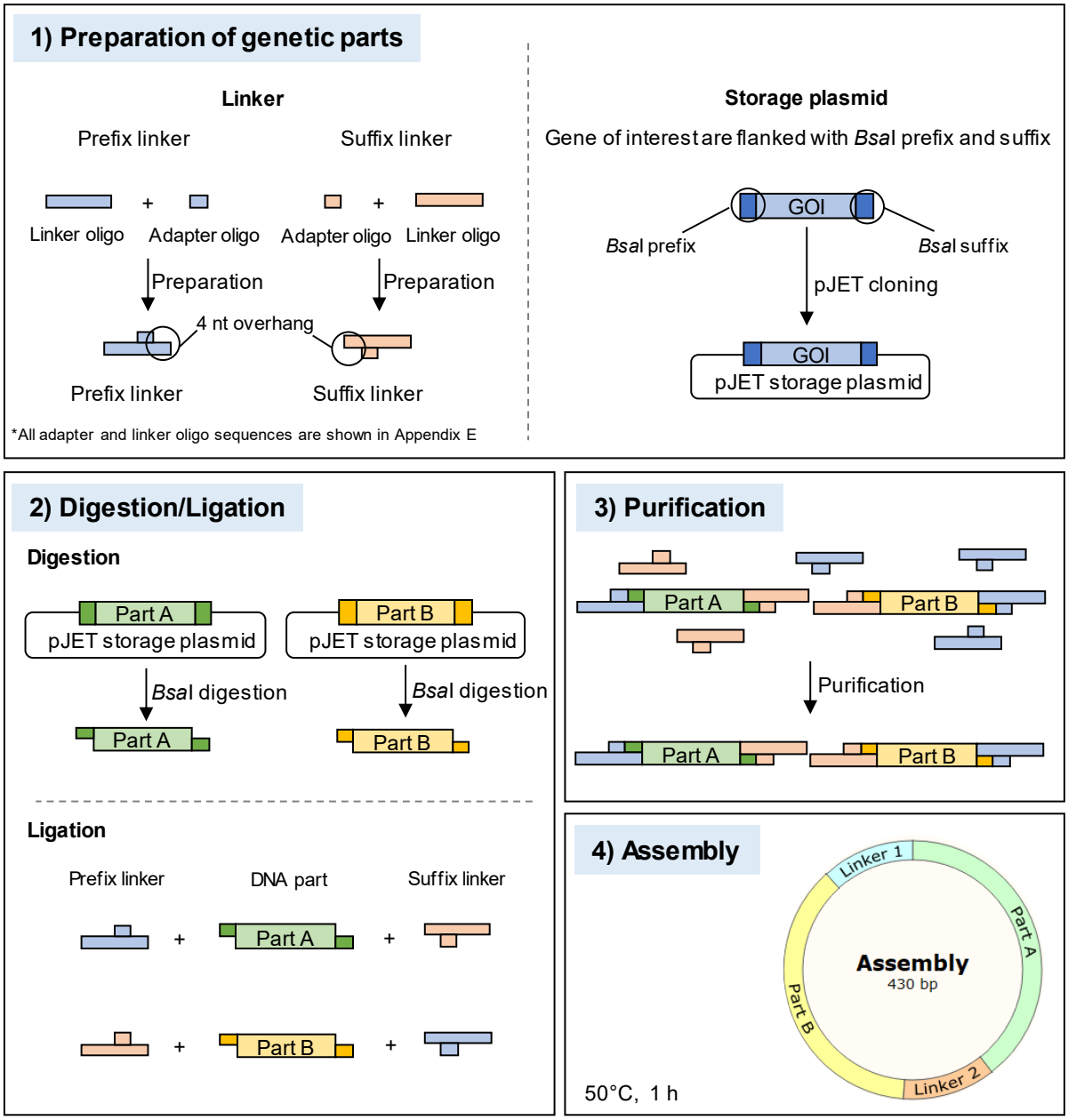

Figure 3.1 The simplified BASIC assembly workflow adapted from Storch *et al.*, 2015
A schematic diagram depicting simplified BASIC assembly workflow 1) Preparation of DNA parts 2) Digestion and ligation 3) Purification 4) Plasmid assembly.

1) Linker preparation

Linkers are DNA parts with a 4-nucleotide overhang compatible with 4-nucleotide overhangs on digested *Bsa*I DNA parts. This allows linkers to consequently ligate to each end of DNA parts of interest (Figure 3.1). The preparation of linkers is as follows (Table 3.11).

Table 3.11 BASIC linker preparation reaction

Component	Volume (µl)	Stock concentration	Final concentration
Annealing buffer*	49	-	-
Linker oligo	0.5	100 µM	1 µM
Adapter oligo	0.5	100 µM	1 µM
Total	50	-	-

*Annealing buffer: 10 mM TRIS-HCl buffer pH 7.9, 100 mM NaCl, and 10 mM $MgCl_2$

All components were mixed together in a 1.5-ml microcentrifuge tube. The tube was then incubated at 95°C in a pre-heated heat block for 2 h. Once the incubation period finished, the heat block was switched off and the tube was left in the heat block until it was cooled down to room temperature then the tube was stored at -20°C. The linker sequences used in this study were listed in the Appendix E.

2) Digestion/ligation

The desirable DNA parts were stored in pJET storage plasmid. Every part, as mentioned, contains *Bsa*I prefix and suffix before and after the sequence of interest. Prior to the assembly, all storage plasmids were digested with *Bsa*I and ligated with the designed linkers to prepare each assembly part (Figure 3.1). The digestion/ligation reaction was prepared separately for each part as follows (Table 3.12). All components were mixed in PCR tube and incubated in a Thermocycler at 37°C for 2 h following with 20°C for 20 min and 65°C for 20 min.

Table 3.12 Digestion and ligation reaction for BASIC assembly

Component	Volume (µl)	Stock concentration	Final concentration
Ultrapure water	12.5	-	-
10xT4 buffer	3	10x	1x
iP Linker	5	1 µM	166.6 nM
iS Linker	5	1 µM	166.6 nM
DNA part (plasmid)	3	-	-
*Bsa*I	1	10 unit/µl	0.33 unit/µl
T4 ligase	0.5	1-3 unit/µl	0.1 unit/µl
Total	30	-	-

3) Purification

Once the digestion and ligation reactions were finished. AmpliCleanTM magnetic bead-based PCR clean-up was used to purify each DNA part from the digestion/ligation reaction (Figure 3.1). The purification was carried out using 50 µl of magnetic beads and according to

manufacturer's protocol. For the final step, purified DNA was eluted in 30 µl of nuclease free water.

4) Assembly

The purified DNA parts were assembled by mixing each part together in a PCR tube. CutSmart buffer (10x) (New England BioLabs (NEB)) was also added to the reaction at the final concentration of 1x (Table 3.13). The reaction was incubated at 50°C for 1 h in a Thermocycler then transformed to *E. coli* DH5α as in Section 3.1.6.

Table 3.13 Assembly reaction for BASIC assembly

Component	Volume (µl)
DNA parts (each)	2.5
CutSmart buffer (10x)	1
Nuclease free water	to 10
Total	10

3.3.13 DNA sequencing

DNA sequencing was used to validate newly constructed plasmids. Plasmids and their corresponding primers were diluted to 100 ng/µl and 3.2 pmol/µl, respectively, and put in separate 1.5-ml microcentrifuge tubes. They were then sent off for Sanger Sequencing by Source BioScience (Nottingham, UK).

3.4 Product analysis

3.4.1 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS was used to detect and quantify volatile bioproducts. In Chapter 4 and 5, samples were analyzed using an Agilent 7890B GC, 7693 autosampler and 5977B MSD system equipped with an HP-5ms column. The initial temperature was set at 40°C held for 3 min before ramping up to 235°C at 30°C/min and held for 4 min before ramping up to the final temperature of 300°C for 2 min at 20°C/min. Helium was used as carrier gas at a rate of 1 ml/min. The injection was set to pulsed split mode. One µl was analyzed for each sample. In Chapter 6, 7 and 8, samples were analyzed using a Hewlett Packard (HP) 6890 Series GC, 5973 Mass Selective Detector equipped a DB-WAXetr column. The injector temperature was set at 250°C. The carrier gas was helium at a rate of 1.5 ml/min. The oven temperature was initially set at 100°C and held for 30 sec, before ramping up to 250°C at 30°C/min and hold for 3 min. The detector was turned off at the elution time of solvent overlay.

3.4.2 High Performance Liquid Chromatography (HPLC)

An Agilent 1200 series HPLC instrument equipped with different columns and a refractive index detector (RID) was used to detect and quantify glucose, *E. coli* fermentation products, sucrose, fructose and octyl glucoside in this study. One ml of samples was centrifuged at 17,000 x*g* for 15 min before transferring to a new 2-ml vial for HPLC analysis. For glucose and fermentation products, where no sucrose supplemented in the media, an Aminex HPX-87H column (Bio-Rad Laboratories) was used to analyse 100 µl with the flow rate and column temperature at 0.6 ml/min and 60°C, respectively. For octyl glucoside detection, twenty µl of samples were analyzed with a Zorbax XDB-C18 column (Agilent). The flow rate was set at 1 ml/min with a column temperature of 30°C (Wang *et al.*, 2012). The quantification of glucose, fructose and sucrose in the same sample was carried out using the HPLC system equipped with HPX-87P (Bio-Rad Laboratories), which allowed the identification of these sugars as they eluted as separate peaks. Twenty µl samples were injected and eluted with flow rate of 0.6 ml/min and column temperature of 85°C. Sulfuric acid (5 mM) was used as the mobile phase for HPX-87H and HPX-87P columns and methanol:H_2O (2:1) was used as the mobile phase for Zorbax XDB-C18 column. Serial dilutions of standards: octyl glucoside or Octyl β-D-glucopyranoside (Sigma Aldrich), glucose (Sigma Aldrich), sucrose (Sigma Aldrich), fructose (Sigma Aldrich), sodium acetate (Sigma Aldrich), sodium lactate (Sigma Aldrich), sodium succinate (Sigma Aldrich), sodium formate (Sigma Aldrich), and absolute ethanol (VWR) were used to determine the amounts of these compounds in the sample.

3.4.3 Mock experiment for 1-octanol and octyl acetate localization

The mock experiment to understand the localization of 1-octanol and octyl acetate was studied in 25 ml M9 minimal medium with 2% (w/v) glucose overlaid with 10% (v/v) hexadecane. Flasks were incubated at 30°C, 150 rpm for 24 h or 48 h, which was the condition for production assay. Hexadecane (100 µl) was obtained directly and injected to GC-MS.

3.4.4 Extraction of intracellular products

To extract intracellular products, chloroform:methanol extraction and glass bead lysis were used. For chloroform:methanol extraction, one ml of pellet suspension (cell pellet from 1 ml of culture resuspended in 1 ml of ultrapure water) was added to 2 ml of chloroform:methanol (2:1) solution in a glass tube with cap. The glass tube was vortexed vigorously for 10 sec. The glass tube was then centrifuged at 4,000 x*g* for 3 min. The chloroform phase (the lowest layer) was then transferred to a new glass tube. The extraction was repeated, and the chloroform phase was transferred to the same glass tube in the previous step. Chloroform phase was then dried under N_2. White pellets were resuspended in 100 µl of appropriate solvent for each product of interest before GC-MS analysis. For glass bead lysis, whole cultures were

centrifuged and washed twice in ultrapure water before resuspending in fresh media. Pellet suspension was transferred to a 2-ml screw-cap vial with around 250 µl-volume of glass beads (Sigma Aldrich). Tissuelyser II (QIAGEN) used to lyse the cells was set at 30 hertz for 6 min for *E. coli* and 15 min (3 cycles) for cyanobacteria. The sample was then centrifuged at 17,000 x*g* for 10 min before the supernatant was obtained for the analysis.

3.4.5 Extraction of extracellular products in the absence of solvent overlay

To extract extracellular products from supernatant in the absence of solvent overlay, solvent extraction was used. One ml of whole cultures was centrifuged at 17,000 x*g* for 10 min. The supernatant was then obtained and transferred to a new 1.5 ml microcentrifuge tube. Appropriate solvent (100 µl or 10% (v/v)) was added to 1 ml of supernatant. The sample was vortexed vigorously for 10 sec. The microcentrifuge tube was centrifuged again for 3 min, and solvent phase was transferred to a vial insert for GC-MS analysis.

3.4.6 Extraction of extracellular products in the presence of solvent overlay

To extract extracellular products from supernatant in the presence of solvent overlay, the similar solvent extraction in Section 3.4.5 was used. However, centrifugation step to obtain the supernatant from whole cultures was repeated twice by first taking the culture and centrifuging to separate cell pellet and supernatant in a microcentrifuge tube. Then, the supernatant was obtained and centrifuged again before transferring only the aqueous phase to a new microcentrifuge tube and adding the fresh solvent for extraction. The repeating step was to limit the amount of overlay that may be left in the aqueous phase.

3.4.7 Extraction of 1-octanol and octyl acetate in the absence of solvent overlay

To extract 1-octanol and octyl acetate from cultivation media in the absence of solvent overlay, hexadecane extraction (see Section 3.4.5) were used to extract the product from supernatant.

3.4.8 Extraction of 1-octanol and octyl acetate in the presence of solvent overlay

Solvent overlay (100 µl) was obtained directly from the cultivation and injected to GC-MS. If the concentration of the products were high. The samples were diluted in the same solvent prior to injection. To investigate if 1-octanol or octyl acetate remained in the aqueous phase when solvent overlay was applied, hexadecane extraction (see Section 3.4.6) was used to extract the products from the aqueous phase in order to understand the liquid phase partitioning due to the difference in solubility of the compounds and the efficiency of the extraction.

3.4.9 Extraction of 1-dodecanol and lauryl acetate in the presence of solvent overlay

To extract 1-dodecanol and lauryl acetate from cultivation media in the presence of solvent overlay, chloroform:methanol extraction (see Section 3.4.4) was used to extract the product from intracellular fraction and dodecane overlay was obtained directly from the cultivation and injected to GC-MS.

3.4.10 Extraction of 1-dodecanol and lauryl acetate in the absence of solvent overlay

To extract 1-dodecanol and lauryl acetate from cultivation media in the absence of solvent overlay, chloroform:methanol extraction (see Section 3.4.4) were used to extract the product from intracellular fraction and supernatant.

3.4.11 Extraction of products from feeding experiment of menthol and eugenol in the absence of solvent overlay

The samples were extracted using chlororform:menthol extraction (see Section 3.4.4) for intracellular fraction and solvent extraction (see Section 3.4.5) for extracellular extraction. Isopropyl myristate and dodecane were used as solvents for menthol and eugenol, respectively.

3.4.12 Analysis of octyl glucoside, sugars and fermentation products

Octyl glucoside, sugars and fermentation products were analyzed with HPLC (see Section 3.4.2). For sugars and fermentation products, only supernatant fraction was analyzed. Whole cultures were centrifuged at 17,000 x*g* for 15 min and the supernatant was transferred to 2-ml vials prior to HPLC analysis. For octyl glucoside, supernatant and cell pellets were analyzed separately. The supernatant fraction was obtained in the same way with sugars and fermentation products analysis, but the sample was diluted in methanol with 2:1 methanol:supernatant ratio prior to HPLC analysis. The cell pellets from centrifugation was extracted using both chloroform:methanol extraction and glass bead lysis (see Section 3.4.4). The cell extract was also diluted in methanol prior to HPLC analysis.

3.5 Statistical analysis

A two-sided student's t-test was used to determine difference between each treatment and/or condition, with asterisks indicating significance (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.005$). All data presented in this thesis are average ± SD from three biological replicates, unless stated otherwise. All individual data are presented accordingly on each bar graph.

Chapter 4
Improved 1-octanol production in *E. coli*

1-Octanol is an attractive biofuel with diesel-like properties and has been successfully produced in genetically engineered microorganisms, *E. coli*, yeast, and cyanobacteria (Akhtar *et al.*, 2015; Henritzi *et al.*, 2018; Yunus & Jones, 2018). As mentioned in Section 2.4, to date, there are four known synthetic metabolic routes for 1-octanol production: (*i*) the reversal of β-oxidation (Dellomonaco *et al.*, 2011), (*ii*) redirecting branched-chain amino acid biosynthesis (Marcheschi *et al.*, 2012), (*iii*) extending the CoA-dependent *n*-butanol pathway (Machado *et al.*, 2012), and (*iv*) the carboxylic acid reductase-dependent pathway (Akhtar *et al.*, 2015; Henritzi *et al.*, 2018; Yunus & Jones, 2018). Among these synthetic metabolic routes, the carboxylic acid reductase-dependent pathway has the advantages over the others for its high product specificity (i.e., 1-octanol accounted for ~70% of the total products) and productivity (Akhtar *et al.*, 2015). However, its yield still remains low and has not yet reached a level where it is cost-efficient for commercialization. It has been hypothesized that this may be due to several factors including its high toxicity to host microorganisms (Yunus & Jones, 2018). In this work, the concept of bioderivatization was proposed to solve this challenge. Carboxylic acid reductase-dependent 1-octanol synthetic pathway was optimized in this chapter. The effects of cultivation conditions (e.g., inducer (IPTG) concentration, cultivation volume and container, etc.) and host backgrounds on 1-octanol production were investigated. Additionally, the limiting step in the pathway was examined. Finally, several superior thioesterases were also screened to achieve a higher titer of 1-octanol.

4.1 *In vivo* biosynthesis of 1-octanol in *E. coli*

The production of 1-octanol from a carboxylic acid reductase-dependent pathway in *E. coli* BL21(DE3) strain was first demonstrated by Akhtar and colleagues (Akhtar *et al.*, 2015) by expressing Tes3, Sfp, and CAR (Figure. 4.1A) in a plasmid (pET-TPC3) under a T7 promoter and AHR in another plasmid under the same T7 promoter. Up to 0.48 mM (62 mg/L) of 1-octanol was produced in 14 h. However, the corresponding yield (12 mg/g glucose) is far lower than the maximum theoretical yield that can be achieved for 1-octanol production (361 mg/mg glucose) (Akhtar *et al.*, 2015), suggesting opportunities for further enhancements in order to take the advantage of the bioderivatization process. In this work, optimization of 1-octanol production was then carried out by first optimizing the strength of plasmid inducer, IPTG. A range of IPTG concentrations (0.1-1 mM) was used to induce the strains carrying the pET-TPC3 plasmid (Figure 4.1B). Hexadecane (10% (v/v)) was used to capture 1-octanol and

to minimize the product loss via volatilization. Figure 4.1C shows that at higher concentrations of IPTG, the concentrations of 1-octanol slightly increased, albeit statistically insignificant. When considering the yield, however, the highest 1-octanol yield was achieved upon induction with 0.75 mM IPTG (Figure 4.1D) with no significant difference in growth (Figure 4.1E) or glucose consumption (Figure 4.1F). Despite this improvement, the 1-octanol titer was still low. This emphasizes the need for further improvements to obtain a high 1-octanol-producing strain.

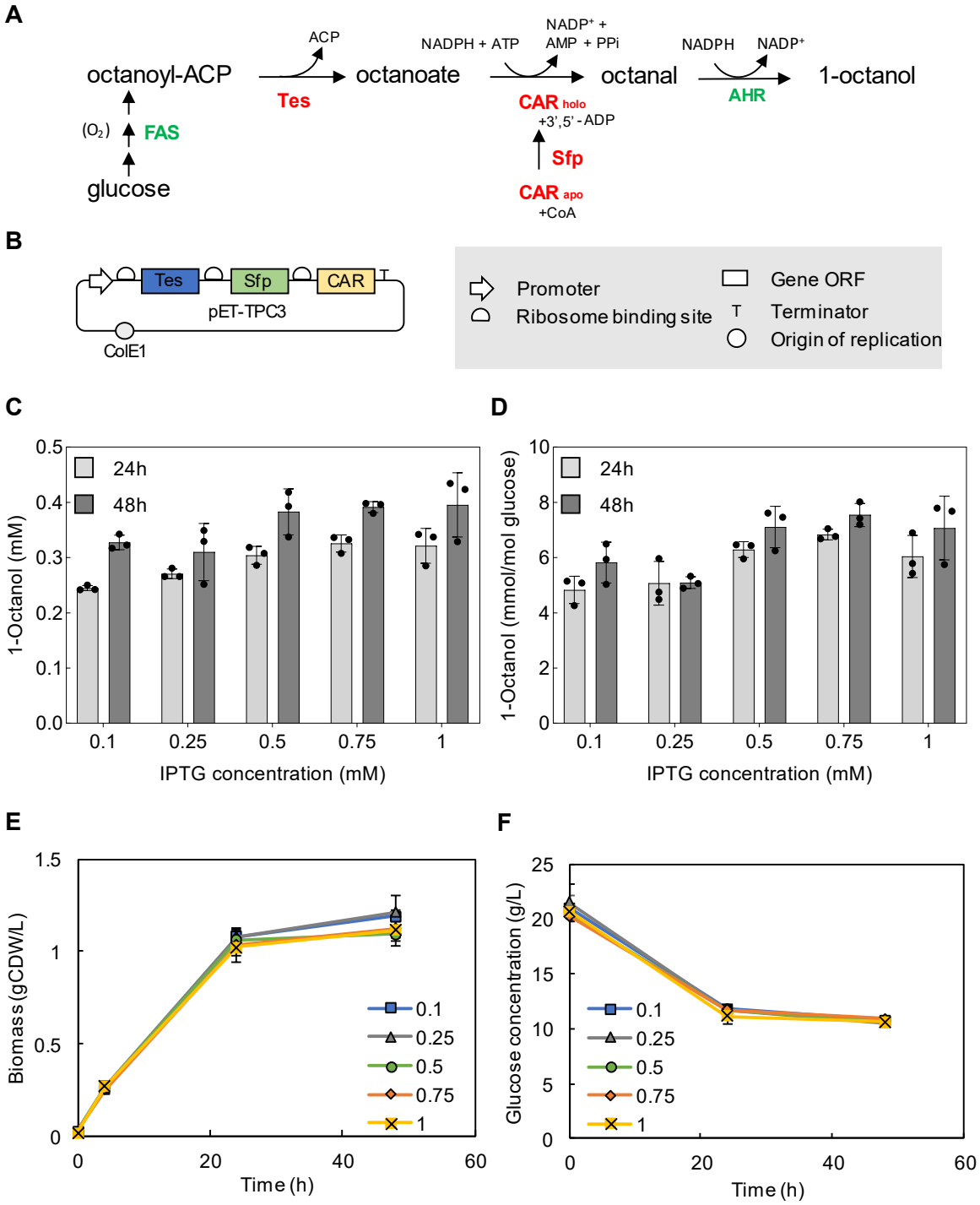

Figure 4.1 Optimization of 1-octanol production by varying IPTG inducer concentrations
(A) Metabolic pathway for *in vivo* production of 1-octanol (TPC3 pathway). (B) A plasmid construct used for 1-octanol production (Akhtar *et al.*, 2015) was transformed to *E. coli* C43(DE3) in this study. 1-Octanol production (C) Titer (D) Yield (E) Biomass accumulation and (F) Glucose consumption when *E. coli* C43(DE3) harboring TPC3 pathway was cultivated in M9 minimal media with different concentrations of IPTG (0.1-1 mM). The source organisms for all enzymes are listed in Appendix J. All data are average of 3 biological replicates and error bars represent standard deviation.

4.2 Cultivation volume affected fermentation of *E. coli* and 1-octanol production

The availability of oxygen in culture systems was one of the important factors that could affect the production (Zimmermann *et al.*, 2006) though the suitable oxygen availability may be variable for divergent synthetic pathways. As a facultative anaerobic microorganism, *E. coli* converts carbon sources to mixtures of products via fermentation which is triggered when oxygen availability is limiting. This response is harnessed by the cells to continue the supply of energy under anaerobic condition where there was no oxygen as an electron receptor (Clark, 1989). The redirecting of carbon sources toward undesirable fermentation products could result in inefficient production of target compounds and the inactivation of the competitive fermentation pathways has been shown to improve the biosynthesis of bioproducts (Jarboe *et al.*, 2010; Vuoristo *et al.*, 2015). Therefore, here, the volume of cultivation flask and liquid culture as two factors that may affect oxygen availability were investigated. Different culture flasks and volumes, such as 100-ml, 250-ml Erlenmeyer flasks, and 250-ml baffled flasks, were used to cultivate *E. coli* C43 (DE3) strains carrying TPC3 pathway in M9 minimal medium with 2% (w/v) glucose. To capture 1-octanol from the liquid culture, samples were overlaid with 10% (v/v) hexadecane. The quantity of three major fermentation products (acetate, lactate, and ethanol) was also monitored at 24 and 48 h.

The results demonstrated that liquid cultures grown in 250-ml baffled flasks showed the highest biomass accumulation over 24 and 48 h (Figure 4.2A). This was presumably due to higher aeration rate provided by the baffled flasks. Surprisingly, this sample consumed less glucose whilst the cultures grown in 100-ml flask used the highest amount of glucose (Figure 4.2B). From the fermentation profile, the results showed that cultures grown in larger flasks accumulated slightly lower fermentation products (Figure 4.2C and 4.2D). Despite this, the titer of 1-octanol was nevertheless found lower in larger flasks (Figure 4.2E), although the difference was less apparent when normalized with the amount of glucose consumption (Figure 4.2F). One possibility is that larger flasks provide bigger surface area which contributes to higher volatilization rate of 1-octanol. In order to cover the surface of the media and minimize the volatilization, more solvent overlay could be applied. However, it would also limit the gas exchange between culture media and environment, lowering the availability of oxygen. To avoid this complication; therefore, all subsequent experiments in this study were conducted with 25 ml media in 100-ml flasks.

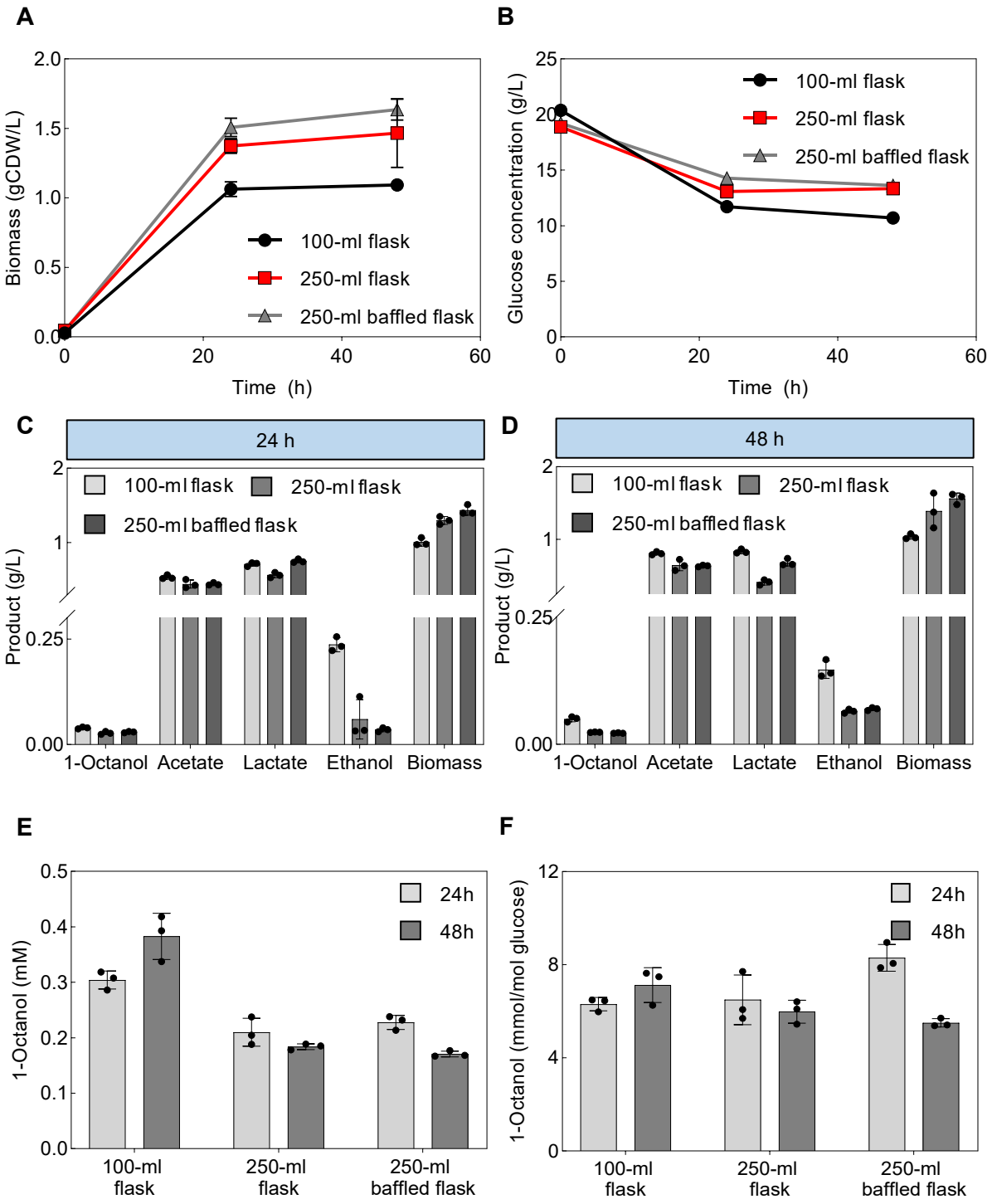

Figure 4.2 1-Octanol production in different volumes of cultivation flasks
(A) Biomass accumulation (B) Glucose consumption. Metabolite and biomass accumulation at (C) 24 h and (D) 48 h. 1-Octanol production (E) Titer (F) Yield. *E. coli* C43(DE3) harboring pET-T7-TPC3 was cultivated in M9 minimal medium with 2% (w/v) glucose, induced with 0.5 mM IPTG and overlaid with 10% (v/v) hexadecane. It should be noted that the data for 100-ml Erlenmeyer flask treatment were from Section 4.1 with the same conditions. All data are average of 3 biological replicates and error bars represent standard deviation.

4.3 Investigation of *E. coli* fermentation fluxes in different *E. coli* backgrounds

As described in previous studies (Mattam & Yazdani, 2013; Jawed *et al.*, 2016), the genetic background of *E. coli* strains plays significant roles in *in vivo* biosynthesis of chemical compounds. For example, it has been shown that butyric acid production when introduced with the same plasmids demonstrated significantly variable titers in different *E. coli* backgrounds (Jawed *et al.*, 2016). Here, two commonly used *E. coli* strains, namely C43(DE3) and BW25113 strains, were investigated for production of 1-octanol by introducing the same pET-pA1lacO-1-TPC3 plasmid where T7 promoter was replaced with pA1lacO-1 promoter to allow the protein expression in *E. coli* BW25113 strain. The *E. coli* strains were cultivated for 48 h in the presence of 10% (v/v) hexadecane overlay. The results showed that 1-octanol was produced significantly higher in the BW25113 strain (Figure 4.3A and 4.3B). Fermentation product profiles also displayed that C43(DE3) strain produced more lactate and accumulated more biomass, suggesting higher carbon diversion from 1-octanol pathway to fermentation products and biomass in this strain (Figure 4.3C and 4.3D). Although further investigations are needed to dissect the effect of genetic differences between C43(DE3) and BW25113 strains on 1-octanol production, the inherent characteristic of BW25113 strain that contributes to higher production of 1-octanol seems to be beneficial for bioderivatization. *E. coli* BW25113 strain was then used for further experiments.

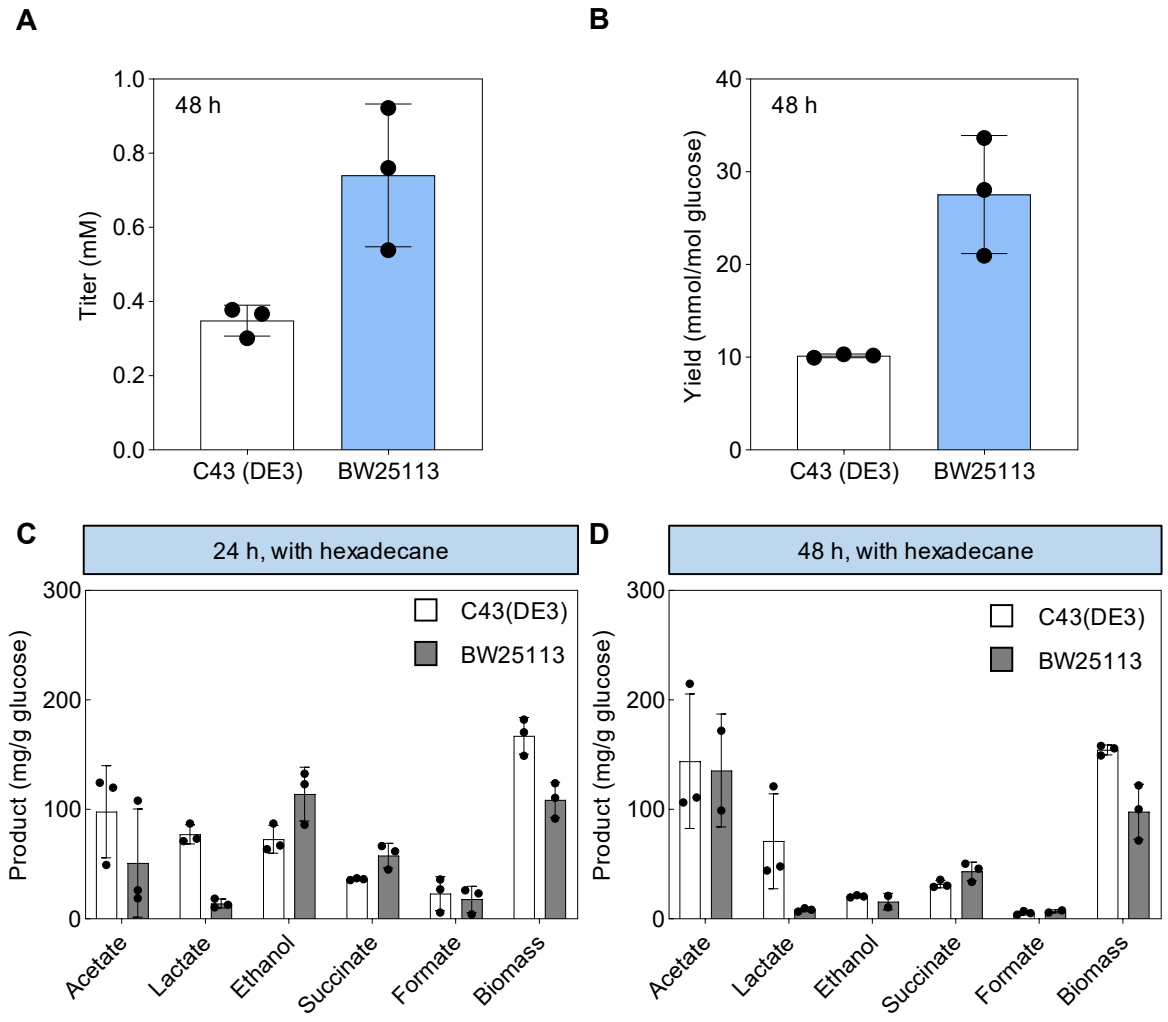

Figure 4.3 1-Octanol production from different *E. coli* backgrounds harboring pET-pA1lacO-1-TPC3
(A) Titer (B) Yield at 48 h. Metabolite and biomass accumulation at (C) 24 h and (D) 48 h. All strains were cultivated in M9 minimal medium, induced with 0.5 mM IPTG and overlaid with 10% (v/v) hexadecane. All data are average of 2 or 3 biological replicates and error bars represent standard deviation.

4.4 Identification of limiting substrate(s) in 1-octanol pathway

Once appropriate cultivation condition and *E. coli* host had been identified, other potential bottlenecks in the pathway which might hinder optimum 1-octanol production were evaluated. It has been previously reported that octanoic acid was a limiting substrate in 1-octanol production in cyanobacteria (Yunus & Jones, 2018). To investigate whether octanoic acid was also the limiting substrate in *E. coli*, octanoic acid with increasing concentrations was supplemented to *E. coli* C43 (DE3) expressing Tes3, Sfp, and CAR. The results showed that 1-octanol titer increased in a linear fashion with octanoic acid concentrations (Figure. 4.4). As octanoic acid was produced by a thioesterase, this suggested that thioesterase (Tes) was presumably a bottleneck enzyme in the carboxylic acid reductase-dependent 1-octanol pathway in *E. coli*.

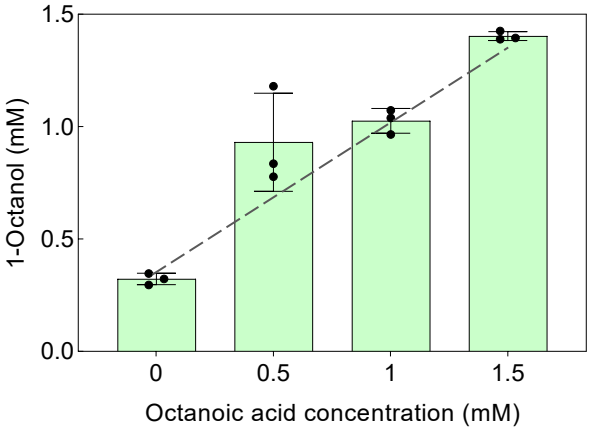

Figure 4.4 Identification of limiting substrate(s) in 1-octanol production by substrate feeding experiment
E. coli C43(DE3) harboring pET-TPC3 plasmid was cultivated in M9 minimal media with different concentrations (0-1.5 mM) of octanoic acid supplementation incubated for 48 h. All data are average of 3 biological replicates and error bars represent standard deviation.

To find a superior thioesterase, here, four C8-preferring thioesterases which have shown high specificity for octanoic acid were selected and evaluated in *E. coli* BW25113. The new thioesterases were originated from *Cuphea palustris* (CpFatB1) (Dehesh *et al.*, 1996) and *Cuphea avigera pulcherrima* (CaFatB3) (Graham & Kleiman, 1992). CpFatB1 was shown to be predominantly active on C8 acyl-ACP (Dehesh *et al.*, 1996) and *C. avigera pulcherrima* seed oils showed high amount of C8:0 fatty acids (75-94% of total fatty acid composition) suggesting the activity of their thioesterases on C8 acyl-ACPs (Graham & Kleiman, 1992; Graham *et al.*, 2016). The chloroplast transit peptide of these thioesterases was removed and in some cases, amino acid mutations were introduced, resulting in thioesterases detailed in Table 4.1 and Figure 4.5. These modifications were conducted by Yunus, 2019. The mutations were introduced according to reported literature to improve C8 activity (Lozada *et al.*, 2018) and the results showed that these newly manipulated thioesterases were highly active toward octanoyl-ACP in cyanobacterial strains (Yunus, 2019). The thioesterases were cloned into a plasmid under pA1lacO-1 promoter, resulting in plasmids similar to constructs shown in Figure 4.1B. To evaluate and optimize the performance of these thioesterases in *E. coli* BW25113, different concentrations of IPTG were used for protein expression. Newly screened thioesterases (‘CpFatB1-4 and ‘CaFatB3-5) yielded higher 1-octanol compared with Tes3; however, the activity is highly dependent on IPTG concentrations (Figure 4.6A-D). The strain carrying ‘CpFatB1-4 together with Sfp and CAR produced the highest amount of 1-octanol at 4.29 mM or 59.48 mmol/mol glucose (Figure 4.6B) at 48 h when induced at 0.05 mM IPTG, which was nearly 7-fold improvement in yield from the first-generation 1-octanol strain under the same conditions (Figure 4.6B). Whilst this same strain, ‘CpFatB1-4, showed growth

defects when induced at high concentration of IPTG (0.2 and 0.5 mM) (Figure 4.6E). This has been speculated to be due to insufficient acyl-ACP for cell membrane biosynthesis as 'CpFatB1-4 was highly active toward acyl-ACP and exhausted this metabolite before it was regenerated (Lozada *et al.*, 2018). The same traits have been observed in cyanobacteria (Yunus, 2019). The second best thioesterase, 'CaFatB3-5, could produce up to 2.90 mM or 44.2 mmol/mol glucose of 1-octanol at 48 h when induced with 0.2 mM IPTG. Interestingly, IPTG concentrations did not affect the growth profile of the strain carrying 'CaFatB3-5 (Figure 4.6F). Altogether, these results demonstrated that optimization of inducer concentrations and the expression of improved thioesterases enabled the production of 1-octanol to reach high production. This optimized system might therefore be adequate to enable clear and logical evaluations of bioderivatization.

Table 4.1 List of thioesterases and modifications

Gene	Source organism	Modification		Reference
		Truncation	Mutation	
Tes3	*Anaerococcus tetradius*	-	-	(Akhtar *et al.*, 2015)
'CpFatB1	*Cuphea palustris*	R120	-	(Dehesh *et al.*, 1996; Yunus, 2019)
'CaFatB3	*Cuphea avigera pulcherrima*	K21	6xHis added N-terminally	(Yunus, 2019)
'CpFatB1-4	*Cuphea palustris*	F114	N122S, I169M	(Lozada *et al.*, 2018)
'CaFatB3-5	*Cuphea avigera pulcherrima*	H112	D120S, I157M	(Yunus, 2019)

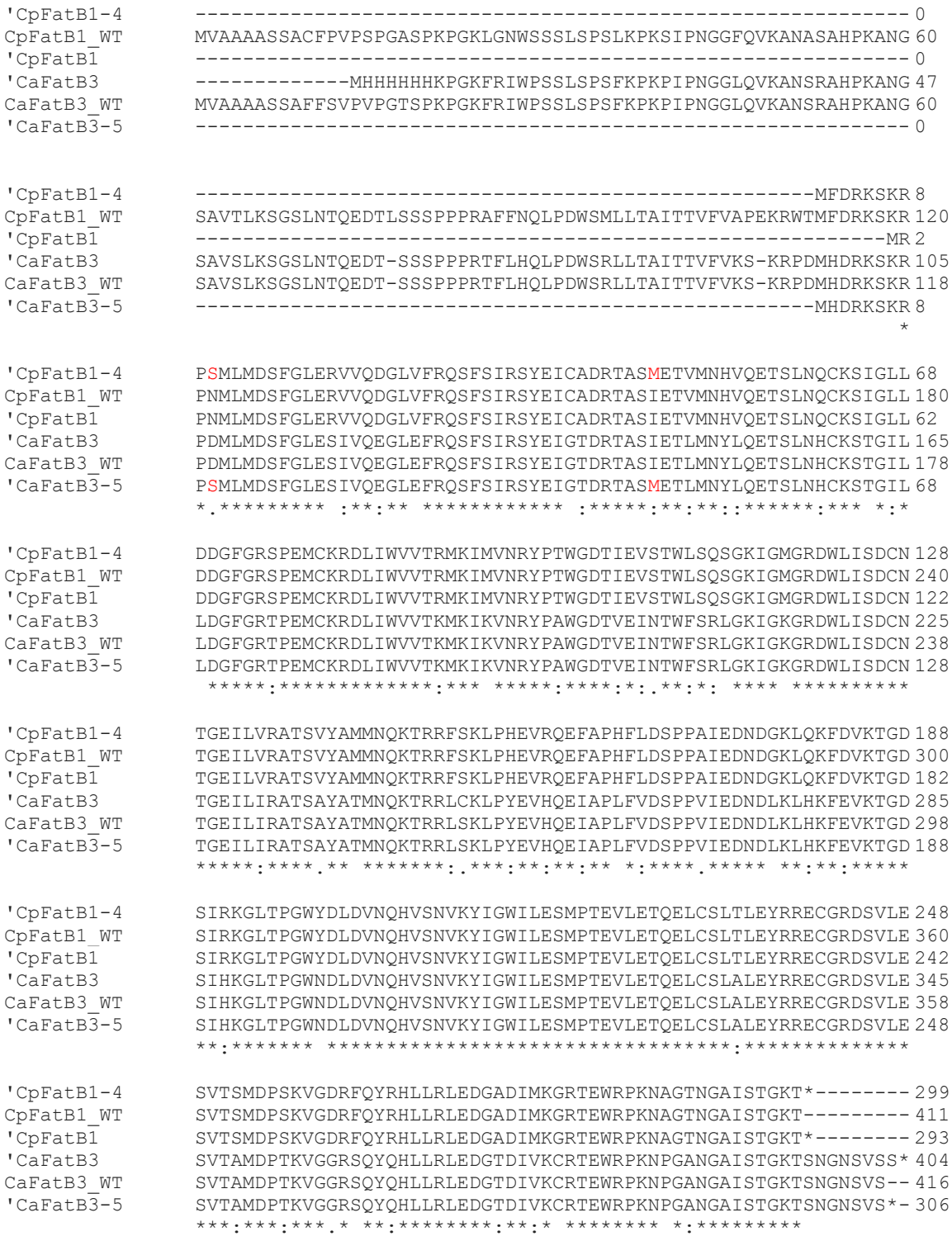

Figure 4.5 Amino acid sequence alignments of thioesterases and modifications (Yunus, 2019)
CpFatB1-wild type (Uniprot Q39554), 'CpFatB1, 'CpFatB1-4, CaFatB3-wild type (Uniprot R4J2L6), 'CaFatB3 and 'CaFatB3-5. Mutated amino acids were highlighted in red.

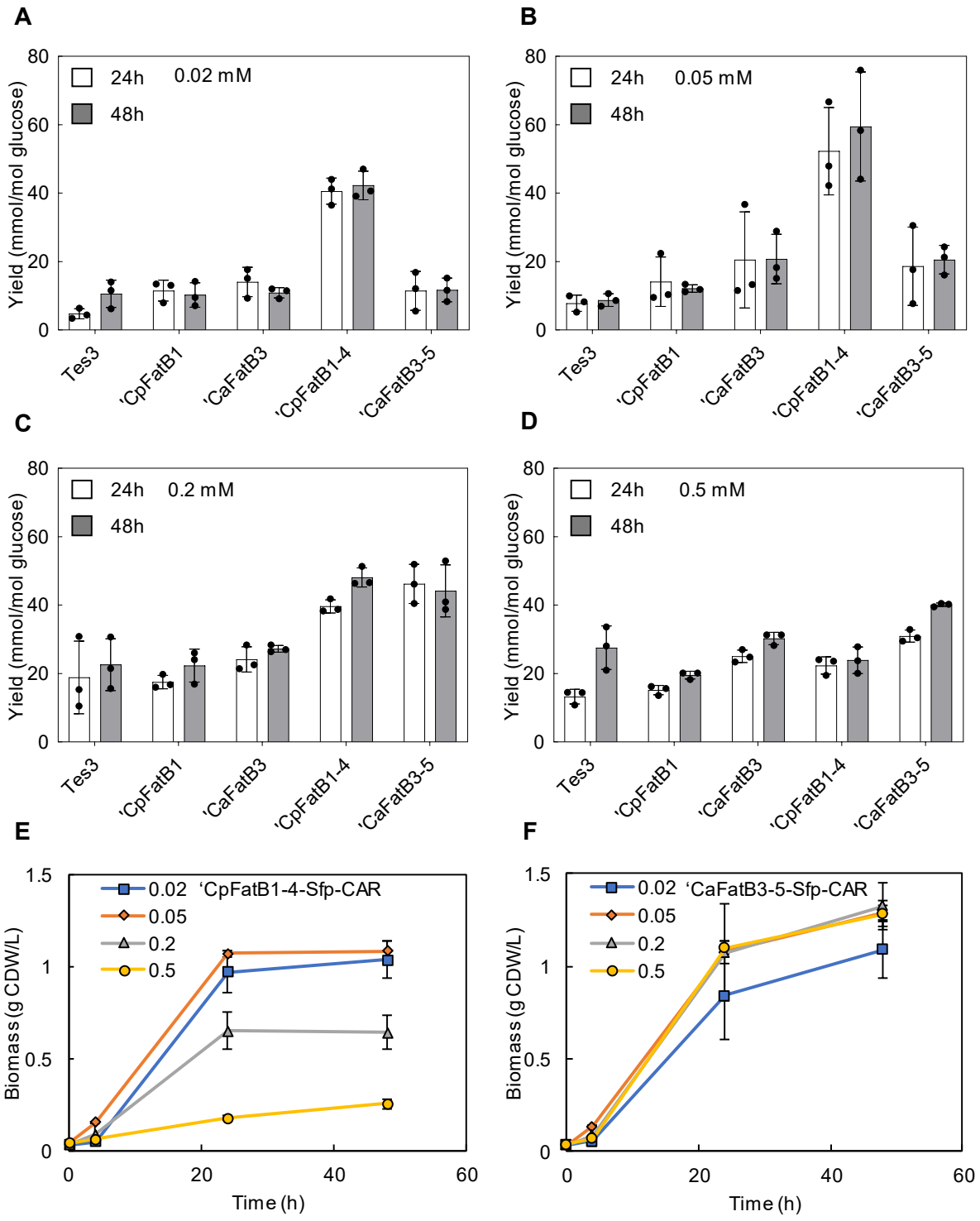

Figure 4.6 Thioesterase selection to improve 1-octanol production at different concentrations of IPTG
E. coli BW25113 strains harboring pET-pA1lacO-1-Tes-Sfp-CAR were cultivated in M9 minimal medium with 2% (w/v) glucose and induced with (A) 0.02 mM (B) 0.05 mM (C) 0.2 mM (D) 0.5 mM IPTG at 24 h and 48 h with 10% (v/v) hexadecane overlay. Biomass accumulation from (E) 'CpFatB1-4-Sfp-CAR and (F) 'CaFatB3-5-Sfp-CAR strains. It should be noted that data for Tes3 with 0.5 mM IPTG at 48 h are also presented in Figure 4.3B. All data are average of 3 biological replicates and error bars represent standard deviation.

4.5 Conclusion

In this work, the 1-octanol pathway was investigated and optimized to enhance the production based on several factors, inducer concentration, cultivation volume, strain background and limiting substrate. The inadequate activity of thioesterase (Tes) was identified as the limiting step in 1-octanol production. Four new C8-specific thioesterases were evaluated and these thioestereases were found to be more effective than the original thioesterase (Tes3). The two best thioesterases (‘CpFatB1-4 and ‘CaFatB3-5) were selected for further studies.

Chapter 5
Production of octyl acetate in *E. coli*

In this chapter, *O*-acetylation of 1-octanol was implemented to evaluate the effect of bioderivatization on bioproduction. Octyl acetate is found in fruits as a flavoring and scented component and is as a commodity in different industries such as fragrance, cosmetic, and food industries. Octyl acetate is synthesized by esterification of 1-octanol with vinyl acetate or acetic acid as the acetyl-donor, catalyzed by enzymes or acids (Tomke & Rathod, 2016; Chandane *et al.*, 2017). Even though this molecule possesses valuable industrial properties, renewable production of octyl acetate has not yet been reported. To understand the benefits of bioderivatization, the toxicity of octyl acetate compared with 1-octanol and the analytical methods to reliably quantify 1-octanol and octyl acetate were first investigated. Subsequently, the first octyl acetate biosynthetic pathway was implemented. Lastly, the effects of bioderivatization on growth and production of target compounds were investigated.

5.1 1-Octanol was more toxic than its ester derivative

In order to understand the effects of bioderivatization on the production of 1-octanol, derivatives with lower toxicity than 1-octanol were first identified. As mentioned earlier (Chapter 2), two reactions, namely *O*-acetylation and *O*-glucosylation, are two natural detoxification mechanisms commonly used by many organisms (Jones & Vogt, 2001; de Roode *et al.*, 2003; Saerens *et al.*, 2010). The simplest ester and glucoside derivatives of 1-octanol, octyl acetate and octyl glucoside, were selected at first. These simple derivatives can be synthesized from 1-octanol in a single reaction step, which would make bioderivatization a feasible and straightforward process to implement and lessen the effort to optimize the pathways (e.g., were multiple enzymes involved).

To compare the toxicity of 1-octanol and octyl acetate, *E. coli* C43 (DE3) strain was cultivated in M9 minimal media with different concentrations of 1-octanol and octyl acetate. Figure 5.1A and 5.1C show that 0.75 mM of 1-octanol was sufficient to inhibit the growth of *E. coli* whilst it required a higher concentration of octyl acetate (Figure 5.1B and 5.1D) to achieve the same effect. When only the growth of the first four hours was taken into account to calculate the specific growth rate of *E. coli* (Figure 5.1E), it was clear that 1-octanol was more toxic than octyl acetate. The data suggested that octyl acetate could potentially be a good candidate for investigation of the bioderivatization concept.

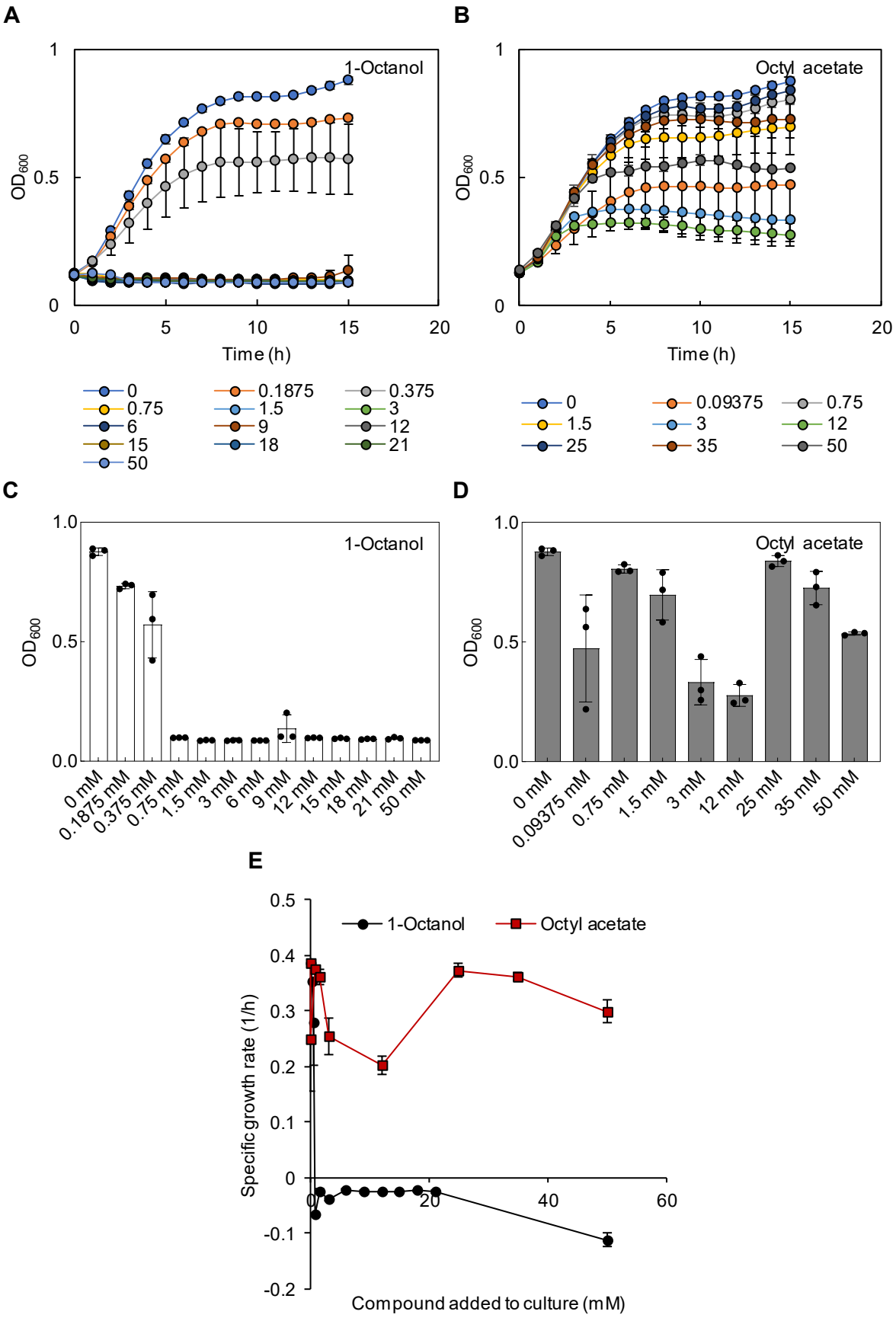

Figure 5.1 Toxicity of 1-octanol and octyl acetate at different concentrations
Average growth curves of *E. coli* C43(DE3) cultivating in M9 minimal media with different concentrations (0-50 mM) of (A) 1-Octanol and (B) Octyl acetate. OD_{600} after 15 h of incubation in (C) 1-Octanol and (D) Octyl acetate. (E)

Specific growth rate determined by calculating the slope of average growth curves within 1-4 h. All data are average of 3 biological replicates and error bars represent standard deviation.

5.2 Analytical methods for 1-octanol and octyl acetate quantification

1-Octanol and octyl acetate have different water solubility, 460-536 mg/L for 1-octanol (Rumble, 2019) and 120-210 mg/L for octyl acetate (Stephenson, 1992). This may result in different partitioning of the products in aqueous and organic phases. In order to accurately quantify the amount of both bioproducts, the % recovery of each compound and how they partition differently were investigated. A mock experiment in 100-ml flask with M9 minimal media spiked with 1-octanol (1 or 3.84 mM) and octyl acetate (1 or 2.9 mM) was prepared. The amount of 1-octanol or octyl acetate partitioned in the aqueous or solvent phase after a 24-48 h incubation with 10% (v/v) hexadecane overlay was quantified. The results showed that none of the compounds was detected in the aqueous phase at the lower concentrations (Figure 5.2A and 5.2B), while ~10% recovery of 1-octanol was found in the aqueous phase and ~80% of both 1-octanol and octyl acetate was found in solvent phase at the higher concentrations (Figure 5.2C and 5.2D). This suggested that most of both compounds migrated to the solvent phase under all tested conditions while 10-20% was lost. The loss was presumably caused by volatilization. As octyl acetate is less water-soluble than 1-octanol; thus, it is less likely to partition into the aqueous phase and may cause volatilization to occur more easily. Therefore, as approximately 80% of both compounds was found in solvent overlay phase, the analytical detection of products was carried out only in the solvent overlay phase.

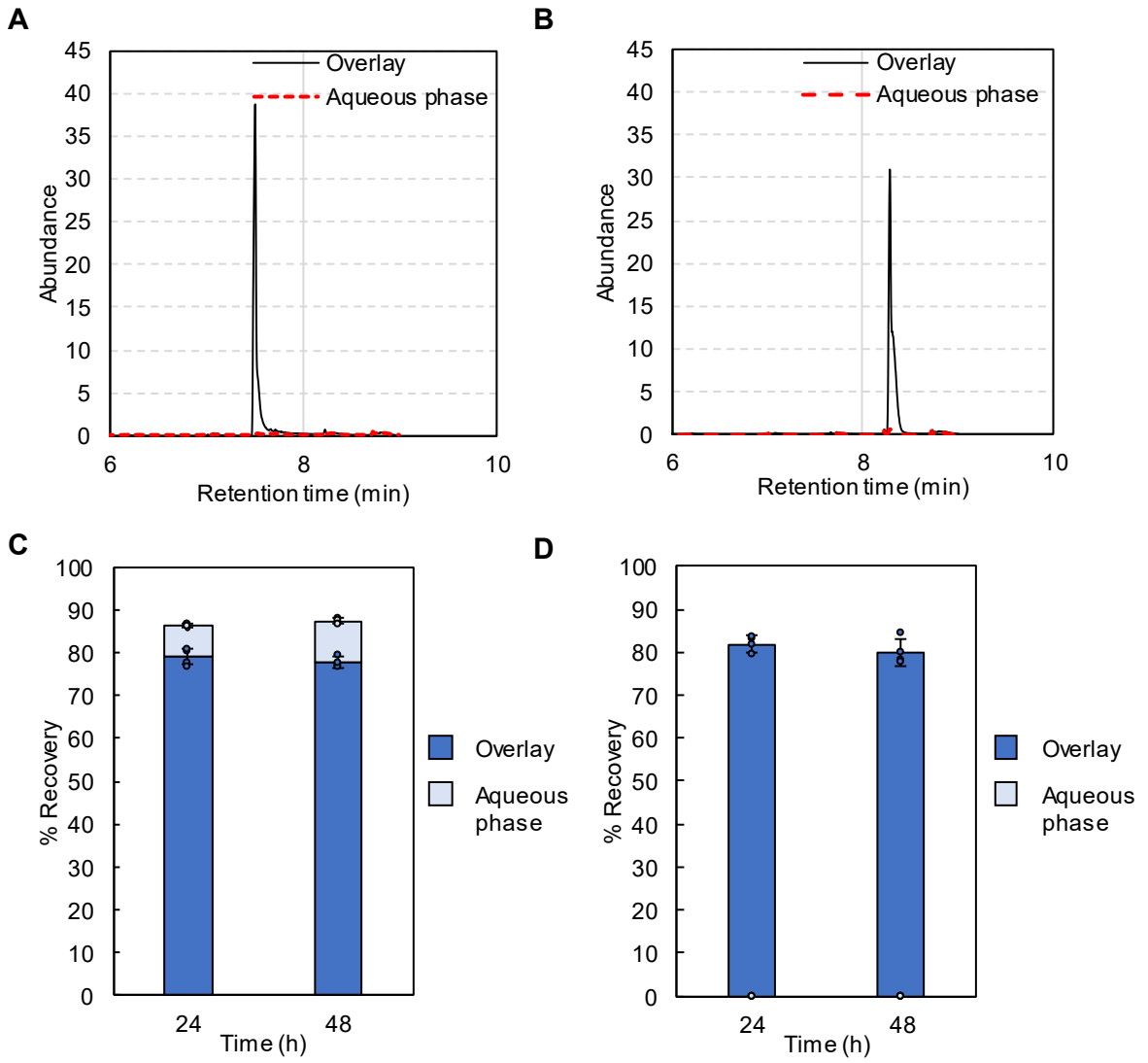

Figure 5.2 Mock experiments to understand localization of 1-octanol and octyl acetate in the presence of solvent overlay

GC-MS chromatograms obtained from hexadecane overlay (overlay) and M9 minimal media (aqueous phase) when spiked with 1 mM of (A) 1-Octanol and (B) Octyl acetate after 24 h incubation. Percent recovery of (C) 1-Octanol and (D) Octyl acetate from hexadecane overlay (overlay) and M9 minimal medium (aqueous phase) when spiked with 500 mg/L of 1-octanol (3.84 mM) or octyl acetate (2.9 mM) after 24-48 h incubation. All data are average of 4 replicates and error bars represent standard deviation.

5.3 ATF1 was the best alcohol acetyltransferase for octyl acetate production

Alcohol acetyltransferase (AAT) is a group of enzymes in the BAHD acyltransferase family and catalyzes ester biosynthesis in organisms via *O*-acetylation of acetyl-CoA donors and aliphatic or aromatic alcohol acceptors with free CoA as a by-product (D'Auria, 2006). Initially, the first-generation 1-octanol pathway, TPC3 (Akhtar *et al.*, 2015), was extended by adding AAT enzymes (Figure 5.3A) in order to implement the first octyl acetate synthetic pathway. Three AATs, namely CAT, SAAT and ATF1, were selected based on the literature. CAT has been reported for ester biosynthesis in *E. coli* (Rodriguez, Tashiro & Atsumi, 2014)

and both SAAT and ATF1 showed activity toward 1-octanol (Aharoni *et al.*, 2000; Rodriguez, Tashiro & Atsumi, 2014; Cumplido-Laso *et al.*, 2012). To evaluate the activity of these AATs, *E. coli* C43(DE) carrying pET-TPC3 plasmid was transformed with a second plasmid harboring different AATs (pCDF-T7-CAT, pCDF-T7-SAAT, or pCDF-T7-ATF1) (Figure 5.3B) and cultivated in M9 minimal medium with 2% (w/v) glucose overlaid with 10% (v/v) hexadecane. The results demonstrated that all three AATs could convert 1-octanol to octyl acetate. The highest octyl acetate titer (0.54 mM or 93.82 mg/L) with a complete conversion of 1-octanol to octyl acetate was achieved by the ATF1 strain after 48 h of incubation (Figure 5.3C and 5.3D). The introduction of AAT did not show marked changes in growth (Figure 5.3E), although a slight improvement in production was observed from strains expressing SAAT and ATF1 (Figure 5.3C). This was speculated to be due to at least 2 possible explanations including (*i*) the hexadecane solvent overlay reduced the toxicity of the products by *in situ* product removal (Rodriguez, Tashiro & Atsumi, 2014; Yunus & Jones, 2018) or (*ii*) the 1-octanol-producing strain reached a final titer of only 0.32 mM, which is lower than the concentration (0.75 mM) that affected the growth of *E. coli*, as shown in Section 5.1. To comprehensively evaluate the impact of bioderivatization, it was considered important to exceed the titer at which 1-octanol affected the growth of the host, at least in the absence of solvent overlay. Therefore, the limiting substrate for octyl acetate pathway was identified in Section 5.4 before the higher-flux strains (*E. coli* BW25113 with newly screened thioesterases, ‘CpFatB1-4 and ‘CaFatB3-5) were investigated in Section 5.5 and 5.6.

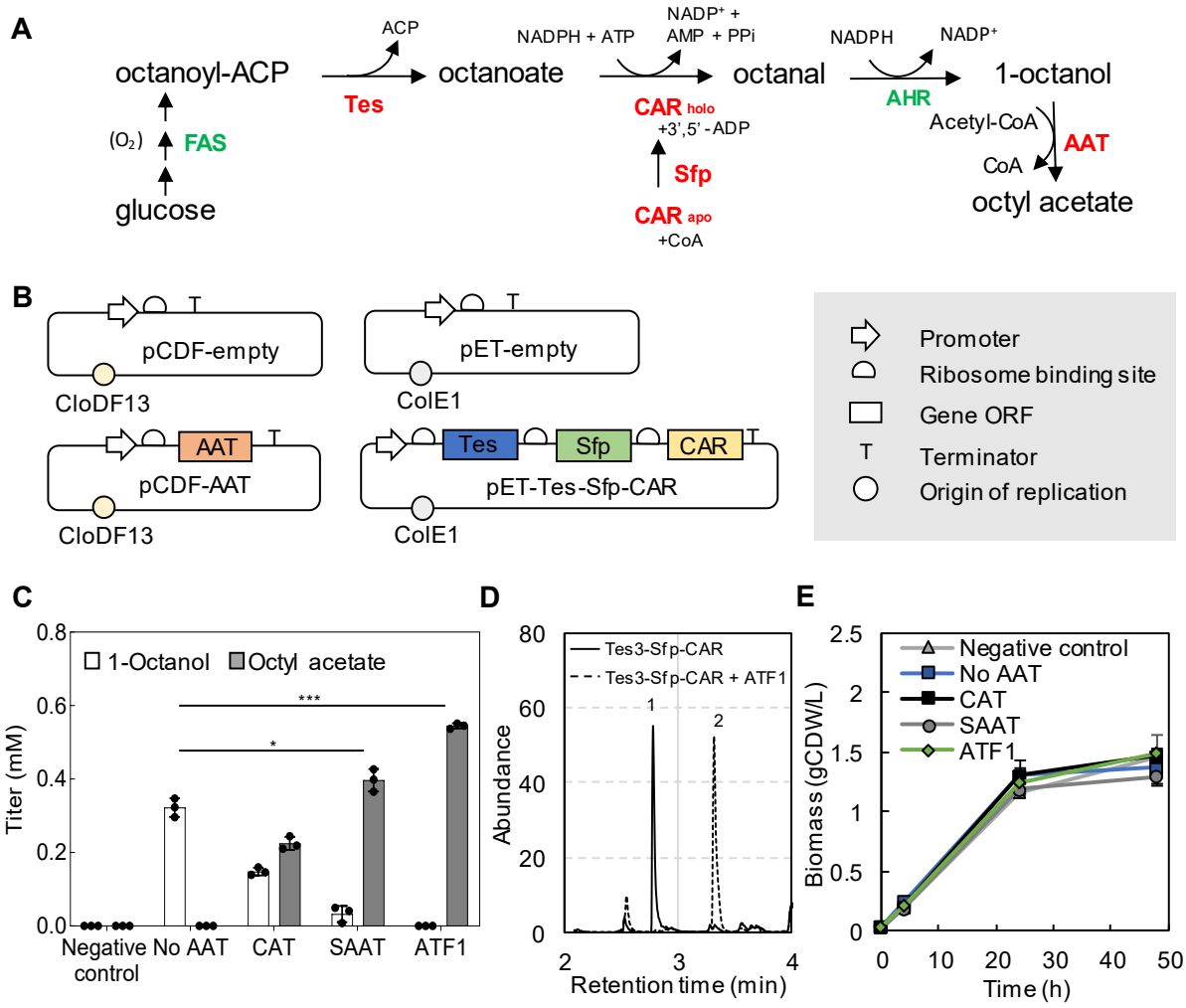

Figure 5.3 Biosynthetic pathway of octyl acetate in engineered *E. coli*
(A) Metabolic pathway for *in vivo* production of octyl acetate extended from an existing 1-octanol pathway. (B) Plasmid constructs used for 1-octanol and octyl acetate production. (C) *In vivo* production of octyl acetate with 3 different AATs; CAT, SAAT and ATF1 at 48 h. (D) Chromatograms of overlay sampled from 1-octanol (Tes3-Sfp-CAR) and octyl acetate (Tes3-Sfp-CAR + ATF1) strains from C. Peak identification: (1) 1-octanol; (2) octyl acetate. (E) Biomass accumulation. *E. coli* C43(DE3) strains harboring Tes3-Sfp-CAR and/or different AATs with a negative control harboring empty plasmids were cultivated in M9 minimal media with 10% (v/v) hexadecane and incubated for 48 h. Asterisk indicates significant difference between 2 treatments (* $P \leq 0.05$ and *** $P \leq 0.005$). All data are average of 3 biological replicates and error bars represent standard deviation.

5.4 AAT activity is not limiting octyl acetate biosynthesis

As mentioned in Section 4.4, octanoic acid was shown to be a limiting substrate for 1-octanol pathway. This indicated that thioesterase (Tes) was a bottleneck enzyme (Figure 4.4). To investigate the limiting step in the octyl acetate pathway, *E. coli* C43(DE3) expressing TPC3 pathway and SAAT was cultivated in M9 minimal media with different concentrations (0-1.5 mM) of exogenously added octanoic acid. Hexadecane (10% (v/v)) was also added on top of culture media. The results showed that a complete conversion of 1-octanol to octyl acetate was achieved (Figure 5.4). This indicated that neither the activity of AAT nor the other

substrate for 1-octanol to octyl acetate conversion, acetyl-CoA, was limiting (Figure 5.4). Thus, it confirmed that the AAT used in this first-generation octyl acetate strain was adequately efficient for the complete conversion of 1-octanol.

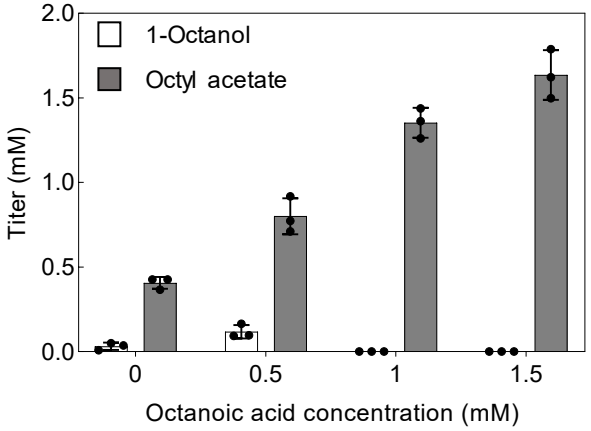

Figure 5.4 Identification of limiting substrate(s) in octyl acetate pathway by substrate feeding experiment
E. coli C43(DE3) harboring Tes3-Sfp-CAR + SAAT was cultivated in M9 minimal media with different concentrations (0-1.5 mM) of octanoic acid supplementation overlaid with 10% (v/v) hexadecane and incubated for 48 h. All data are average of 3 biological replicates and error bars represent standard deviation.

5.5 The octyl acetate strain showed better growth and consumed more glucose

In previous experiments (Figure 5.3C), hexadecane was used to capture 1-octanol or octyl acetate from the liquid culture. The use of hexadecane; however, masked the effect of bioderivatization on cellular growth (Figure 5.3E). Although the toxicity test (Figure 5.1) clearly showed that 1-octanol was more toxic than octyl acetate, the effect of these chemicals on cellular growth when produced *in vivo* remained elusive. Here, three *E. coli* strains, negative control, 1-octanol (Tes3-Sfp-CAR), and octyl acetate (Tes3-Sfp-CAR + SAAT) strains, were cultivated in the absence of hexadecane overlay. The results showed that 1-octanol-producing strain (Tes3-Sfp-CAR) showed noticeably lower cell density (Figure 5.5A and 5.5E) and glucose consumption (Figure 5.5C) compared to the octyl acetate-producing strain (Tes3-Sfp-CAR + SAAT). This indicated that *in vivo* bioderivatization of toxic compound was beneficial for cellular growth. Indeed, in the presence of additional 1.5 mM octanoic acid, the phenotype became more apparent (Figure 5.5B, 5.5D and 5.5F). However, it was also noticeable that the presence of 1.5 mM octanoic acid itself was already detrimental to the growth of a negative control (compare Figure 5.5A and 5.5B). To avoid this issue, additional strains in which Tes3 was replaced with a superior thioesterase, ‘CpFatB1-4 (Figure 4.6), were created. Strains carrying pET-pA1lacO-1-‘CpFatB1-4-Sfp-CAR and pCDF-pA1lacO-1-empty (negative control) or pCDF-pA1lacO-1-ATF1 were cultivated in M9 minimal medium without solvent overlay and

induced with 0.05 mM IPTG. Enhanced growth (Figure 5.6A) and glucose consumption (Figure 5.6B) of octyl acetate-producing strain compared to 1-octanol-producing strain were also observed as similarly shown in Figure 5.5.

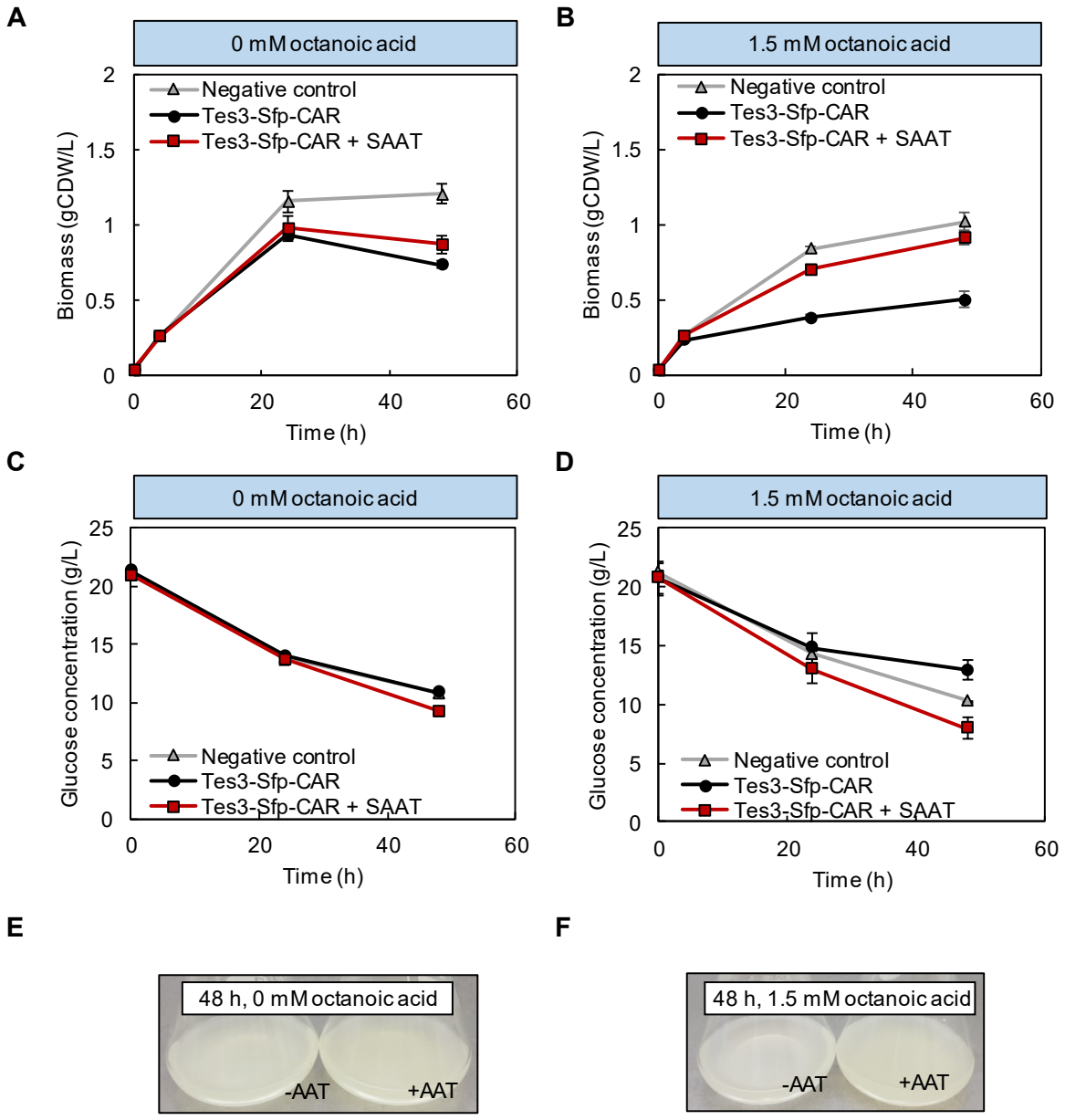

Figure 5.5 Growth and glucose consumption comparison between low-flux 1-octanol and octyl acetate strains in the absence of solvent overlay
Biomass accumulation and glucose consumption of *E. coli* C43 (DE3) fed with (A and C) 0 mM and (B and D) 1.5 mM octanoic acid in the absence of solvent overlay. Three strains were tested, negative control (gray triangles), Tes3-Sfp-CAR (black circles), and Tes3-Sfp-CAR + SAAT (red squares). Photographs of liquid cultures taken at 48 h when supplemented with (E) 0 mM octanoic acid and (F) 1.5 mM octanoic acid in the absence of solvent overlay. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.5 mM IPTG was used to induce the cultures. All data are average of 3 biological replicates and error bars represent standard deviation.

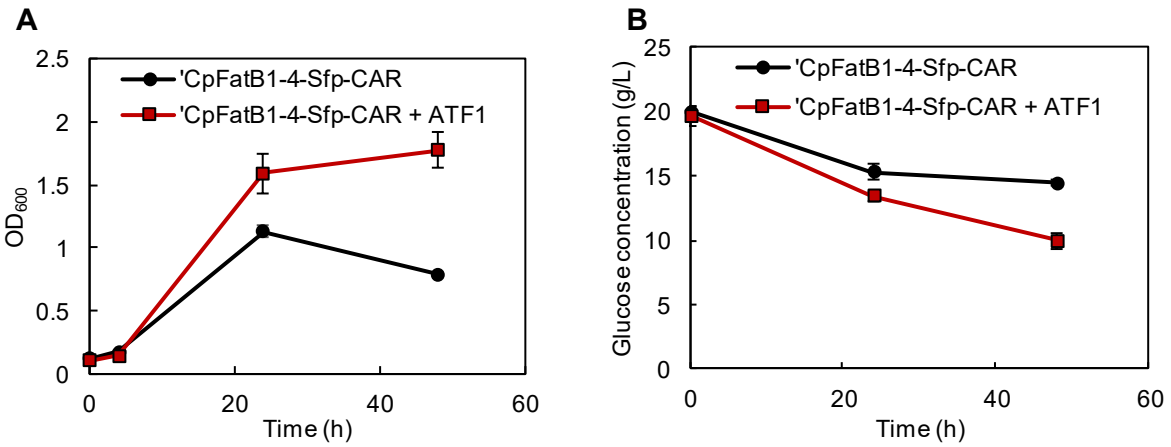

Figure 5.6 Growth and glucose consumption comparison between high-flux 1-octanol and octyl acetate strains in the absence of solvent overlay

(A) Biomass accumulation of *E. coli* BW25113 strains 'CpFatB1-4-Sfp-CAR (black circles) and 'CpFatB1-4-Sfp-CAR + ATF1 (red squares) and (B) Glucose consumption when cultivated in the absence of solvent overlay. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.05 mM IPTG was used to induce the cultures. All data are average of 3 biological replicates and error bars represent standard deviation

Overall, these results suggest that bioderivatization via *O*-acetylation enhances both growth and metabolism of host strains, in the absence of solvent overlay. Next, the effect of bioderivatization on bioproduction was evaluated.

5.6 Octyl acetate strains display enhanced yield and productivity of C8 products

To investigate whether bioderivatization influences the productivity of 1-octanol or not, the effect of ATF1 in the 'CpFatB1-4 and 'CaFatB3-5 strains was evaluated in the presence of solvent overlay at three different IPTG levels (0.05, 0.2 and 0.5 mM). A number of observations were made as many different combinations of induction/thioesterase were investigated. Firstly, cellular growth and glucose consumption were positively influenced by ATF1 in some of the combinations (e.g., 'CafatB3-5 at 0.2 and 0.5 mM IPTG) but not in others (e.g., none of the strains at 0.05 mM IPTG) (Figure 5.7). No differences were observed at low IPTG induction as also observed in low-flux strains (Section 5.3), in spite of a substantial impact on growth and glucose consumption that were observed in the absence of solvent overlay in the previous experiment (Figure 5.6). Hence, this also confirmed that solvent overlay partially diminished cellular growth and metabolism defects caused by the pathway and/or its product, at least when compared with the same strain cultured in the absence of solvent overlay. This is in line with what has been shown previously (Yunus & Jones, 2018).

From a productivity perspective, bioderivatization via *O*-acetylation had no impact on yield (Figure 5.8) or titer (Figure 5.9) at the lowest protein expression inducer level (0.05 mM

IPTG) for both strains. Nevertheless, almost all IPTG/strain/time combinations showed both improved yield and titer when ATF1 was co-expressed at the higher IPTG levels (0.2 and 0.5 mM). Interestingly, the combinations of IPTG level at 0.2 and 0.5 mM and strain sampled at 24 and 48 h except one ('CpFatB3-5, 0.2 mM, 48 h), had increased productivity, even with the variable impacts on growth or glucose consumption (Figure 5.7). This suggests that the effect of *O*-acetylation was partially independent of any effect on cellular activity. I.e. the effect of *O*-acetylation on product toxicity may not have been the only reason for the improved productivity. This additional positive effect can possibly be explained by *O*-acetylation enhancing product removal, either (*i*) by enhancing the compatibility with native efflux transporter(s) or, more likely, (*ii*) by enhancing product solubility in the solvent overlay and thereby facilitating the transfer away from the cell. This would sequentially reduce the product concentration in aqueous phase near the cells, which consequently affects the toxicity.

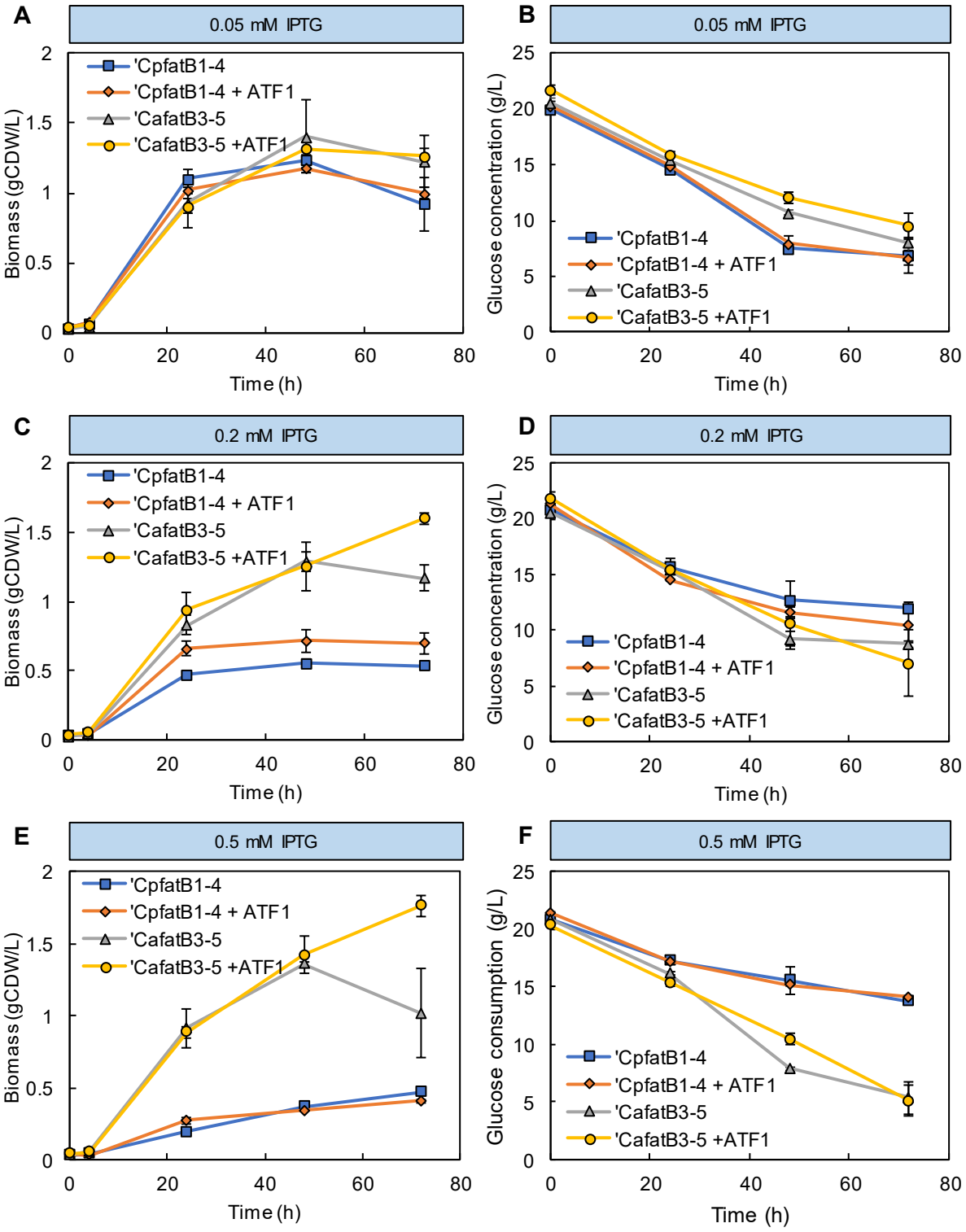

Figure 5.7 Biomass accumulation and glucose consumption of 1-octanol and octyl acetate producing strains in the presence of solvent overlay

E. coli BW25113 strains 'CpFatB1-4-Sfp-CAR, 'CpFatB1-4-Sfp-CAR + ATF1, 'CaFatB3- 5-Sfp-CAR and, 'CaFatB3-5-Sfp-CAR + ATF1 were cultivated in M9 minimal medium with 2% (w/v) glucose and 10% (v/v) hexadecane overlay. Different concentrations of IPTG were used to induce the cultures (A-B) 0.05, (C-D) 0.2 and, (E-F) 0.5 mM. (A, C, E) Biomass accumulation and (B, D, F) Glucose consumption were monitored. All data are average of 3 biological replicates and error bars represent standard deviation.

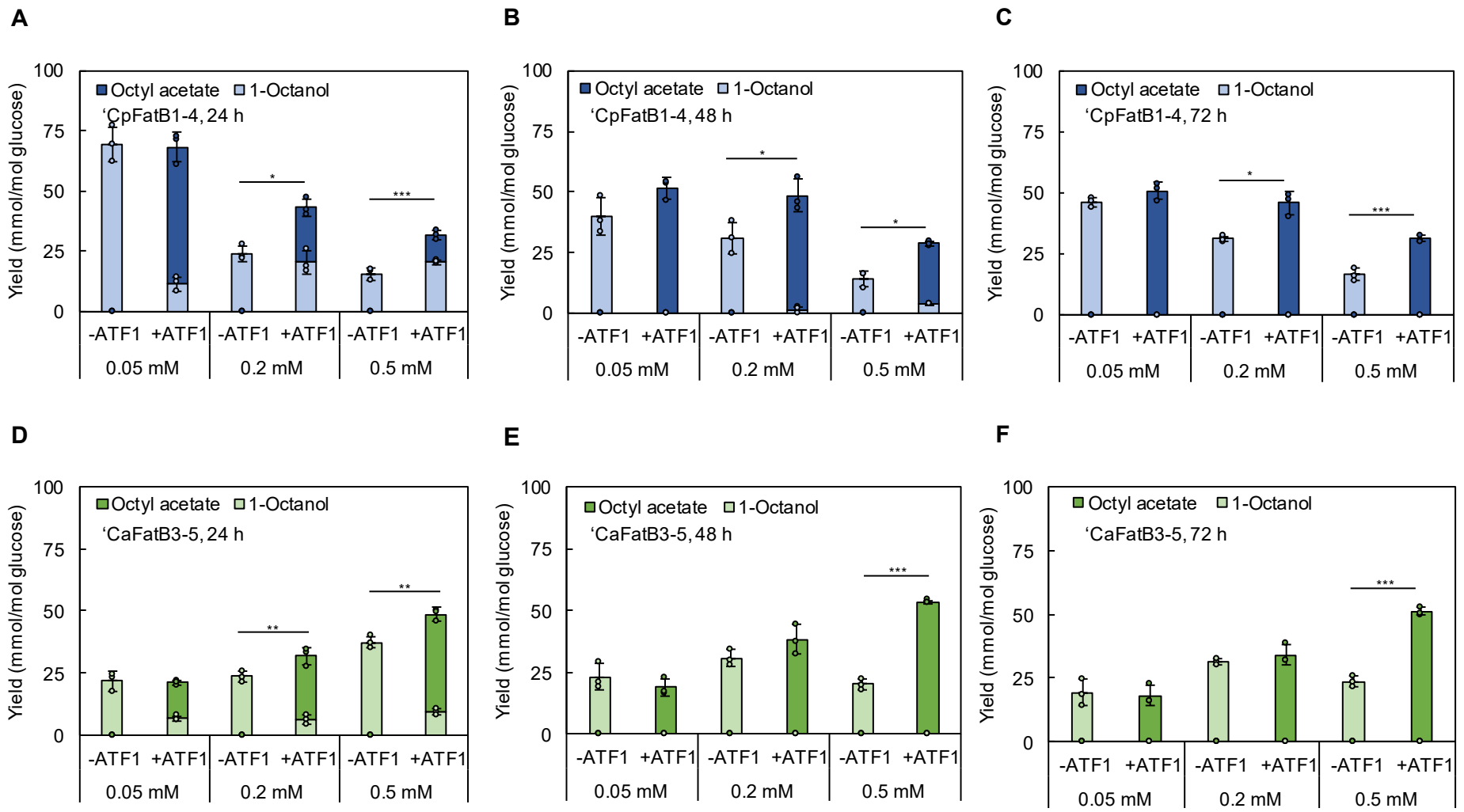

Figure 5.8 Comparison of *in vivo* 1-octanol and octyl acetate production (yield) from *E. coli* BW25113 in the presence of solvent overlay

E. coli BW25113 strains 'CpFatB1-4-Sfp-CAR and 'CpFatB1-4-Sfp-CAR + ATF1 were sampled at (A) 24 h, (B) 48 h, (C) 72 h. Similarly, strains 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1 were sampled at (D) 24 h, (E) 48 h and (F) 72 h. All strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 10% (v/v) hexadecane overlay. Different concentrations of IPTG were used to induce the cultures (0.02, 0.2 and, 0.5mM). Asterisk indicates significant difference between 2 treatments (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.005$). All data are average of 3 biological replicates and error bars represent standard deviation.

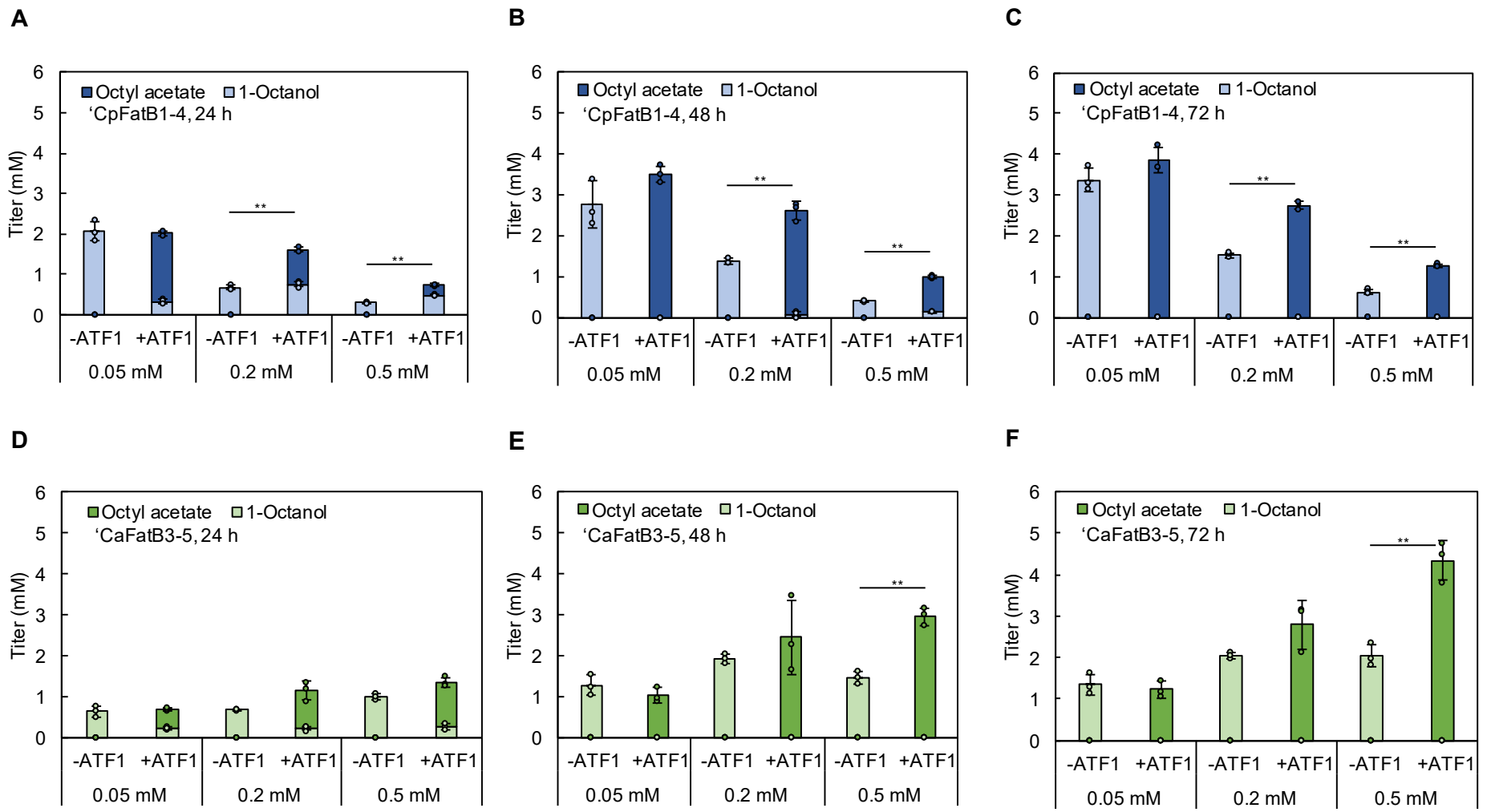

Figure 5.9 Comparison of *in vivo* 1-octanol and octyl acetate production (titer) from *E. coli* BW25113 in the presence of solvent overlay

E. coli BW25113 strains 'CpFatB1-4-Sfp-CAR and 'CpFatB1-4-Sfp-CAR + ATF1 were sampled at (A) 24 h, (B) 48 h, (C) 72 h. Similarly, strains 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1 were sampled at (D) 24 h, (E) 48 h and (F) 72 h. All strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 10% (v/v) hexadecane overlay. Different concentrations of IPTG were used to induce the cultures (0.02, 0.2 and, 0.5mM). ** indicates significant difference between 2 treatments ($P \leq 0.01$). All data are average of 3 biological replicates and error bars represent standard deviation.

The lack of bioderivatization effect on C8 productivity at the low (0.05 mM) IPTG induction level might be explained by an imbalance between pathway catalysts, complicated by the contrasting impact of IPTG on the expression of two different thioesterases. This difference is best illustrated by the change in the product ratio between strains with and without *O*-acetylation in response to the concentration of IPTG used (Figure 5.10).

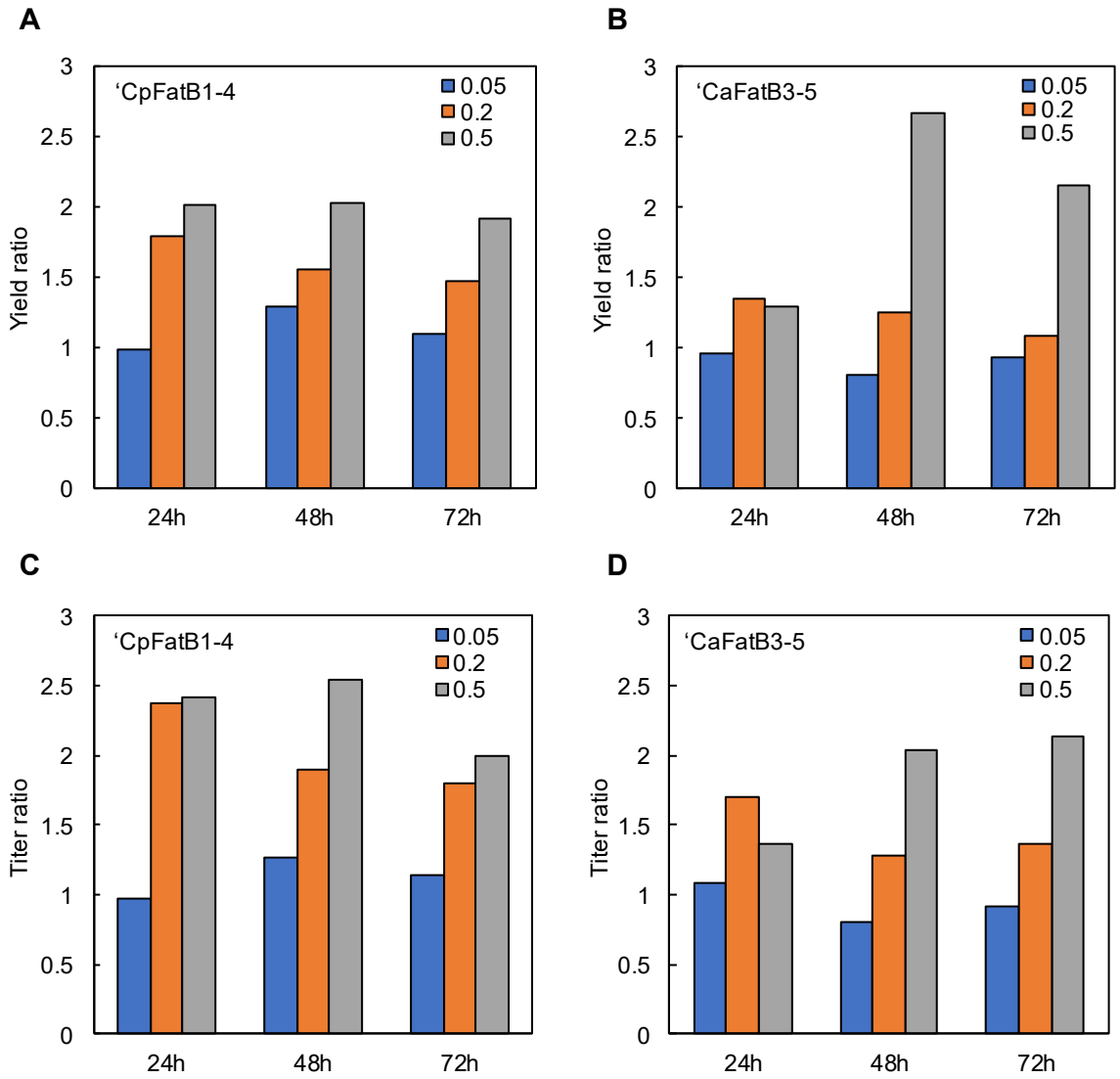

Figure 5.10 The product ratio between octyl acetate and 1-octanol producing strains
The yield or titer ratio between *E. coli* strains (A and C) 'CpFatB1-4-Sfp-CAR and 'CpFatB1-4-Sfp-CAR + ATF1 and (B and D) 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1 when cultivated in M9 minimal medium with 2% (w/v) glucose and 10% (v/v) hexadecane overlay. Different concentrations of IPTG were used to induce the cultures (0.05, 0.2 and 0.5 mM). The ratio was calculated from total products (titer/yield) from octyl acetate strain divided by product from 1-octanol strain to observe fold change in response to IPTG induction level.

To comprehensively understand the system and gain insights for further improvements, fermentation products (acetate, lactate, ethanol, succinate and formate) from *E. coli* BW25113 strains with 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1 were

also monitored in comparison with *E. coli* BW25113 parental strain without plasmids. The most noticeable change was the amount of acetate that showed significant reduction in the strains harboring synthetic pathways compared with the parental strain. This is likely due to the low acetyl-CoA availability when the synthetic pathways were expressed as there was a pull flux toward fatty acid biosynthesis which utilizes acetyl-CoA as a precursor (see Section 2.3.1). Moreover, octyl acetate strain showed lower acetate as acetyl-CoA is also one of the substrates for ATF1 (Figure 5.11). Despite efforts to optimize the bioproduction systems, biomass still remained as the largest 'competitor' to the target pathway.

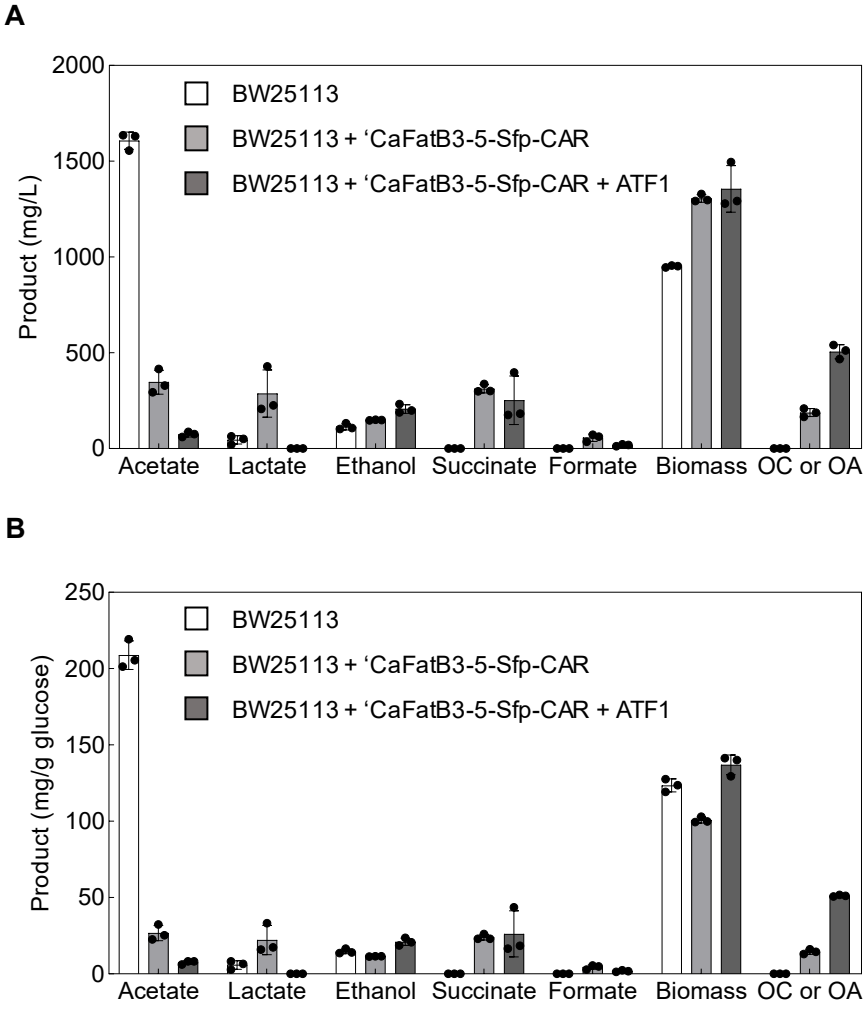

Figure 5.11 Fermentation products and biomass accumulation
(A) Fermentation product and biomass accumulation from *E. coli* BW25113 parental strain (no plasmids and no IPTG induction) and BW25113 strains with 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1 when cultivated in M9 minimal medium with 2% (w/v) glucose and 10% (v/v) hexadecane overlay for 48 h. (B) Fermentation product and biomass accumulation when normalized with glucose consumption. OC: 1-octanol from 'CaFatB3-5-Sfp-CAR strain; OA: octyl acetate from 'CaFatB3-5-Sfp-CAR + ATF1 strain. All data are average of 3 biological replicates and error bars represent standard deviation.

To confirm that the product measurements only from the overlay phase were representative of the total bioproduction, two *E. coli* strains, 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1, were cultivated in the best IPTG induction level (0.5 mM) and the distribution of products between the aqueous and solvent phases was quantified. Similar to what observed in the mock experiments (Figure 5.2), the majority of 1-octanol and all octyl acetate was found in the solvent phase (Figure 5.12). With regards to 1-octanol in the aqueous phase, the results did not change the conclusion on bioderivatization made earlier as there were still improvements in production.

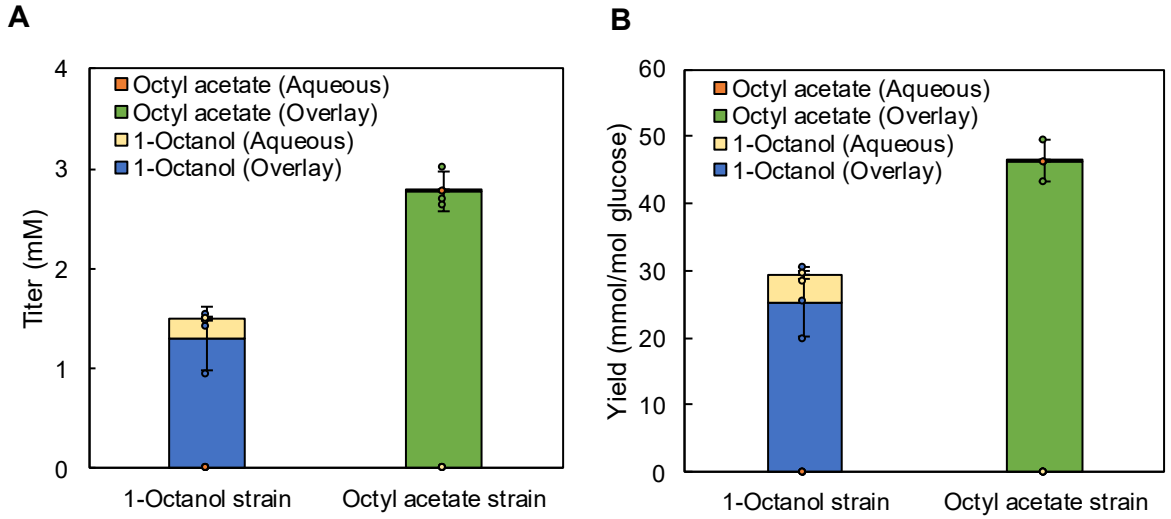

Figure 5.12 Liquid phase partitioning of 1-octanol and octyl acetate accumulating in cultures with the engineered strains

(A) Titer and (B) Yield of 1-octanol and octyl acetate obtained from strains 'CaFatB3-5-Sfp-CAR and 'CaFatB3-5-Sfp-CAR + ATF1. The strains were cultivated in M9 medium with 2% (w/v) glucose induced with 0.5 mM IPTG, overlaid with 10% (v/v) hexadecane, and incubated for 48 h. 1-Octanol or octyl acetate obtained from the aqueous phase was extracted with 10% (v/v) hexadecane prior to GC-MS analysis. All data are average of 3 biological replicates and error bars represent standard deviation.

As cyanobacteria are photoautotrophic microorganisms, they are able to convert sunlight and CO_2 to desirable products. This characteristic has made cyanobacteria an attractive host microorganism for bioproduction via synthetic pathways over the last decade (Atsumi, Higashide & Liao, 2009; Yu *et al.*, 2013; Gomaa, Al-Haj & Abed, 2016; Carroll *et al.*, 2018). Bioderivatization of 1-octanol via *O*-acetylation was also applied to cyanobacterial bioproduction by one of our lab members (Yunus, 2019) with the same pathway enzymes shown in this chapter. It was demonstrated that bioderivatization of 1-octanol to octyl acetate has shown positive impacts on *Synechocystis* sp. PCC 6803 in improving both growth and production of C8 products (Sattayawat, Yunus & Jones, 2020).

5.7 Conclusion

In this work, octyl acetate was demonstrated to be less toxic than 1-octanol. Analytical methods for 1-octanol and octyl acetate were also established. A synthetic metabolic pathway for octyl acetate biosynthesis pathway from renewable substrate was thereafter implemented by extending the 1-octanol pathway with alcohol acetyltransferase (AAT). Three AAT candidates (CAT, SAAT and ATF1) were evaluated and all three were able to convert 1-octanol into octyl acetate. The best AAT for this reaction was ATF1, which enabled the production up to 0.54 mM octyl acetate with the first-generation strains. Octanoic acid was also identified as a limiting substrate for octyl acetate production similar to what has been shown in the previous chapter. This suggested that current AATs used in this study possessed adequate activity for all available 1-octanol. *O*-acetylation of 1-octanol to octyl acetate mitigated the toxic effects on growth and metabolism in the absence of solvent overlay. Moreover, in the presence of solvent overlay, octyl acetate strains showed improved productivity (titer and yield) in most cases compared with 1-octanol strains with variable impact on growth and metabolism suggesting that the effect of bioderivatization was not solely from reduced toxicity, but partially also from the enhanced product removal. The improvement was approximately 2 and 1.6-fold higher in titer and yield, respectively. Altogether, the results support that bioderivatization holds potential as a strategy to enhance the bioproduction of toxic and poorly soluble chemicals.

Chapter 6
Production of octyl glucoside in *E. coli* and cyanobacteria

In Chapter 5, the concept of bioderivatization was systematically studied in two bacterial strains with 1-octanol as the product and *O*-acetylation as the bioderivatization method. The results suggested that the concept could be used to enhance bioproduction of toxic or poorly soluble compounds. However, the concept was only tested with a single product and a single method of bioderivatization. In order to expand the investigation into the bioderivatization concept and to understand how generic and applicable it may be, the study was extended by evaluation of an alternative conjugate type, glucose. In this chapter, bioderivatization through *O*-glucosylation was therefore investigated in both *E. coli* and *Synechocystis* sp. PCC 6803.

6.1 1-Octanol was more toxic than its glucoside derivative

Similar to the study with octyl acetate, the toxicity of 1-octanol was first evaluated compared to its simplest glucoside derivative, octyl glucoside. Octyl glucoside has been used for a long time as a solubilizing detergent in biological applications and scientific studies (Saito & Tsuchiya, 1984). Figure 6.1 shows that octyl glucoside was less toxic than 1-octanol as *E. coli* could grow at 2.5 mM octyl glucoside (Figure 6.1B and 6.1D) but was completely inhibited with 0.75 mM of 1-octanol (Figure 6.1A and 6.1C). This suggests that octyl glucoside is a potential candidate derivative for bioderivatization of 1-octanol.

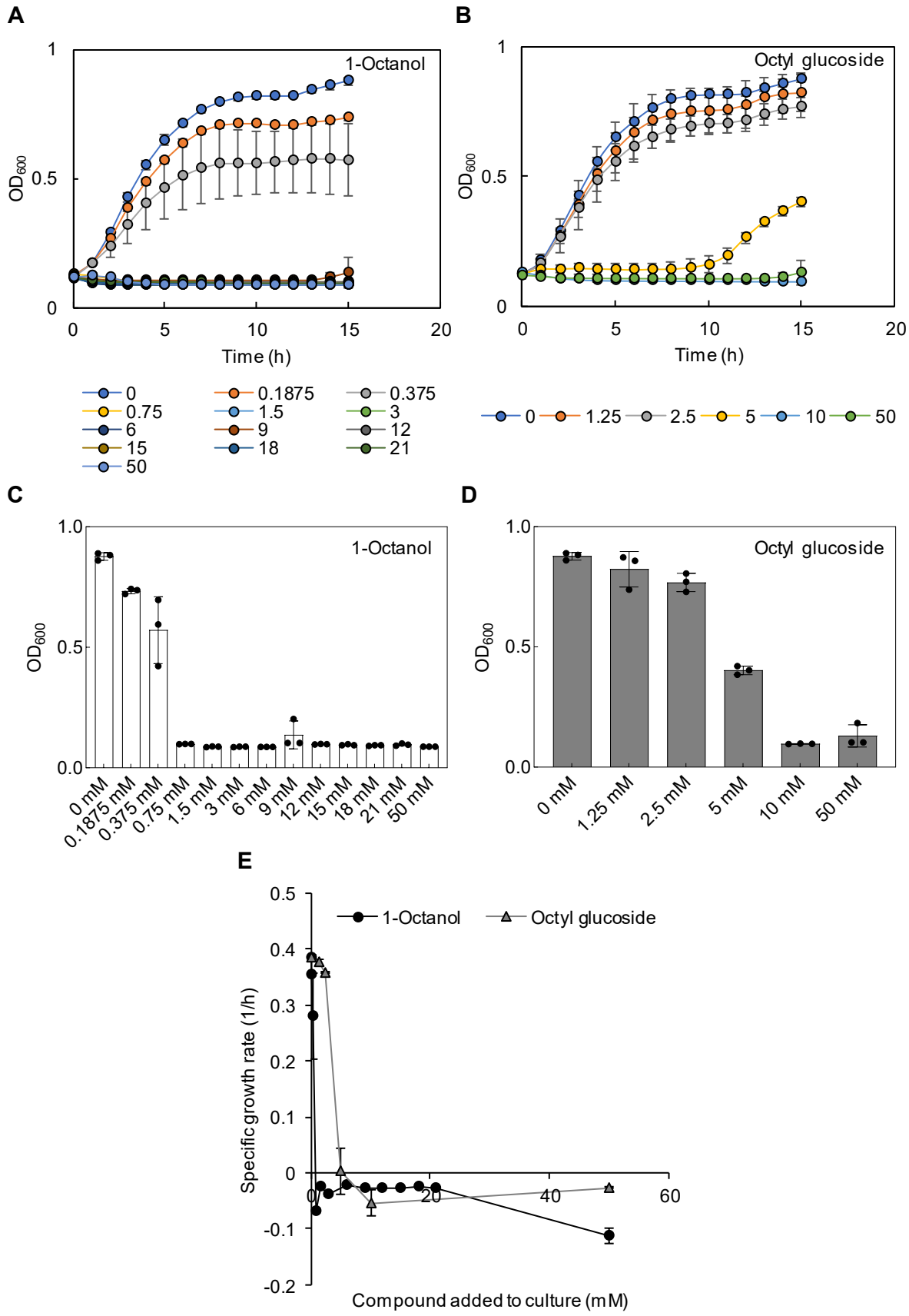

Figure 6.1 Toxicity of 1-octanol and octyl glucoside at different concentrations
Average growth curves of *E. coli* C43(DE3) cultivating in M9 minimal media with different concentrations (0-50 mM) of (A) 1-Octanol and (B) Octyl glucoside. OD_{600} after 15 h of incubation in (C) 1-Octanol and (D) Octyl glucoside. (E) Specific growth rate determined by calculating the slope of average growth curves within 1-4 h. It should be

noted that 1-octanol toxicity results are also presented in Section 5.1 and the experiments were carried out at the same time. All data are average of 3 biological replicates and error bars represent standard deviation.

6.2 MtH2 efficiently converted 1-octanol into octyl glucoside in *E. coli* and octyl glucoside was exclusively found in the liquid medium

Similar to the octyl acetate metabolic pathway, only one reaction step was needed to extend the 1-octanol pathway by the introduction of a UDP-glucose:glycosyltransferase (GT) (Figure 6.2A). GTs catalyze the transfer of monosaccharides from an activated sugar donor (usually UDP-glucose) onto various aglycon acceptors (Lairson *et al.*, 2008). They are generally active on a wider range of substrates and play an important role in determining the chemical diversity of glucosides in plants. In order to find a suitable GT displaying activity also toward 1-octanol, five previously studied GTs from different organisms were selected and characterized (Table 6.1). Some of them (AdGT4 and VvGT1) were selected based on their activity toward longer-chain alcohols (Bönisch *et al.*, 2014; Yauk *et al.*, 2014) and the rest of them were selected randomly from reported literature. Moreover, expression of AdGT4 in transgenic petunia flowers was reported to catalyze the formation of glycosylated 1-octanol (Yauk *et al.*, 2014).

Table 6.1 List of glycosyltransferases (GTs) used in this study

Name	Source organism	Reference
AdGT4	*Actinidia deliciosa*	(Yauk *et al.*, 2014)
AtGT1	*Arabidopsis thaliana*	(Lim *et al.*, 2002)
MtG1	*Medicago truncatula*	(Shao *et al.*, 2005)
MtH2	*Medicago truncatula*	(Li *et al.*, 2007)
VvGT1	*Vitis vinifera*	(Ford, Boss & Hæj, 1998)

To make octyl glucoside, the ‘CaFatB3-5 thioesterase (Tes), phosphopantetheniyl transferase (Sfp), and carboxylic acid reductase (CAR) were expressed in a pET-based plasmid whilst the GT was expressed in a pCDF-based plasmid (Figure 6.2B). This plasmid expression system is similar to the system that has been used for octyl acetate production (Chapter 5, Figure 5.3B). Strains were cultivated for 48 h and octyl glucoside from the supernatant was measured at 24 h and 48 h. Out of five GTs tested, octyl glucoside was only detected in the culture media with the strain carrying MtH2 (Figure 6.2C and 6.2D). The titer and yield reached 0.73 mM (214 mg/L) and 14.84 mmol/mol glucose, respectively after 48 h of incubation. Interestingly, AdGT4, which has been shown in the literature to catalyze 1-octanol as a substrate, did not yield any detectable octyl glucoside. This could be due to two possibilities: (*i*) the HPLC detection limit for octyl glucoside was not low enough to detect octyl glucoside produced from AdGT4 strain or (*ii*) AdGT4 and the rest of the GTs that did not show

octyl glucoside production were not properly expressed. To confirm the latter possibility, SDS-PAGE analysis is one of the most common techniques to validate the expression of recombinant proteins.

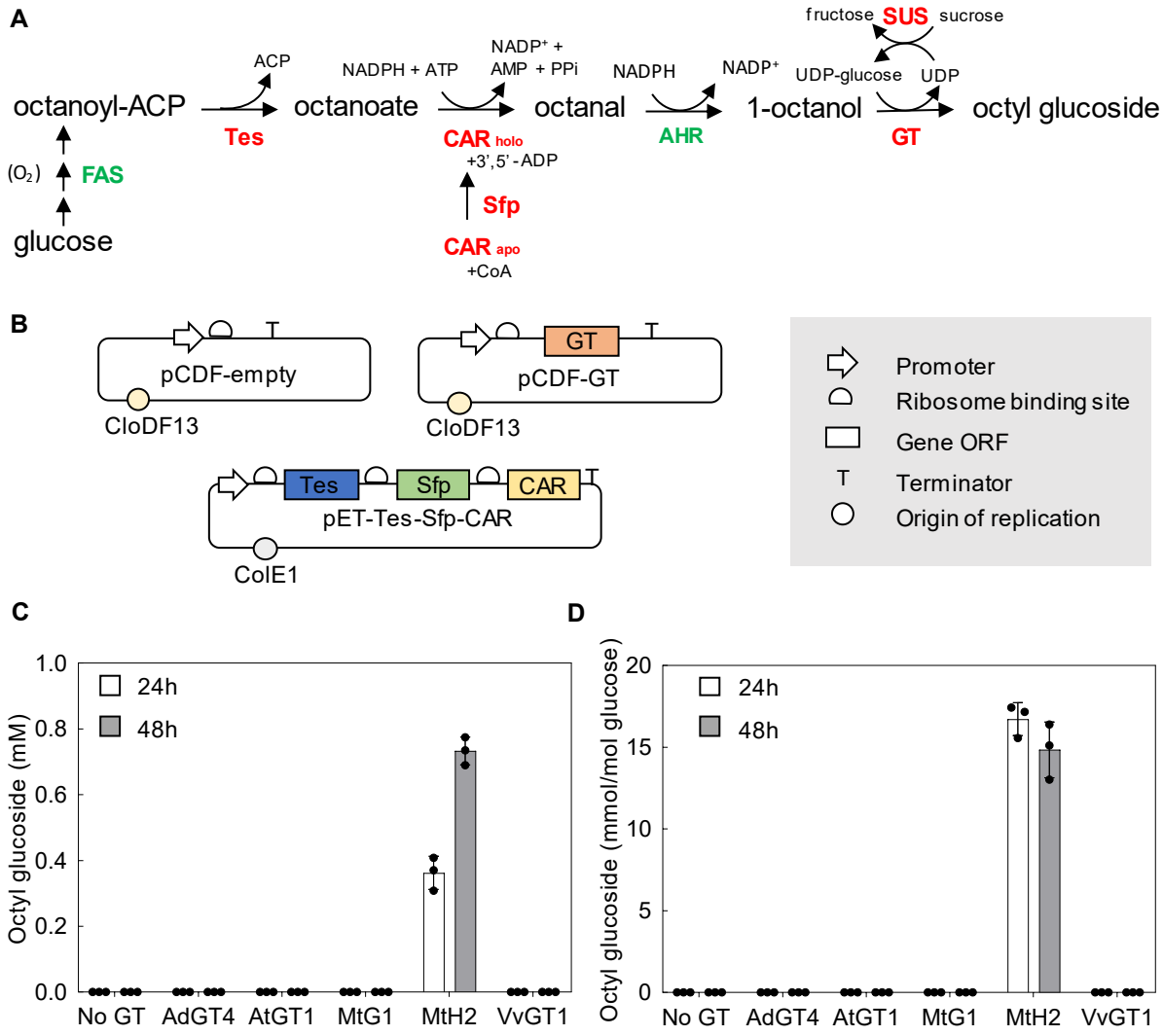

Figure 6.2 Biosynthesis of octyl glucoside with different glycosyltransferases (GTs)
(A) Metabolic diagram for octyl glucoside production by the extension of 1-octanol pathway (B) Schematic diagram depicting plasmid constructs used in *E. coli* octyl glucoside production. Selection of GT for octyl glucoside production with 'CaFatB3-5-Sfp-CAR, 'CaFatB3-5-Sfp-CAR + AdGT4, 'CaFatB3-5-Sfp-CAR + AtGT1, 'CaFatB3-5-Sfp-CAR + MtG1, 'CaFatB3-5-Sfp-CAR + MtH2, and 'CaFatB3-5-Sfp-CAR + VvGT1 in the absence of solvent overlay. (C) Titer (D) Yield. *E. coli* BW25113 strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.5 mM IPTG was used to induce the cultures. All data are average of 3 biological replicates and error bars represent standard deviation.

To investigate if any of the octyl glucoside from this engineered strain remained inside the cells, the cell pellet was extracted with chloroforom:methanol. Although highly soluble in water (5.5 g/L) (Focher *et al.*, 1989), octyl glucoside contains a hydrophilic head and a hydrophobic tail (Figure 6.3A). It was possible that octyl glucoside could partition to both methanol/aqueous and chloroform layers. The octyl glucoside was therefore monitored in both layers. Figure 6.3B and Figure 6.3C show that only a very small amount of octyl glucoside was

detected from the chloroform layer, whilst none was observed from the methanol/aqueous layer. Likewise, when the cell pellet was lysed with glass beads and resuspended in M9 minimal medium, no octyl glucoside was detected (Figure 6.3D). This experiment suggested that all of the octyl glucoside was naturally excreted by the cells and exclusively accumulated in the liquid medium. To my knowledge, the mechanisms for octyl glucoside transportation across bacterial cell membrane are still unknown. However, published reports on glucoside production in *E. coli* suggest that a diverse range of glucosides all accumulated in the extracellular fractions, including geranyl glucoside, cinnamyl alcohol glucoside, and fisetin glucoside (Huang *et al.*, 2016; Pandey *et al.*, 2016; Zhou *et al.*, 2017). Secretion of bioproducts to culture media is a useful property for bio-based production as this bypasses the need to harvest cells and extract the products (Lee *et al.*, 2016) and allows the cells to be continuously used, thereby potentially reducing the overall production cost.

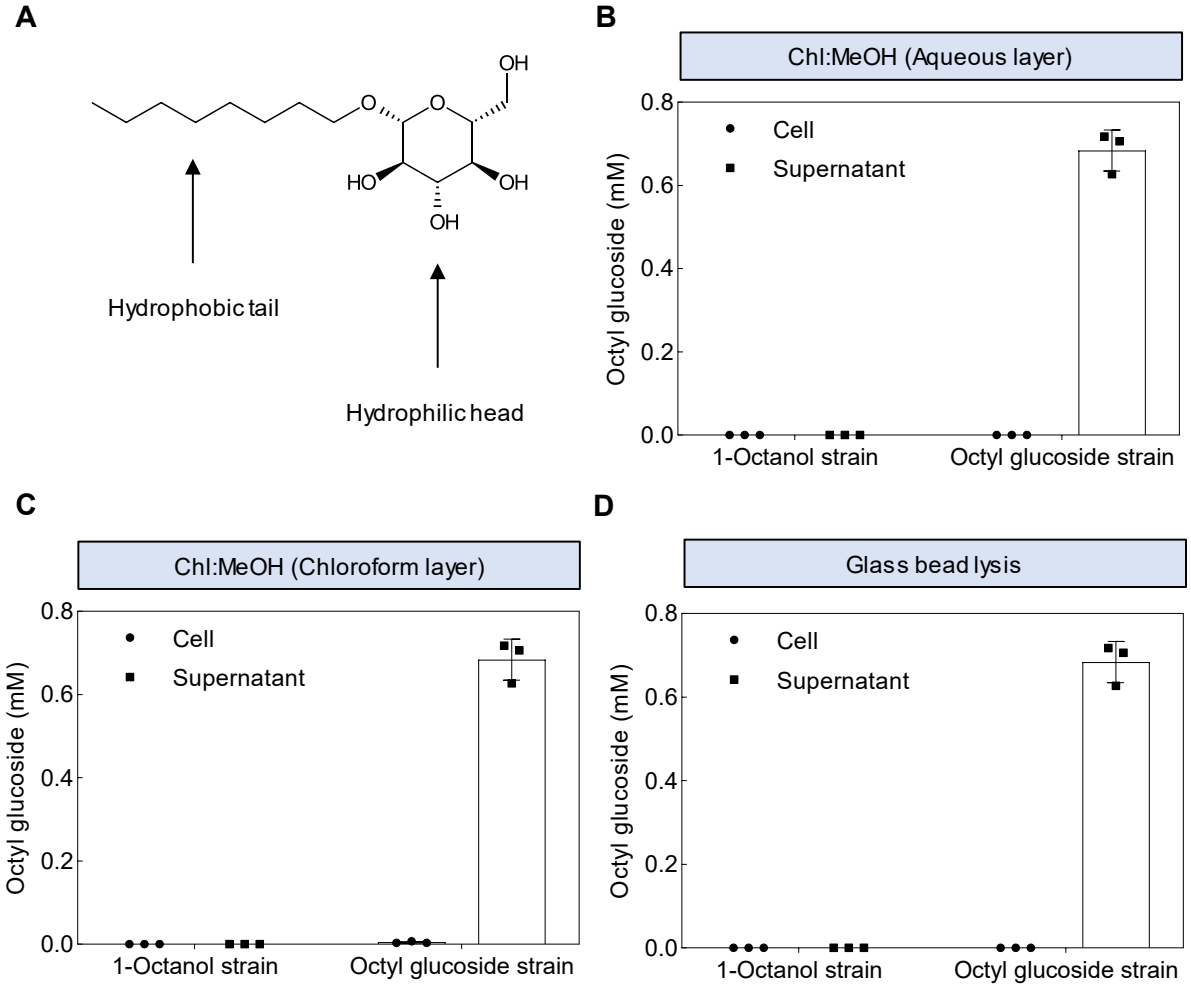

Figure 6.3 Localization of octyl glucoside from different cell lysis methods
(A) Chemical structure of octyl glucoside. The production from first-generation octyl glucoside strains. The supernatant was analyzed with HPLC for extracellular product quantification. For intracellular product quantification, the cells were lysed using Chloroform:methanol extraction in (B) aqueous phase (C) chloroform

phase and (D) Glass bead lysis. All data are average from 3 biological replicates and the error bars represent standard deviation.

6.3 AtSUS1 enhanced octyl glucoside production

Above, it was shown that MtH2 efficiently converted 1-octanol into octyl glucoside (Figure 6.2C and 6.2D). MtH2 originates from *Medicago truncatula* and has been described as an (iso)flavonoid glycosyltransferase displaying activity toward UDP-glucose and a wide range of flavonoid substrates, such as kaempferol, quercetin, isoliquiritigenin, biochanin A, and genistein (Li *et al.*, 2007). Apart from an aglycon receptor, glycosyltransferases also require an activated sugar donor (Breton *et al.*, 2006; Schwab, Fischer & Wüst, 2015). The production of octyl glucoside in Section 6.2 relies on naturally available UDP-glucose as a sugar donor. In bacteria, this metabolite is used as one of the precursors for cell wall biosynthesis, thus all bacteria synthesize UDP-glucose at a basal level (Ruffing & Chen, 2006). It has been reported that GT activity was inadequate due to inhibition of GT activity by free UDP and/or the low natural availability of UDP-glucose (Masada *et al.*, 2007; Terasaka *et al.*, 2012; Huang *et al.*, 2016). To overcome such issues, there are several previous reports on the use of sucrose synthase (SUS) to enhance glucoside formation such as geranyl glucoside and curcumin monoglucoside or diglucoside (Masada *et al.*, 2007; Terasaka *et al.*, 2012; Huang *et al.*, 2016). Sucrose synthase (SUS) is an enzyme that catalyzes a reversible reaction between sucrose and free UDP to UDP-glucose and fructose (Geigenberger & Stitt, 1993). In this study, AtSUS1 from *Arabidopsis thaliana* (Zheng *et al.*, 2011) was co-expressed with MtH2 to enhance production by (*i*) increasing UDP-glucose availability and (*ii*) reducing the inhibitory effect of UDP by *in situ* regeneration of UDP-glucose. In order to use SUS in this manner, sucrose needs to be supplemented to the media as one of the substrates for SUS (Masada *et al.*, 2007; Terasaka *et al.*, 2012); however, this also complicates interpretation as it potentially also may act as a carbon substrate. A set of plasmids was constructed to investigate the use of AtSUS1. The pET-based 1-octanol plasmid (pET-‘CaFatB3-5-Sfp-CAR) and pCDF-based MtH2 plasmid (pCDF-MtH2) from earlier experiment were used. New pCDF-based constructs harboring AtSUS1 only or MtH2 with AtSUS1 were constructed (Figure 6.4A). When combined, it was found that octyl glucoside production was enhanced by the addition of AtSUS1 at both concentrations (15 and 100 mM) of sucrose supplementation (Figure 6.4B). It was also noticeable that the supplementation of 100 mM sucrose enhanced product yield even further than 15 mM (Figure 6.4B).

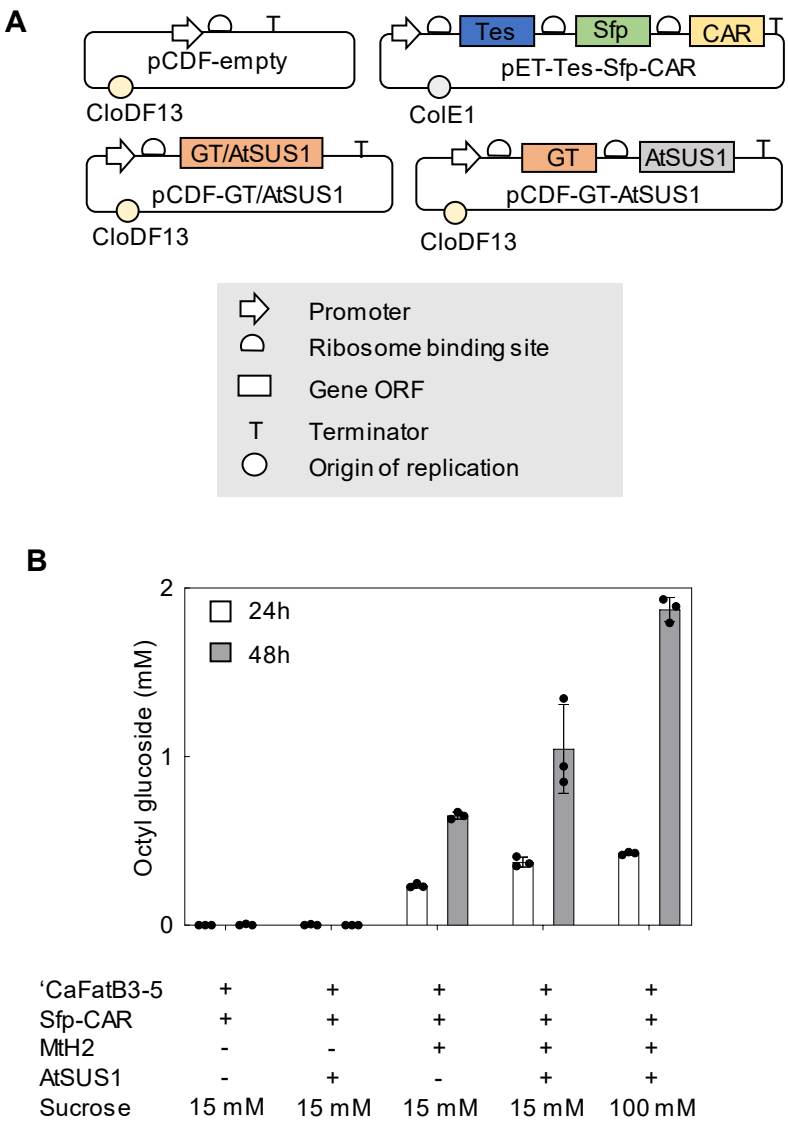

Figure 6.4 The use of sucrose synthase (SUS) to enhance octyl glucoside production in *E. coli*
(A) Schematic diagram depicting plasmid constructs used (B) Octyl glucoside production in the absence of hexadecane overlay. Octyl glucoside production with strains 'CaFatB3-5-Sfp-CAR, 'CaFatB3-5-Sfp-CAR + AtSUS1, 'CaFatB3-5-Sfp-CAR + MtH2, and 'CaFatB3-5-Sfp-CAR + MtH2 + AtSUS1, supplemented with 15 mM and 100 mM sucrose. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose, 0.5 mM IPTG was used to induce the cultures and 15 or 100 mM sucrose was supplemented at the time of induction. All data are average of 3 biological replicates and error bars represent standard deviation.

Even though octyl glucoside can be recovered and quantified from the aqueous phase, in order to understand the effect of bioderivatization on the bioproduction system also 1-octanol needed to also be accurately quantified. The use of solvent overlay was therefore used to capture 1-octanol as mentioned earlier in previous chapters. The mock experiment to understand the localization of octyl glucoside in the presence of solvent overlay was therefore carried out. Octyl glucoside (500 mg/L) was spiked to M9 minimal medium with 2% (w/v) glucose and overlaid with 10% (v/v) hexadecane for 24 h. The results suggested that despite the presence of 10% (v/v) hexadecane overlay, all octyl glucoside was only recovered from

the aqueous phase (Figure 6.5). This is interesting given that octyl glucoside contains both hydrophilic and hydrophobic tails (Figure 6.3A).It is speculated to be due to its very high water solubility (5.5 g/L) (Focher *et al.*, 1989).

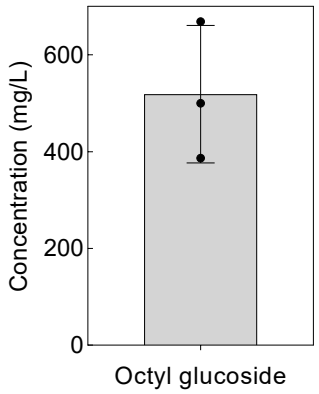

Figure 6.5 The mock experiment to understand the localization of octyl glucoside in the presence of solvent overlay
M9 minimal medium with 2% (w/v) glucose was spiked with 500 mg/L of octyl glucoside overlaid with 10% (v/v) hexadecane and incubated for 24 h. Octyl glucoside was quantified from aqueous phase. All data are average of 3 replicates and error bars represent standard deviation.

6.4 Octyl glucoside producing strains carrying both MtH2 and AtSUS1 showed improved production

In previous experiments, it was shown that octyl glucoside was secreted and accumulated solely in the aqueous culture medium (Figure 6.3) and that the use of hexadecane overlay did not affect the quantification of octyl glucoside in the aqueous phase (Figure 6.5). Therefore, hexadecane was used as an overlay of the cultures in order to determine whether any 1-octanol was excreted too quickly and thereby evaded glycosylation. Surprisingly, more octyl glucoside was produced in the presence of solvent overlay than in its absence (compare Figure 6.4B and 6.6B). As the solvent overlay had no impact on the growth of these strains, this could not be explained by any differential toxicity. An alternative possibility is that the solvent overlay captures 1-octanol, minimizes the loss of 1-octanol from volatilization and enables re-uptake by the cells and conversion into octyl glucoside in the stationary phase. The results showed that both the titer and yield of 1-octanol was improved after both 24 h and 48 h, in a strain combining the 1-octanol pathway with both MtH2 and AtSUS1 compared with the strain carrying only the 1-octanol pathway (Figure 6.6A-D). The strain carrying 1-octanol pathway with both MtH2 and AtSUS1 also showed higher biomass accumulation, though not significantly different (Figure 6.6E).

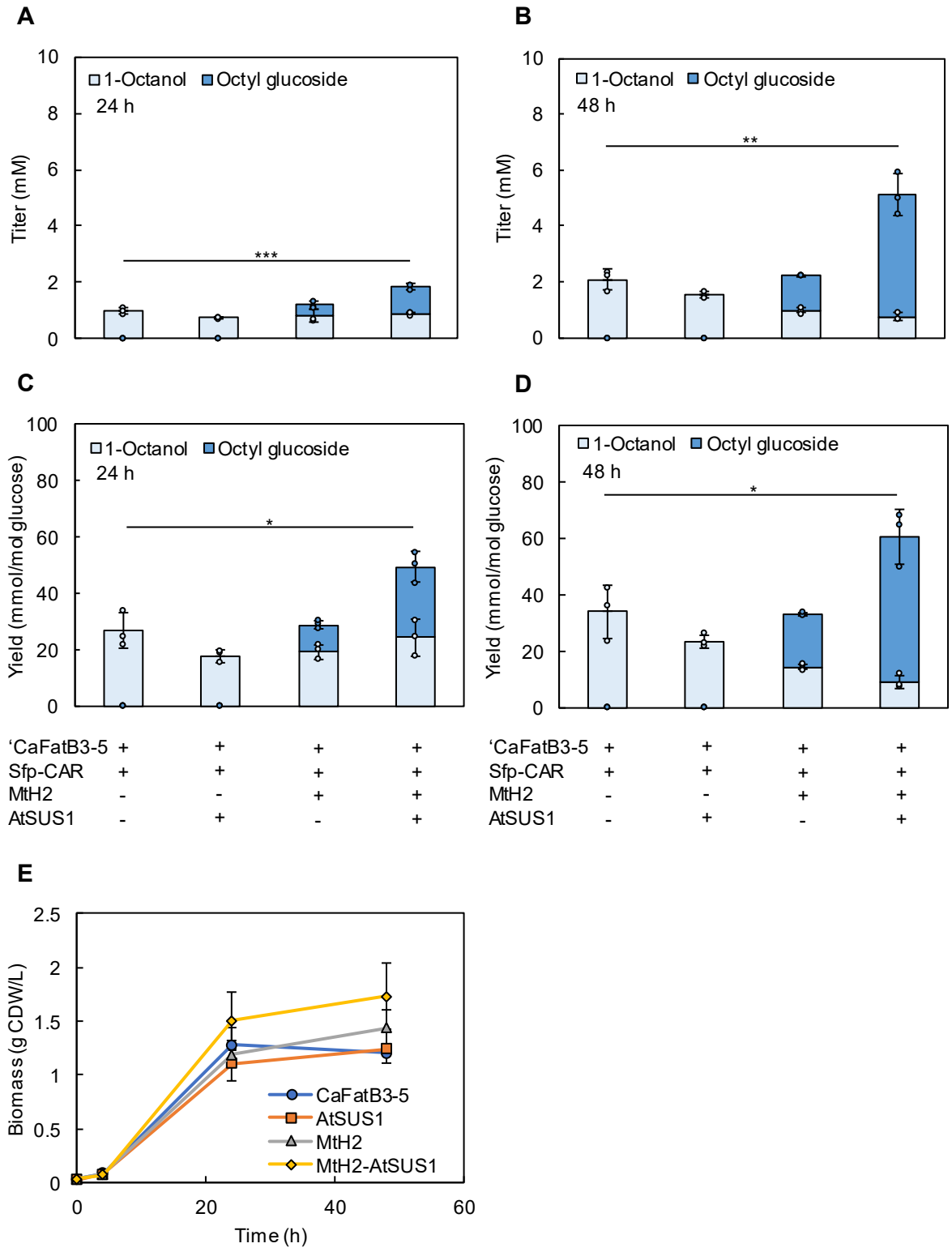

Figure 6.6 Improved productivity from octyl glucoside producing strains

In vivo 1-octanol and octyl glucoside production in the presence of solvent overlay with 15 mM sucrose supplementation. Titer at (A) 24 h (B) 48 h and Yield at (C) 24 h and (D) 48 h (E) Biomass accumulation. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose, 0.5 mM IPTG was used to induce the cultures and 15 mM sucrose was supplemented at the time of induction. The cultures were overlaid with 10% (v/v) hexadecane. Asterisk indicates significant difference between 2 treatments (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.005$). All data are average of 3 biological replicates and error bars represent standard deviation.

6.5 Octyl glucoside was less toxic than 1-octanol in cyanobacteria

In order to evaluate the ubiquity and generality of the concept, the octyl glucoside bioproduction system was also evaluated in a second host organism, *Synechocystis* sp. PCC 6803, similarly to what was previously described with *O*-acetylation in Chapter 5. The toxicity of 1-octanol and octyl glucoside was first investigated in shake flasks in the absence and presence of solvent overlay. The results showed that octyl glucoside was less toxic than 1-octanol in cyanobacteria when solvent overlay was not used (Figure 6.7A). However, the toxicity of 1-octanol was less apparent in the presence of solvent overlay (Figure 6.7B), in agreement with a previous study (Yunus & Jones, 2018).

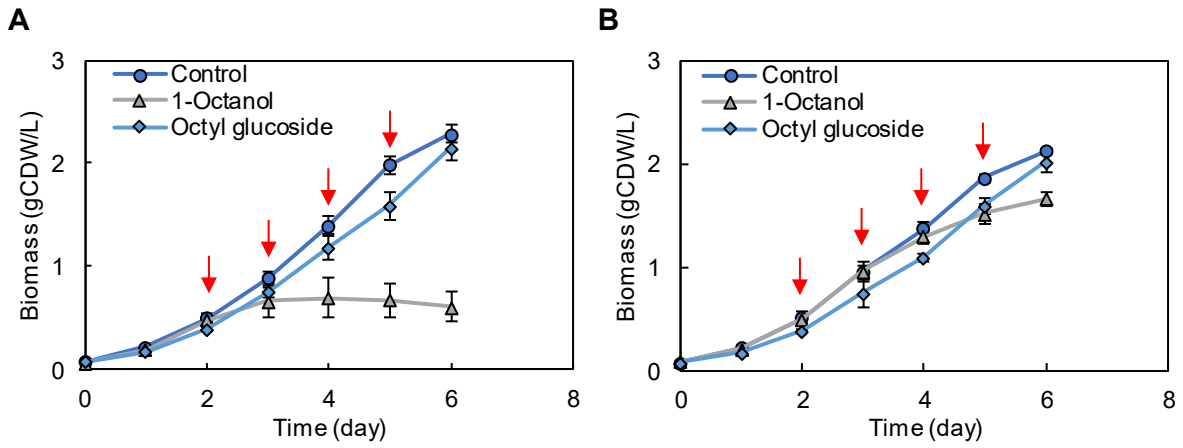

Figure 6.7 Toxicity of 1-octanol and octyl glucoside in *Synechocystis* sp. PCC 6803
Synechocystis sp. PCC 6803 Δ*aas* strain was cultured in BG11-Co with the addition of 25 mg/L of each compound on Day 2 onwards (indicated by red arrows) in (A) the absence of solvent overlay and (B) the presence of 10% (v/v) hexadecane overlay. It should be noted that octyl glucoside data are from the experiments conducted at a different time. All data are average from 3 biological replicates and the error bars represent standard deviation.

6.6 The octyl glucoside pathway was functional in *Synechocystis* sp. PCC 6803

The pathway for octyl glucoside production shown previously in Section 6.2 was transferred to the same *Synechocystis* sp. PCC 6803 Δ*aas* (acyl-ACP synthetase deficient) strain having an Sfp-CAR expression cassette integrated into the DNA chromosome under nickel-inducible promoter (Yunus, 2019). The RSF1010 plasmid used to transform this strain therefore only contained the C8-specific thioesterase (Tes) alone or in combination with the MtH2 (Figure 6.9A). Similar to what was observed in *E. coli*, the C8-specific thioesterases used in this study needed to be induced at different optimal concentrations of cobalt (6.25 µM for ‘CaFatB3-5 and 625 nM for ‘CpFatB1-4). These optimal concentrations of inducer for each thioesterase in cyanobacteria were shown previously by one of our lab members (Yunus, 2019). By combining 2 thioesterases, ‘CaFatB3-5 and ‘CpFatB1-4, with MtH2, octyl glucoside

was detected at 1.57 mM (459 mg/L) and 1.47 mM (429 mg/L) after 10 days, respectively (Figures 6.9B and 6.9D) in the presence of solvent overlay.

The results showed different patterns of biomass accumulation and production on two thioesterases, ‘CaFatB3-5 and ‘CpFatB1-4, used in this study. The biomass accumulation rates of the 1-octanol and octyl glucoside strains did not differ significantly (Figure 6.8). In contrast, the choice of thioesterase had a significant impact on the titer and yield of 1-octanol and octyl glucoside. For ‘CaFatB3-5, when induced at a high inducer concentration, the molar titers showed improvement in octyl glucoside strain in most cases (Figure 6.9B). However, the yields showed no difference except on day 4 (Figure 6.9C). For ‘CpFatB1-4, both titers and yields of octyl glucoside strain were less than those of 1-octanol strain, though only significantly on day 8 (Figure 6.9D and 6.9E). These differences may be due to the different optimal inducer concentrations for thioesterase and glycosyltransferase. To be specific, it is speculated that ‘CpFatB1-4 thioesterase requires a low induction level; however, MtH2 requires a high induction level. Therefore, in this experiment where cobalt was added in favor of only the thioesterase resulted in low expression level of glycosyltransferase, which in turn, resulted in low glycosylation. The cultivation for ‘CpFatB1-4 strains was extended to 20 days without the addition of fresh media to understand whether the production of octyl glucoside could catch up with 1-octanol or not. The results showed that after 20 days of incubation, octyl glucoside was produced at similar titer and yield with 1-octanol (Figure 6.9F and 6.9G). This may indicate that the two systems containing different numbers of genes in the same operon could not be fairly compared together as it may result in different amount of expressed protein. It is interesting to note, however, that the conversion of 1-octanol to octyl glucoside was complete. One possible explanation for the enhanced conversion is that the intracellular availability of UDP-glucose is better in *Synechocystis* sp. PCC 6803 than in *E. coli* (Figure 6.6A-D) and that the supply of 1-octanol is the main limiting factor for the overall pathway.

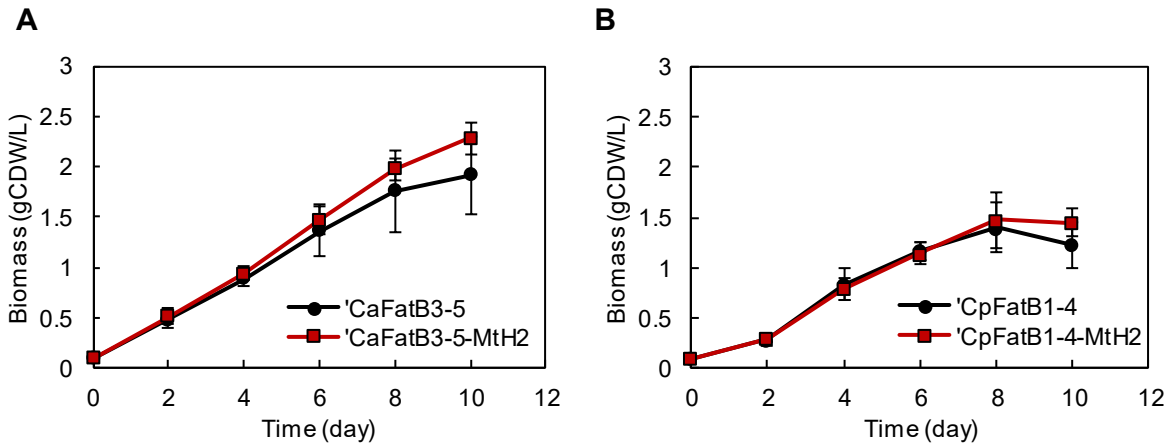

Figure 6.8 Biomass accumulation from 1-octanol and octyl glucoside strains
Synechocystis sp. PCC 6803 Δ*aas* with integrated *sfp-car* expressing (A) 'CaFatB3-5 (B) 'CpFatB1-4 with or without MtH2 were cultivated in BG11-Co in the presence of 30% (v/v) hexadecane overlay. Nickel (15 μM) and cobalt (6.25 μM for 'CaFatB3-5 and 625 nM for 'CpFatB1-4) were used to induce the strains on Day 2. All data are average from 3 biological replicates and the error bars represent standard deviation.

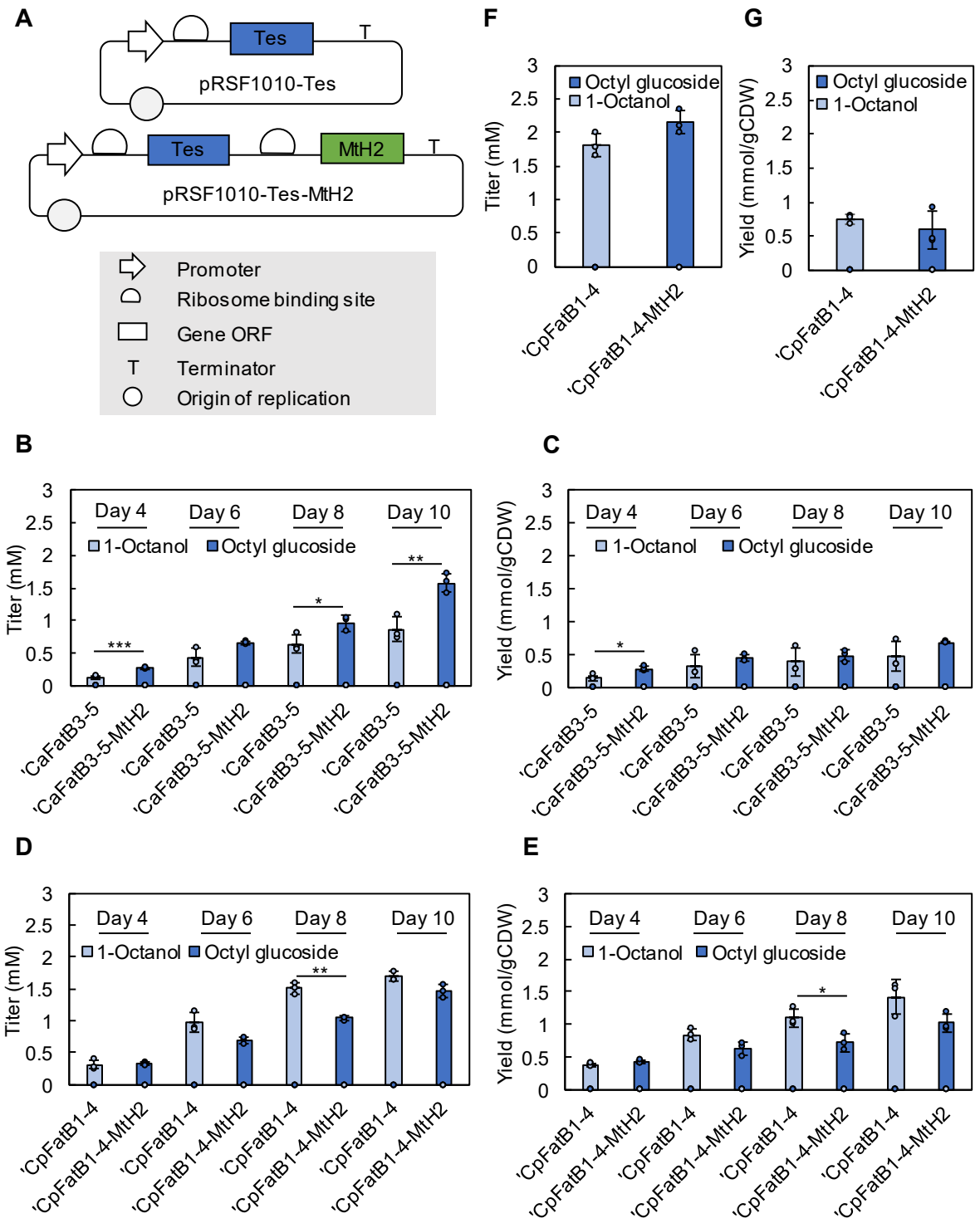

Figure 6.9 Octyl glucoside production in *Synechocystis* sp. PCC 6803 with 2 different C8-preferring thioesterases

(A) Schematic diagram of plasmid constructs used in octyl glucoside production in *Synechocystis* sp. PCC 6803 Δ*aas* with integrated *sfp-car* (B) Titer and (C) Yield from 'CaFatB3-5 strains. (D) Titer and (E) Yield from 'CpFatB1-4 strains. (F) Titer and (G) Yield from 'CpFatB1-4 strains on Day 20. All strains were cultivated in BG11-Co and overlaid with 30% (v/v) hexadecane on Day 2. Nickel (15 μM) and cobalt (6.25 μM for 'CaFatB3-5 and 625 nM for 'CpFatB1-4) were also used to induce the strains on Day 2. Asterisk indicates significant difference between 2 treatments (* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.005$). All data are average from 3 biological replicates and the error bars represent standard deviation.

Similar to the *E. coli* study reported in Chapter 5, 1-octanol accumulating in *Synechocystis* sp. PCC 6803 cultures was also quantified from both the aqueous and overlay phases to accurately evaluated the benefits of bioderivatization. The result showed that only a small amount of 1-octanol was detected in aqueous phase (3.71% of total titer), lower than what was observed from the *E. coli* experiment (compare Figure 6.10 and Figure 5.12). This was probably because 30% (v/v) of solvent overlay was used in cyanobacterial experiments instead of 10% (v/v) and resulted in more 1-octanol partitioned into the organic phase.

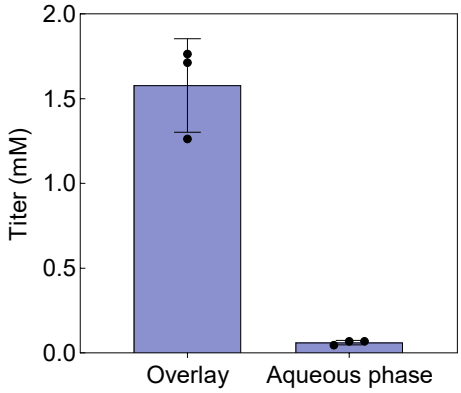

Figure 6.10 1-Octanol recovered from overlay and aqueous phases in cyanobacterial 1-octanol strain
Synechocystis sp. PCC 6803 Δ*aas* with integrated *sfp-car* strain expressing 'CpFatB1-4 was cultivated in BG11-Co induced with 625 nM cobalt and 15 µM nickel and overlaid with 30% (v/v) hexadecane on Day 2. The aqueous phase was extracted with 10% (v/v) hexadecane prior to GC-MS analysis. All data are average from 3 biological replicates and the error bars represent standard deviation.

6.7 Octyl glucoside strain showed improvements in cell growth in the absence of solvent overlay

As mentioned earlier, solvent overlay masked the toxic effect of 1-octanol to the host microorganism. A similar experiment to Section 6.6 ('CpFatB1-4 and 'CpFatB1-4-MtH2 strains) was carried out in the absence of solvent overlay. The results clearly indicated that *O*-glucosylation rescued the growth from 1-octanol toxicity (Figure 6.11A and 6.11B). Even though it has been shown that 1-octanol production could not be accurately quantified without the solvent overlay, samples from the aqueous phase of 'CpFatB1-4 strains were subjected to product quantification. Only 0.13 mM (16.9 mg/L) of 1-octanol was recovered from the aqueous phase after 10 days of incubation (Figure 6.11C) compared to 1.58 mM (205.8 mg/L) accumulating in the hexadecane phase when solvent overlay was used (Figure 6.10). This demonstrates that a large amount of 1-octanol was lost during cultivation and emphasizes the need of solvent overlay to capture even partially soluble products (alcohols), let alone those that are highly water-insoluble (e.g., esters). In the absence of solvent overlay, octyl glucoside

1.67 mM (488 mg/L) was detected in the aqueous phase after 10 days of incubation or 0.68 mmol/gCDW (Figure 6.11D and 6.11E). It has been shown that there was no substantial difference in octyl glucoside production when comparing between treatments with and without solvent overlay (Figure 6.9D, 6.9E and 6.11D, 6.11E) unlike what was found with *E. coli* (Section 6.3 and 6.4). This observation is most likely explained by the fact that the overall production rate in cyanobacteria is substantially lower (14-fold) than in *E. coli*, therefore, enabling the complete conversion of 1-octanol into octyl glucoside before it was lost through volatilization.

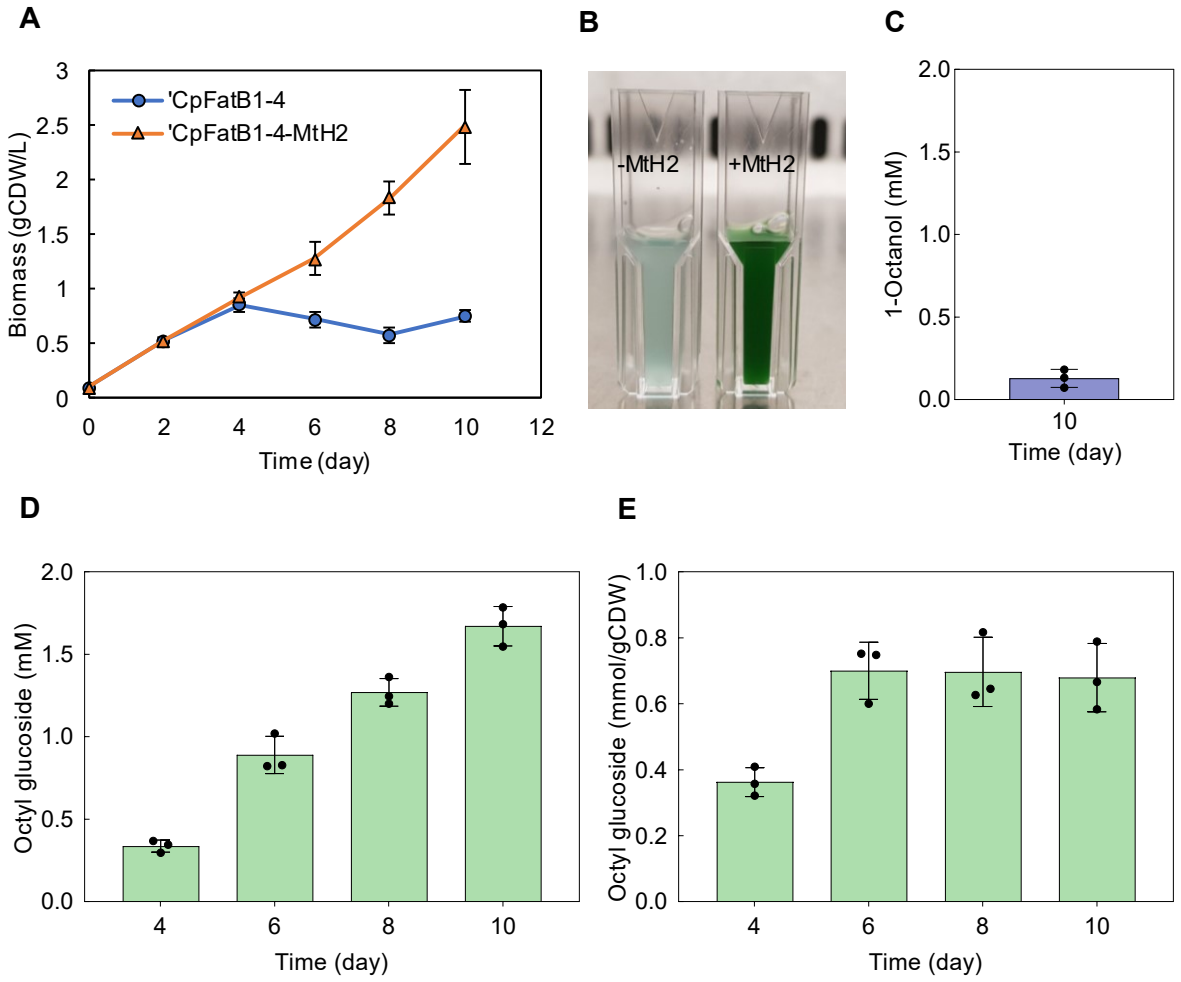

Figure 6.11 Octyl glucoside production in the absence of solvent overlay in 'CpFatB1-4 strains
(A) Biomass accumulation and (B) a photograph of 1-octanol and octyl glucoside strains on Day 10 (C) 1-Octanol from aqueous phase of 1-octanol strain on Day 10 (D) Titer (E) Yield. All strains were cultivated in BG11-Co induced with 625 nM cobalt and 15 µM nickel on Day 2 in the absence of solvent overlay. All data are average from 3 biological replicates and the error bars represent standard deviation.

6.8 AtSUS1 did not improve octyl glucoside production in cyanobacteria

The use of AtSUS1 and sucrose supplementation was found to enhance octyl glucoside production in *E. coli* in Section 6.3 and 6.4. Although the conversion of 1-octanol into octyl glucoside was complete, the effect of a SUS- and sucrose-dependent UDP-glucose recycling on octyl glucoside production was evaluated also in cyanobacteria. Cyanobacteria natively synthesize sucrose under high salt conditions (Blumwald & Tel-Or, 1982); however, sucrose accumulation was also observed when *Synechocystis* sp. PCC 6803 was cultivated in salt-free condition (Kirsch *et al.*, 2018). Therefore, the addition of sucrose to culture media may not be essential if naturally synthesized sucrose is intracellularly available and can be utilized to improve the production of octyl glucoside. In this study, a strain carrying 3 genes, Tes, MtH2, and AtSUS1, was constructed and cultivated along with a respective 1-octanol strain as a negative control in both the absence and presence of sucrose (0 and 100 mM) supplementation. It is worth noting that exogenously supplemented sucrose could be taken up by *Synechocystis* sp. PCC 6803 (Mikkat, Effmert & Hagemann, 1997). The results showed that titer of octyl glucoside from the strains carrying Tes-MtH2-AtSUS1 were slightly lower ($P \leq 0.01$) compared with strains expressing only Tes-MtH2. The Tes-MtH2-AtSUS1 strain produced 0.96 mM (0.52 mmol/gCDW) octyl glucoside (Figure 6.12A and 6.12C) whilst the Tes-MtH2 strain produced up to 1.57 mM (0.69 mmol/gCDW) (Figure 6.9B and 6.9C) after 10 days of incubation, without the addition of sucrose. However, it should be noted that the two experiments were conducted at a different time. It was also observed that when cultures were supplemented with 100 mM sucrose, the production was considerably lower than without the supplementation (Figure 6.12A-D), this could be due to osmotic stress caused by sucrose supplementation. Though biomass accumulation was not affected by sucrose addition (Figure 6.12E and 6.12F).

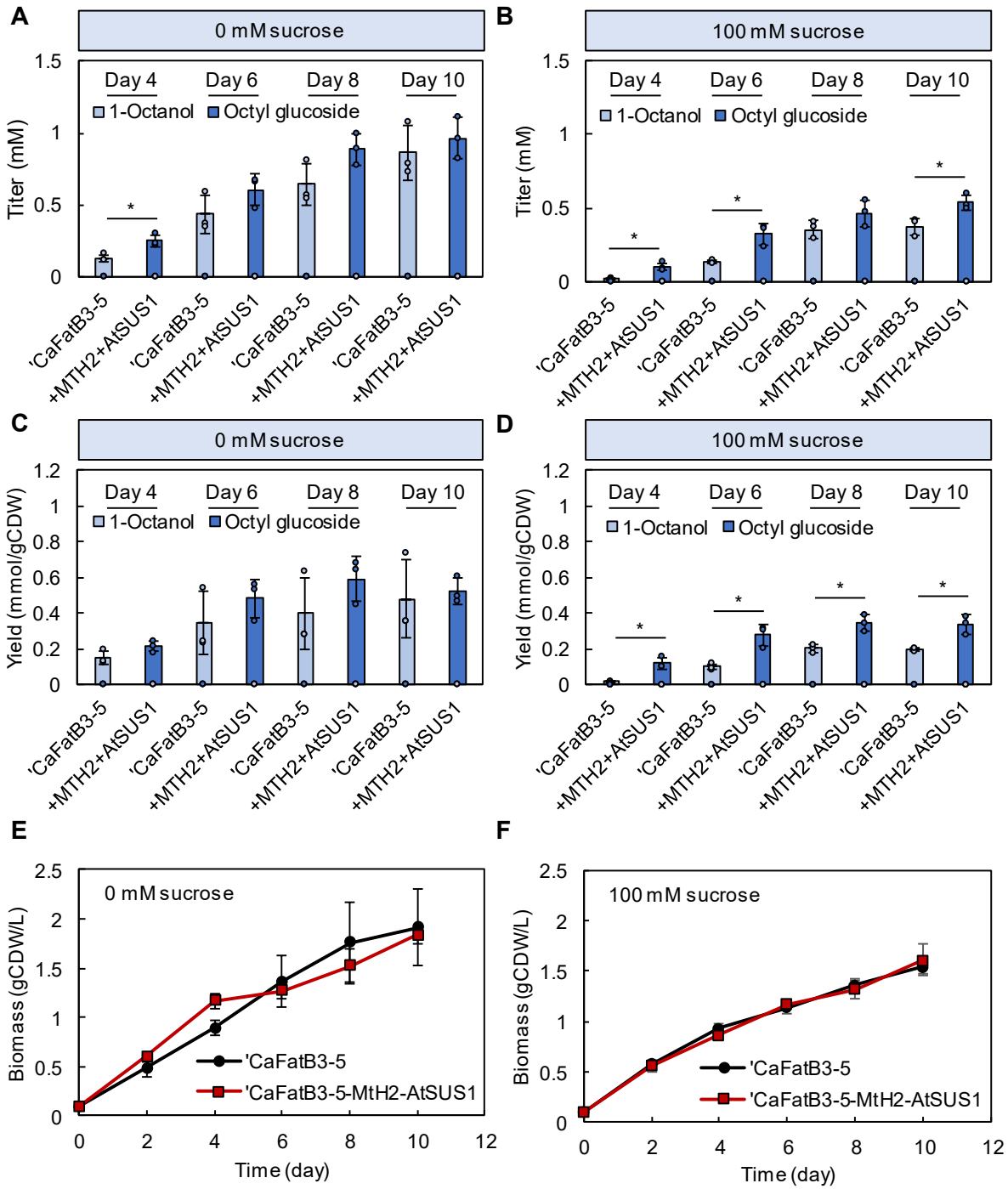

Figure 6.12 The expression of AtSUS1 in *Synechocystis* sp. PCC 6803 for enhancement of octyl glucoside production

Synechocystis sp. PCC 6803 Δ*aas* with integrated *sfp-car* strain expressing 'CaFatB3-5 and 'CaFatB3-5-MtH2-AtSUS1 were supplemented with sucrose at 0 mM (panels on the left) and 100 mM (panels on the right) at the same time of induction on Day 2. (A) (B) Titer, (C) (D) Yield, and (E), (F) Biomass were monitored. All strains were cultivated in BG11-Co induced with 6.25 µM cobalt and 15 µM nickel and overlaid with 30% (v/v) hexadecane on Day 2. Asterisk indicates significant difference between 2 treatments (* $P \leq 0.05$). It should be noted that the data for 'CaFatB3-5 with 0 mM sucrose are also presented in Figure 6.9. All data are average from 3 biological replicates and the error bars represent standard deviation.

The low titer and yield of octyl glucoside from experiments in this section suggested that expression of more than one genes on a one-plasmid system under the same operon reduced

the amount of total or each recombinant protein, which may depend on several factors. The similar effects have been seen in the production of methyl laurate where the expression level of one gene was weakened by an introduction of another gene into the same operon and resulted in lower production titers (Yunus *et al.*, 2020). In order to fairly compare the plasmid system expressing one gene with a system expressing more than one genes, the amount of each protein may need to be quantified and normalized with the product. Selected Reaction Monitoring (SRM) proteomics is one of the techniques used to quantify selected proteins within a mixture and offers highly specific, sensitive, and reproducible results (Picotti & Aebersold, 2012; Schumacher *et al.*, 2014). The use of this technique has been demonstrated previously including the quantification of expressed proteins in cyanobacteria (Vuorijoki *et al.*, 2016; Yunus & Jones, 2018; Yunus *et al.*, 2020)

Altogether, it suggests that bioderivatization has the potential to enhance at least some systems in which product toxicity places excessive limits on bio-based production of valuable chemicals. In most cases, the growth defect caused by target products was overcome either by adding solvent overlay or by bioderivatization and one may argue that the enhancement can then be implemented by simply adding solvent overlay. The increased water solubility of octyl glucoside relative to 1-octanol and the small difference in toxicity between the two compounds support that enhanced product solubility also plays an important role. Hence, bioderivatization does not improve productivity solely by mitigating product toxicity. Regardless, and most importantly, the addition of solvent overlay alone did not result in the same level of improvement as bioderivatization. Thus, even if the use of solvent overlay can help overcome the growth defect, bioderivatization achieves the same effect and also improves productivity.

6.9 Conclusion

In this study, octyl glucoside was shown to be less toxic than 1-octanol and presented as a good candidate to extend the evaluation of the bioderivatization concept with respect to the derivatization method. A synthetic metabolic pathway for octyl glucoside biosynthesis was developed in *E. coli* and then transferred to *Synechocystis* sp. PCC 6803. Interestingly, the water solubility of this second form of bioderivatization is contrasting to the first one evaluated, yet to some degree, it had at least under some conditions the same positive impact on bioproduction. In *E. coli*, 0.73 mM octyl glucoside was produced by combining the 1-octanol pathway deploying the ‘CaFatB3-5 thioesterase and the MtH2 glycosyltransferase, after 48 h of incubation. AtSUS1 was shown to enhance the production when cultures were supplemented with sucrose and resulted in the titer of 4.39 mM octyl glucoside after 48 h of incubation in the presence of solvent overlay. Interestingly, all octyl glucoside was excreted to

the culture media. Subsequently, the same pathway was transferred to cyanobacteria, and a selection of different pathway components was evaluated. Both tested thioesterases were effective, resulting in 1.57 mM (‘CaFatB3-5) and 1.47 mM (‘CpFatB1-4) octyl glucoside after 10 days of incubation in the presence of solvent overlay. In cyanobacteria, *O*-glucosylation improved C8 bioproduction only under some conditions. We speculate that this may be due to the use of strains with suboptimal expression of pathway enzymes in cyanobacteria. In the absence of solvent overlay, nevertheless, the 1-octanol producing strain showed clear growth defects in contrast with the octyl glucoside strain. This emphasized the need to further optimize and investigate the production system of octyl glucoside vs 1-octanol in cyanobacteria.

Chapter 7
1-Dodecanol and lauryl acetate production in *E. coli* and cyanobacteria

In previous chapters, it was shown that bioderivatization of 1-octanol could improve growth, metabolism, and production of target compounds in *E. coli*. It was also demonstrated that bioderivatization of 1-octanol had positive impacts on cyanobacterial growth and production in some cases. In order to generalize this concept further and to gain more fundamental knowledge of the concept, bioderivatization of a new toxic chemical, 1-dodecanol, was evaluated.

7.1 1-Dodecanol is highly toxic to *Synechocystis* sp. PCC 6803

1-Dodecanol is a C12 fatty alcohol used in different commercial applications, such as detergents, emulsifiers, lubricants, and cosmetics. Its biosynthesis has been previously demonstrated in *E. coli* (Hamilton-Kemp *et al.*, 2005; Liu *et al.*, 2016b; Opgenorth *et al.*, 2019). Previous studies have shown that 1-dodecanol is not particularly toxic to *E. coli* even at high concentrations (>40 g/L) (Liu *et al.*, 2016b), whereas it is highly toxic to cyanobacteria even at concentrations as low as 20 mg/L (Kämäräinen *et al.*, 2012). Given the differential sensitivity between the two bacteria to this fatty alcohol, the decision was made to primarily focus on cyanobacteria as the target host, with the assumption that a large difference in toxicity is important to observe an impact from bioderivatization. To confirm that there was a difference in response to the alcohol and its corresponding derivative, the study commenced by evaluating the toxicity of 1-dodecanol compared to its ester derivative, lauryl acetate, in both *E. coli* and *Synechocystis* sp. PCC 6803. Indeed, when *E. coli* cultures were spiked with 200 mg/L of 1-dodecanol, no growth defect was observed (Figure 7.1A and 7.1B). For cyanobacteria, Figure 7.2A shows that, in the absence of solvent overlay, the growth of *Synechocystis* sp. PCC 6803 was already inhibited by 1-dodecanol at low concentration (20 mg/L), whereas lauryl acetate only slightly affected the growth of *Synechocystis* sp. PCC 6803 (Figure 7.2B and 7.2E). This toxicity study indicated that 1-dodecanol was more toxic than lauryl acetate. However, in the presence of solvent overlay, the toxic effect of 1-dodecanol was no longer observed (Figure 7.2C), similar to what was found previously with 1-octanol. The strains spiked with 1-dodecanol or lauryl acetate did not show growth defects (Figure 7.2C, 7.2D, and 7.2F). This study is in agreement with previous studies where the presence of 1-dodecanol at only 20 mg/L completely prevented the growth of two cyanobacterial strains

(*Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942) (Kämäräinen *et al.*, 2012). Altogether, it suggests that bioderivatization of 1-dodecanol to lauryl acetate in cyanobacteria can potentially be used as a case study to understand the extent to which the bioderivatization concept can be applied for a wider range of biotechnological products.

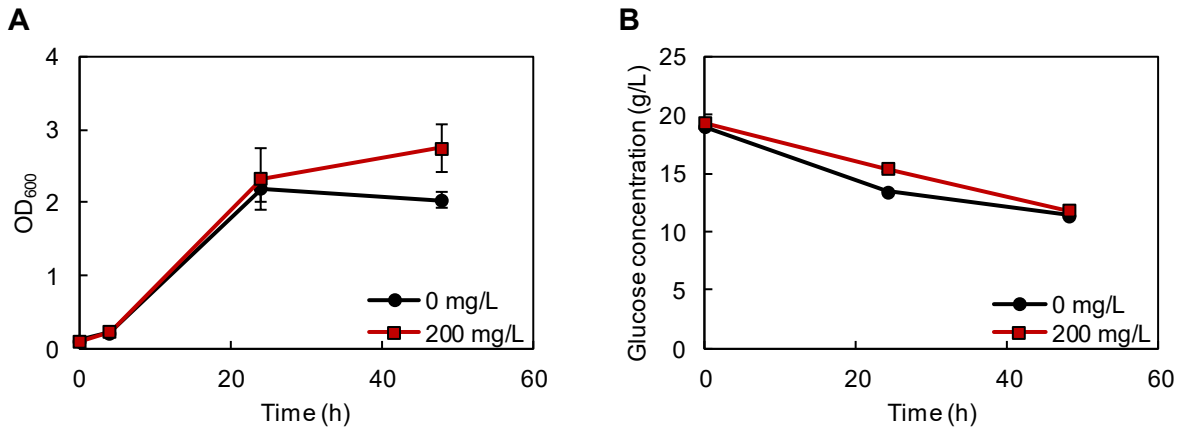

Figure 7.1 Toxicity study of 1-dodecanol in *E. coli*
(A) Average growth curves and (B) Glucose consumption when *E. coli* BW25113 was cultivated in M9 minimal medium with 2% (w/v) glucose and spiked with 0 or 200 mg/L of 1-dodecanol at the beginning of the cultivation. All data are average from 3 biological replicates and the error bars represent standard deviation.

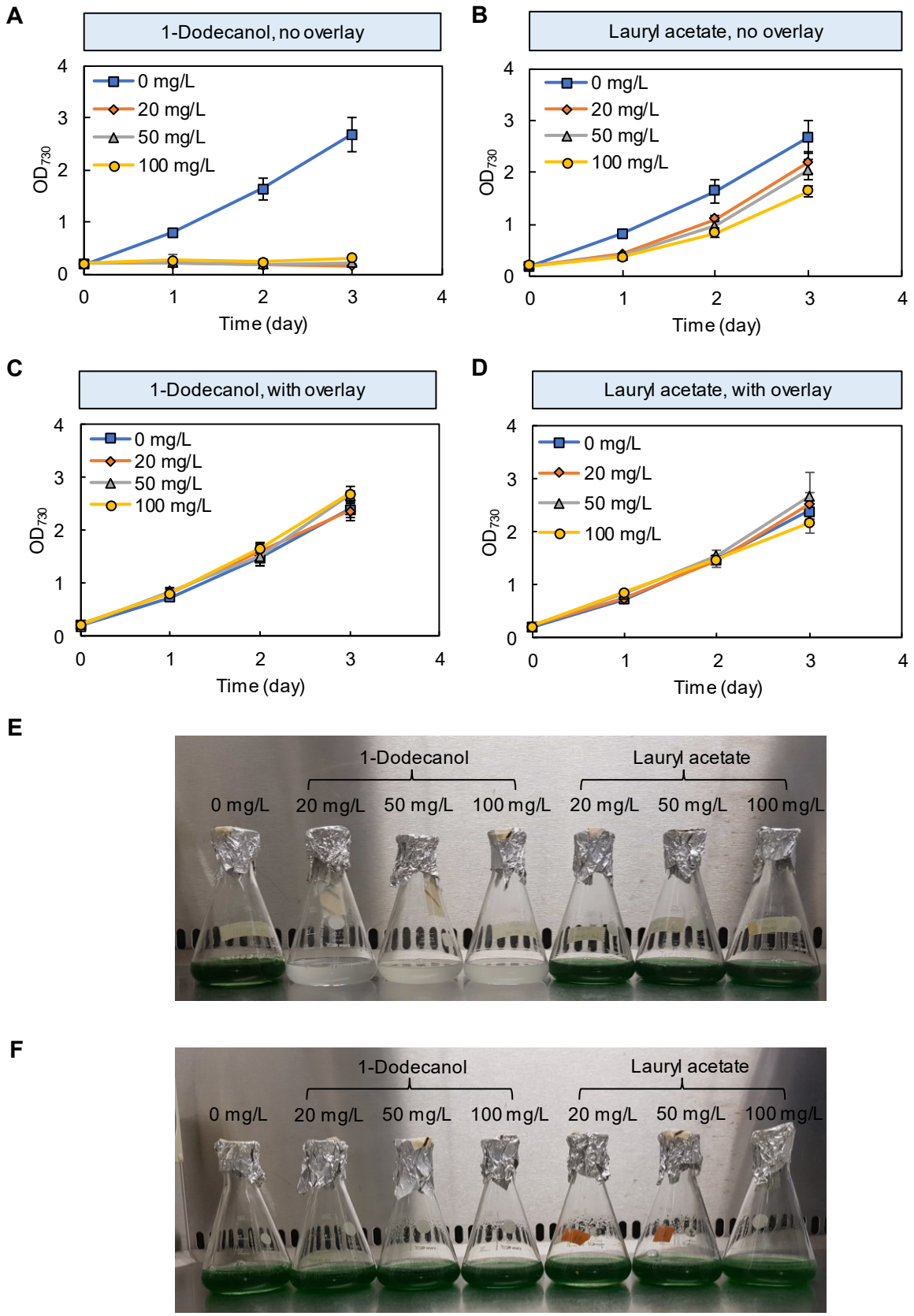

Figure 7.2 Toxicity of 1-dodecanol and lauryl acetate in *Synechocystis* sp. PCC 6803
The growth of *Synechocystis* sp. PCC 6803 when spiked with (A), (C) 1-dodecanol and (B), (D) lauryl acetate at the beginning of the cultivation. Photographs of cyanobacterial cultures on day 10 in the (E) absence and (F) presence of dodecane solvent overlay. All data are average from 3 biological replicates and the error bars represent standard deviation.

7.2 Expression of Tes12, Sfp, CAR and ATF1 resulted in extracellular 1-dodecanol and lauryl acetate

Even though cyanobacteria were the selected target host for bioproduction, the pathways to 1-dodecanol and lauryl acetate were first implemented in *E. coli* in order to verify that the metabolic pathways were working as expected (Figure 7.3A). Implementing synthetic metabolic pathways in a fast-growing microbe such as *E. coli* prior to cyanobacteria can be advantageous, especially if the pathways have not previously been evaluated in microbial hosts. Here, a pathway similar to the pathway used for 1-octanol production (see Chapter 4) was reconstructed for 1-dodecanol production. The C8-specific thioesterase was replaced with 'UcFatB1 thioesterase from *Umbellularia californica* (hereafter Tes12), which has shown specificity toward dodecanoyl-ACP (Voelker *et al.*, 1992; Yuan, Voelker & Hawkins, 1995) and has been used for production of dodecanoic acid in both *E. coli* (Lennen *et al.*, 2010) and cyanobacteria (Yunus *et al.*, 2020). The carboxylic acid reductase (CAR) enzyme remained the same as it has already shown a broad range in its substrate specificity (Akhtar, Turner & Jones, 2013). For ester biosynthesis, two alcohol acetyltransferase (AAT) enzymes, namely SAAT and ATF1, were selected based on their efficient activity toward 1-octanol in Chapter 5. ATF1 has also been reported to catalyze the formation of lauryl acetate from 1-dodecanol and acetyl-CoA (Guo, Pan & Li, 2015). Prior to *in vivo* production, the performance of three different solvent overlays (dodecane, hexadecane, and isopropyl myristate) to capture 1-dodecanol and lauryl acetate was evaluated. Dodecane was the only solvent that allowed the quantification of both compounds, at least using the standard GC-MS with DB-WAXetr column. Dodecane overlay was therefore used in the production experiments.

In the production experiments, *E. coli* BW25113 expressing Tes12, Sfp, and CAR and/or AAT enzymes (Figure 7.3B) were cultivated in M9 minimal medium with 2% (w/v) glucose. The strains were induced with 0.5 mM IPTG and overlaid with 10% (v/v) dodecane. The results showed that 1-dodecanol was detected at a concentration of 0.32 mM (59.3 mg/L) from a strain expressing only the 1-dodecanol pathway after 48 h of incubation (Figure 7.3D). When the 1-dodecanol pathway was co-expressed with ATF1, lauryl acetate accumulated to a concentration of 0.21 mM (47.7 mg/L) after 48 h of incubation (Figure 7.3D). However, when the alcohol acetyltransferase (AAT) was changed to SAAT, no lauryl acetate was detected (Figure 7.3C and 7.3D). Thus, at this point, a method for detection of both the alcohol and corresponding ester, and metabolic pathways for biosynthesis of lauryl acetate in *E. coli* were demonstrated. However, the results also showed that *O*-acetylation did not improve the productivity of 1-dodecanol. On the bioderivatization perspective, this could be due two possible explanations: (*i*) at concentration of 59.3 mg/L, 1-dodecanol was not toxic to *E. coli*;

hence, no benefit from bioderivatization was observed (Figure 7.1) or (*ii*) the 1-dodecanol and lauryl acetate pathways were suboptimal, and optimization of the system was needed before further investigations. For example, ATF1, which is the only AAT able to catalyze 1-dodecanol in this study, could not convert all available 1-dodecanol to lauryl acetate (Figure 7.3C and 7.3D).

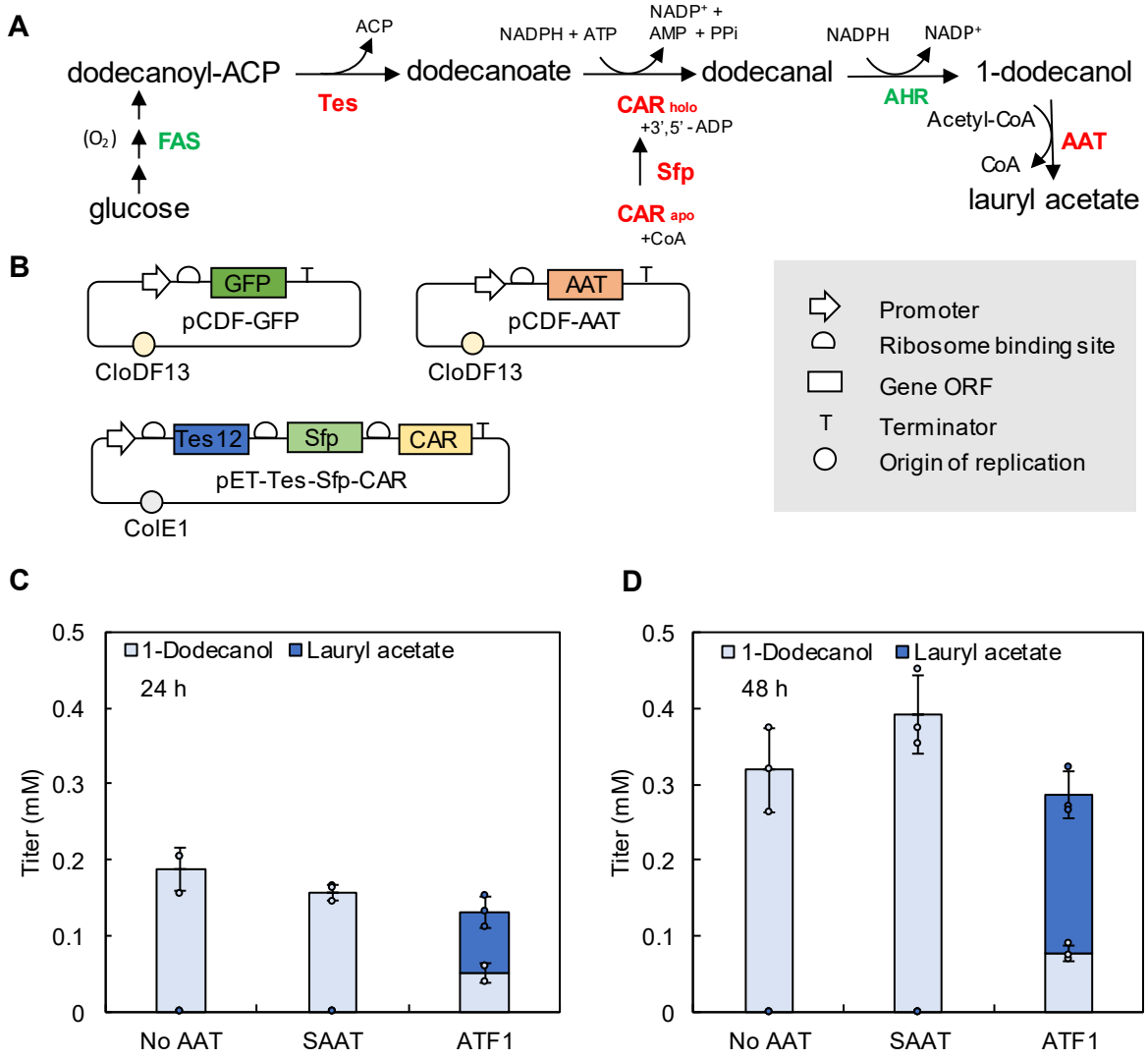

Figure 7.3 Biosynthesis of 1-dodecanol and lauryl acetate in *E. coli* via carboxylic acid reductase-dependent pathway

(A) Schematic diagram of 1-dodecanol and lauryl acetate biosynthetic pathways (B) Plasmid constructs used in the production. Titers at (C) 24 h (D) 48 h. *E. coli* BW25113 strains expressing 1-dodecanol pathway and 2 different AATs, SAAT and ATF1, were cultivated in M9 minimal medium with 2% (w/v) glucose induced at 0.5 mM IPTG and overlaid with 10% dodecane. A strain expressing 1-dodecanol pathway and GFP was used as a negative control (No AAT). All data are average from 3 biological replicates and the error bars represent standard deviation.

7.3 Localization of 1-dodecanol and lauryl acetate in *E. coli*

As shown in Section 7.2, the productivity of 1-dodecanol and lauryl acetate was low. Both 1-dodecanol and lauryl acetate have long hydrophobic alkyl-chains, C12 and C14, respectively, which may likely remain partially inside the cells (Guo, Pan & Li, 2015; Liu *et al.*, 2016; Yunus & Jones, 2018). This would prevent accurate quantification of the products if conducted only from the extracellular fractions. Therefore, in this study, the localization of 1-dodecanol and lauryl acetate was investigated both in the presence and absence of solvent overlay. 1-Dodecanol and lauryl acetate-producing strains cultivated in the absence of solvent overlay for 48 h were investigated in comparison with the samples at 48 h from the previous experiment (Section 7.2) in the presence of solvent overlay. In the absence of solvent overlay, cell pellets (intracellular fraction) and supernatant (extracellular fraction) were separated and analyzed where in the presence of solvent overlay, extracellular products were analyzed directly from the solvent overlay. In the presence of solvent overlay, both 1-dodecanol and lauryl acetate were secreted to culture media; however, in the absence of solvent overlay, both 1-dodecanol and lauryl acetate remained intracellularly (Figure 7.4A and 7.4B). This indicates that when solvent overlay was used, all products were simultaneously extracted during the incubation, resulting in the accumulation of all products in the solvent overlay. Therefore, 1-dodecanol and lauryl acetate production in the presence of solvent overlay could be accurately quantified solely from the overlay.

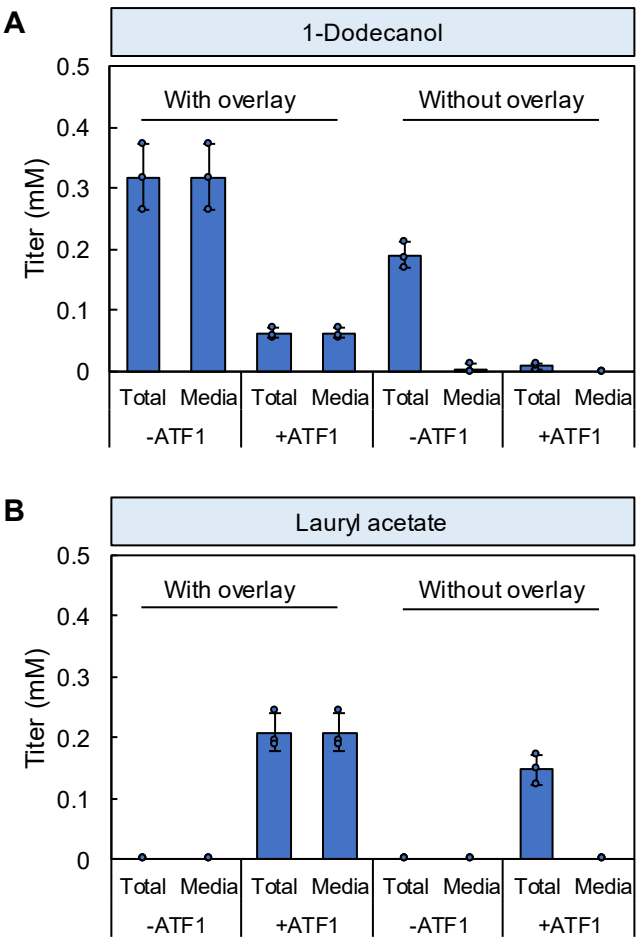

Figure 7.4 Localization of 1-dodecanol and lauryl acetate from *E. coli in vivo* production
(A) 1-Dodecanol (B) Lauryl acetate quantification from different fractions. 1-Dodecanol and lauryl acetate producing strains were cultivated in the presence and absence of 10% (v/v) dodecane overlay for 48 h. Chloroform:methanol extraction was used to extract the products from cell pellets from both treatments and supernatant from the treatment without solvent overlay. It should be noted that the data from media fractions with solvent overlay also presented in Figure 7.3. All data are average from 3 biological replicates and the error bars represent standard deviation.

7.4 The deletion of *fadD* did not improve 1-dodecanol and lauryl acetate

Although bioderivatization of 1-dodecanol in *E. coli* as host microorganism was not the focus in this chapter, the low product titers prompted further optimization as there was room for improvements. One possibility could be due to the recycling of a substrate for CAR enzyme, dodecanoic acid, via fatty acid degradation pathway (β-oxidation pathway). FadD is a fatty acyl-CoA synthetase that catalyzes the activation of free fatty acids into acyl-CoAs before entering *E. coli* β-oxidation cycle (Black *et al.*, 1992; Lennen & Pfleger, 2013; Ford & Way, 2015). The inactivation of *fadD* has been shown to result in free fatty acid accumulation in stationary phase of *E. coli* growth (Pech-Canul *et al.*, 2011). To investigate whether the deletion of *fadD* could improve the production of 1-dodecanol and lauryl acetate or not, *E. coli* BW25113 with *fadD* deletion was studied compared with *E. coli* BW25113 strain (shown in

Section 7.2). Even though the deletion of *fadD* gene was reported to improve total medium-long chain fatty acid production in *E. coli* including slight improvement in dodecanoic acid titer (Cao *et al.*, 2016), 1-dodecanol and lauryl acetate production in *fadD* deletion strains in this study did not show improvements (Figure 7.3C, 7.3D and Figure 7.5A, 7.5B). It was speculated that the inactivation of *fadD* did not completely block β-oxidation pathway and some fraction of dodecanoic acid still entered the degradation cycle. Knocking out both *fadD* and *fadE* might result in higher free fatty acids accumulation (Steen *et al.*, 2010) and therefore providing more precursors for 1-dodecanol or lauryl acetate production. Alternatively, there are several options that may involve in enhancing the production of 1-dodecanol and lauryl acetate. For example, a search for higher-performance C12-preferring thioesterase and AAT could be one of the strategies.

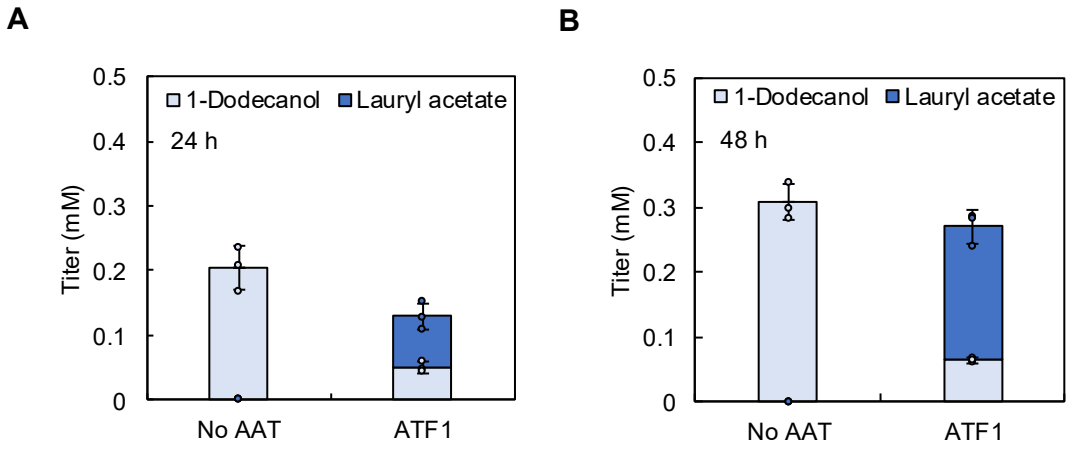

Figure 7.5 The production of 1-dodecanol and lauryl acetate in the *fadD* deletion strains
Titers at (A) 24 h (B) 48 h. *E. coli* JW1994-1 Δ*fadD* strains expressing 1-dodecanol pathway and/or ATF1 were cultivated in M9 minimal medium with 2% (w/v) glucose induced at 0.5 mM IPTG and overlaid with 10% dodecane. All data are average from 3 biological replicates and the error bars represent standard deviation.

7.5 ATF1 could not be expressed in *Synechocystis* sp. PCC 6803

As mentioned earlier, the target host organism of this study was cyanobacteria since the toxic effects of 1-dodecanol on cell growth in this microorganism were more pronounced. The same set of genes for 1-dodecanol and lauryl acetate production from previous experiments in *E. coli* was then transferred to a cyanobacterial host, *Synechocystis* sp. PCC 6803 Δ*aas* with *sfp* and *car* genes integrated in the chromosome. The plasmid constructs were designed similarly to the system used for octyl glucoside production in cyanobacteria in Chapter 6 (Figure 7.6A). Several attempts to obtain the first-generation strain harboring Tes12 and ATF1 under a cobalt-inducible promoter failed as only a few colonies were seen on the transformation plates and none of them grew after reinoculations in liquid media. This is in line with the results from one of our lab members where the attempts to transform ATF1 for octyl

acetate production in cyanobacteria was not successful. It was speculated that the expression of ATF1 in *Synechocystis* sp. PCC 6803 was the culprit. Therefore, to vary the expression levels of ATF1, five different choices of RBS strength were studied (Table 7.1). These five RBSs were previously evaluated for fatty acid production in cyanobacteria. The results demonstrated a correlation between protein quantification and fatty acid production where the strength of RBSs were ranging from RBS No. 2, 3, 1, 5, and 4, respectively from low to high (Yunus & Jones, 2018). The transformation results showed that different numbers of colonies were observed on transformation plates even without the addition of inducer (Figure 7.6B). After several attempts to reinoculate the transformants in 6-well plates, strains carrying Tes12 and ATF1 with RBS No. 1 and 2, but not 3, 4, and 5, were able to grow. They were then inoculated in BG11-Co liquid media for the production experiment and compared with 1-dodecanol producing strain. 1-Dodecanol was detected from the overlay of a strain expressing Tes12 after 10 days of incubation. However, no lauryl acetate was detected from strains expressing Tes12 and ATF1. It was noticed that 1-dodecanol was also detectable in cultures with these strains, albeit at a much lower concentration than in strains lacking ATF1 (Figure 7.6C). Nevertheless, these results suggest that the 1-dodecanol pathway was functional in these strains. To make things more complicated, a couple of more transformation attempts following this experiment were made; however, the results shown in Figure 7.6C could not be reproduced. The strains carrying ATF1 somehow seemed to be unstable. These experiments suggest that ATF1 may affect the stability of cyanobacteria and could be due to its (*i*) activity or (*ii*) intracellular localization in cyanobacteria. As shown in previous sections and literature, ATF1 is a highly active and a known broad-range specific alcohol acetyltransferase (Rodriguez, Tashiro & Atsumi, 2014). One possible explanation for the difficulty with transformation is that leaky expression of ATF1 may catalyze the acetylation of native metabolites that disturb cyclic homeostasis or result in the accumulation of toxic metabolites. However, it remains unknown which compound(s) this could be. Moreover, ATF1 was reported to localize to the endoplasmic reticulum (ER) and lipid droplets (LD) of yeast cells (Lin & Wheeldon, 2014). *In silico* analysis of signalling peptides using the online prediction tool SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/) indicated that no signalling peptides are present in the ATF1 protein sequence. Indeed, the expression of ATF1 was successfully achieved in *E. coli* as shown previously in this study and also in the literature (Rodriguez, Tashiro & Atsumi, 2014). Nevertheless, removal of the N- and/or C-terminal helices reported to be essential for ER and LD association in yeast (Lin & Wheeldon, 2014) would be a next logical step to pursue ATF1 expression in cyanobacteria. A search for alternative AATs with appropriate substrate specificity is also an option.

Alternatively, *O*-glucosylation as the other bioderivatization method could be implemented instead of *O*-acetylation. A glycosyltransferase that catalyzes 1-dodecanol to lauryl glucoside was recently reported from *Mentha x piperita* or peppermint (Sun, 2019).

Table 7.1 List of ribosome binding site sequences used in this study (Yunus, 2019)

RBS No.	Sequence	Source/ID
1	ATCACACAGGAC	BBa_B0033
2	AAAGAGGGGAAA	BBa_B0064
3	AAAGAGGAGAAA	BBa_B0034
4	ATCACAAGGAGG	Shine-Dalgarno (SD) *E. coli* consensus
5	ATTAGTGGAGGT	(Heidorn *et al.*, 2011)

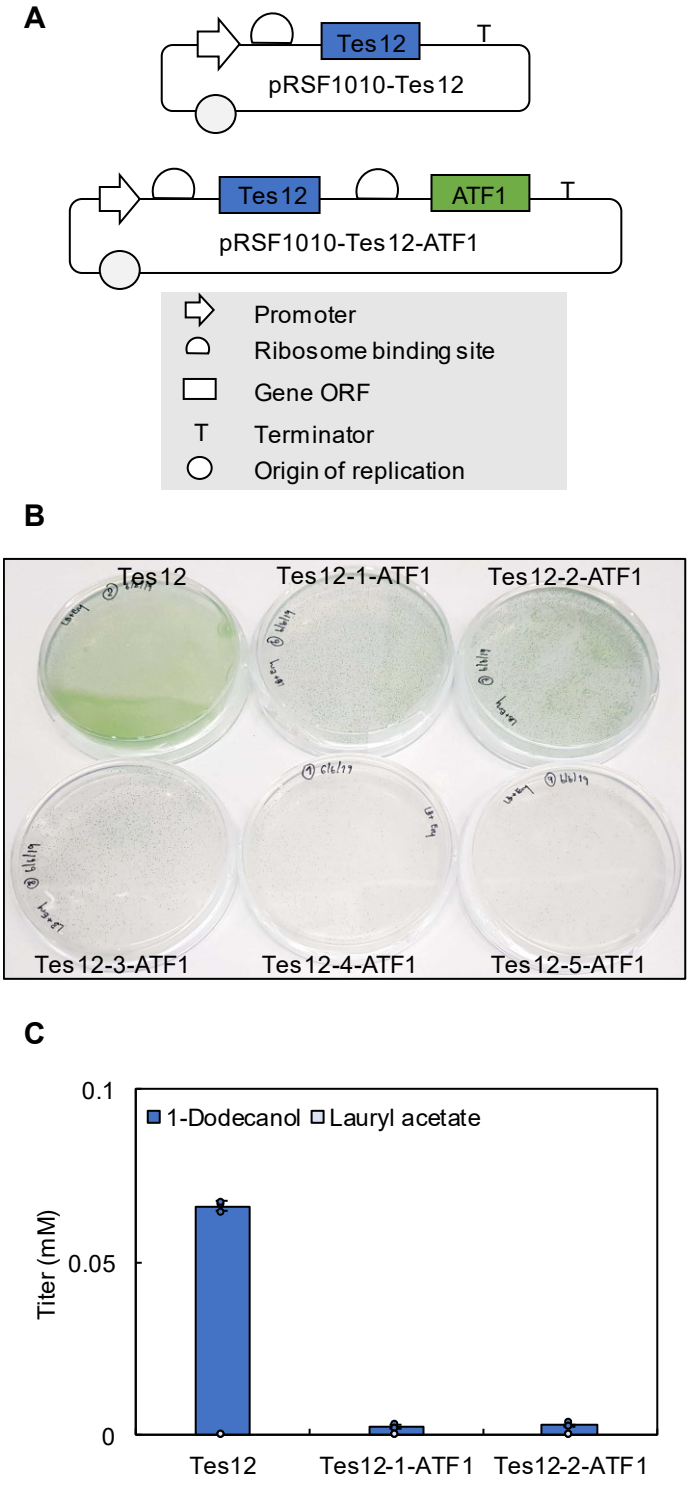

Figure 7.6 Production of 1-dodecanol and lauryl acetate in cyanobacteria with varying RBS strength in front of *atf1*

(A) Schematic diagram of plasmid constructs used for 1-dodecanol and lauryl acetate production in *Synechocystis* sp. PCC 6803 Δ*aas* with integrated *sfp-car*. (B) A photograph of transformation plates with different RBSs in front of *atf1*. (C) Product titer after 10 days of incubation. All strains were cultivated in BG11-Co induced with 5 μM cobalt and 15 μM nickel and overlaid with 10% (v/v) dodecane on Day 2. All data are average from 3 biological replicates and the error bars represent standard deviation.

7.6 Conclusion

In this work, further explorations of bioderivatization were investigated. The toxicity assay of 1-dodecanol showed that 1-dodecanol was not highly toxic to *E. coli*. However, it was highly toxic to cyanobacteria unlike its ester derivative, lauryl acetate. The differential toxicity of 1-dodecanol to *E. coli* and cyanobacteria was speculated to be due to the difference in cell membrane of these bacteria. 1-Dodecanol and lauryl acetate pathways were implemented in *E. coli* but the production of 1-dodecanol was low. This may be due to the low activity of thioesterase used. Moreover, when SAAT and ATF1 were used for lauryl acetate production, only ATF1 could convert 1-dodecanol and acetyl-CoA to lauryl acetate and a complete conversion of 1-dodecanol was not observed suggesting inefficient activity of ATF1. Several attempts to express ATF1 in *Synechocystis* sp. PCC 6803 were not successful or resulted in genetically unstable strains. This emphasizes the need for further evaluations of ATF1 and alcohol acetyltransferase in cyanobacteria in future. Alternatively, evaluation of 1-dodecanol bioderivatization via *O*-glucosylation could be implemented instead of *O*-acetylation to solve the ATF1 issues. From the perspective of evaluating the utility of bioderivatization concept with biotechnological products other than 1-octanol, it is also worthwhile to consider alternative products that are not straight-chain fatty alcohols.

Chapter 8
Can the bioderivatization concept also be used for other chemical products?

In previous chapters, the concept of bioderivatization was investigated by evaluating the effects of converting toxic straight-chain alcohols to their corresponding less toxic derivatives, hydrophobic esters or hydrophilic glucosides. In most cases, bioderivatization enhanced growth, metabolism and/or product yield. To understand if the bioderivatization strategy has a more broadly applicable utility when applied to other chemical compounds, the strategy was also evaluated with several other high-value chemicals using *O*-acetylation (Table 8.1). The target chemicals were selected based on several factors, for example (*i*) their value in the market, the availability of (*ii*) the corresponding chemical standards and (*iii*) the (bio)synthetic pathways in nature. Like with 1-octanol, the primary toxicity of the compounds and their derivatives to *E. coli* was first evaluated and the results are described in this chapter. Subsequently, screening of efficient key enzymes responsible for bioderivatization of some high value chemicals was carried out *in vivo*, resulting in the identification of promising active enzymes for the conversion of menthol and eugenol to their respective ester derivatives.

Table 8.1 List of chemicals and their derivatives of interest in this study

Chemical	Class of compound	Market price* (£/kg)	Derivative of interest
Menthol	Monoterpenoid	54	Menthyl acetate
Eugenol	Phenolic alcohol	75	Eugenyl acetate
Linalool	Monoterpenoid	56.7	Linalyl acetate, linalyl butyrate
Vanillin	Phenolic aldehyde	51	Vanillin acetate
Quercetin	Flavonol	2,140	-
Cinnamyl alcohol	Aromatic alcohol	46.8	Cinnamyl acetate

*Market price obtained from Sigma Aldrich supplier, food grade (FG) chemicals with synthetic sources except for quercetin (only HPLC grade standard is available).

8.1 Menthol

Menthol is a cyclic monoterpene alcohol (Figure 8.1A (*i*)) and a main contributor for distinctive smell and flavor of plants in genus *Mentha* or commonly known as mint plants. Menthol occurs naturally in eight different stereoisomers, but the most commonly known isomer is (-)-menthol or l-menthol (Eccles, 1994). Menthol is a high-value chemical as it is widely used for several purposes such as in oral hygiene products, pharmaceuticals, and food

additives. However, menthol is traditionally obtained from mint plants, which may not be sufficient for its current high consumption. Menthol has also previously been reported to have antibacterial and anti-plasmid (elimination of plasmid from the cell) activities (Schelz, Molnar & Hohmann, 2006; Landau & Shapira, 2012).

Menthyl acetate (Figure 8.1A (*ii*)) is also found naturally in mint plants, although not a major constituent like menthol (Croteau & Hooper, 1978; Khan & Abourashed, 2010; Tsai *et al.*, 2013; Karami *et al.*, 2017). Menthyl acetate was chosen in this study as it represents the simplest ester derivatives of menthol. To evaluate their toxicity, different concentrations of menthol or menthyl acetate were added into *E. coli* culture. The results showed that 2.5 mM menthol already inhibited the *E. coli* growth (Figure 8.1B and 8.1C) whilst menthyl acetate did not inhibit the *E. coli* growth at any given concentrations (Figure 8.1D). This indicated that menthol is more toxic than menthyl acetate.

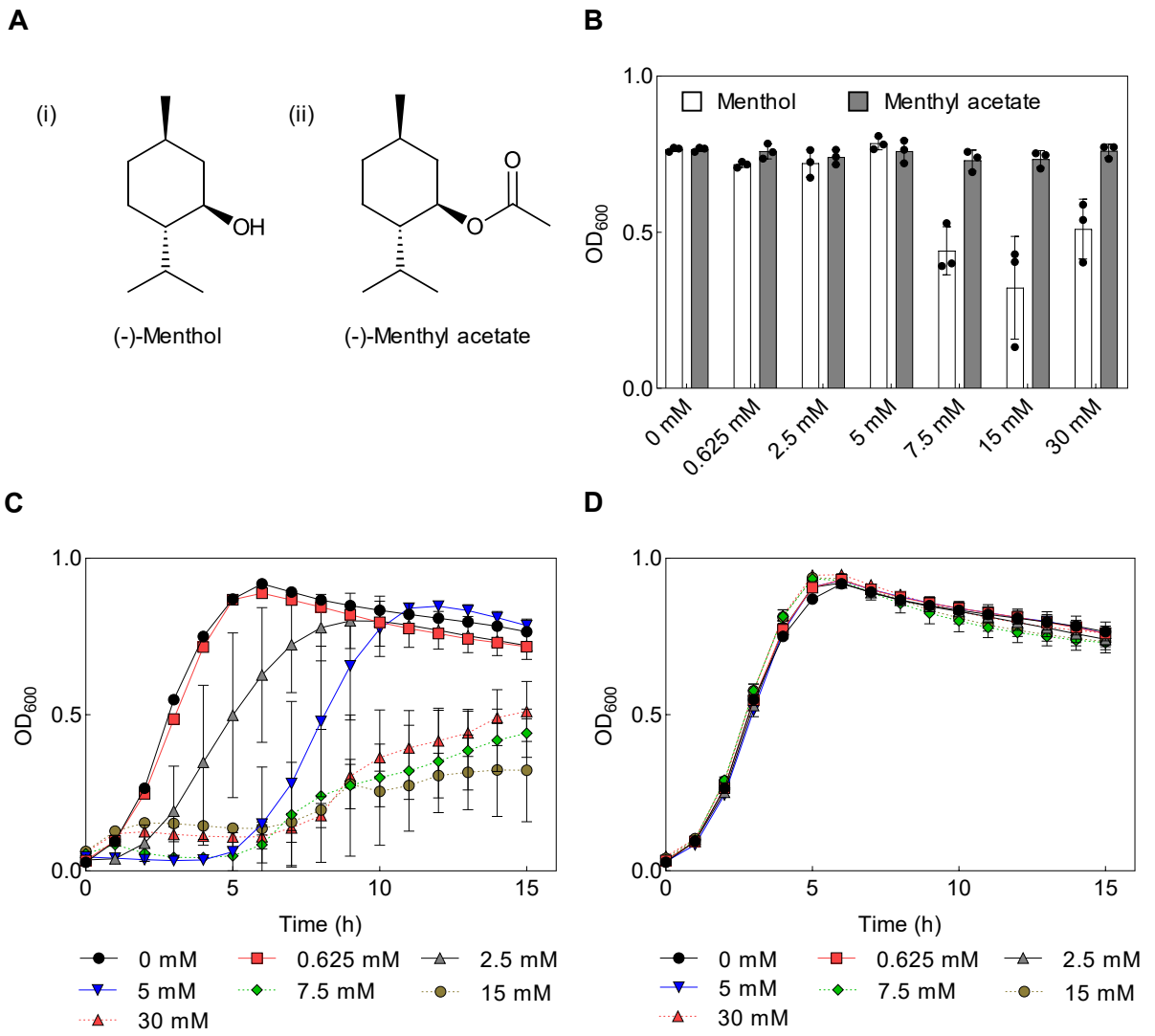

Figure 8.1 Toxicity assay of menthol and menthyl acetate to *E. coli*
(A) Chemical structures of (*i*) (-)-menthol and (*ii*) (-)-menthyl acetate. (B) OD_{600} after 15 h of incubation from *E. coli* BW25113 when cultivated in LB media in 24-well plates with different concentrations of menthol and menthyl acetate. Average growth curves in (C) Menthol (D) Menthyl acetate. The plates were incubated in a plate reader at 37°C, 432 rpm for 15 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

The toxicity assay showed that menthol was more toxic to *E. coli* than menthyl acetate, indicating menthol as another suitable candidate for bioderivatization. Feeding experiment of menthol to strains carrying AATs was then carried out to screen for AAT enzymes that is active on menthol. However, it should be noted that if the compound is not fully assimilated by the cells or highly volatile, the obvious effect may not be seen due to the loss of compound through volatilization. *E. coli* BW25113 strains carrying five different AAT enzymes, CAT, SAAT, ATF1, VAAT, and VpAAT1, and GFP (as a negative control) (Figure 8.2A) were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.5 mM IPTG for 2 h. The cultures were then spiked with 5 mM menthol in the absence of solvent overlay under the assumption that *E. coli*

would naturally assimilate the alcohol. To quantify the product, culture media and cell pellets were extracted with isopropyl myristate and chloroform:methanol, respectively, after 48 h of incubation and analyzed using GC-MS. The feeding experiment showed that ATF1 was the only AAT enzyme that catalyzed *O*-acetylation of menthol to enable measurable menthyl acetate accumulation (Figure 8.2B). To my knowledge, this AAT has not been reported for activity on menthol. Enzymatic *O*-acetylation of (-)-menthol to menthyl acetate in peppermint has been reported suggesting an existing AAT responsible for this reaction from peppermint plants (Croteau & Hooper, 1978). Results from gas chromatography analysis also revealed that only around 50% of spiked menthol was recovered from the cultures even from the negative control strain where no conversion was expected (Figure 8.2C and 8.2D). This indicated that in the absence of solvent overlay, around half of the spiked menthol was loss during cultivation, presumably as a result of volatilization – menthol is known to be a volatile substance (Beigi, Torki-Harchegani & Pirbalouti, 2018). Only a small amount of menthyl acetate was found in the extracellular fraction (0.1 mM) of the strain expressing ATF1 (Figure 8.2D), whilst the majority (0.34 mM) was detected inside the cells (Figure 8.2C) – presumably as hydrophobic compounds tend to remain inside the cells. Curiously, only ~0.58 mM of menthol remained in this strain (Figure 8.2D). It was speculated that menthyl acetate was more volatile than menthol; therefore, the loss of menthyl acetate was more pronounced during incubation. To understand the effect of bioderivatization of menthol to menthyl acetate on growth, the cell density of each strain was measured every 24 h. The results showed no growth difference was observed between strains with and without *O*-acetylation, although it was obvious that the addition of menthol alone affected biomass accumulation (Figure 8.2E). Altogether, these experiments indicated that ATF1 was reasonably active on menthol and that further optimization would be needed to investigate the effect of bioderivatization on menthol.

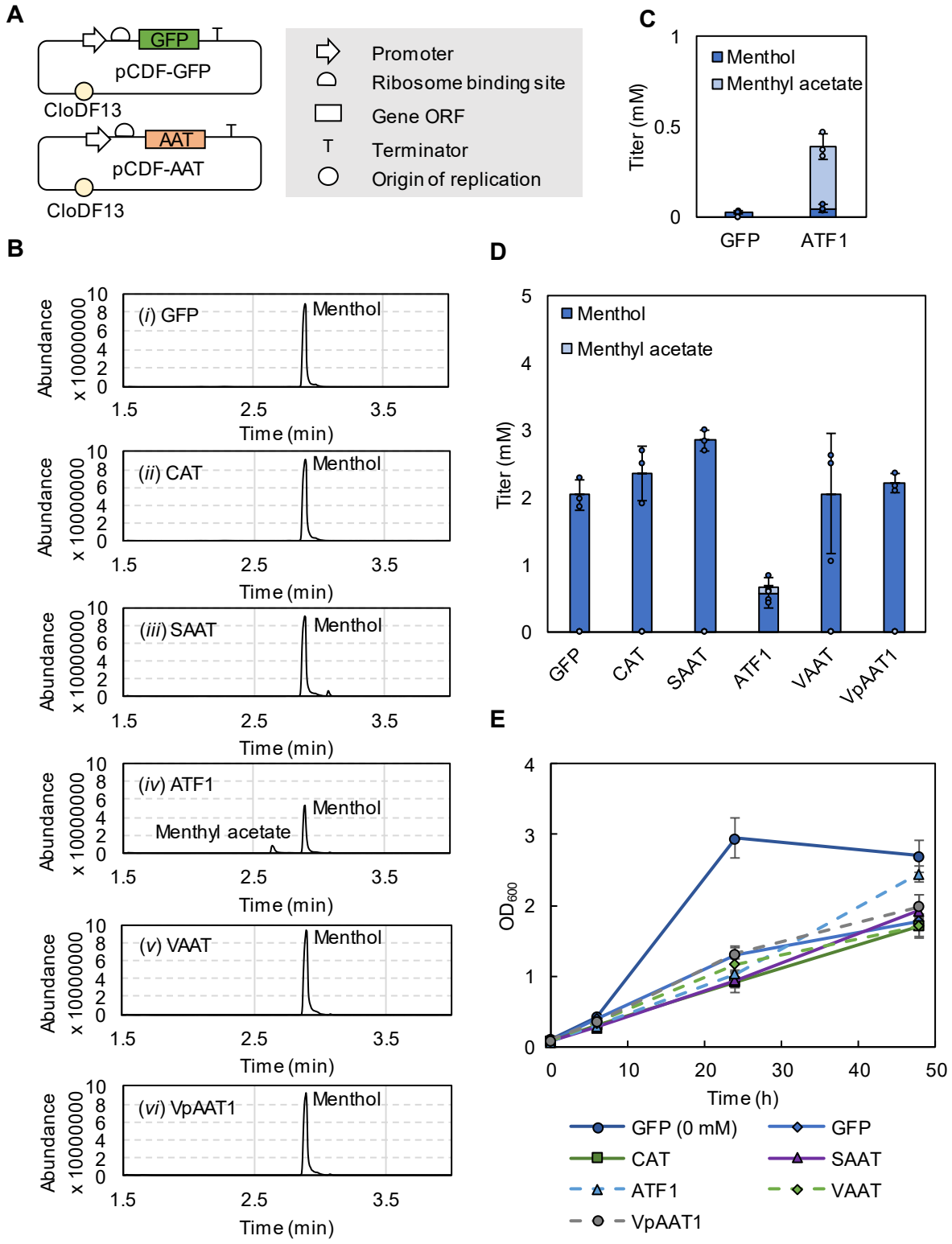

Figure 8.2 The feeding experiment of menthol

(A) Schematic diagram of constructs used in this experiment (B) Chromatograms obtained from GC-MS of samples extracted from extracellular fractions at 48 h from (*i*) GFP (*ii*) CAT (*iii*) SAAT (*iv*) ATF1 (*v*) VAAT and (*vi*) VpAAT1. Product titers from (C) Intracellular and (D) Extracellular fractions. (E) Biomass accumulation. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.5 mM IPTG was used to induce the cultures in the absence of solvent overlay at 30°C, 150 rpm. Menthol (5 mM) was spiked to the cultures at 2 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.2 Eugenol

Eugenol, a major active ingredient found in Clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry.), is a high-value phenolic alcohol (Figure 8.3A(*i*)) with several applications in pharmaceutical, food, and cosmetic industries (Chaieb *et al.*, 2007; Han & Parker, 2017; Mohammadi Nejad, Özgüneş & Başaran, 2017; Barboza *et al.*, 2018). Eugenol is currently obtained from plant sources (Khalil *et al.*, 2017). With its antimicrobial properties and its wide range uses, this compound can serve as an interesting target. Eugenyl acetate (Figure 8.3A(*ii*)) is an intended ester derivative of eugenol in this study. Eugenyl acetate is also found as one of the component in clove essential oil (Chaieb *et al.*, 2007) and was also reported to possess antibacterial properties (Chiaradia *et al.*, 2012). Here, the toxicity of eugenol and its ester derivative, eugenyl acetate, was first investigated (Figure 8.3B-D). The results showed that eugenyl acetate was less toxic than eugenol. To find a suitable enzyme for bioderivatization of eugenol, *E. coli* BW25113 strains harboring different AATs (Figure 8.4A) were cultivated in M9 minimal medium with 2% (w/v) glucose and spiked with 2.5 mM eugenol without solvent overlay. Quantification of the products from the culture medium and cell pellet was done similarly to menthol feeding experiment in Section 8.1, except that dodecane, not isopropyl myristate, was used for extraction of extracellular fraction. The whole-cell biotransformation of eugenol showed that four AAT enzymes, CAT, SAAT, ATF1, and VAAT, were able to convert eugenol to eugenyl acetate, VpAAT1 was the only AAT that was not active toward eugenol (Figure 8.4B). CAT was the most active AAT on eugenol. However, a complete conversion was not observed (Figure 8.4C). Almost all eugenol and eugenyl acetate were found in extracellular fraction (Figure 8.4D). Growth differences were not observed from strains with and without *O*-acetylation though eugenol obviously affected cellular growth of the strains (Figure 8.4E).

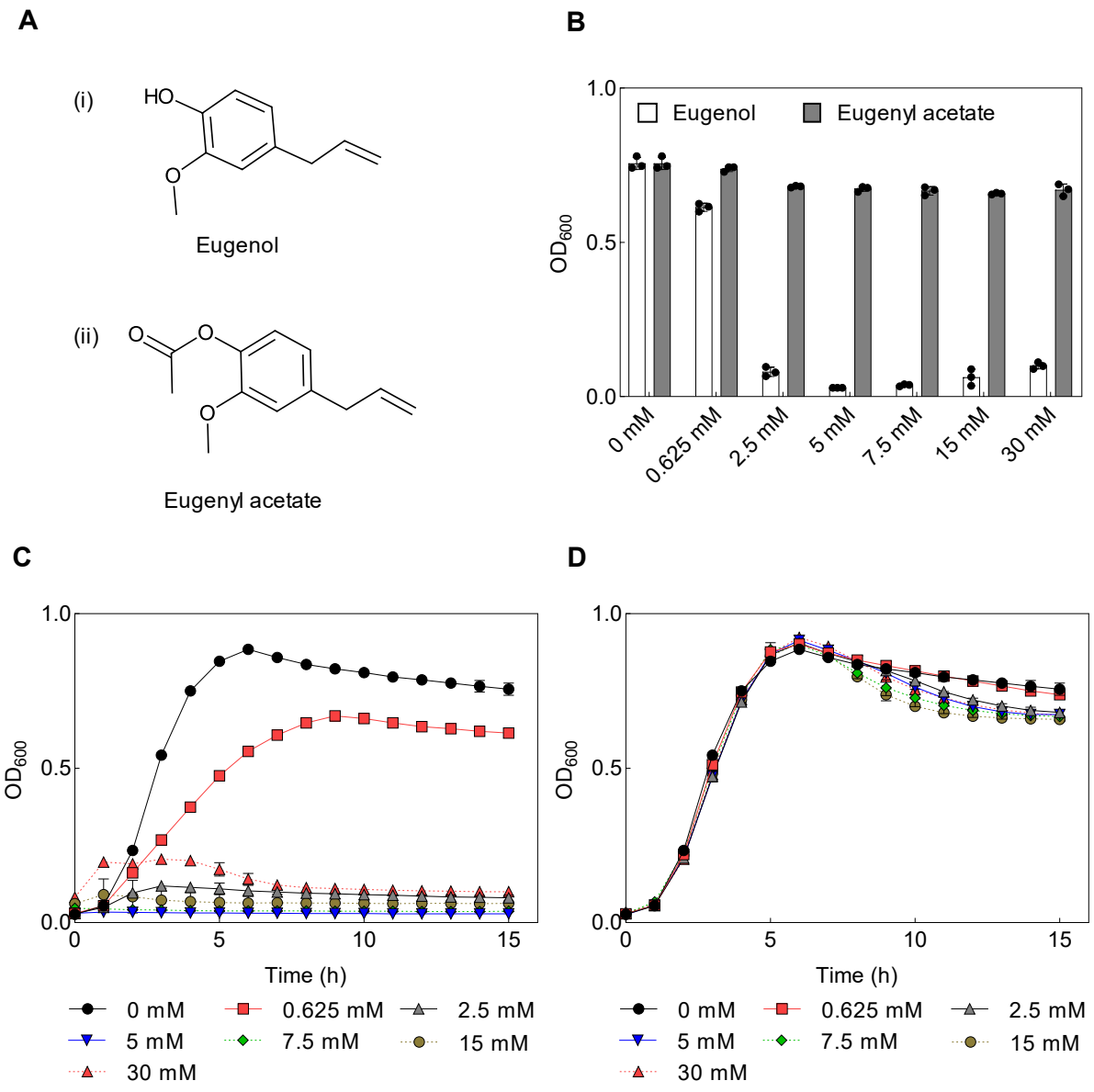

Figure 8.3 Toxicity assay of eugenol and eugenyl acetate to *E. coli*
(A) Chemical structures of (*i*) eugenol and (*ii*) eugenyl acetate. (B) OD_{600} after 15 h of incubation from *E. coli* BW25113 when cultivated in LB media in 24-well plates with different concentrations of eugenol and eugenyl acetate. Average growth curves in (C) Eugenol (D) Eugenyl acetate. The plates were incubated in a plate reader at 37°C, 432 rpm for 15 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

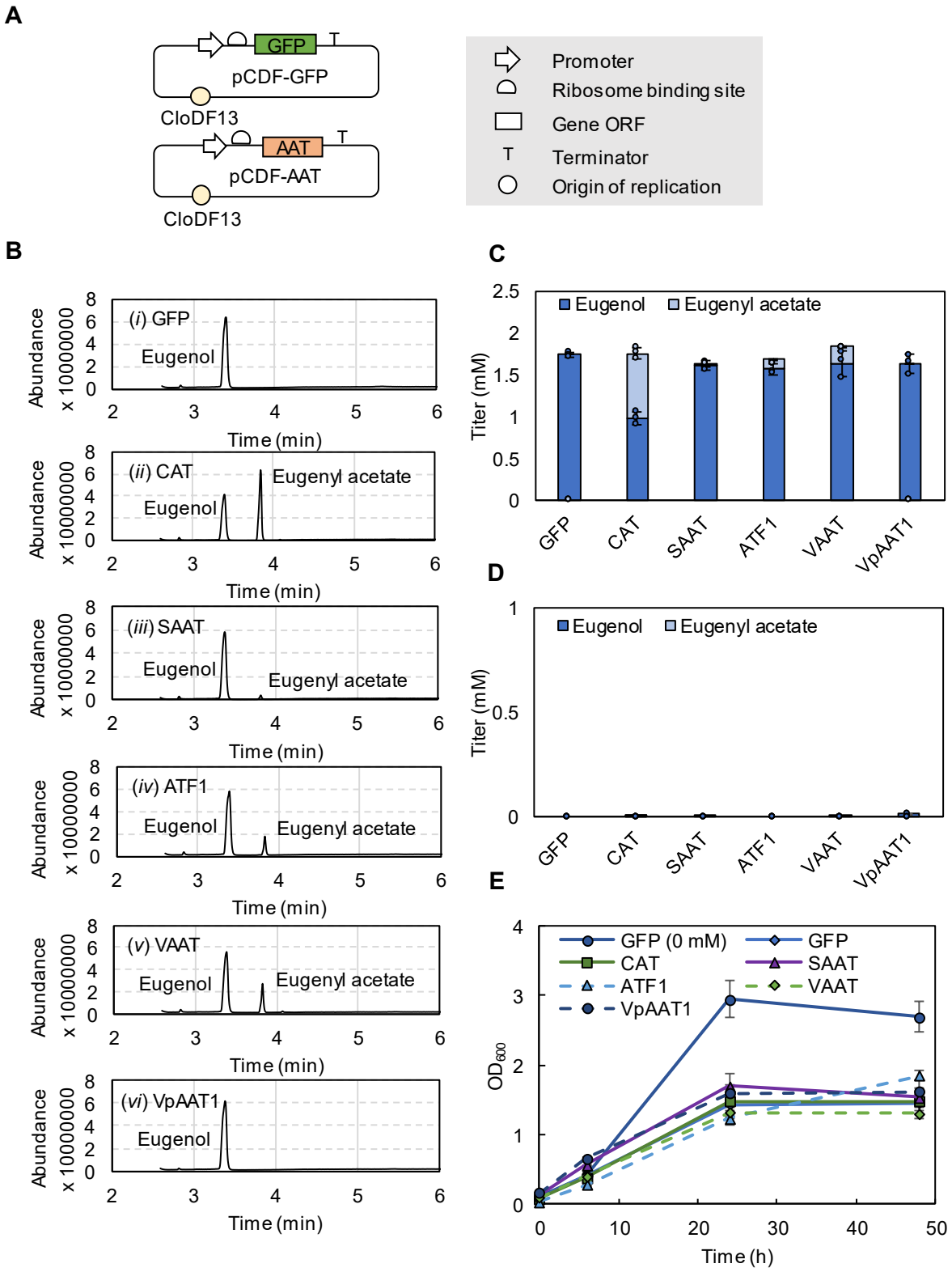

Figure 8.4 The feeding experiment of eugenol

(A) Schematic diagram of plasmid constructs used in this experiment (B) Chromatograms obtained from GC-MS of samples from extracellular fractions at 48 h from (*i*) GFP (*ii*) CAT (*iii*) SAAT (*iv*) ATF1 (*v*) VAAT and (*vi*) VpAAT1. (C) Product titers from extracellular and (D) Intracellular fractions. (E) Biomass accumulation. *E. coli* strains were cultivated in M9 minimal medium with 2% (w/v) glucose and 0.5 mM IPTG was used to induce the cultures in the absence of solvent overlay at 30°C, 150 rpm. Eugenol (2.5 mM) was spiked to the cultures at 2 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.3 Linalool

Linalool is an acyclic monoterpene (Figure 8.5A(*i*)) that is commonly found as a main component of plant floral scents and essential oils (Pichersky *et al.*, 1994; Dudareva & Pichersky, 2000). This compound has been used industrially as a fragrant ingredient, flavoring agent and essential oil constituent for medicinal purposes and has been regarded as Generally Recognized As Safe (GRAS). Moreover, linalool has been shown to possess antibacterial properties (Fisher & Phillips, 2006). Presently, linalool is obtained from plant extracts and this may not sufficiently reach high commercial demands. Renewable production of linalool in *E. coli* via synthetic pathways was achieved previously (Thanasomboon *et al.*, 2012; Mendez-Perez *et al.*, 2017). Linalyl acetate (Figure 8.5A(*ii*)) and linalyl butyrate (Figure 8.5A(*iii*)) were selected as ester derivatives of linalool. Linalyl acetate also has pleasant odors described as floral, sweet, and citric and can be directly synthesized from linalool by different chemical reactions such as transesterification of linalool with ethyl acetate (d'Acampora Zellner *et al.*, 2006; Martín *et al.*, 2007). Linalyl butyrate contributes to odors in some plants such as sour guava and has also been described as sweet (Cuadrado-Silva, Pozo-Bayón & Osorio, 2017; Liu *et al.*, 2017). However, linalyl butyrate has not been exploited much in research. Primary toxicity assay indicated that linalool was more toxic than its ester derivatives, linalyl acetate and linalyl butyrate (Figure 8.5B-E). The high toxicity of linalool toward *E. coli* growth shown in this study is in line with its antimicrobial properties (Fisher & Phillips, 2006; Herman, Tambor & Herman, 2016). Given its high toxicity, linalool and its ester derivatives are therefore demonstrated as potential candidates for bioderivatization.

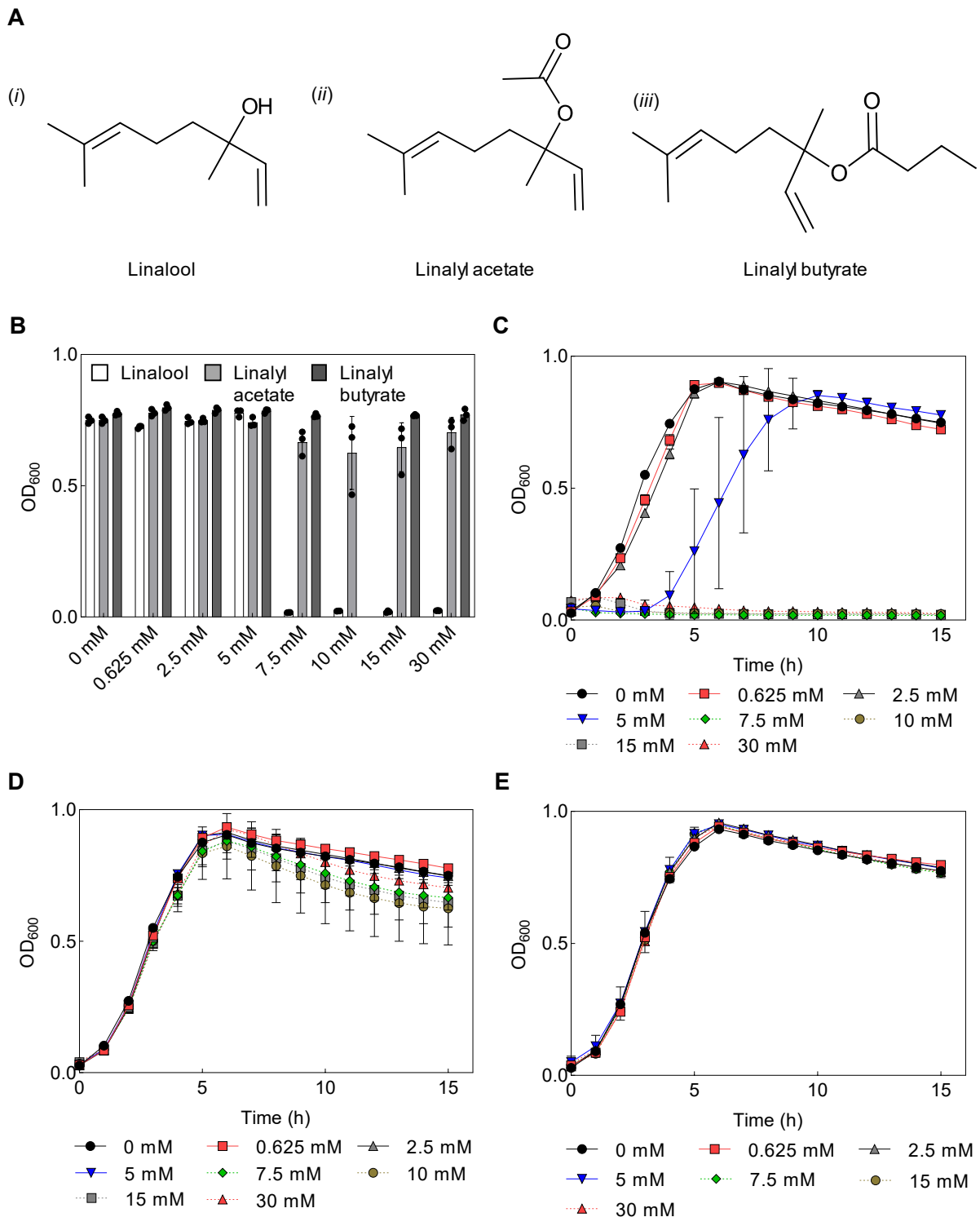

Figure 8.5 Toxicity assay of linalool, linalyl acetate and linalyl butyrate to *E. coli*
(A) Chemical structures of (*i*) linalool, (*ii*) linalyl acetate and (*iii*) linalyl butyrate. (B) OD_{600} after 15 h of incubation from *E. coli* BW25113 when cultivated in LB media in 24-well plates with different concentrations of linalool, linalyl acetate, and linalyl butyrate. Average growth in (C) Linalool (D) Linalyl acetate (E) Linalyl butyrate. The plates were incubated in a plate reader at 37°C, 432 rpm for 15 h. It should be noted that linalyl butyrate at 10 mM was not tested. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.4 Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a phenolic aldehyde (Figure 8.6A(*i*)) that is one of the most widely used compounds in flavor and fragrance industries. It was traditionally obtained from beans of *Vanilla planifolia* and *V. tahitensus*, but chemical synthesis such as from guaiacol and lignin has currently been used to supplement the high demand (Rao & Ravishankar, 2000). Vanillin and its synthesis have been studied broadly and microbial bioproduction of vanillin has been reported in both *E. coli* and yeast (Hansen *et al.*, 2009; Ni *et al.*, 2015). However, the production of bio-vanillin is facing challenges including toxicity to recombinant hosts (Fitzgerald *et al.*, 2004; Luziatelli *et al.*, 2019). Vanillin acetate (4-Formyl-2-methoxyphenyl acetate) (Figure 8.6A(*ii*)) was selected as an ester derivative of vanillin in this study. For the toxicity assay, vanillin and vanillin acetate standards were in crystal forms and impossible to dissolve in water, therefore, they were dissolved in ethanol and added to the culture media. The negative control was also spiked with the same amount of ethanol. The toxicity comparison showed that both vanillin and vanillin acetate had similar toxic effects on growth of *E. coli* (Figure 8.6B-D). Thus, these compounds may not be a good set of candidates for bioderivatization. However, vanillin-β-D-glucoside biosynthesis in *Schizosaccharomyces pombe* yeast has been implemented to convert vanillin to a non-toxic derivative by introduction of a GT from *A. thaliana* (Hansen *et al.*, 2009). The results demonstrated a slight improvement in total titer from vanillin glucoside strain compared with vanillin strain. Therefore, it would be of interest to also evaluate the toxicity of this glucoside derivative in *E. coli* in future.

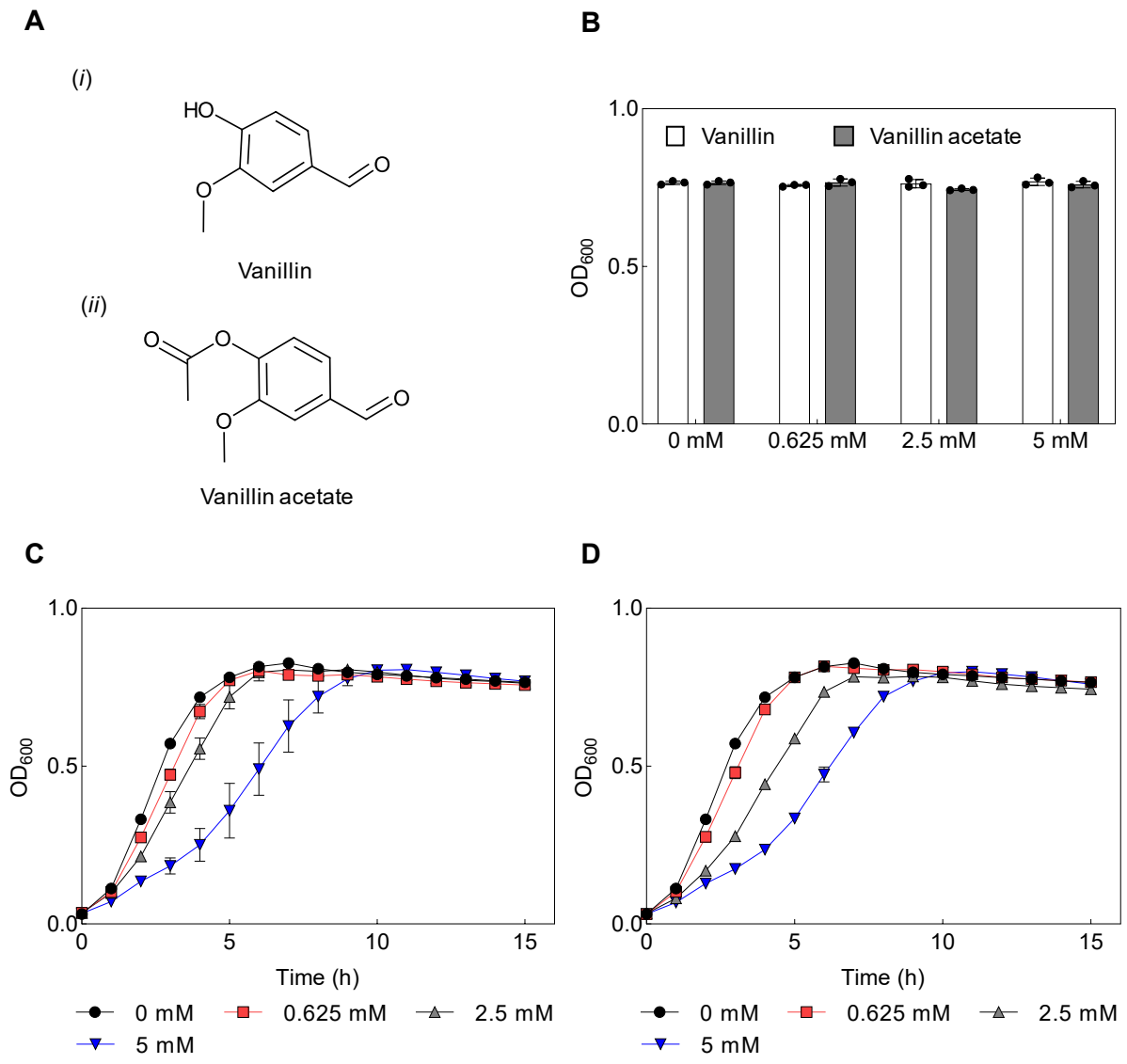

Figure 8.6 Toxicity assay of vanillin and vanillin acetate to *E. coli*
(A) Chemical structures of (*i*) vanillin and (*ii*) vanillin acetate. (B) OD_{600} after 15 h of incubation from *E. coli* BW25113 when cultivated in LB media in 24-well plates with different concentrations of vanillin and vanillin acetate. Average growth curves in (C) Vanillin (D) Vanillin acetate. The plates were incubated in a plate reader at 37°C, 432 rpm for 15 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.5 Quercetin

Quercetin is a flavonol (Figure 8.7A) with well-documented medicinal properties including anti-carcinogen, anti-inflammatory and antiviral activities (Davis, Murphy & Carmichael, 2009; Li *et al.*, 2016). Though biosynthesis of quercetin in microorganisms via synthetic pathways has not been reported, glucosides derived from quercetin have been investigated and known GTs catalyzing the reaction have also been described (He, Wang & Dixon, 2006; Yoon *et al.*, 2012; Xia & Eiteman, 2017). The quercetin standard is a yellow powder that turned milky liquid when dissolved in water (Figure 8.7B), suggesting it is poorly

soluble in water (Wang *et al.*, 2016). As this could interfere with OD measurements, a blank (LB liquid medium spiked with different concentrations of quercetin) was prepared separately for each concentration. It was shown that normal growth trends were monitored from OD measurements at low concentrations of quercetin (0.156-0.625 mM) (Figure 8.7C-E). However, the same was not observed when 2.5 mM was used (Figure 8.7F). Moreover, the treatments turned brown after 20 h of incubation (Figure 8.7B), likely due to oxidation reactions (Zenkevich *et al.*, 2007; Zhou & Sadik, 2008) and this further affected the optical density measurement. Quercetin was reported to be instable under *E. coli* growth conditions (Stahlhut *et al.*, 2015). Therefore, OD measurements were not taken for further considerations as optical density could not be used as an indicator for toxicity. In future, other indicators such as glucose consumption may be a more suitable option for the toxicity study of this compound. Nevertheless, it should be noted that toxicity of quercetin to *E. coli* was reported in previous work. There was no significant difference in specific growth rate and only 20% reduction in OD_{600} was observed when *E. coli* was cultivated in minimal sucrose medium with 0.15 and 1.5 g/L of quercetin (De Bruyn *et al.*, 2015) suggesting that quercetin did not highly affect the growth of *E. coli*. Even though, the browning effects or OD measurement complication was not mentioned.

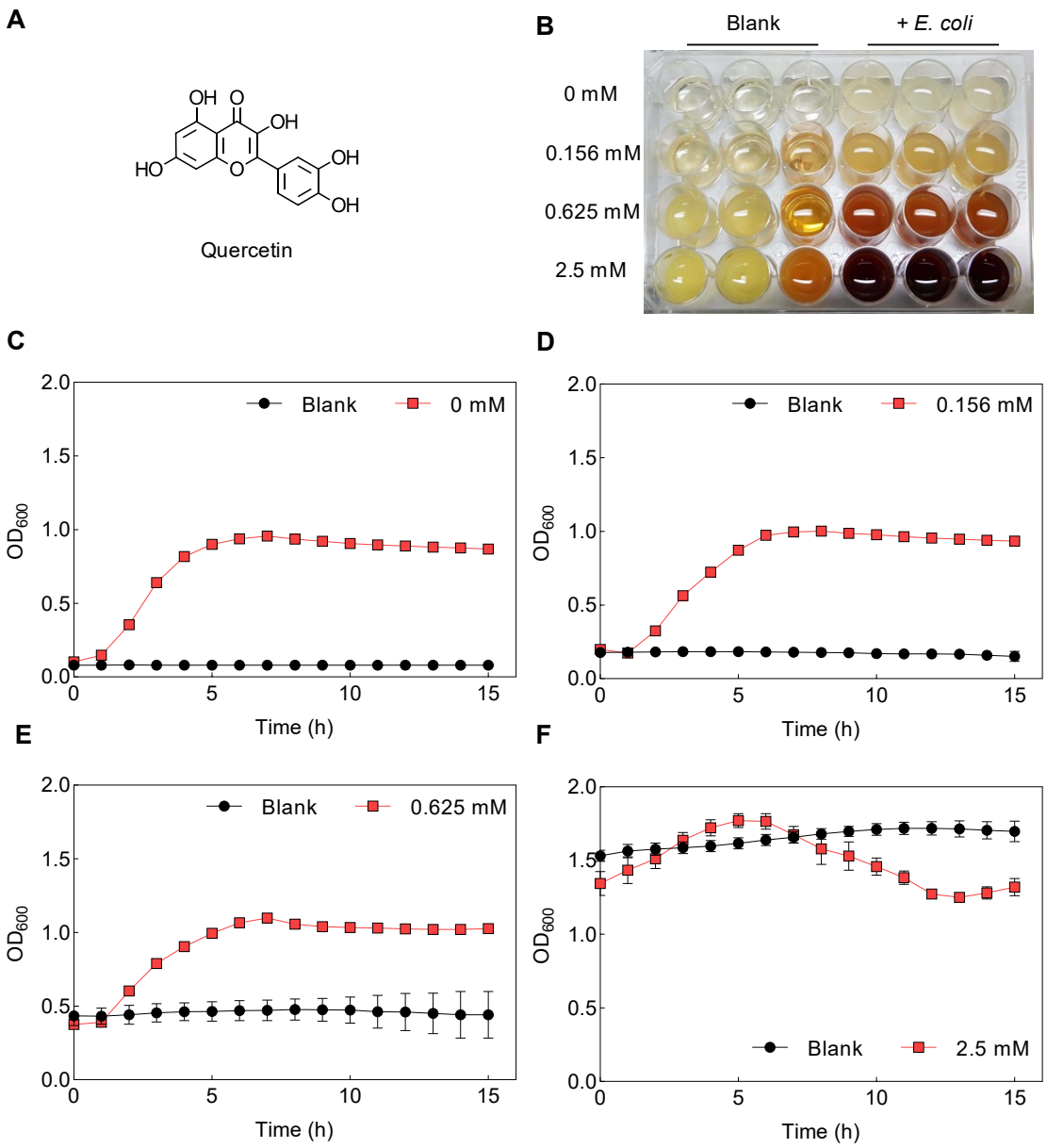

Figure 8.7 Toxicity assay of quercetin to *E. coli*
(A) Chemical structures of quercetin (B) A photograph of microtiter plate after 20 h of incubation. Toxicity of quercetin at (C) 0 mM (D) 0.156 mM (E) 0.625 mM (F) 2.5 mM when cultivated in LB media in 24-well plates with different concentrations of quercetin. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.6 Cinnamyl alcohol

Cinnamyl alcohol is an aromatic alcohol (Figure 8.8A(*i*)) used in fragrance and flavoring industries for cinnamon notes. This chemical is traditionally obtained from chemical reduction of cinnamaldehyde (Gallezot & Richard, 1998). Biosynthesis of cinnamyl alcohol and its glucoside derivative called rosin has been demonstrated in *E. coli* via an amino acid derived pathway with known GTs that catalyze the glycosylation reaction (Zhou *et al.*, 2017; Klumbys

et al., 2018). Cinnamyl alcohol synthetic pathway from L-phenylalanine only requires two recombinant proteins, phenylalanine ammonia lyase (PAL) and CAR. PAL catalyzes L-phenylalanine to cinnamic acid and CAR catalyzes cinnamic acid to cinnamaldehyde prior to the activity of native AHR for cinnamyl alcohol formation (Klumbys *et al.*, 2018). The final two steps of this pathway are similar to alcohol synthetic pathways used in this study; thus, it could reduce the effort to optimize the system if cinnamyl alcohol is characterized as a suitable candidate. Cinnamyl acetate (Figure 8.8A(*ii*)), the simplest ester derivative of cinnamyl alcohol, is an attractive compound itself as it is used to give spicy and floral aromas. The toxicity assay showed that cinnamyl alcohol was more toxic than cinnamyl acetate at 5 mM. However, it was also observed that cultures with cinnamyl acetate exhibited reduction in growth (Figure 8.8B-D), which indicated negative effects of cinnamyl acetate. This is in contrast with menthyl acetate, eugenyl acetate, linalyl acetate, and linalyl butyrate where the growth was markedly different compared with negative controls.

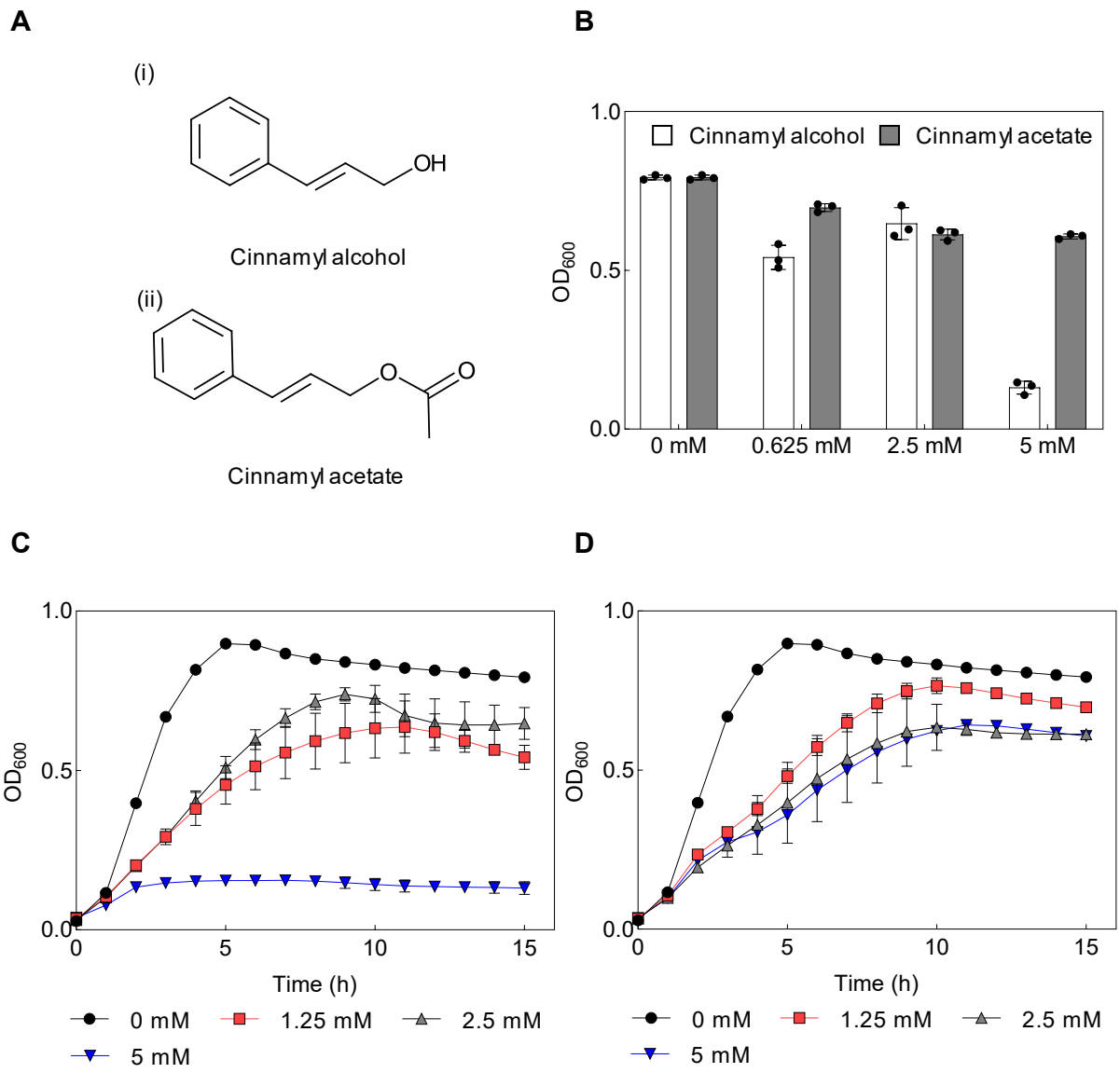

Figure 8.8 Toxicity assay of cinnamyl alcohol and cinnamyl acetate to *E. coli*
(A) Chemical structures of (*i*) cinnamyl alcohol and (*ii*) cinnamyl acetate. (B) OD_{600} after 15 h of incubation from *E. coli* BW25113 when cultivated in LB media in 24-well plates with different concentrations of cinnamyl alcohol and cinnamyl acetate. Average growth curves in (B) Cinnamyl alcohol (C) Cinnamyl acetate. The plates were incubated in a plate reader at 37°C, 432 rpm for 15 h. All data are average from 3 biological replicates and the error bars represent standard deviation.

8.7 Conclusion

A handful number of highly attractive compounds from different classes were tested for their toxicity compared with their ester derivatives to fundamentally identify new candidates and potentially expand the concept of bioderivatization. It was demonstrated that menthol, eugenol, and linalool were more toxic than their ester derivatives. Cinnamyl alcohol and cinnamyl acetate were both toxic to *E. coli* with the cinnamyl alcohol slightly more toxic at 5 mM. Vanillin showed similar toxicity to vanillin acetate; thus, it may not be a suitable candidate.

The toxicity of quercetin was studied; however, its physical properties and instability prevented the use of OD as a growth indicator. Therefore, other indicators may be needed to understand the toxicity of this compound in future. Moreover, the feeding experiment was carried out for menthol and eugenol to gain initial knowledge on available AATs activity toward the potential chemicals. It was shown that currently available AATs were able to catalyze *O*-acetylation of both compounds. In future, AAT screening of linalool could be conducted similarly to feeding experiments of menthol and eugenol. Furthermore, extended AAT and GT libraries could be useful for future utility of bioderivatization. Throughout this work, it was demonstrated that efficiency of these key enzymes was one of the factors influencing effects of bioderivatization. Therefore, to have such libraries on enzyme activity and substrate preference could be beneficial for future applications of bioderivatization.

Chapter 9
Conclusions and recommendations for future work

9.1 Conclusions

Bio-based production from renewable resources is an alternative to petroleum-based production. However, this biotechnology faces several challenges including the toxicity of bioproducts to host microorganisms and the cost of separation and purification processes. In this work, bioderivatization was proposed as a new biological strategy to overcome such challenges. It is defined as an *in vivo* transformation of toxic chemicals into less toxic derivatives. The transformation aims to alleviate the toxicity of target bioproducts and potentially enable the cost-effective downstream processes. The newly proposed concept was evaluated mainly on toxicity mitigation in this study.

In Chapter 4, 1-octanol was proposed as the first proof-of-principle bioproduct. The existing 1-octanol pathway from Akhtar *et al.*, 2015 was optimized to obtain high product titers and allow comprehensive evaluation of the concept. The key limitation of the pathway was identified to be the thioesterase enzyme. With newly screened C8-specific thioestereases, the optimized 1-octanol strain produced the highest titer of 4.29 mM (59.48 mmol/mol glucose) 1-octanol which is around 7-fold higher in yield compared to the first-generation strain in the same conditions. The optimization in this study also improved the yield of 1-octanol to 43 mg/g glucose in comparison with the previous work on 1-octanol production via the carboxylic acid reductase-dependent pathway by Akhtar *et al.*, 2015 where the yield was reported at 12 mg/g glucose.

In Chapter 5, the toxicity of 1-octanol and its ester derivative, octyl acetate, was evaluated. It was shown that octyl acetate was less toxic than 1-octanol. Then, octyl acetate biosynthetic pathway was implemented in *E. coli* by extending one reaction step from 1-octanol pathway. Three alcohol acetyltransferases (AAT), CAT, SAAT, and ATF1, were co-expressed with 1-octanol pathway to convert 1-octanol to octyl acetate. The first-generation octyl acetate strain expressing ATF1 produced up to 0.54 mM octyl acetate. However, solvent overlay used to capture the volatile products masked the toxicity effects and resulted in no growth differences observed. Similar to what was found in 1-octanol pathway, thioesterase was also identified as a limiting enzyme for octyl acetate pathway. Growth and glucose consumption were assessed in the absence of solvent overlay and it showed that octyl acetate-producing strain exhibited greater biomass accumulation and glucose consumption presenting the first evidence of positive impact from bioderivatization. Lastly, high-flux octyl acetate strains produced higher titers and yields than 1-octanol strains in most conditions with

variable effects on growth and metabolism suggesting that the positive impact of bioderivatization was not solely from reduced toxicity, but also partially from product removal.

In Chapter 6, another strategic reaction of bioderivatization, *O*-glucosylation, was investigated. The toxicity of octyl glucoside was evaluated and shown to be less toxic than 1-octanol. Synthetic octyl glucoside pathway was then implemented in *E. coli*. The use of sucrose synthase (SUS) to enhance octyl glucoside production was also investigated and it showed that octyl glucoside production increased when SUS expression and sucrose supplementation were applied. From a bioderivatization perspective, octyl glucoside-producing strain (with SUS expression and sucrose supplementation) showed higher titers and yields of C8 products compared with 1-octanol-producing strain, which is in line with observations from Chapter 5. Octyl glucoside pathway was then transferred to *Synechocystis* sp. PCC 6803 as another production host. Octyl glucoside was produced successfully in cyanobacteria. *O*-glucosylation showed positive impact on titers and yields under some conditions. However, the evaluation of bioderivatization was challenging as it showed that expression of more than one proteins in a one-plasmid system seemed to affect the amount of each and total protein and, thereby interfered with result interpretation. Even though, it was shown that production of octyl glucoside clearly rescued cyanobacterial growth from 1-octanol toxicity in the absence of solvent overlay.

Another toxic chemical, 1-dodecanol, was investigated in Chapter 7. 1-Dodecanol did not show high toxicity on *E. coli*; however, this compound was highly toxic to cyanobacteria. 1-Dodecanol and its ester derivative, lauryl acetate, were produced in *E. coli* by expression of Tes12, Sfp, CAR with and without ATF1. In the case of *E. coli*, bioderivatization did not show positive effects which may be because (*i*) the pathway components were suboptimal or (*ii*) 1-dodecanol is not highly toxic to *E. coli*. The pathways were transferred to *Synechocystis* sp. PCC 6803 as a focused host in this chapter. Unfortunately, it was shown that ATF1 could not be expressed in cyanobacteria or the expression resulted in unstable strains. Therefore, further investigations on ATF1 need to be carried out in order to efficiently produce lauryl acetate in cyanobacteria.

In Chapter 8, a range of commercially attractive chemicals from different chemical classes were selected and investigated as candidates for bioderivatization. Menthol, eugenol and linalool showed to be more toxic than their ester derivatives, menthyl acetate, eugenyl acetate, linalyl acetate, and linalyl butyrate. Thus, these compounds hold potential as suitable candidates for the concept. However, vanillin and cinnamyl alcohol showed similar toxic effects toward growth compared to their ester derivatives, vanillin acetate and cinnamyl acetate, respectively. Toxicity of quercetin in this study was not conclusive as the OD measurement could not be used as cellular activity indicator.

In summary, the results presented in this work support the concept of bioderivatization as a potential biotechnological strategy to overcome the aforementioned challenges in renewable production of toxic and/or poorly soluble compounds with 1-octanol as a proof-of-principle where implementation of *O*-acetylation and *O*-glucosylation could enhance the production around 2-fold in the optimal conditions compared with strains without bioderivatization. Further investigations on other candidates also present fundamental knowledge on the concept that could be useful in future utility of bioderivatization.

9.2 Recommendations for future work

Indeed, bioderivatization is a newly proposed biotechnological strategy, the investigation can be extended to thoroughly evaluate the impact of this concept on a broader perspective. Some of the recommendations have been mentioned throughout this thesis.

In Chapter 6, although octyl glucoside pathway was successfully transferred to cyanobacteria and the production was observed, it showed that expression of two genes on the same plasmid may result in lowering the amount of each protein. Further optimization of the system may be needed before the comprehensive investigation of bioderivatization could take place. This could be further optimized, for example, by integrating one gene into the genome and expressing another gene on the plasmid. Another option could be to monitor protein expression as well as the production to fairly compare the systems.

In Chapter 7, the expression of ATF1 in cyanobacteria was not successful, one of the speculations was because of the membrane-association property of ATF1. The essential domains for ATF1 membrane-association were reported in literature (Lin & Wheeldon, 2014) and could potentially be a next step for further investigation. Searching for other C12-preferring AATs is also an option. A plant-derived AAT enzyme able to catalyze 1-dodecanol and acetyl-CoA was previously reported; however, the activity on 1-dodecanol of this enzyme was shown to be ~20-fold lower than of ATF1 (Ding *et al.*, 2016). Alternatively, *O*-glucosylation could be another strategy to implement bioderivatization of 1-dodecanol to avoid the issue with ATF1.

In Chapter 8, it was shown that toxicity of quercetin on *E. coli* growth could not be assessed by OD measurements. If this chemical is still considered interesting for further investigation, other indicators such as glucose consumption should be considered. Moreover, different classes of petroleum- or plant-derived chemicals were also investigated in this chapter in order to investigate whether bioderivatization could be applied to other toxic or poorly soluble chemicals or not. Preliminary work suggested that one of the time-consuming steps was to identify the genetic parts for the novel synthetic pathways, especially the key enzymes for bioderivatization, AATs and GTs. As shown throughout this thesis, effective AATs and GTs are an important factor influencing effects of bioderivatization. Therefore, it would be

useful to have libraries of AATs and GTs on their activity and substrate specificity. In the case of AATs, a previous study on phylogenetic analysis of AATs showed that similarities of amino acid sequences could not be used to predict the activity or substrate preference of the enzymes (Beekwilder *et al.*, 2004), this could complicate the search even further. Creation of a GT library regarding their activity and substrate preference was demonstrated by Caputi *et al*., 2008 where 107 GTs from *A. thaliana* were screened for their activity on model terpenoids. In this work, they were able to identify GTs with high activity on terpenoids of interest. It is worth noting that rapid screening of enzyme activity is also needed to conduct such screenings. In the same study, an initial screening assay based on TLC system was also demonstrated. Furthermore, rapid screening methods for ester biosynthesis were developed for AATs (Lin, Zhu & Wheeldon, 2016; Löbs *et al.*, 2016). It could be useful to have such assays to facilitate library generation of AATs and GTs.

In this work, the effect of bioderivatization on toxicity was systematically evaluated. However, its effect on downstream separation and purification processes, which is another challenge in bio-based production (see Section 2.1.3), has yet to be studied. As bioderivatization modifies functional groups on targeted compounds; therefore, it can be used to design the derivative that benefits the downstream processes. In this case, the method used for product:process separation directly influences the choice of bioderivatization strategy whether to opt for more hydrophobic or more hydrophilic derivatives. For example, distillation is arguably the most common method used for low molecular weight bioproduct recovery from fermentation (Kujawska *et al.*, 2015; Kiss *et al.*, 2016). This technology relies on different levels of volatility of chemicals. However, distillation is not suitable for compounds with high boiling points as they would require more energy demand (Oudshoorn, Van Der Wielen & Straathof, 2009; Kiss *et al.*, 2016). Esters are known to be more volatile and less water-soluble compared to their corresponding alcohols and esterification is also used to separate carboxylic acids from fermentation processes. For instance, esterification of lactic acid with methanol or ethanol prior to separation via distillation is used to obtain high-purity product. To recover lactic acid at the end of the processes, corresponding esters are hydrolyzed with water (Datta & Henry, 2006). Esterification was also demonstrated to facilitate the separation of mixed acids via reactive distillation (Orjuela *et al.*, 2011). Therefore, bioderivatization via *O*-acetylation may be more suitable, if distillation is a method for downstream processes. Moreover, liquid-liquid extraction (LLE) or solvent extraction is also used to separate bio-based chemicals from fermentation broth (van den Berg *et al.*, 2013; de Jesus *et al.*, 2019) as it is more suitable for high boiling points or temperature-sensitive compounds (Kiss *et al.*, 2016). The use of this method to simultaneously extract bioproducts during fermentation (also known as *in situ* product removal (ISPR)) has also shown to benefit the bio-based production as it reduced product inhibition on microbial hosts (Buque-Taboada *et al.*, 2006). Energetic cost of liquid-

liquid extraction is also less than distillation, for example, in the case of biobutanol, liquid-liquid extraction requires around four times less energy demand than distillation (Garcia-Chavez *et al.*, 2012; Kiss *et al.*, 2016). To maximize the efficiency of liquid-liquid extraction, final products of interest must be compatible with the choice of extraction solvent. To give an example, different solvents and ratios have been investigated for biobutanol extraction and briefly reviewed (Kujawska *et al.*, 2015). Therefore, it is a case-by-case consideration whether a more hydrophobic or hydrophilic derivatives would be useful for the separation and purification processes. Altogether, bioderivatization may open an opportunity to reduce the cost of downstream bioprocesses; however, the effect has yet to be evaluated.

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Appendices

Appendix A- List of *E. coli* strains used for production in this study

Strain No.	*E. coli* strain	ColE1 plasmid	CloDF13 plasmid
Chapter 4			
1	C43(DE3)	pET-TPC3	-
2	BW25113	pET-pA1lacO-1-TPC3	-
3	BW25113	pET-pA1lacO-1-'CpFatB1-Sfp-CAR	-
4	BW25113	pET-pA1lacO-1-'CaFatB3-Sfp-CAR	-
5	BW25113	pET-pA1lacO-1-'CpFatB1-4-Sfp-CAR	-
6	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	-
Chapter 5			
7	C43(DE3)	pET-T7-empty	pCDF-T7-empty
8	C43(DE3)	pET-TPC3	pCDF-T7-CAT
9	C43(DE3)	pET-TPC3	pCDF-T7-SAAT
10	C43(DE3)	pET-TPC3	pCDF-T7-ATF1
11	BW25113	pET-pA1lacO-1-'CpFatB1-4-Sfp-CAR	pCDF-pA1lacO-1-empty
12	BW25113	pET-pA1lacO-1-'CpFatB1-4-Sfp-CAR	pCDF-pA1lacO-1-ATF1
13	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-empty
14	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-ATF1
Chapter 6			
15	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-empty
16	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-AdGT4
17	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-AtGT1
18	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-MtG1
19	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-MtH2
20	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-VvGT1
21	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-AtSUS1
22	BW25113	pET-pA1lacO-1-'CaFatB3-5-Sfp-CAR	pCDF-pA1lacO-1-MtH2-AtSUS1
Chapter 7			
23	BW25113	pET-pA1lacO-1-Tes12-Sfp-CAR	pCDF-pA1lacO-1-GFP
24	BW25113	pET-pA1lacO-1-Tes12-Sfp-CAR	pCDF-pA1lacO-1-SAAT
25	BW25113	pET-pA1lacO-1-Tes12-Sfp-CAR	pCDF-pA1lacO-1-ATF1
Chapter 8			
26	BW25113	-	pCDF-pA1lacO-1-CAT
27	BW25113	-	pCDF-pA1lacO-1-SAAT
28	BW25113	-	pCDF-pA1lacO-1-ATF1
29	BW25113	-	pCDF-pA1lacO-1-VAAT
30	BW25113	-	pCDF-pA1lacO-1-VpAAT1

Appendix B List of *Synechocystis* sp. PCC 6803 strains used in this study

Strain No.	Cyanobacterial Strain	RSF1010-based plasmid
Chapter 6		
1	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-'CpFatB1-4
2	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-'CpFatB1-4-MtH2
3	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-'CaFatB3-5
4	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-'CaFatB3-5-MtH2
5	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-'CaFatB3-5-MtH2-AtSUS1
Chapter 7		
6	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12
7	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12-1-ATF1
8	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12-2-ATF1
9	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12-3-ATF1
10	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12-4-ATF1
11	*Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR	pRSF1010-Pcoa-Tes12-5-ATF1

* *Synechocystis* sp. PCC 6803- *Δaas* -PnrsB-Sfp-CAR obtained from Yunus, 2019

Appendix C- Summary of plasmid construction via traditional restriction cloning

Plasmid used	Ligated fragment		Relevant information
	Plasmid backbone	Insert	
pCDF-CAT	pCDF-GFP (*Bsa*I)	*cat* (*Bsa*I)	A plasmid encoding CAT protein
pCDF-SAAT	pCDF-GFP (*Bsa*I)	*saat* (*Bsa*I)	A plasmid encoding SAAT protein
pCDF-ATF1	pCDF-GFP (*Bsa*I)	*atf1* (*Bsa*I)	A plasmid encoding ATF1 protein
pCDF-empty	pCDF-GFP (*Bsa*I)	-	A negative control plasmid
pET-empty	pET-TPC3 (*Nco*I and *Avr*II)	-	A negative control plasmid

GFP gene was amplified from pJET-GFP using primers with *Nco*I and *Avr*II restriction sites to construct pCDF-GFP plasmid. The amplified *GFP* was then cloned into pCDF-Ahr_{his} by replacing Ahr_{his} with *GFP*. *cat* gene was amplified from pACYC-petF-fpr using primers with *Bsa*I prefix and suffix, while SAAT and ATF1 were order as a gBlock from IDT technology. All three genes were cloned into pCDF-GFP. To construct empty plasmids, pET-TPC4 was digested with *Nco*I and *Avr*II to remove TPC4 cassette and pCDF-GFP was digested with *Bsa*I to remove *GFP*. Both backbones were recirculated using NEB Quick Blunting™ and Quick Ligation™ Kits after restriction digestion.

Appendix D- List of primers used in this study

Name	Gene	Template	Primer sequence (5' – 3')
For restriction cloning			
-	*cat*	pACYC-petF-fpr[a]	Forward: TCTGGTGGGTCTCTGTCCatggagaaaaaaatcactggatataccaccg Reverse: CGATAGGTCTCCCGAGCCttacgccccgccctgc
-	*saat*	pJET-SAAT	Forward: TCTGGTGGGTCTCTGTCCATG Reverse: CGATAGGTCTCCCGAGCC
-	*atf1*	pJET-ATF1	Forward: TCTGGTGGGTCTCTGTCCATG Reverse: CGATAGGTCTCCCGAGCC
-	*gfp*	pJET-GFP	Forward: GGCCATGGTCTGGTGGGTCTCTGTCC Reverse: GGCCTAGGCGATAGGTCTCCCGAGCC
-	*tes3*	pET-TPC3	Forward: TCTGGTGGGTCTCTGTCCATGAAATTTAAAAAAAAATTTAAAATTGGGCGGATGCACG Reverse: CGATAGGTCTCCCGAGCCTTACACGTTAGTTTTAATTTTCCCCAAACAGTAGTCC
-	*sfp-car*	pET-TPC3	Forward: TCTGGTGGGTCTCTGTCCATGAAGATCTACGGCATATACATGGACC Reverse: CGATAGGTCTCCCGAGCCgtggcagcagcctagggaattcttacagc
For insert validation			
pJET-forward	Inserted gene	pJET-plasmid	CGACTCACTATAGGGAGAGCGGC
pJET-reverse	Inserted gene	pJET-plasmid	AAGAACATCGATTTTCCATGGCAG
PB66	Inserted gene	CloDF13-based	GGTCGTCGGTTCAGGGCAGG
PB88	Inserted gene	ColE1-based	GCGTATCACGAGGCCCTTTCG
PB174	KanR	ColE1-based	GTTGGTAGCTCTTGATCCGGC
PB175	GFP	ColE1-based	CTGTTGCCCGTCTCACTGG
IY100	bom-rop	ColE1-based	CGGTTCCTGGCCTTTTGCTGG
IY216	Inserted gene	ColE1-based	GTAAGCAGACAGTTTTATTGTTCATG
PB217	Inserted gene	RSF1010-based	AATCCCAGGGTGTGGTAATG
IY152	Inserted gene	RSF1010-based	ggctttctacgggttcgctgcgag

Appendix E- BASIC Linker sequences

Mixed linkers	Adapter		Linker	
	Name	Sequence (5'-3')	Name	Sequence (5'-3')
1P	1P-A	TTTATTGAACTA	1P-L	GGACTAGTTCAATAAATACCCTCTGACTGTCTCGGAG
2P	2P-A	TTCTTATTACCT	2P-L	GGACAGGTAATAAGAACTACACGACTGGATACTGACT
3P	3P-A	TGTTATTACAGA	3P-L	GGACTCTGTAATAACAATACCGATAAAGCAACGAGTG
5P	5P-A	TTGATTTATCCT	5P-L	GGACAGGATAAATCAACTCGTAAGCAATACTGTCTGT
1MP	1MP-A	TCTGGTGGGT/iMe-dC/TCT	1MP-L	GGACAGAGACCCACCAGATAATAGTGTTTCCACGAAGTG
2MP	2MP-A	AACTTCGGAATC	2MP-L	GGACGATTCCGAAGTTACACCAGATTGGACTGTTATTAC
LRBS1-1P	LRBS1-1P-A	ATCACACAGGACTA	LRBS1-1P-L	GGACTAGTCCTGTGTGATTTACAACTGATACTTACCTGA
LRBS1-2P	LRBS1-2P-A	AAAGAGGGGAAATA	LRBS1-2P-L	GGACTATTTCCCCTCTTTTTACAACTGATACTTACCTGA
LRBS1-3P	LRBS1-3P-A	AAAGAGGAGAAATA	LRBS1-3P-L	GGACTATTTCTCCTCTTTTTACAACTGATACTTACCTGA
LRBS1-4P	LRBS1-4P-A	ATCACAAGGAGGTA	LRBS1-4P-L	GGACTACCTCCTTGTGATTTACAACTGATACTTACCTGA
LRBS1-5P	LRBS1-5P-A	ATTAGTGGAGGTTA	LRBS1-5P-L	GGACTAACCTCCACTAATTTACAACTGATACTTACCTGA
LRBS2-3P	LRBS2-3P-A	AAAGAGGAGAAATA	LRBS2-3P-L	GGACTATTTCTCCTCTTTTTTCTGCTACCCTTATCTCAG
LRBS2-4P	LRBS2-4P-A	ATCACAAGGAGGTA	LRBS2-4P-L	GGACTACCTCCTTGTGATTTTCTGCTACCCTTATCTCAG
LRBS3-5P	LRBS3-5P-A	ATTAGTGGAGGTTA	LRBS3-5P-L	GGACTAGTCCTGTGTGATTTACAACTGATACTTACCTGA
1S	1S-A	TGTCGTAAGTAA	1S-L	CTCGTTACTTACGACACTCCGAGACAGTCAGAGGGTA
2S	2S-A	TTTCACACCGAT	2S-L	CTCGATCGGTGTGAAAAGTCAGTATCCAGTCGTGTAG
3S	3S-A	TAGTGCCGTGAT	3S-L	CTCGATCACGGCACTACACTCGTTGCTTTATCGGTAT
5S	5S-A	GGCACTACTTCT	5S-L	CTCGAGAAGTAGTGCCACAGACAGTATTGCTTACGAG
1MS	1MS-A	CGAGTTCTTACC	1MS-L	CTCGGGTAAGAACTCGCACTTCGTGGAAACACTATTA
2MS	2MS-A	CGATAGGT/iMe-dC/TCC	2MS-L	nTATCGGTAATAACAGTCCAATCTGGTGT
LRBS1-XS	LRBS1-1S-A	GACGGTGTTCAA	LRBS1-XS-L	CTCGTTGAACACCGTCTCAGGTAAGTATCAGTTGTAA
LRBS2-XS	LRBS2-2S-A	CCAATAGTAACA	LRBS2-XS-L	CTCGTGTTACTATTGGCTGAGATAAGGGTAGCAGAAA
LRBS3-XS	LRBS3-3S-A	GCCTCGGTAAAT	LRBS3-XS-L	CTCGATTTACCGAGGCTGAATAAGGATTACTTTCCGT

Appendix F-Plasmids used for construct preparation in this study

No.	Prefix	Plasmid	Suffix	Plasmid generated	Relevant information
1	3P	pJET-ColE1-rop-bom	1S	pET-pA1lacO-1-GFP	A plasmid used as a backbone for BASIC assembly with ColE1 origin of replication, pA1lacO-1 promoter and kanamycin resistance
	1P	pJET-pA1lacO-1	2MS		
	2MP	pJET-GFP	1MS		
	1MP	pJET-termB15	2S		
	2P	pJET-Kan	3S		
2	1P	pJET-CloDF-Spec	2S	pCDF-pA1lacO-1-GFP	A plasmid used as a backbone for BASIC assembly with CloDF origin of replication, pA1lacO-1 promoter and spectinomycin resistance
	2P	pJET-pA1lacO-1	2MS		
	2MP	pJET-GFP	1MS		
	1MP	pJET-termB15	1S		
3	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-TPC3	A plasmid encoding Tes3, Sfp and CAR
	LRBS1-3P	pJET-Tes3	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
4	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-‘CpFatB1-Sfp-CAR	A plasmid encoding ‘CpFatB1, Sfp and CAR
	LRBS1-3P	pJET-‘CpFatB1	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
5	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-‘CaFatB3-Sfp-CAR	A plasmid encoding ‘CaFatB3, Sfp and CAR
	LRBS1-3P	pJET-‘CaFatB3	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
6	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-‘CpFatB1-4-Sfp-CAR	A plasmid encoding ‘CpFatB1-4, Sfp and CAR
	LRBS1-3P	pJET-‘CpFatB1-4	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
7	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-‘CaFatB3-5-Sfp-CAR	A plasmid encoding ‘CaFatB3-5, Sfp and CAR
	LRBS1-3P	pJET-‘CaFatB3-5	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
8	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-ATF1	A plasmid encoding ATF1

	LRBS1-3P	pJET-ATF1	2S		
9	2P	pCDF-pA1lacO-1-GFP	2S	pCDF-pA1lacO-1-empty	A negative control plasmid.
10	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-AdGT4	A plasmid encoding AdGT4
	LRBS1-3P	pJET-AdGT4	2S		
11	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-AtGT1	A plasmid encoding AtGT1
	LRBS1-3P	pJET-AtGT1	2S		
12	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-MtG1	A plasmid encoding MtG1
	LRBS1-3P	pJET-MtG1	2S		
13	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-MtH2	A plasmid encoding MtH2
	LRBS1-3P	pJET-MtH2	2S		
14	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-VvGT1	A plasmid encoding VvGT1
	LRBS1-3P	pJET-Vv	2S		
15	2P	pCDF-pA1lacO-1-GFP	LRBS1-XS	pCDF-pA1lacO-1-MtH2-AtSUS1	A plasmid encoding MtH2 and AtSUS1
	LRBS1-3P	pJET-MtH2	LRBS2-XS		
	LRBS2-3P	pUCIDT-AtSUS1	2S		
16	2P	pCDF-GFP	LRBS1-XS	pCDF-pA1lacO-1-AtSUS1	A plasmid encoding AtSUS1
	LRBS1-3P	pUCIDT-AtSUS1	2S		
17	1P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-‘CpFatB1-4	A plasmid encoding ‘CpFatB1-4
	LRBS1-4P	pJET-‘CpFatB1-4	1S		
18	1P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-‘CpFatB1-4-MtH2	A plasmid encoding ‘CpFatB1-4 and MtH2
	LRBS1-4P	pJET-‘CpFatB1-4	LRBS2-XS		
	LRBS2-4P	pJET-MtH2	1S		
19	1P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-‘CaFatB3-5	A plasmid encoding ‘CaFatB3-5
	LRBS1-4P	pJET-‘CaFatB3-5	1S		
20	1P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-‘CaFatB3-5-MtH2	A plasmid encoding ‘CaFatB3-5 and MtH2
	LRBS1-4P	pJET-‘CaFatB3-5	LRBS2-XS		
	LRBS2-4P	pJET-MtH2	1S		
21	1P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS		

	LRBS1-4P	pJET-‘CaFatB3-5	LRBS2-XS	pRSF1010-Pcoa-‘CaFatB3-5-MtH2-AtSUS1	A plasmid encoding ‘CaFatB3-5, MtH2 and AtSUS1
	LRBS2-4P	pJET-MtH2	LRBS3-XS		
	LRBS3-5P	pUCIDT-AtSUS1	1S		
22	2P	pET-pA1lacO-1-GFP	LRBS1-XS	pET-pA1lacO-1-Tes12-Sfp-CAR	A plasmid encoding Tes12, Sfp and CAR
	LRBS1-3P	pJET-Tes12	LRBS2-XS		
	LRBS2-3P	pJET-Sfp-CAR	2S		
23	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12	A plasmid encoding Tes12
	LRBS1-5P	pJET-Tes12	2S		
24	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12-1-ATF1	A plasmid encoding Tes12 and ATF1 with RBS 1
	LRBS1-5P	pJET-Tes12	LRBS2-XS		
	LRBS2-1P	pJET-ATF1	2S		
25	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12-2-ATF1	A plasmid encoding Tes12 and ATF1 with RBS 2
	LRBS1-5P	pJET-Tes12	LRBS2-XS		
	LRBS2-2P	pJET-ATF1	2S		
26	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12-3-ATF1	A plasmid encoding Tes12 and ATF1 with RBS 3
	LRBS1-5P	pJET-Tes12	LRBS2-XS		
	LRBS2-3P	pJET-ATF1	2S		
27	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12-4-ATF1	A plasmid encoding Tes12 and ATF1 with RBS 4
	LRBS1-5P	pJET-Tes12	LRBS2-XS		
	LRBS2-4P	pJET-ATF1	2S		
28	2P	pRSF1010-Ery-Pcoa-GFP	LRBS1-XS	pRSF1010-Pcoa-Tes12-5-ATF1	A plasmid encoding Tes12 and ATF1 with RBS 5
	LRBS1-5P	pJET-Tes12	LRBS2-XS		
	LRBS2-5P	pJET-ATF1	2S		

Appendix I- Biomass calibration curves (*E. coli* and cyanobacteria)

E. coli strains, C43 (DE3) and BW25113, were cultivated in M9 minimal medium for 24 h and *Synechocystic* sp. PCC 6803 was cultivated in BG11-Co for 7 days before OD was then measured and diluted down to the desirable range. The cells were harvested by centrifugation and washed with ultrapure water twice. The culture for each OD was then filtered through filter paper using a pump. Prior to this step, the filter papers were dried at 60°C overnight and weighed. The cells on filter papers were then dried at 60°C for 24 h and weighed again. The calibration curves obtained from each strain were shown below.

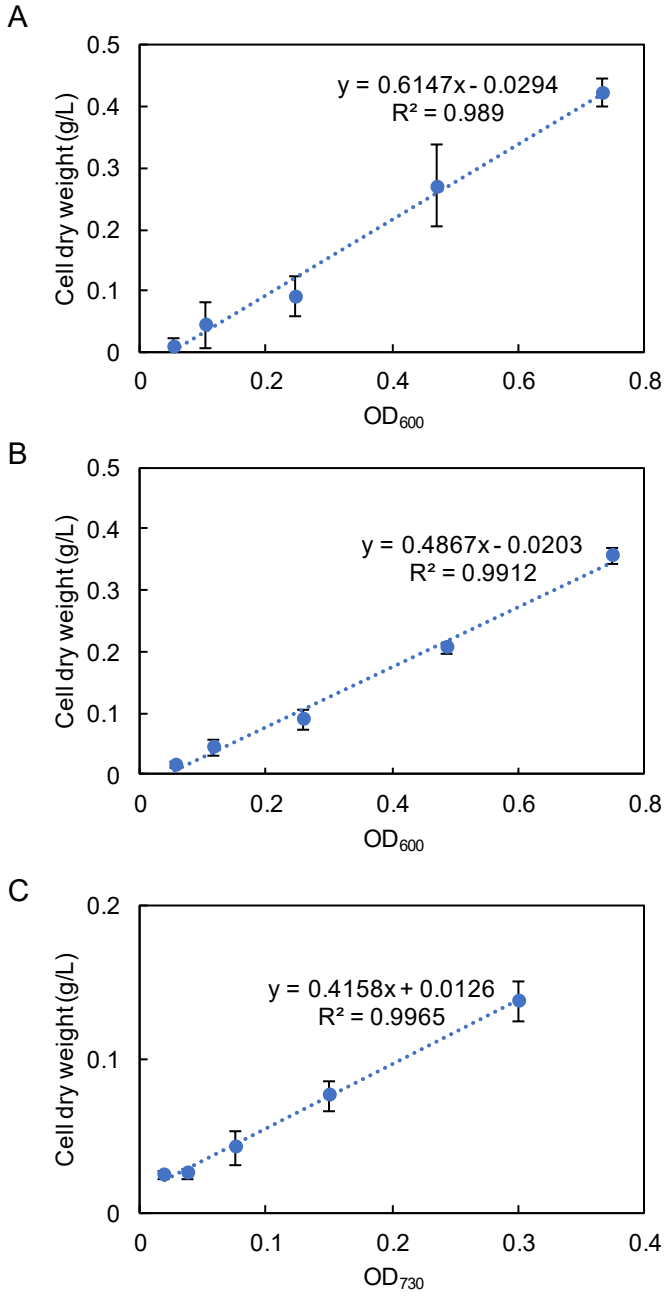

Figure A. Calibration curves to convert optical density (OD) to cell dry weight (gCDW/L)

(A) *E. coli* C43 (DE3) (B) *E. coli* BW25113 (C) *Synechocystis* sp. PCC 6803. All data are average from 3 technical replicates and the error bars represent standard deviation.

Appendix J- List of enzymes used in this study and their sources

Gene	Source organism	UniProtKB	Reference
tes3	*Anaerococcus tetradius*	C2CIR4	(Akhtar *et al.*, 2015)
sfp	*Bacillus subtilis*	P39135	(Akhtar *et al.*, 2015)
car	*Mycobacterium marinum*	B2HN69	(Akhtar *et al.*, 2015)
cat	*Escherichia coli*	P62577	(Rottig & Steinbuchel, 2013)
saat	*Fragaria ananassa cv. Elsanta*	Q9FVF1	(Aharoni *et al.*, 2000)
atf1	*Saccharomyces cerevisiae*	P40353	(Verstrepen *et al.*, 2004)
CpFatB1 (wild type)	*Cuphea palustris*	Q39554	(Dehesh *et al.*, 1996)
CaFatB3 (wild type)	*Cuphea avigera pulcherrima*	R4J2L6	(Graham & Kleiman, 1992)
'CpFatB1-4	*Cuphea palustris*	-	(Lozada *et al.*, 2018)
'CaFatB3-5	*Cuphea avigera pulcherrima*	-	(Yunus, 2019)
AdGT4	*Actinidia deliciosa*	A0A077EMP8	(Yauk *et al.*, 2014)
VvGT1	*Vitis vinifera*	P51094	(Ford, Boss & Hæj, 1998)
AtGT1	*Arabadopsis thaliana*	Q9M156	(Lim *et al.*, 2002)
MtG1	*Medicago truncatula*	Q5IFH7	(Shao *et al.*, 2005)
MtH2	*Medicago truncatula*	A6XNC5	(Mashek, Li & Coleman, 2007)
AtSUS1	*Arabidopsis thaliana*	P49040	(Zheng *et al.*, 2011)
VAAT	*Fragaria vesca*	Q8GTM5	(Beekwilder *et al.*, 2004)
VpAAT1	*Vasconcellea pubescens*	D0QJ94	(Balbontín *et al.*, 2010)

If the genes were from foreign organisms, codon optimization was carried out prior to synthesis in order to express in bacteria.

Appendix K- List of gene sequences used in this study

Gene	Sequence
tes3	ATGAAATTTAAAAAAAAATTTAAAATTGGGCGGATGCACGTTGACCCCTTTAACTACATTAGT ATGCGCTATCTGGTTGCCTTGATGAATGAAGTGGCTTTTGATCAAGCCGAAATTTTGGAAAA AGATATTGACATGAAAAACCTGCGTTGGATTATTTATAGTTGGGATATTCAGATTGAAAATAA CATTCGCCTGGGGGAAGAAATTGAAATTACCACTATTCCCACCCACATGGATAAATTTTATG CTTACCGGGACTTTATTGTTGAAAGCCGCGGAAATATTTTAGCCCGTGCTAAAGCCACCTTT TTGTTAATGGATATTACTCGCCTGCGTCCCATTAAAATTCCCCAAAATCTGTCTTTGGCCTAT GGCAAAGAAAACCCCATTTTTGATATTTACGACATGGAAATTCGGAACGATCTGGCTTTTAT TCGCGACATTCAGTTACGTCGGGCCGATCTGGACAATAACTTTCACATTAACAACGCCGTG TACTTTGATTTGATTAAAGAAACCGTTGATATTTACGATAAAGACATTTCCTACATTAAATTGA TTTACCGGAACGAAATTCGCGATAAAAAACAAATTCAGGCTTTTGCCCGTCGGGAAGATAAA AGTATTGACTTTGCCCTGCGTGGCGAAGATGGTCGGGACTACTGTTTGGGGAAAATTAAAA CTAACGTGTAA

sfp	ATGAAGATCTACGGCATATACATGGACCGCCCCCTAAGCCAGGAAGAGAACGAGCGCTTT ATGAGCTTCATCTCCCCGGAGAAGCGTGAAAAATGTCGCCGTTTCTACCACAAAGAAGAT GCCCATCGCACTTTACTCGGTGATGTTTTGGTCCGATCTGTCATTAGCCGTCAATACCAAC TCGATAAAAGCGACATTCGTTTCTCGACTCAAGAATACGGCAAACCCTGCATCCCAGACCT ACCGGATGCCCACTTTAACATTTCCCACAGTGGCCGGTGGGTGATCTGTGCCTTTGACTC CCAACCGATTGGAATTGACATTGAAAAGACCAAACCCATTAGTTTAGAAATAGCTAAACGT TTCTTCTCTAAGACAGAATATAGCGATCTCCTGGCCAAAGACAAGGACGAGCAAACTGACT ACTTCTATCATTTGTGGAGCATGAAAGAATCCTTTATCAAACAAGAGGGCAAAGGTCTTTC ACTGCCTTTAGACTCTTTTTCTGTCCGTTTACATCAGGACGGCCAGGTCAGTATTGAGCTT CCGGATTCGCATTCCCCCTGTTATATCAAAACGTACGAAGTAGATCCGGGCTACAAAATG GCGGTCTGCGCAGCGCACCCCGATTTCCCGGAAGATATTACGATGGTTTCTTACGAAGAA CTACTCTAA
car	ATGACCGGCTATGCGGATCGTCCGGCACTGGCACAACGTAGCGTGGAATTTGTGACCGA TGCGGGCACCGGTCATACCACCCTGCGTCTGCTGCCGCATTTTGAAACCATTAGCTATGG CGAACTGTGGGATCGTATTAGCGCGCTGGCCGATGTTCTGAGCACCGAACAGACCGTGA AACCGGGCGATCGTGTGTGCCTGCTGGGCTTTAACAGCGTGGATTATGCGACCATTGATA TGACCCTGGCACGTCTGGGTGCTGTCGCTGTCCCGCTGCAGACCTCTGCTGCGATTACC CAGCTGCAGCCGATTGTGGCGGAAACCCAGCCGACCATGATTGCGGCGAGCGTGGATGC CCTGGCCGATGCGACCGAACTGGCACTGAGTGGTCAAACGGCTACGCGTGTGCTGGTGT TTGATCATCATCGTCAGGTGGATGCGCATCGTGCGGCGGTTGAAAGCGCGCGTGAACGT CTGGCCGGTAGCGCGGTGGTTGAAACCCTGGCCGAAGCGATTGCGCGTGGTGATGTGC CGCGTGGTGCGAGCGCGGGTAGCGCACCGGGCACCGATGTGAGCGATGATAGCCTGGC CCTGCTGATTTATACCTCTGGTAGTACGGGTGCGCCGAAAGGCGCCATGTATCCGCGTCG TAACGTGGCGACCTTTTGGCGTAAACGTACCTGGTTTGAAGGCGGCTATGAACCGAGCAT TACCCTGAACTTTATGCCGATGAGCCATGTGATGGGCCGTCAGATTCTGTATGGCACCCT GTGCAACGGCGGCACCGCGTATTTTGTGGCGAAAAGCGATCTGAGCACCCTGTTTGAAG ATCTGGCCCTGGTGCGTCCGACCGAACTGACCTTCGTCCCGCGTGTTTGGGATATGGTGT TCGATGAATTTCAGAGCGAAGTGGATCGTCGTCTGGTGGATGGCGCGGATCGTGTTGCG CTGGAAGCGCAGGTGAAAGCGGAAATTCGTAACGATGTGCTGGGCGGTCGTTATACCTCT GCTCTGACGGGTTCTGCTCCGATTAGCGATGAAATGAAAGCGTGGGTGGAAGAACTGCT GGATATGCATCTGGTGGAAGGCTATGGCAGCACCGAAGCGGGCATGATTCTGATTGATG GCGCGATTCGTCGTCCGGCGGTGCTGGATTATAAACTGGTGGATGTTCCGGATCTGGGC TATTTTCTGACCGATCGTCCGCATCCGCGTGGCGAACTGCTGGTGAAAACCGATAGCCTG TTTCCGGGCTATTATCAGCGTGCGGAAGTGACCGCGGATGTGTTTGATGCGGATGGCTTT TATCGCACCGGCGATATTATGGCGGAAGTGGGCCCGGAACAGTTTGTGTATCTGGATCGT CGTAACAACGTGCTGAAACTGAGCCAGGGCGAATTTGTTACCGTGAGCAAACTGGAAGCG GTGTTTGGCGATAGCCCGCTGGTGCGTCAGATTTATATTTATGGCAACAGCGCGCGTGCG TATCTGCTGGCCGTGATTGTGCCGACCCAGGAAGCGCTGGACGCGGTCCCGGTTGAAGA ACTGAAAGCGCGTCTGGGTGACTCTCTGCAGGAAGTGGCGAAAGCGGCGGGTCTGCAGA GCTATGAAATTCCGCGCGATTTTATTATCGAAACCACCCCGTGGACCCTGGAAAACGGCC TGCTGACGGGTATTCGTAAACTGGCCCGTCCGCAGCTGAAAAAACATTATGGTGAACTGC TGGAACAAATTTATACCGATCTGGCCCACGGCCAGGCGGATGAACTGCGTAGCCTGCGT CAGAGCGGTGCGGATGCGCCGGTGCTGGTGACCGTTTGTCGTGCGGCTGCAGCTCTGCT GGGTGGTAGCGCGAGCGATGTGCAGCCGGATGCGCATTTCACCGATCTGGGTGGTGATA

	GCCTGAGCGCCCTGAGCTTTACCAACCTGCTGCATGAAATCTTTGATATTGAAGTGCCGG TGGGCGTGATTGTGAGCCCGGCGAACGATCTGCAGGCGCTGGCCGATTATGTGGAAGCG GCGCGTAAACCGGGTAGCAGCCGTCCGACCTTTGCGAGCGTGCATGGCGCGAGCAACG GCCAGGTGACCGAAGTGCATGCGGGCGATCTGAGCCTGGATAAATTTATTGATGCGGCG ACCCTGGCCGAAGCCCCGCGTCTGCCGGCTGCAAATACCCAGGTGCGTACCGTGCTGCT GACCGGTGCGACCGGCTTTCTGGGCCGTTACCTGGCCCTGGAATGGCTGGAACGTATGG ATCTGGTTGATGGCAAACTGATTTGCCTGGTGCGTGCCAAAAGCGATACCGAAGCGCGTG CGCGTCTGGATAAAACCTTTGATAGCGGCGATCCGGAACTGCTGGCCCATTATCGTGCGC TGGCCGGCGATCATCTGGAAGTGCTGGCCGGTGATAAAGGCGAAGCGGATCTGGGCCTG GATCGTCAGACCTGGCAACGCCTGGCAGATACCGTGGATCTGATTGTTGACCCGGCTGC CCTGGTGAATCATGTGCTGCCGTATAGCCAGCTGTTTGGCCCGAATGCGCTGGGCACCG CTGAACTGCTGCGCCTGGCTCTGACCAGCAAAATTAAACCGTATAGCTACACCAGCACCA TTGGCGTGGCGGATCAGATTCCGCCGAGCGCGTTTACCGAAGATGCGGATATTCGTGTG ATTAGCGCGACCCGTGCGGTGGATGATAGCTATGCGAACGGCTATAGCAACAGCAAATG GGCGGGTGAAGTGCTGCTGCGTGAAGCGCATGATCTGTGCGGTCTGCCGGTGGCGGTG TTTCGTTGCGATATGATCCTGGCAGACACGACCTGGGCGGGTCAGCTGAACGTGCCGGA TATGTTTACCCGTATGATTCTGTCTCTGGCAGCTACGGGTATCGCACCGGGTAGCTTTTAT GAACTGGCCGCGGATGGTGCGCGTCAGCGTGCGCATTATGATGGCCTGCCGGTGGAATT TATTGCGGAAGCGATTAGCACCCTGGGCGCGCAGAGCCAGGATGGCTTTCATACCTATCA TGTGATGAATCCGTATGATGATGGCATTGGCCTGGATGAATTTGTGGATTGGCTGAACGA AAGCGGCTGCCCGATTCAGCGTATTGCGGATTATGGCGATTGGCTGCAGCGTTTTGAAAC CGCGCTGCGCGCTCTGCCGGATCGTCAGCGTCATAGCAGCCTGCTGCCGCTGCTGCATA ACTATCGTCAGCCGGAACGTCCGGTGCGTGGTAGCATTGCGCCGACCGATCGCTTTCGT GCGGCCGTGCAGGAAGCGAAAATTGGCCCGGATAAAGATATTCCGCATGTGGGTGCGCC GATTATTGTGAAATATGTGAGCGATCTGCGCCTGCTGGGCCTGCTGTAA
cat	ATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAAC ATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATAT TACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACA TTCTTGCCCGCCTGATGAATGCTCATCCGGAGTTCCGTATGGCAATGAAAGACGGTGAGC TGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTT TTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAA GATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGT TTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATAT GGACAACTTCTTCGCCCCCGTTTTCACTATGGGCAAATATTATACGCAAGGCGACAAGGT GCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTCTGTGATGGCTTCCATGTCGGCAG AATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAA
saat	ATGGAGAAGATTGAAGTGTCGATTAATTCGAAACACACCATCAAGCCGAGCACCTCCTCC ACCCCTCTTCAACCGTACAAACTCACGTTACTGGACCAATTAACGCCGCCAGCATATGTTC CAATTGTGTTTTTTTATCCGATTACAGATCACGACTTTAACCTTCCGCAGACTTTGGCCGAT CTGCGTCAAGCGCTGAGCGAAACACTGACGCTGTATTATCCACTGAGCGGCCGCGTCAA GAACAACCTGTACATTGACGATTTTGAAGAGGGGGTGCCGTACCTGGAAGCTCGTGTGAA TTGCGACATGACGGATTTCCTCCGTCTGCGTAAGATCGAGTGCTTAAACGAATTTGTCCCT ATTAAGCCGTTTAGTATGGAAGCAATTTCGGATGAGCGCTATCCGCTGCTTGGGGTTCAA GTAAACGTATTTGATTCCGGTATTGCGATCGGGGTCAGTGTCTCCCATAAACTGATCGAC

	GGCGGGACCGCTGATTGCTTCCTGAAGTCGTGGGGAGCGGTGTTCCGCGGATGCCGCG AGAACATTATCCATCCGTCCCTCTCGGAAGCAGCATTGCTTTTTCCGCCACGTGACGACC TCCCGGAAAAATATGTAGATCAAATGGAAGCGTTATGGTTCGCGGGTAAAAAGGTTGCGA CCCGTCGTTTCGTGTTTGGTGTGAAAGCTATCTCATCTATTCAGGATGAAGCAAAATCTGA GAGCGTACCAAAGCCGAGTCGCGTGCACGCCGTCACGGGTTTTTTGTGGAAGCATCTGA TCGCGGCGAGTCGTGCACTGACCTCAGGTACTACTTCTACTCGCCTGAGTATCGCCGCCC AAGCGGTTAATTTGCGCACGCGCATGAATATGGAAACCGTACTGGATAACGCGACGGGTA ATCTGTTTTGGTGGGCGCAGGCGATCCTCGAGTTAAGCCATACCACTCCAGAGATCAGCG ATCTGAAACTGTGTGATCTGGTGAACCTGTTGAACGGCAGCGTTAAGCAGTGCAATGGGG ATTATTTTGAAACCTTTAAAGGTAAAGAAGGTTATGGCCGTATGTGCGAGTACCTTGATTTT CAGCGGACCATGAGTTCCATGGAACCTGCACCGGATATTTACCTGTTTTCTTCTTGGACGA ACTTTTTCAACCCCTTGGATTTTGGCTGGGGCCGCACCTCTTGGATTGGGGTTGCGGGCA AGATTGAAAGCGCATCTTGCAAATTTATTATCCTTGTCCCGACACAGTGTGGCTCAGGCAT CGAAGCGTGGGTAAATCTGGAGGAAGAAAAAATGGCGATGCTTGAACAGGACCCCCATTT CCTGGCTCTTGCGTCTCCCAAAACGTTGATTTAA
atf1	ATGAACGAGATCGATGAGAAGAATCAAGCACCGGTCCAGCAAGAGTGTTTGAAGGAGATG ATTCAAAATGGGCATGCACGTCGCATGGGTTCTGTCGAAGATTTGTATGTAGCACTGAAC CGTCAGAACTTATACCGTAATTTTTGCACCTACGGGGAGTTATCTGACTATTGTACCCGTG ATCAATTGACTCTTGCCTTACGTGAAATCTGCCTGAAGAATCCCACATTGTTACACATCGT ACTGCCAACCCGTTGGCCAAATCACGAGAATTACTATCGCTCCAGTGAGTACTACTCACG CCCTCATCCCGTGCACGATTATATCTCTGTGCTTCAAGAACTGAAATTATCAGGCGTAGTC CTTAATGAACAACCGGAGTATTCTGCGGTTATGAAGCAGATTTTGGAGGAGTTTAAGAATA GTAAGGGCTCTTATACAGCGAAAATCTTTAAATTAACTACTACTTTAACAATTCCGTATTTC GGTCCTACGGGGCCCTCATGGCGTCTGATTTGCTTACCGGAAGAGCACACTGAAAAATGG AAAAAGTTCATCTTCGTTTCTAATCATTGTATGTCCGATGGCCGCTCAAGTATCCATTTCTT CCACGACCTGCGTGATGAATTGAATAATATTAAGACACCACCTAAAAAGTTGGATTACATC TTTAAGTATGAGGAAGACTACCAGTTACTGCGTAAATTGCCCGAGCCTATCGAGAAAGTAA TTGATTTTCGTCCCCCGTACCTTTTCATCCCGAAATCTCTTTTAAGCGGTTTTATCTATAAT CACCTGCGTTTCAGTTCGAAGGGAGTGTGTATGCGTATGGACGATGTGGAGAAGACGGA CGATGTTGTCACCGAAATTATCAACATCAGCCCTACGGAATTTCAAGCTATCAAGGCCAAT ATTAAATCTAATATTCAAGGCAAATGCACAATCACTCCGTTTTTGCATGTTTGCTGGTTTGT CTCACTGCACAAATGGGGAAAATTCTTTAAACCATTAAATTTCGAGTGGCTTACTGACATTT TCATTCCAGCCGACTGCCGTTCTCAGTTACCTGACGATGATGAGATGCGTCAGATGTACC GTTATGGAGCAAACGTTGGATTCATCGACTTCACACCGTGGATTTCCGAGTTCGACATGAA TGACAACAAGGAAAATTTTTGGCCCCTTATCGAACATTACCATGAAGTAATTTCGGAAGCA CTGCGCAATAAGAAGCACCTGCATGGGTTAGGCTTCAATATTCAGGGGTTCGTCCAGAAA TATGTAAATATCGATAAGGTGATGTGTGATCGTGCCATTGGTAAGCGTCGTGGCGGAACA CTGCTGTCCAACGTCGGTTTGTTCAACCAGTTAGAAGAACCTGATGCGAAATATTCAATCT GTGACTTAGCTTTCGGGCAATTTCAGGGATCGTGGCATCAGGCTTTTAGCTTGGGCGTAT GTTCAACCAATGTAAAAGGCATGAACATCGTTGTTGCTAGTACAAAAAATGTGGTTGGCTC TCAAGAAAGTCTTGAGGAACTTTGCTCTATTTATAAAGCCCTGTTGTTGGGGCCCTAA
CpFatB1 (wild type)	ATGCGGCCAAACATGTTAATGGATAGCTTTGGGCTGGAACGGGTGGTTCAGGACGGCCT GGTGTTTCGGCAATCATTCAGCATTCGCAGTTATGAAATCTGTGCGGATCGCACCGCTTC CATCGAAACTGTGATGAACCACGTTCAGGAAACCAGCCTGAATCAATGTAAAAGTATTGGC

	TTACTGGATGACGGGTTTGGCCGTTCCCCCGAAATGTGCAAACGGGATCTGATTTGGGTG GTTACCCGCATGAAAATCATGGTGAACCGTTATCCGACCTGGGGCGATACTATTGAAGTT AGCACCTGGCTGAGTCAGTCCGGTAAAATTGGTATGGGGCGGGATTGGCTGATCTCTGA CTGCAACACCGGTGAAATTTTGGTGCGCGCAACTAGCGTTTACGCCATGATGAATCAGAA AACCCGCCGTTTTAGTAAATTGCCCCATGAAGTGCGTCAAGAATTTGCGCCGCACTTCTTA GATTCCCCACCCGCTATCGAAGATAATGACGGTAAACTGCAAAAATTCGATGTGAAAACC GGGGACTCTATTCGCAAAGGGTTGACTCCTGGCTGGTATGATTTAGACGTTAATCAGCAT GTGTCTAACGTTAAATACATTGGCTGGATTCTGGAATCAATGCCCACCGAAGTGCTGGAAA CTCAAGAATTGTGTAGCCTGACCTTGGAATATCGTCGCGAATGCGGGCGTGATAGTGTGT TGGAAAGCGTGACCTCAATGGACCCCTCCAAAGTGGGTGACCGCTTTCAATACCGTCACT TGTTACGGCTGGAAGATGGTGCGGACATCATGAAAGGGCGCACCGAATGGCGTCCCAAA AATGCGGGCACTAACGGTGCTATTAGTACCGGTAAAACTTGA
CaFatB3 (wild type)	ATGCATCATCACCACCACCATAAGCCAGGCAAATTCCGTATTTGGCCGTCGAGTCTGTCG CCTTCATTTAAGCCGAAGCCGATCCCCAACGGTGGACTTCAAGTAAAGGCAAATTCGCGC GCTCACCCGAAGGCCAACGGCTCTGCCGTCTCACTGAAAAGTGGTTCGCTTAATACACAA GAAGATACATCATCTAGTCCCCCGCCACGCACTTTTTTACACCAGTTGCCTGATTGGTCCC GTTTATTAACCGCTATTACGACCGTCTTCGTAAAGAGCAAACGCCCTGATATGCATGACCG CAAATCGAAGCGTCCGGACATGCTGATGGACAGTTTTGGGCTGGAGTCCATCGTCCAAGA AGGATTAGAGTTCCGTCAGTCCTTTAGCATCCGTTCATACGAGATCGGCACGGATCGTAC CGCTTCCATCGAGACGCTTATGAACTATTTACAGGAAACGTCCTTAAATCATTGCAAAAGT ACCGGCATTCTGTTAGATGGATTCGGACGTACGCCTGAGATGTGCAAGCGTGATTTAATC TGGGTCGTGACTAAAATGAAGATCAAAGTGAATCGCTACCCGGCGTGGGGGGACACGGT GGAAATTAATACGTGGTTTAGCCGCTTGGGAAAAATTGGCAAAGGTCGCGATTGGCTTAT CAGCGACTGTAATACGGGTGAAATCCTGATCCGTGCAACAAGCGCATACGCTACTATGAA CCAGAAGACCCGTCGTTTATGCAAGTTGCCATACGAAGTACACCAGGAAATCGCGCCGTT GTTCGTTGACAGCCCCCCTGTTATTGAGGACAATGATCTTAAGTTACATAAGTTTGAGGTA AAAACAGGCGACTCCATTCACAAAGGGCTGACCCCCGGATGGAATGATCTGGACGTCAAT CAACATGTAAGCAACGTGAAGTATATTGGGTGGATCTTAGAATCAATGCCTACGGAAGTTT TAGAAACCCAAGAATTGTGTTCCTTAGCGCTGGAGTATCGTCGTGAATGCGGGCGCGACT CCGTACTGGAATCAGTCACGGCCATGGACCCCACTAAGGTGGGTGGACGTTCGCAATAT CAACACTTGCTTCGTCTTGAAGACGGTACCGACATTGTAAAATGTCGTACCGAGTGGCGT CCTAAAAACCCTGGTGCAAACGGTGCAATTTCGACAGGCAAGACCTCGAACGGCAATTCA GTTTCTTCCTAGG
'CpFatB1-4	ATGTTCGATCGTAAATCAAAACGGCCATCCATGTTAATGGATAGCTTTGGGCTGGAACGG GTGGTTCAGGACGGCCTGGTGTTTCGGCAATCATTCAGCATTCGCAGTTATGAAATCTGT GCGGATCGCACCGCTTCCATGGAAACTGTGATGAACCACGTTCAGGAAACCAGCCTGAAT CAATGTAAAAGTATTGGCTTACTGGATGACGGGTTTGGCCGTTCCCCCGAAATGTGCAAA CGGGATCTGATTTGGGTGGTTACCCGCATGAAAATCATGGTGAACCGTTATCCGACCTGG GGCGATACTATTGAAGTTAGCACCTGGCTGAGTCAGTCCGGTAAAATTGGTATGGGGCGG GATTGGCTGATCTCTGACTGCAACACCGGTGAAATTTTGGTGCGCGCAACTAGCGTTTAC GCCATGATGAATCAGAAAACCCGCCGTTTTAGTAAATTGCCCCATGAAGTGCGTCAAGAAT TTGCGCCGCACTTCTTAGATTCCCCACCCGCTATCGAAGATAATGACGGTAAACTGCAAAA ATTCGATGTGAAAACCGGGGACTCTATTCGCAAAGGGTTGACTCCTGGCTGGTATGATTT AGACGTTAATCAGCATGTGTCTAACGTTAAATACATTGGCTGGATTCTGGAATCAATGCCC

	ACCGAAGTGCTGGAAACTCAAGAATTGTGTAGCCTGACCTTGGAATATCGTCGCGAATGC GGGCGTGATAGTGTGTTGGAAAGCGTGACCTCAATGGACCCCTCCAAAGTGGGTGACCG CTTTCAATACCGTCACTTGTTACGGCTGGAAGATGGTGCGGACATCATGAAAGGGCGCAC CGAATGGCGTCCCAAAAATGCGGGCACTAACGGTGCTATTAGTACCGGTAAAACTTGA
'CaFatB3-5	ATGTTCGATCGTAAATCAAAACGGCCATCCATGTTAATGGATAGCTTTGGGCTGGAACGG GTGGTTCAGGACGGCCTGGTGTTTCGGCAATCATTCAGCATTCGCAGTTATGAAATCTGT GCGGATCGCACCGCTTCCATGGAAACTGTGATGAACCACGTTCAGGAAACCAGCCTGAAT CAATGTAAAAGTATTGGCTTACTGGATGACGGGTTTGGCCGTTCCCCCGAAATGTGCAAA CGGGATCTGATTTGGGTGGTTACCCGCATGAAAATCATGGTGAACCGTTATCCGACCTGG GGCGATACTATTGAAGTTAGCACCTGGCTGAGTCAGTCCGGTAAAATTGGTATGGGGCGG GATTGGCTGATCTCTGACTGCAACACCGGTGAAATTTTGGTGCGCGCAACTAGCGTTTAC GCCATGATGAATCAGAAAACCCGCCGTTTTAGTAAATTGCCCCATGAAGTGCGTCAAGAAT TTGCGCCGCACTTCTTAGATTCCCCACCCGCTATCGAAGATAATGACGGTAAACTGCAAAA ATTCGATGTGAAAACCGGGGACTCTATTCGCAAAGGGTTGACTCCTGGCTGGTATGATTT AGACGTTAATCAGCATGTGTCTAACGTTAAATACATTGGCTGGATTCTGGAATCAATGCCC ACCGAAGTGCTGGAAACTCAAGAATTGTGTAGCCTGACCTTGGAATATCGTCGCGAATGC GGGCGTGATAGTGTGTTGGAAAGCGTGACCTCAATGGACCCCTCCAAAGTGGGTGACCG CTTTCAATACCGTCACTTGTTACGGCTGGAAGATGGTGCGGACATCATGAAAGGGCGCAC CGAATGGCGTCCCAAAAATGCGGGCACTAACGGTGCTATTAGTACCGGTAAAACTTGA
AdGT4	ATGGGCTCGGCGGGCATGCCTGAAAAGCCCCACGCAGTGTGCCTGCCGTATCCGGCGC AGGGCCACATCACGCCGATGTTAAAACTCGCGAAATTGCTGCACTCAAAAGGTTTTCACG TTACATTTGTAAACACGGAATTCAATCATAAACGTCTGTTGAAATCTCGTGGCCCTGATTCA TTGACCGGCCTGTCGAGCTTCCGTTTCGAGACAATTCCAGACGGTCTGCCTGAATCTGAT CTGGATGCAACTCAGTTCATCCCTAGTCTCTGCGAATCAACCCGTAAAAATTGTCTGGGCC CGTTCCGTCAACTGCTGGGCAAACTGAACAATACAGTGAGTTCTGGGGTGCCGCCAGTCT CCTGCGTTGTTAGTGATGGCGTGATGTCCTTTTCCCTGGATGCCGCAGAGGAACTGGGCA TCCCGCAGGTCTTATTTTGGACGACGAGTGTTTGTGGGTTTATGGCCTACGTGCACTATC GTAATCTGATCGAAAAAGGATATACGCCGCTGAAGGACGTTTCGTACGTGACAAATGGCT ATTTAGATACAGTTATCGACTGGATTCCGGGTATGGAGGGTATTCGGCTTAAAGATCTGCC GTCGTTTCTGCGTACCACTGACCCTAACGATATCATGTTAGATTTTGTACTGAGCGAAACC AAGAACACCCACCGTTCGTCTGCTATTATCTTCAATACCTTCGATAAACTCGAACATCAAG TACTGGAACCTCTTGCGTCTATGTTTCCGCCTATTTACACCATTGGCCCTCTGAACTTGCT GATGAATCAGATCAAGGAAGAAAGCCTTAAAATGATTGGCAGCAATTTGTGGAAAGAGGA ACCAATGTGTATCGAATGGTTGAATTCAAAAGAACCTAAAAGTGTTGTGTACGTGAACTTT GGATCAATTACGGTCATGACACCGAACCAGCTGGTAGAATTCGCATGGGGTCTGGCGAAC AGCAATCAGTCATTTCTGTGGATTATTCGCCCAGACCTGGTGGTCGGCGAGTCGGCGGTT CTGCCGCCTGAATTTGTTGCGGTTACCAAAGAACGTGGTATGCTGGCAAGCTGGGCCCC GCAGGAAGAGGTGCTGGCGCACAGCTCCGTTGGCGGTTTTCTGACCCATTGTGGTTGGA ACTCTACCTTAGAAAGCATTAGCTCTGGTGTTGCCGTGGTGTGTTGGCCGTTTTTCGCCG AGCAGCAGACAAATTGCTGGTACTGTTGCGGGGAACTGGGGATTGGTATGGAAATCGACA GCGATGTGAAGCGCGAAGAAGTTGAGCGGCTCGTCCGGGAATTGATGGTTGGTGAAAAA GGCAAAGAAATGAAAGAACGCGCCATGGGCTGGAAACGCCTGGCAGAAGAAGCGACCCA AAGCTCCTCCGGCAGCTCGTTTTTAAATCTGGACAAACTGGTCCACCAGGTATTGCTGTC CCCGCGTCCG

VvGT1	ATGTCACAGACGACAACCAATCCTCATGTTGCCGTGCTGGCTTTCCCGTTTAGCACCCAT GCCGCACCGTTACTGGCCGTCGTGCGTCGTTTGGCGGCGGCGGCCCCGCACGCCGTTT TTTCATTCTTTAGCACGAGCCAGAGCAATGCTAGCATCTTCCATGACAGTATGCATACAAT GCAGTGTAACATTAAGTCCTACGACGTTTCAGATGGCGTCGCAGAAGGATATGTGTTCGC AGGACGTCCGCAAGAAGACATTGAACTGTTCATGCGCGCGGCTCCAGAATCGTTCCGTCA AGGCATGGTTATGGCGGTGGCGGAGACGGGCCGTCCAGTGTCTTGCTTAGTAGCAGATG CGTTTATCTGGTTCGCCGCTGATATGGCCGCGGAGATGGGCGTGGCCTGGCTTCCGTTTT GGACGGCAGGCCCTAACTCCTTGAGCACCCATGTGTACACCGATGAAATTCGCGAAAAAA TTGGTGTGTCGGGAATTCAGGGGCGCGAAGATGAACTTTTGAATTTCATTCCGGGAATGT CGAAAGTACGCTTTCGTGACCTGCAGGAAGGAATTGTCTTCGGGAATTTAAATTCCTTATT CTCTCGCATGCTGCATCGCATGGGTCAAGTTTTGCCAAAAGCGACAGCGGTATTTATTAAC AGTTTCGAAGAATTGGACGATTCCCTGACGAACGATCTGAAAAGCAAACTCAAAACTTATC TGAACATCGGACCGTTTAACTTAATCACCCCTCCCCCTGTGATTCCGAACACCACAGGCT GCCTGCAATGGTTAAAAGAACGCAAACCGACCTCGGTAGTCTATATTTCATTTGGCACAGT TACGACTCCACCGCCGGCCGAGCTGGTGGCGCTCGCCGAAGCGCTCGAGGCGTCCCGT GTGCCGTTTATTTGGAGCTTGCGCGATAAAGCCCGCGTCCATCTGCCGGAAGGATTCTTA GAAAAAACCCGCGGCTATGGTATGGTTGTGCCCTGGGCGCCGCAGGCGGAAGTACTTGC ACACGAGGCGGTGGGTGCGTTTGTGACGCATTGTGGTTGGAACTCTCTGTGGGAATCGG TGGCTGGCGGCGTGCCGCTGATTTGTCGCCCATTCTACGGCGATCAACGCTTAAATGGG CGCATGGTGGAAGATGCCTTGGAGATCGGGGTGCGCATTGAAGGTGGCGTCTTTACCGA AAGTGGACTGATGTCATGTTTCGATCAGATTTTATCTCAAGAAAAGGGTAAAAAATTGCGT GAAAATTTAGGCGCCCTCCGTGAGACTGCCGATCGTGCGGTCGGTCCCAAAGGCAGTAG CACCGAGAATTTTAAGACCCTGGTGGACCTTGTGTCCAAACCGAAAGACGTT
AtGT1	ATGGAAGAATCTAAAACCCCGCACGTTGCTATCATCCCGTCTCCGGGTATGGGTCACCTG ATCCCGCTGGTTGAATTCGCTAAACGTCTGGTTCACCTGCACGGTCTGACCGTTACCTTC GTTATCGCTGGTGAAGGTCCGCCGTCTAAAGCTCAGCGTACCGTTCTGGACTCTCTGCCG TCTTCTATCTCTTCTGTTTTCCTGCCGCCGGTTGACCTGACCGACCTGTCTTCTTCTACCC GTATCGAATCTCGTATCTCTCTGACCGTTACCCGTTCTAACCCGGAACTGCGTAAAGTTTT CGACTCTTTCGTTGAAGGTGGTCGTCTGCCGACCGCTCTGGTTGTTGACCTGTTCGGTAC CGACGCTTTCGACGTTGCTGTTGAATTCCACGTTCCGCCGTACATCTTCTACCCGACCAC CGCTAACGTTCTGTCTTTCTTCCTGCACCTGCCGAAACTGGACGAAACCGTTTCTTGCGAA TTCCGTGAACTGACCGAACCGCTGATGCTGCCGGGTTGCGTTCCGGTTGCTGGTAAAGA CTTCCTGGACCCGGCTCAGGACCGTAAAGACGACGCTTACAAATGGCTGCTGCACAACAC CAAACGTTACAAAGAAGCTGAAGGTATCCTGGTTAACACCTTCTTCGAACTGGAACCGAAC GCTATCAAAGCTCTGCAGGAACCGGGTCTGGACAAACCGCCGGTTTACCCGGTTGGTCC GCTGGTTAACATCGGTAAACAGGAAGCTAAACAGACCGAAGAATCTGAATGCCTGAAATG GCTGGACAACCAGCCGCTGGGTTCTGTTCTGTACGTTTCTTTCGGTTCTGGTGGTACCCT GACCTGCGAACAGCTGAACGAACTGGCTCTGGGTCTGGCTGACTCTGAACAGCGTTTCCT GTGGGTTATCCGTTCTCCGTCTGGTATCGCTAACTCTTCTTACTTCGACTCTCACTCTCAG ACCGACCCGCTGACCTTCCTGCCACCCGGCTTCCTGGAACGTACTAAAAAACGTGTTCGT GCTAAATGGCAGCCGCTGAACATC
MtG1	ATGTCTATGAGCGATATCAACAAAAACTCGGAACTGATCTTTATCCCGGCCCCGGGTATTG GTCATCTGGCGAGCGCGCTGGAATTTGCGAAACTTTTAACAAACCACGACAAGAATCTGT ATATTACCGTGTTTTGCATCAAGTTCCCGGGTATGCCTTTTGCTGATAGCTACATTAAGTCA

	GTCCTGGCGTCCCAACCTCAGATTCAGCTGATTGACCTGCCTGAAGTGGAACCTCCGCCG CAGGAACTGCTTAAGAGCCCGGAATTTTACATCTTGACCTTCCTGGAGAGTTTAATCCCGC ACGTGAAAGCAACGATTAAAACAATCCTGTCTAACAAGGTAGTTGGCCTGGTTTTGGATTT TTTCTGCGTTTCCATGATCGATGTCGGCAACGAGTTTGGCATTCCGTCCTATCTGTTTTTA ACGTCGAACGTGGGCTTTCTGTCACTGATGCTGTCGCTCAAAAACCGTCAGATTGAGGAG GTCTTTGATGACAGCGACCGTGATCACCAGCTTCTGAATATCCCTGGAATCAGCAACCAG GTTCCCAGTAATGTGTTGCCTGACGCGTGTTTCAACAAGGATGGTGGTTATATTGCCTATT ATAAATTGGCAGAACGTTTTCGCGATACAAAAGGCATCATTGTTAACACCTTTTCTGACCT GGAACAGAGTAGCATTGATGCGTTGTATGATCATGATGAAAAAATCCCTCCGATCTATGCC GTGGGTCCGCTGCTGGATTTGAAAGGCCAACCCAACCCGAAACTGGACCAAGCCCAGCA TGACCTGATTCTGAAATGGTTGGATGAGCAACCCGACAAATCTGTAGTGTTTCTGTGCTTC GGATCGATGGGCGTCAGCTTTGGCCCTTCTCAGATTCGTGAGATTGCGCTGGGCCTGAAA CACTCTGGTGTCCGTTTTCTCTGGTCAAACAGTGCTGAGAAAAAAGTATTCCCGGAAGGTT TTCTGGAGTGGATGGAACTGGAAGGCAAAGGGATGATTTGTGGCTGGGCACCGCAGGTT GAAGTACTGGCACATAAGGCGATCGGCGGTTTCGTTAGCCACTGTGGTTGGAACAGTATC CTGGAATCGATGTGGTTTGGGGTCCCGATCCTGACTTGGCCTATCTACGCGGAACAGCAG CTGAATGCGTTTCGGTTAGTTAAAGAATGGGGAGTGGGCCTGGGTTTGCGTGTGGACTAC CGCAAAGGAAGCGACGTCGTCGCTGCGGAAGAAATTGAAAAAGGCCTGAAAGATCTGAT GGATAAAGACAGTATTGTCCATAAAAAAGTACAGGAAATGAAGGAGATGAGTCGGAACGC AGTGGTAGATGGTGGTTCGAGTCTGATTAGTGTAGGCAAACTCATTGATGATATTACCGG CTCTAAT
MtH2	ATGGGTAACTTTGCCAATCGCAAACCGCATGTAGTGATGATCCCATATCCGGTACAGGGC CATATTAATCCGCTGTTCAAATTGGCGAAGCTGCTGCATCTGCGCGGTTTTCACATTACAT TTGTAAACACCGAGTATAACCATAAGCGCTTGCTCAAGTCCCGTGGTCCAAAAGCATTTGA TGGTTTCACCGATTTCAATTTTGAGAGCATCCCGGATGGTTTAACGCCGATGGAAGGCGA CGGCGATGTGAGCCAGGATGTTCCTACCCTGTGTCAGTCTGTACGCAAAAACTTTCTCAA GCCGTATTGCGAGTTACTCACTCGCCTGAACCATTCCACCAACGTGCCACCGGTCACTTG TCTCGTTAGCGACTGCTGTATGTCATTCACGATCCAGGCGGCTGAAGAATTTGAGCTGCC GAACGTGCTTTATTTTAGCAGTAGTGCCTGCTCACTGCTGAACGTAATGCATTTTCGTTCA TTCGTGGAACGTGGAATCATTCCGTTCAAAGACGAAAGCTACTTGACCAATGGCTGCCTG GAGACCAAAGTCGATTGGATCCCTGGGCTTAAAAACTTTCGCCTGAAAGATATTGTCGACT TTATTCGTACCACTAATCCGAATGATATTATGCTGGAGTTTTTTATTGAGGTAGCGGATCG GGTGAACAAAGATACCACCATTCTCCTCAATACCTTTAACGAGCTGGAAAGCGACGTGAT CAACGCACTGTCGAGTACTATTCCTTCGATTTATCCGATCGGTCCGTTACCGTCATTGTTA AAACAGACCCCGCAAATCCACCAGTTAGACAGCTTGGATAGCAATCTCTGGAAGGAGGAT ACCGAATGTCTTGACTGGCTGGAGTCAAAGGAGCCGGGGTCGGTGGTTTATGTGAATTTC GGCTCTATTACGGTGATGACGCCAGAACAGCTGTTAGAATTCGCCTGGGGGCTGGCAAAT TGTAAAAAATCTTTTCTGTGGATTATCCGCCCGGATCTGGTGATTGGTGGCTCAGTCATCT TCAGTAGTGAATTCACCAACGAAATTGCAGATCGTGGTCTGATTGCGTCTTGGTGCCCCC AGGACAAAGTCCTGAATCATCCGTCAATCGGTGGGTTTCTGACTCATTGCGGTTGGAACA GTACAACCGAGTCGATTTGTGCGGGCGTCCCAATGTTGTGTTGGCCTTTTTTTGCGGACC AACCGACAGATTGTCGTTTTATTTGTAACGAATGGGAAATTGGCATGGAAATTGATACGAA CGTGAAACGTGAAGAGCTGGCCAAACTGATTAACGAAGTCATTGCCGGCGATAAGGGCAA

	AAAAATGAAACAGAAAGCCATGGAGCTGAAAAAAAAGGCAGAAGAGAATACGCGCCCAG GGGGGTGCAGCTACATGAATCTGAACAAAGTTATCAAAGATGTGTTGTTGAAGCAAAAC
AtSUS1	ATGGCTAACGCTGAACGTATGATCACCCGTGTTCACTCTCAGCGTGAACGTCTGAACGAA ACCCTGGTTTCTGAACGTAACGAAGTTCTGGCTCTGCTGTCTCGTGTTGAAGCTAAAGGTA AAGGTATCCTGCAGCAGAACCAGATCATCGCTGAATTCGAAGCTCTGCCGGAACAGACCC GTAAAAAACTGGAAGGTGGTCCGTTCTTCGACCTGCTGAAATCTACCCAGGAAGCTATCG TTCTGCCGCCGTGGGTTGCTCTGGCTGTTCGTCCGCGTCCAGGTGTGTGGGAGTACCTG CGTGTTAACCTGCACGCTCTGGTTGTTGAAGAACTGCAGCCGGCTGAATTCCTGCACTTC AAAGAAGAACTGGTTGACGGTGTTAAAAACGGTAACTTCACCCTGGAACTGGACTTCGAA CCGTTCAACGCTTCTATCCCGCGTCCGACCCTGCACAAATACATCGGTAACGGTGTTGAC TTCCTGAACCGTCACCTGTCTGCTAAACTGTTCCACGACAAAGAATCTCTGCTGCCGCTG CTGAAATTCCTGCGTCTGCACTCTCACCAGGGTAAAAACCTGATGCTGTCTGAAAAAATCC AGAACCTGAACACCCTGCAGCACACCCTGCGTAAAGCTGAAGAATACCTGGCTGAACTGA AATCTGAAACCCTGTACGAAGAATTCGAAGCTAAATTCGAAGAAATCGGTCTGGAACGTG GTTGGGGTGACAACGCTGAACGTGTTCTGGACATGATCCGTCTGCTGCTGGACCTGCTG GAAGCTCCGGACCCGTGCACCCTGGAAACCTTCCTGGGTCGTGTTCCGATGGTTTTCAAC GTTGTTATCCTGTCTCCGCACGGTTACTTCGCTCAGGACAACGTTCTGGGTTACCCGGAC ACCGGTGGTCAGGTTGTTTACATCCTGGACCAGGTTCGTGCTCTGGAAATCGAAATGCTG CAGCGTATCAAACAGCAGGGTCTGAACATCAAACCGCGTATCCTGATCCTGACCCGTCTG CTGCCGGACGCTGTTGGTACCACCTGCGGTGAACGTCTGGAACGTGTTTACGACTCTGAA TACTGCGACATCCTGCGTGTTCCGTTCCGTACCGAAAAAGGTATCGTTCGTAAATGGATCT CTCGTTTCGAAGTTTGGCCGTACCTGGAAACCTACACCGAAGACGCTGCTGTTGAACTGT CTAAAGAACTGAACGGTAAACCGGACCTGATCATCGGTAACTACTCTGACGGTAACCTGG TTGCTTCTCTGCTGGCTCACAAACTGGGTGTTACCCAGTGCACCATCGCTCACGCTCTGG AAAAAACCAAATACCCGGACTCTGACATCTACTGGAAAAAACTGGACGACAAATACCACTT CTCTTGCCAGTTCACCGCTGACATCTTCGCTATGAACCACACCGACTTCATCATCACCTCT ACCTTCCAGGAAATCGCTGGTTCTAAAGAAACCGTTGGTCAGTACGAATCTCACACCGCTT TCACCCTGCCGGGTCTGTACCGTGTTGTTCATGGCATCGACGTATTCGACCCAAAATTCA ACATCGTTTCTCCGGGTGCTGACATGTCTATCTACTTCCCGTACACCGAAGAAAAACGTCG TCTGACCAAATTCCACTCTGAAATCGAAGAACTGCTGTACTCTGACGTTGAAAACAAAGAA CACCTGTGCGTTCTGAAAGACAAAAAAAAACCGATCCTGTTCACCATGGCTCGTCTGGAC CGTGTTAAAAACCTGTCTGGTCTGGTTGAATGGTACGGTAAAAACACCCGTCTGCGTGAA CTGGCTAACCTGGTTGTTGTTGGTGGTGACCGTCGTAAAGAATCTAAAGACAACGAAGAA AAAGCTGAAATGAAAAAAATGTACGACCTGATCGAAGAATACAAACTGAACGGTCAGTTCC GTTGGATCTCTTCTCAGATGGACCGTGTTCGTAACGGTGAACTGTACCGTTACATCTGCG ACACAAAGGGTGCGTTCGTTCAGCCGGCGCTGTACGAGGCGTTCGGTCTGACCGTTGTT GAAGCTATGACCTGCGGTCTGCCGACCTTCGCTACCTGCAAAGGTGGTCCGGCTGAAAT CATCGTTCACGGTAAATCTGGTTTCCACATCGACCCGTACCACGGTGACCAGGCTGCTGA CACCCTGGCTGACTTCTTCACCAAATGCAAAGAAGACCCGTCTCACTGGGACGAAATCTC TAAAGGTGGTCTGCAGCGTATCGAAGAAAAATACACCTGGCAGATCTACTCTCAGCGTCT GCTGACCCTGACCGGTGTTTATGGTTTTTGGAAACACGTAAGCAACCTGGACCGTCTGGA AGCTCGTCGTTACCTGGAAATGTTCTACGCTCTGAAATACCGTCCGCTGGCTCAGGCTGT TCCGCTGGCTCAGGACGACTAA

VAAT	ATGGAAAAAATTGAAGTTTCGATTATCTCAAAACATACGATCAAACCGAGTACCTCTTCGA GCCCCCTGCAGCCTTACAAGTTAACCCTGCTGGACCAGCTTACCCCGCCGAGTTACGTCC CTATGGTGTTTTTCTATCCCATCACTGGGCCTGCAGTTTTTAACCTGCAGACATTGGCCGA CCTTCGCCATGCCTTGTCAGAGACGTTAACCCTTTATTATCCCTTGTCTGGGCGCGTGAA GAATAATCTGTACATCGACGACTTCGAGGAAGGCGTGCCTTACTTAGAAGCACGTGTTAAT TGCGATATGAATGACTTTCTGCGCTTGCCTAAAATCGAATGTCTGAACGAATTCGTCCCCA TTAAACCATTTAGCATGGAGGCCATTTCGGATGAACGCTATCCTTTATTAGGTGTGCAGGT TAATATTTTTAATTCAGGAATCGCAATTGGCGTATCGGTTTCACATAAGCTTATCGACGGG CGTACTTCAGATTGCTTCCTGAAGTCCTGGTGTGCAGTTTTCCGTGGGTCGCGCGACAAA ATCATCCATCCGAACTTGTCTCAAGCGGCCTTACTTTTTCCTCCACGCGACGATTTGCCCG AGAAATACGCGCGTCAGATGGAGGGGTTATGGTTCGTAGGTAAGAAAGTTGCCACACGC CGTTTTGTCTTTGGAGCTAAAGCGATCTCAGTGATTCAAGACGAGGCTAAGAGCGAAAGC GTACCCAAGCCATCCCGTGTACAGGCAGTCACTTCATTTTTATGGAAACACTTAATTGCTA CCTCTCGCGCACTGACGTCTGGCACTACAAGCACGCGTCTTAGTATTGCCACACAGGTAG TAAACATTCGCTCTCGTCGCAATATGGAAACGGTCTGGGACAACGCGATCGGCAACTTGA TTTGGTTTGCTCCTGCGATCTTAGAGCTGTCCCACACAACTCTTGAGATTTCTGACTTAAA ACTGTGCGATTTGGTTAATTTACTTAATGGCAGCGTTAAGCAATGCAATGGAGACTACTTT GAGACATTCATGGGAAAGGAGGGGTATGGAAGCATGTGTGAATACTTAGACTTTCAGCGC ACGATGTCGTCTATGGAACCCGCTCCAGAAATCTACTTATTTACGTCGTGGACAAATTTTT TCAACCAACTTGATTTCGGTTGGGGGCGTACATCATGGATTGGGGTTGCAGGGAAGATTG AGTCGGCCTTTTGTAACCTTACTACATTGGTCCCGACCCCATGCGACACAGGTATCGAGG CTTGGGTAAATCTGGAAGAAGAAAAAATGGCAATGCTGGAGCAGGACCCGCAATTTCTTG CTCTTGCATCGCCGAAGACCCTGATCAGCCGTTATTAA
VpAAT1	ATGGCAGAAAAGGCGTCTTCTCTTATGTTTAACGTGCGCCGTCATGAGCCAGAGCTTATC ACACCTGCCAAGCCAACACCGCGTGAGATCAAACTGTTGTCGGATATCGATGACCAAGAT GGCCTGCGTTTCCAGGTACCCATTATCCAGTTTTACAAGAATAATTCCTCGATGCAGGGTA AGAACCCTGCCAAAATTATCAAATCAGCCTTAGCGGAGACTTTGGTACACTATTATCCACT TGCAGGGCGCTTACGCGAAGGGTTCGGTCGCAAGTTAATGGTCGAGTGTACGGGGGAAG GTATCCTTTTTATTGAAGCTGATGCGGACGTTACATTACACGAATTCGGCGATGATTTGCC CCCACCTTTTCCGTGCCTTGTGGAGTTGCTGTATGACGTGCCTGGCTCTAGTGGCATTAT CGATACACCGCTTTTGTTGATCCAGGTAACACGCTTAAAATGCGGAGGTTTCATTTTCGCC TTGCGCTTAAACCATACCATGAGCGATGCGAGTGGGCTGGTGCAATTCATGACGGCAGTC GGCGAGATGGCTCGTGGACAACGTTCTTTGTCCATTCAGCCAGTATGGGAGCGTCATTTA TTAAATGCGCGTGATCCACCGCGTGTAACCCATATTCACCACGAATACGATGATTTGGAG GACACTAAAGGTACCATTATTCCATTAGACGATATGGTGCATCGCTCGTTCTTTTTCGGCC CATCTGAGATGGCAGCAATTCGTCGCCTGGTACCGGCGCACTTCCACCGCTCTACGACTA GCGAGGTTTTAACAGCATATTTGTGGCGCTGCTACACTATTGCGCTTCAACCAGACCCAG AGGAGGAAATGCGTGTAATCTGCGTTGTCAATTCCCGTACCAAACTTAACCCACCCCTTCC TACAGGGTTTTACGGTAATGGAATTGCTTTCCCCGCTGCTATCAGCCAGGCGAAGAAAATT TGTGAAAATCCGTTTGGATATACCTTACAGCTGGTAAAGCAGACCAAAGTTGATGTGACCG AGGAATATATGCGCAGCGCGGCCGATTTGATGGCTATGAAGGGACGTCCGCACTTCACT GTTGTTCGTCGCTACATGGTAAGTGACGTAACACGTGCAGGGTTTGGATTGGTGGACTTT GGATGGGGCCGTCCTGAACCGGTGTACGGTGGGCCTGCAAAAGGGGGAGTGGGGCCCA TTCCGGGAGTAACCTCTTTTTTTGTACCATTCAAAAATCGTAAGGGCGAAAAAGGCATTGT

	CGTGCCAACATGCCTGCCTACGCCGGCGATGGAACGTTTTGCTAAACTTATGAATGAAAT TCTTCAGAATCAATTACTGGTTTCCGCCGAAGAAAACAAGTCAGTGTTTATCGTCAGCGCG ATCTAA
GFP	ATGCGTAAAGGCGAAGAACTGTTCACGGGCGTAGTTCCGATTCTGGTCGAGCTGGACGG CGATGTGAACGGTCATAAGTTTAGCGTTCGCGGTGAAGGTGAGGGCGACGCGACCAACG GCAAACTGACCCTGAAGTTCATCTGCACCACCGGTAAACTGCCGGTGCCTTGGCCGACCT TGGTGACGACGTTGACGTATGGCGTGCAGTGTTTTGCGCGTTATCCGGACCACATGAAAC AACACGATTTCTTCAAATCTGCGATGCCGGAGGGTTACGTCCAGGAGCGTACCATTTCCT TCAAGGATGATGGCactTACAAAACTCGCGCAGAGGTTAAGTTTGAAGGTGACACGCTGGT CAATCGTATCGAATTGAAGGGTATCGACTTTAAAGAGGATGGTAACATTCTGGGCCATAAA CTGGAGTATAACTTCAACAGCCATAATGTTTACATTACGGCAGACAAGCAAAAGAACGGCA TCAAGGCCAATTTCAAGATTCGCCACAATGTTGAGGACGGTAGCGTCCAACTGGCCGACC ATTACCAGCAGAACACCCCAATTGGTGACGGTCCGGTTTTGCTGCCGGATAATCACTATC TGAGCACCCAAAGCGTGCTGAGCAAAGATCCGAACGAAAAACGTGATCACATGGTCCTGC TGGAATTTGTGACCGCTGCGGGCATCACCCACGGTATGGACGAGCTGTATAAGCGTCCGt aa
Tes12	ATGTTGGAATGGAAACCCAAGCCCAAACTTCCACAGTTACTGGACGATCATTTCGGCCTTC ACGGCCTTGTTTTCCGTCGTACGTTCGCTATTCGTTCCTACGAAGTTGGCCCAGATCGCA GCACCTCCATTTTGGCTGTGATGAACCACATGCAGGAAGCGACCCTGAATCACGCAAAAT CCGTCGGTATTTTGGGAGATGGATTCGGAACAACTTTGGAGATGAGTAAACGCGACCTGA TGTGGGTAGTACGCCGTACTCACGTAGCTGTTGAACGTTACCCTACTTGGGGAGACACAG TAGAGGTTGAGTGTTGGATCGGTGCCTCAGGCAATAACGGAATGCGCCGTGACTTTTTGG TACGCGACTGTAAAACTGGAGAGATCCTGACACGTTGCACATCGTTAAGCGTGCTGATGA ACACGCGCACGCGTCGTCTGTCGACGATCCCGGATGAAGTGCGCGGAGAGATCGGACC GGCGTTTATCGATAATGTTGCGGTTAAAGATGATGAAATCAAAAAGTTACAAAAACTGAAT GATTCGACGGCAGATTACATTCAGGGGGGATTAACTCCACGTTGGAACGACTTAGACGTT AATCAACACGTGAATAATTTAAAATATGTCGCTTGGGTCTTCGAGACAGTCCCGGATTCTA TTTTCGAATCTCACCACATCAGTTCTTTCACATTAGAGTACCGCCGTGAATGCACACGCGA TTCCGTACTGCGTAGCCTTACTACCGTATCGGGTGGCAGCAGCGAGGCCGGTTTAGTATG CGACCATCTTCTTCAGTTGGAGGGGGGCTCAGAAGTGTTGCGCGCCCGTACGGAGTGGC GTCCCAAACTTACGGATTCGTTCCGTGGTATTAGCGTAATTCCGGCAGAACCGCGCGTGT AACCTAGG

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