

Rapid Design, Construction, and Validation of Synthetic Metabolic Pathways in a Modular *Escherichia coli* (chassis) Cell

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

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December 2020

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Acknowledgements

First, I want to sincerely thank my advisor Dr. Cong T. Trinh for his mentorship during my dissertation. His guidance was invaluable for my development as an engineer and research success. I would also like to thank my committee members, Dr. Brian H. Davison, Dr. Eric T. Boder, and Dr. Josh K. Michener for their participation in this process, and for providing guidance and insightful perspectives to improve the way I approached science. I want to thank the Bredesen Center for inviting me to pursue my doctorate at the University of Tennessee (UTK), particularly, Dr. Lee Riedinger and Dr. Suresh Babu for his dedication to the program and students, as well as Wanda Davis, Jessica Garner, and Allie Burns for their support and assistance. I am grateful for the Department of Chemical Engineering at UTK, the BioEnergy Science Center (BESC), and the Center for Bioenergy Innovation (CBI) at Oak Ridge National Lab (ORNL) for allowing me to broaden my horizons and develop my research with numerous precious opportunities to interact with other researchers from diverse backgrounds.

I would also like to thank Dr. Jin-Ho Seo, my master's degree advisor, at Seoul National University for his guidance and support, without which I never would have decided to study abroad. Special acknowledgement is required for Trinh lab members, in particular Caleb Walker, Brian Mendoza, Sergio Garcia, Hyeongmin Seo, Donovan Layton, Adam Thompson, Brandon Wilbanks, Taylor Weiskittel, Katherine Krouse, Jared Clements, John Caruthers, Neel Patel, Nicholas Zheng, and Seunghyun Ryu as well as my friends, and family who supported me through this process. Thanks for always being there.

Abstract

Current strain development has been hindered by the vast biochemical space in nature. The concept of modular cell design has been invented to enable rapid and predictable construction of multiple optimal production strains for efficient production of a large variety of biochemicals with minimal experimental effort. While modular cell design principles have been successfully validated in some cases, its development is still limited by the small library of the production modules demonstrated.

The goals of this thesis are i) to establish a framework for rapid design, construction, and validation of production modules to explore a large space of molecules (e.g., a library of esters), and ii) to demonstrate the modular cell design principles as a rapid strain development platform for production of molecules of interest. This dissertation consists of five chapters. **Part I** presents a framework for rapid design and construction of biosynthetic pathways for combinatorial biosynthesis of C4-derived esters including butyl acetate, ethyl butyrate, and butyl butyrate. **Part II** presents the *de novo* biosynthesis of lactate esters as green solvents as well as identification and alleviation of the most limiting enzymatic steps in lactate esters biosynthesis. **Part III** presents the development of a computer-guided rational protein engineering protocol for improved production of designer esters. **Part IV** presents the establishment of a 96-well plate-based high throughput ester synthesis screening platform. **Part V** presents the evaluation of growth selection of an efficient butyl acetate production module in a modular (chassis) cell.

Using knowledge gained from ester biosynthesis, the work presented will facilitate future strain development efforts for production of a large space of biofuels and biochemicals in a more systematic and efficient fashion.

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1 Introduction

This part includes excerpts from the review published by:

Lee, J.W., Trinh, C.T. Towards renewable flavors, fragrances, and beyond, *Curr. Opin. Biotechnol.*, **61**:168-180.

JWL and CTT reviewed, drafted, and edited the manuscript. **JWL** visualized ideas and concepts. CTT conceptualized ideas, administrated the project and acquired funding.

Esters are ubiquitous in nature and often responsible for the characteristic fragrances of fruits and flowers [1]. Esters comprise of carboxylic acid and alcohol moieties, that can be linear, branched, saturated, unsaturated, aromatic or any features with carbon chain lengths up to C18 and higher [2]. With a diversity of chemical moieties, esters can make up a large space of unique molecules that have broad applications as flavors, fragrances, cosmetics, pharmaceuticals, green solvents, and advanced biofuels [2-5].

According to the BCC Research's recent report, the global market for flavors and fragrances was valued at US\$26 billion in 2015 and is expected to reach US\$37 billion by 2021 [6]. Cosmetics takes an even larger share of global market valued at over US\$500 billion in 2017, and its market is expected to reach over US\$805 billion by 2023 [7]. The increasing preference for using natural and sustainable products pushes large fragrance and ingredient firms to find alternative sources for production of natural ingredient [8]. Since microbes have long been exploited for production of foods and beverages [9] and biochemicals [10], harnessing microbial cell factories for production of natural esters from renewable feedstocks is primed to be a promising sustainable alternative.

In nature, microbes and plants possess diverse metabolic pathways to provide numerous

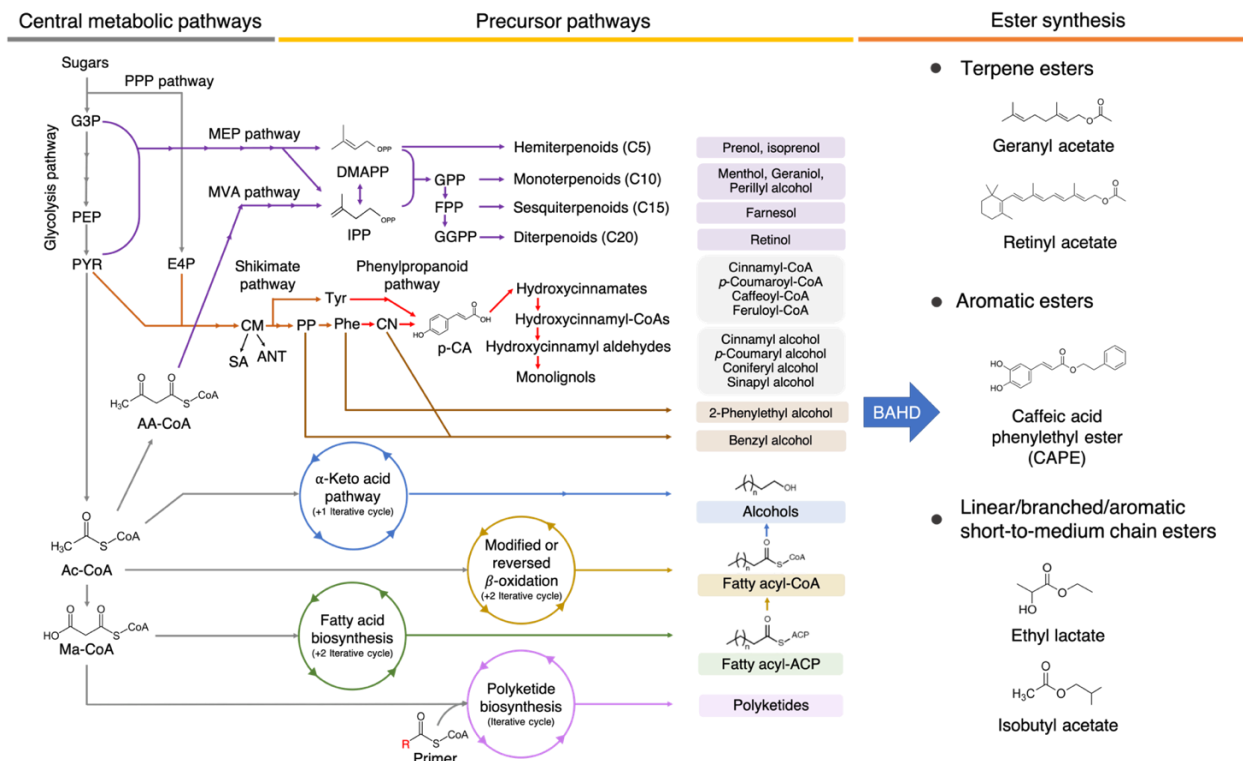


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precursor metabolites for ester biosynthesis (Figure 1-1). For instance, to make linear (carbon) chain esters, linear short-to-long fatty acyl-CoAs and alcohols can be derived from the fatty acid biosynthesis and reversed β -oxidation pathways [11]. For branched chain esters, enzymatic on of α -keto acids by branched-chain keto acid dehydrogenase complex (KDHC) and α -keto acid pathways can be exploited to generate branched acyl-CoAs [12] and alcohols [13], respectively. For aromatic esters, aryl-CoAs and alcohols can be synthesized from the shikimate and phenylpropanoid pathways [14]. For terpene esters, methylerythritol phosphate (MEP) and mevalonate (MVA) pathways can supply various chain length of terpenols [15]. Like the fatty acid biosynthesis cycles, the polyketide biosynthesis pathways can also be employed to generate a large and diverse library of linear, branched, and cyclic CoAs and alcohols [16]. By harnessing these naturally existing biosynthesis pathways, novel microbial cell factories have been developed to produce various natural esters (Table 1-1).

Current production of natural products relies on chemical extraction of natural sources such as plants. This traditional technology faces several challenges. The low content and purity of the ingredients of interest derived from the natural sources limit large-scale production and quality control, and hence cause increasing concerns for their environmental impact [17]. Furthermore, chemical extraction method has an inherent disadvantage in production of volatile compounds due to products loss resulting in a low recovery rate [18]. Harnessing microbial cell factories for natural ester production can potentially overcome many challenges present in the traditional approach. First, ester microbial biosynthesis enables industrial-scale production of pure compounds [19]. Second, the microbial conversion route can save time and cost due to higher production, faster growth of microbes than plants, and ease of ester recovery from fermentation [5, 20]. Third, ester microbial production can utilize abundant, renewable and/or sustainable feedstocks from biologi-

Table 1-1: Summary of microbial production of various esters by BAHD acyltransferases.

Products	Uses	Ref.	Products	Uses	Ref.
<i>Short-to-medium chain esters</i>			<i>Short-to-medium chain esters (Continued)</i>		
Ethyl acetate	Fuels, Solvents, Flavors, Fragrances	[4, 21, 22]	Ethyl valerate	Fuels, Solvents, Flavors, Fragrances	[23]
Propyl acetate		[4]	Isobutyl valerate		[24]
Butyl acetate		[25]	Amyl valerate		[23]
Butyl acetate		[4]	Ethyl hexanoate		[23]
Isobutyl acetate		[2, 4, 20, 25-27]	Isobutyl hexanoate		[24]
2-methyl-1-butyl acetate		[27]	<i>Aromatic esters</i>		
Amyl acetate		[23]	Benzyl acetate	Fuels, Solvents, Flavors, Fragrances	[4, 25]
Isoamyl acetate		[2, 4, 20, 27]	Benzyl lactate		[4]
Hexyl acetate		[23]	2-Phenylethyl acetate		[2, 25] [28]
Ethyl propionate		[29]	2-Phenylethyl isobutyrate		[2]
Propyl propionate		[23]	Methyl anthranilate	Flavors, Fragrances	[30]
Isobutyl propionate		[24]	Ethyl benzoate	Fuels, Solvents, Flavors, Fragrances	[31]
Ethyl lactate		[4]	Butyl benzoate		[31]
Propyl lactate		[4]	Isoamyl benzoate		[31]
Butyl lactate		[4]	2-Phenylethyl benzoate		[31]
Isobutyl lactate		[4]	Caffeic acid phenethyl ester (CAPE)	Drug candidates	[3, 31]
Isoamyl lactate		[4]	<i>Terpene esters</i>		
Ethyl butyrate		[32]	Geranyl acetate	Jet fuels, Favors, Fragrances, Pharmaceuticals, Biopesticides, Precursor chemicals	[33]
Butyl butyrate		[23, 32, 34]	Geranyl acetate		[5]
Butyl octanoate		[35]	Geranylgeranyl acetate		[36]
Isobutyl butyrate		[24, 32]	Perillyl acetate		[37]
Isobutyl isobutyrate		[2]	Farnesyl acetate		[38]
Isoamyl isobutyrate		[2]	Retinyl acetate	Drugs, Cosmetics, Food additives.	[39]

cal wastes such as carboxylic acids [23, 24] to lignocellulosic biomass [40]. Lastly, the well-established pathways for generating acyl-CoAs and alcohols [41] as the precursors for ester biosynthesis can be leveraged to quickly develop microbial cell factories for ester production.

In contrast to the traditional approach, the high volatility of esters become advantageous in the microbial conversion route [29]. Esters can be readily secreted outside of cells and easily removed from the fermentation broth by gas stripping or dual-phase separation [42]. *In situ* extraction and fermentation help overcome the product toxicity and hence improve final product titer, rate, and yield [42]. For instance, while cell growth was inhibited by 3 g/L of isobutyl acetate [2], gas stripping and dual-phase separation approaches enabled the isobutyl acetate microbial production to reach final titers of ~36 g/L (42% of theoretical maximum yield) [20] and ~17.2 g/L (80% of theoretical maximum yield) [2], respectively. Likewise, ineffective production of geraniol caused by its anti-microbial activity [43] has recently been overcome using the ‘detoxification via esterification’ strategy [5] where instead of geraniol, its esterified derivative geranyl acetate was produced with better properties [44] and lower toxicity [45]. The strategy comprises of two processes including conversion of toxic geraniol into less toxic geranyl acetate by AAT and simultaneous removal of geranyl acetate from the medium with dual-phase separation. This strategy enables to achieve high production of geraniol acetate (~4.8 g/L) with high purity without any additional strain engineering such as expression of efflux pumps, changes in membrane properties, and activation of stress response genes. Taken together, microbial production of esters coupled with *in situ* product removal (ISPR) approach such as gas stripping or dual-phase separation offers an efficient ester production platform.

Despite of these benefits, the development of microbial cell factories for efficient ester biosynthesis is currently limited by the availability of enzymes responsible for the condensation

of precursors into esters. To address this issue, efforts have been made by exploring and engineering various ester-producing enzymes [35, 42, 46]. The most-studied family of enzymes for ester synthesis is BAHD acyltransferase family [46]. In plants, the members of this family play an important role in the formation of a wide range of secondary metabolites [47]. The other well-characterized member of this family is alcohol acyltransferase (AAT, EC 2.3.1.84), for instance, ATF1 of *S. cerevisiae* [48]. AATs catalyze the transfer of the acyl group from an acyl-CoA (donor) to an alcohol (acceptor) [25]. Thus, the final products can be diverse, depending on the substrate specificity of AAT towards acyl-CoAs and alcohols [49]. Although ATF1 has been widely used in production of various acetate esters [2, 23, 24], it has some drawbacks in microbial production of esters. For example, ATF1 could not catalyze other acyl-CoAs than acetyl-CoA [48] and showed the very poor solubility in prokaryotes like *E. coli* [50]. Further, its high K_M values for alcohol substrates (i.e., ~20.2 mM for isobutanol and ~26.0 mM for isoamyl alcohol) can lead to the inefficient ester production associated with the cytotoxicity from high-level alcohol production [20]. Moreover, its precise rational design is limited by the lack of available 3D crystal structure.

Like the combinatorial chemical synthesis [51], recent breakthroughs in metabolic engineering and synthetic biology have enabled novel design of microbial cell factories by harnessing their unique and diverse cellular metabolisms to synthesize a large space of molecules with complex structures and many enzymatic reaction steps [52]. The concept of modular cell design, inspired by modern engineering disciplines and natural systems, has recently been proposed to enable rapid and predictable construction of multiple production strains to achieve superior performance in production of biochemicals such as esters with minimal experimental effort [52, 53]. Each optimal production strain can be obtained by assembling a modular (chassis) cell with an exchangeable production module(s) in a plug-and-play fashion. Because a modular

(chassis) cell contains core metabolic phenotypes shared among production modules, the desirable phenotype of each production strain is determined by the production module, i.e., auxiliary regulatory and metabolic pathways engineered to couple with the modular cell to produce a desirable molecule. Based on the modular cell design principle, the best production strains or modules can be screened or selected based on the growth coupled to product formation phenotypes (Figure 1-2) [54].

As the functional groups of esters are important for determining their chemical properties, a large space of esters can be generated by combining i) acyl-CoA synthesis submodule, ii) alcohol synthesis submodule, and iii) ester condensation submodule in a systematic way. Recently, the concept of modular cell design for combinatorial biosynthesis of esters has been demonstrated for the production of butyrate esters [32]. In this work, by using a combination of the butyl-CoA plus AAT module and various alcohol production modules, the *de novo* production platform of butyrate esters was successfully established for the first time. Furthermore, this study demonstrated the growth-coupled ester production using modular cell design principles. However, while modular cell design principles have been successfully validated in some cases, its development is still limited by the small library of demonstrated production modules. Specifically, the current limitations in the rapid strain development for efficient production of various esters are i) lack of a framework for rapid design and construction of various ester production modules, ii) incompatibility between production modules and a modular (chassis) cell majorly due to the insoluble expression of heterologous enzymes [50], and iii) the lack of an efficient ester-producing enzymes pool [23].

Presented in this work is my efforts i) to develop a framework for rapid construction of production modules to explore a large space of molecules such as a large array of esters, and ii) to

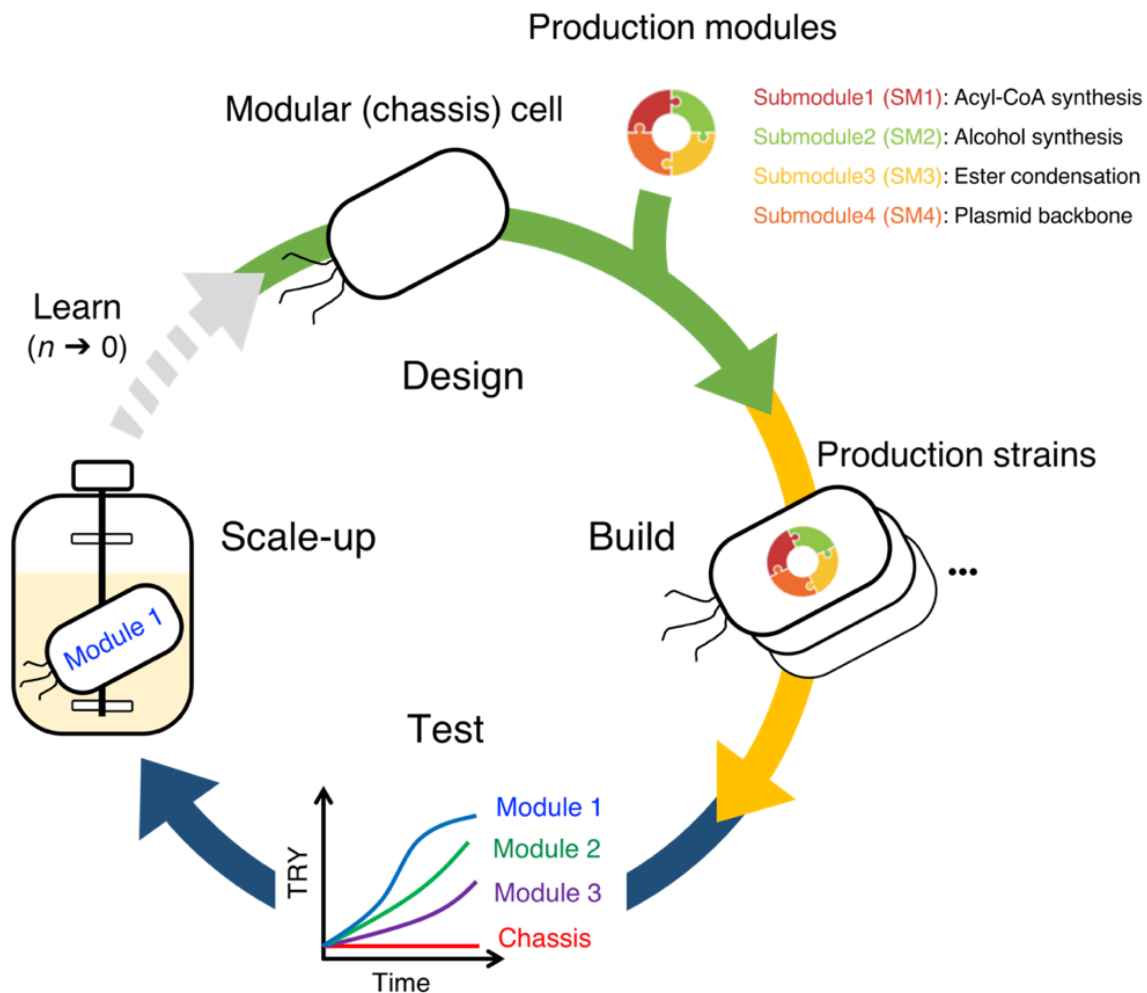


Figure 1-2: Representative presentation of the modular cell design principle for efficient combinatorial production of esters. TRY stands for titer, rate, and yield.

demonstrate the modular cell design principles as a rapid strain development platform for efficient production of molecules of interest. This dissertation consists of five main chapters. **Part I** presents the combinatorial biosynthesis of C4-driven esters as a case study of rapid design and construction of synthetic metabolic pathways. This chapter describes the establishment of a framework for rapid design and construction of synthetic metabolic pathways and the demonstration of one-step fermentative production of C4-derived esters from glucose. **Part II** presents the biosynthesis of lactate esters as a case study of expanding a library of esters that can be produced by microbes from renewable resources using modular design principles. This chapter describes direct fermentative production of lactate esters from glucose as well as identification and alleviation of the most limiting enzymatic step in biosynthesis of lactate esters using genetic parts. **Part III** presents rational engineering of an alcohol acyltransferase for production of designer esters as a case study of computer-guided novel enzyme design. This chapter describes the development a computer-guided rational protein engineering protocol for altering the substrate specificity of a chloramphenicol acetyltransferase. **Part IV** presents the development of a 96-well plate-based high-throughput ester biosynthesis screening platform as a case study of developing a rapid enzyme substrate profiling platform. This chapter describes the development of a microplate-based platform for cell culturing as well as colorimetric quantification of esters. **Part V** presents the growth selection of efficient AATs or ester production modules in a modular (chassis) cell. As a proof-of-concept, this chapter describes the growth selection of an efficient butyl acetate production module with AAT variants in a modular (chassis) cell. This work offers a blueprint for rapid development of efficient production strains of a large space of biofuels and biochemicals from renewable resources with a case study of production of various esters.

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2 One-Step Modular Fermentative Production of C4-Derived Esters

2.1 Abstract

Medium chain esters are considered as fuel additives due to its favorable properties as drop-in fuels. Here, we reported rapid development of a microbial platform for one-step fermentative production of C4-derived esters including butyl acetate, ethyl butyrate, and butyl butyrate from glucose using a modular *Escherichia coli*. Specifically, to achieve efficient production of C4-derived ester, we applied a series of design and engineering approaches including modular pathway design, optimization of induction conditions, and a combinatorial solubilization of pathway enzymes. Finally, the final engineered strains produced 441.4 ± 40.9 mg/L of butyl acetate (9.2% of maximum theoretical yield), 408.9 ± 44.3 mg/L of ethyl butyrate (8.5% of maximum theoretical yield), and 449.6 ± 43.0 mg/L of butyl butyrate (10.0% of maximum theoretical yield) with the selectivity of 91.74%, 85.51%, and 53.52%, respectively. This study provides a framework for efficient microbial combinatorial biosynthesis of various natural esters from renewable biomass.

2.2 Introduction

Microbial platforms for production of biochemicals and fuels from renewable biomass have been considered as a promising alternative to the chemical processes to mitigate climate change and enhance oil independency [1]. For alternative fuels, while alcohols such as ethanol, butanol or isobutanol have taken a large portion of this sector, medium chain esters have recently been attracting attention as drop-in fuels due to its favorable properties such as high energy density [2], high hydrophobicity [3], and good compatibility with current infrastructures including engines, transport, and storage density [4, 5]. Specifically, the medium-chain esters (C6-C10) such as ethyl valerate (C7) [6], butyl butyrate (C8) [5, 7], butyl valerate (C9) [4], and pentyl valerate (C10) [4]

are good fuel additives for gasolines and butyl butyrate (C8) [5, 7] and ethyl octanoate (C10) [7] are also considered as an alternative jet fuel.

Compared to acetate esters such as isobutyl acetate [3, 8] and isoamyl acetate [3], the microbial production of C4-derived esters with high specificities such as ethyl butyrate [9] and butyl butyrate [10] has been less successful majorly due to i) the low substrate preference of alcohol acetyltransferases (AATs), the key enzyme in ester biosynthesis [1, 11], towards butyl-CoA [12] and ii) the low availability of endogenous butyl-CoA [13, 14]. Specifically, although AATs exhibit their activity towards medium-chain acyl-CoAs like butyl-CoA, due to their substrate promiscuity [15-17], the low selective production has been the significant challenge in production of C4-derived esters [9, 10]. Further, the substrate promiscuity of AATs can be more problematic in intracellular environments where medium-chain acyl-CoAs like butyl-CoA present at a low abundance as intermediates of β -oxidation [14, 18, 19]. In other words, because butyl-CoA can be outcompeted by other acyl-CoAs that are abundant or have a higher affinity to AATs [20-22], to achieve efficient C4-derived esters, the AATs with a high substrate preference towards medium-chain acyl-CoAs/alcohols need to be identified and harnessed.

To date, *Escherichia coli* have been considered as a model bacterial chassis in the laboratories as well as a workhorse in the industries due to its rapid growth rate, abundant available genetic engineering tools, cost-effectiveness, high yield of the recombinant proteins, and easy scale-up process [23]. Further, because *E. coli* has well-established platforms for production of the precursor metabolites of esters such as various acyl-CoAs and alcohols [24], by using a combinatorial synthesis approach, a large array of diverse esters with various industrial applications such as flavors, fragrances, solvents, lubricants, coatings, and drop-in fuels can be produced from renewable resources [25] in a renewable and sustainable way.

However, *E. coli* also offers some challenges in expression of active enzymes due to lack of post-translational machinery and the formation of inclusion bodies [26]. Given that the maximum possible metabolic flux ($J_{\max}$) is determined by the product of the turnover number (k_{cat}) and the concentration of active enzyme ($[E]_{\text{active}}$) for the weakest pathway enzyme ($J_{\max} = k_{\text{cat}} \times [E]_{\text{active}}$) [27, 28], all pathway enzymes should be expressed in an active form to obtain the metabolic flux of the given pathway. Thus, to address this problem, several metabolic engineering strategies including fine-tuning of the synthetic pathways [29] and combinatorial pathway optimization [30-32] have been proposed. However, despite these efforts, because some proteins are inherently prone to aggregate in *E. coli* like an AAT [3, 33], we need the strategies to express these enzymes in an active form prior to the pathway optimization.

In this work, to develop a microbial platform for efficient one-step modular fermentative biosynthesis pathway of C4-derived esters in the engineered *E. coli* modular cell from glucose, we employed the following design and engineering strategies. First, the biosynthesis pathways of C4-derived esters were constructed via a combinatorial modular pathway design. Second, the culture conditions for expression of pathway enzymes including culture temperatures and the concentration of inducer were optimized. Third, to improve the soluble expression of alcohol acetyltransferase (AAT), a combinatorial protein solubilization including codon optimization, the use of fusion tags, co-expression of chaperones, and/or the combination thereof was identified. Finally, to evaluate the performance of the final strains, we characterized them in anaerobic bottles with pH-adjustment. This study offers a framework that can be applied to produce a large variety of natural esters from renewable resources.

2.3 Methods

2.3.1 Strains and plasmids

Strains and plasmids used in this study are presented in Table 2-1 and Table 2-2. Briefly, *E. coli* TOP10 strain was used for molecular cloning. Except for EcJWBA15-17, TCS083 (DE3) $\Delta fadE$ described previously [9] was used for production of C4-derived esters. For EcJWBA15-17, TCS095 (DE3) [34] was used as a host strain. A set of duet vectors including pACYCDuet-1, pETDuet-1, and pRSFDuet-1 were used as plasmid backbones for constructing a library of BA, EB, and BB production modules. The origins of replication and the copy number of pACYCDuet-1, pETDuet-1, and pRSFDuet-1 are as follows: P15A, ~10; ColE1, ~40; RSF1030, ~100, respectively [30]. The codon-optimized *S. cerevisiae* *ATF1* ($ATF1_{Sc}^{opt}$), cultivated strawberry (*F. ananassa*) *SAAT* ($SAAT_{Fa}^{opt}$), *Candida boidinii* *fdh* (fdh_{Cb}^{opt}), and *C. acetobutylicum* *adhE2* ($adhE2_{Ca}^{opt}$) were synthesized by the U.S. Department of Energy (DOE) Joint Genome Institute (JGI). The enzymes used in this study are presented in Table 2-3 and the list of codon optimized gene sequences is presented in Table 2-4.

2.3.2 Culture conditions

For molecular cloning and seed cultures, LB (Lysogeny Broth) medium was used. For ester production, TBD₅₀ medium, TB (Terrific Broth) with 50 g/L glucose was used (without supplementation with glycerol). For all cultures, 30 µg/mL chloramphenicol (Cm), 50 µg/mL kanamycin (Kan), and/or 100 µg/mL ampicillin (Amp) was added to the medium where applicable. For seed cultures, 1% (v/v) of stock cells were grown overnight in 5 mL of LB medium with appropriate antibiotics. For ester production in conical tubes, seed cultures were prepared as described in seed cultures. Next, 1% (v/v) of seed cultures inoculated in 500 mL baffled flask con-

Table 2-1: A list of strains used in this study. Key strains are in bold.

Strains	Plasmid 1	Plasmid 2	Plasmid 3
EcJWBA1	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pETD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Amp ^R	-
EcJWBA2	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Kan ^R	-
EcJWBA3	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pACYCD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Cm ^R	-
EcJWBA4	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Kan ^R	-
EcJWBA5	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pACYCD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Cm ^R	-
EcJWBA6	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pETD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ; Amp ^R	-
EcJWBA7	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA8	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::malE_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA9	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::nusA_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA10	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::trx_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA11	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ^{opt} ::fdh _{Cb} -P _{T7lac} ::trx_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA12	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::malE_adhE2 _{Ca} ^{opt} ::fdh _{Cb} -P _{T7lac} ::trx_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA13	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::nusA_adhE2 _{Ca} ^{opt} ::fdh _{Cb} -P _{T7lac} ::trx_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWBA14	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::trx_adhE2 _{Ca} ^{opt} ::fdh _{Cb} -P _{T7lac} ::trx_ATF1 _{Sc} ^{opt} ; Kan ^R	-
EcJWEB1	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pETD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Amp ^R	-
EcJWEB2	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	-
EcJWEB3	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pACYCD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Cm ^R	-
EcJWEB4	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pRSFD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	-
EcJWEB5	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pACYCD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Cm ^R	-
EcJWEB6	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pETD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Amp ^R	-
EcJWEB7	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	pACYC P _{araB} ::groES::groEL; Amp ^R
EcJWBB1	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pETD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Amp ^R	-
EcJWBB2	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	-
EcJWBB3	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pACYCD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Cm ^R	-
EcJWBB4	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	-

Table 2-1: Continued

Strains	Plasmid 1	Plasmid 2	Plasmid 3
EcJWBB5	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pACYCD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Cm ^R	-
EcJWBB6	pRSFD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Kan ^R	pETD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Amp ^R	-
EcJWBB7	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	pACYC P _{araB} ::groES::groEL; Amp ^R
EcJWBB8	pACYCD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Cm ^R	pRSFD P _{T7lac} ::Trx _{adhE2Ca^{opt}} ::fdh _{Cb} -P _{T7lac} ::SAAT _{Fa} ; Kan ^R	
EcJWATF1	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	-	-
EcJWATF1 ^{opt}	pET29 P _{T7lac} ::atf1 _{Sc^{opt}} ; Kan ^R	-	-
EcJWATF1 ^{MBP}	pET29 P _{T7lac} ::malE _{atf1Sc} ; Kan ^R	-	-
EcJWATF1 ^{NusA}	pET29 P _{T7lac} ::nusA _{atf1Sc} ; Kan ^R	-	-
EcJWATF1 ^{Trx}	pET29 P _{T7lac} ::trx _{atf1Sc} ; Kan ^R	-	-
EcJWATF1 ^{Chp1}	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	pACYC P _{araB} ::tig; Cm ^R	-
EcJWATF1 ^{Chp2}	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	pACYC P _{araB} ::groES::groEL; Cm ^R	-
EcJWATF1 ^{Chp3}	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	pACYC P _{pzt-1} ::groES::groEL::tig; Cm ^R	-
EcJWATF1 ^{Chp4}	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	pACYC P _{araB} ::dnaK::dnaJ::grpE; Cm ^R	-
EcJWATF1 ^{Chp5}	pET29 P _{T7lac} ::atf1 _{Sc} ; Kan ^R	pACYC P _{araB} ::dnaK::dnaJ::grpE-P _{pzt-1} ::groES::groEL; Cm ^R	-
EcJWC4SAAT	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Sc} ; Kan ^R	-
EcJWC4SAAT ^{opt}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Fa^{opt}} ; Kan ^R	-
EcJWC4SAAT ^{MBP}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::malE _{SAAT_{Fa}} ; Kan ^R	-
EcJWC4SAAT ^{NusA}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::nusA _{SAAT_{Fa}} ; Kan ^R	-
EcJWC4SAAT ^{Trx}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::trx _{SAAT_{Fa}} ; Kan ^R	-
EcJWC4SAAT ^{Chp1}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Sc} ; Kan ^R	pACYC P _{araB} ::tig; Cm ^R
EcJWC4SAAT ^{Chp2}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Sc} ; Kan ^R	pACYC P _{araB} ::groES::groEL; Cm ^R
EcJWC4SAAT ^{Chp3}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Sc} ; Kan ^R	pACYC P _{pzt-1} ::groES::groEL::tig; Cm ^R
EcJWC4SAAT ^{Chp4}	pETD P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} -P _{T7lac} ::ter _{Td} ; Amp ^R	pET29 P _{T7lac} ::SAAT _{Sc} ; Kan ^R	pACYC P _{araB} ::dnaK::dnaJ::grpE; Cm ^R

taining 50 ml of TBD₅₀ medium with appropriate antibiotics. The cells were aerobically grown in shaking incubators at 28°C or 37°C, 200 rpm and induced at an O.D.₆₀₀ of 0.6~0.8 with various concentrations of IPTG, arabinose (if applicable), and/or 5 ng/ml of tetracycline (if applicable). After 2 hours of induction, the cultures in the baffled flasks were distributed into 15 mL conical centrifuge tubes (Cat. #339650, Thermo Scientific, MA, USA) with a working volume of 5 mL. Then, each tube was overlaid with 1ml hexadecane (20% (v/v)) for *in situ* ester recovery and capped to generate anaerobic conditions. Finally, the tubes were grown for another 18 hours on a 75° angled platform in shaking incubators at 28°C or 37°C, 200 rpm. The remained cultures in the baffled flasks were induced for 2 additional hours and then the cells were harvested for SDS-PAGE analysis.

For ester production in anaerobic bottles with pH-adjustment, the induced cultures were prepared as described in ester production in conical tubes. To generate the anaerobic conditions, the induced cultures were transferred into anaerobic bottles with a working volume of 100 mL. Then, each anaerobic bottle was overlaid with 20 ml hexadecane 20% (v/v) for *in situ* ester recovery and sealed with a rubber stopper inside the anaerobic chamber. The headspace of the anaerobic bottles was vacuumed and replaced by an anaerobic mix of 90% N₂, 5% H₂, and 5% CO₂ inside the anaerobic chamber. Finally, the anaerobic bottles were grown for another 90 hours in shaking incubators at 28°C or 37°C, 200 rpm. The culture medium and hexadecane overlay samples were taken through the rubber stopper via a syringe and needle by maintaining the ratio of 5:1. The culture pH was adjusted to around 7 using 10 M NaOH every 24 hours. All experiments were performed in biological triplicates.

2.3.3 Protein expression and SDS-PAGE analysis

The cells were collected from the culture by centrifugation and resuspended in 1X PBS

(Phosphate Buffered Saline) buffer (pH 7.4) at the final O.D.₆₀₀ of 10. Cell pellets were disrupted using the B-PER complete reagent (Cat. #89822, Thermo Scientific, MA, USA), according to the manufacturer's instruction. Total and soluble fractions were separated by centrifugation for 20 min at 4°C. The resulting samples were mixed with 6X SDS (sodium dodecyl sulfate) sample buffer, heated at 95°C for 5 min, and analyzed by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) using Novex™ 14% Tris-Glycine protein gels (Cat. #XP00145BOX, Thermo Scientific, MA, USA). Protein bands were visualized with Coomassie Brilliant Blue staining.

2.3.4 Determination of cell concentrations

The optical density was measured at 600 nm using a spectrophotometer (GENESYS 30, Thermo Scientific, IL, USA). The dry cell mass was determined by multiplying the optical density of culture broth with a pre-determined conversion factor, 0.48 g/L/O.D.

2.3.5 High performance liquid chromatography (HPLC)

Metabolites and doped alcohols were quantified by using the Shimadzu HPLC system (Shimadzu Inc., MD, USA) equipped with the Aminex HPX-87H cation exchange column (BioRad Inc., CA, USA) heated at 50°C. A mobile phase of 10 mN H₂SO₄ was run at a flow rate of 0.6 mL/min. Detection was made with the reflective index detector (RID).

2.3.6 Gas chromatography coupled with mass spectroscopy (GC/MS)

All esters were quantified by GC/MS. For GC/MS analysis, the cultures were centrifuged at 15,000 x g for 2 min and the hexadecane overlays were used for quantification of esters. The samples were prepared by diluting hexadecane extracts from the cultures with hexadecane containing internal standard (isoamyl alcohol) in a 1:1 (v/v) ratio. Then, 1 µL of samples were directly injected into a gas chromatograph (GC) HP 6890 equipped with the mass selective detector

(MS) HP 5973. For the GC system, helium was used as the carrier gas at a flow rate of 0.5 mL/min and the analytes were separated on a Phenomenex ZB-5 capillary column (30 m x 0.25 mm x 0.25 μ m). The oven temperature was programmed with an initial temperature of 50°C with a 1°C/min ramp to 58°C. Next a 25°C/min ramp was deployed to 235°C and then a 50°C/min ramp was deployed to 300°C. Finally, a temperature of 300°C was held for 2 minutes to elute any residual non-desired analytes. The injection was performed using the splitless mode with an initial injector temperature of 280°C.

For the MS system, a selected ion monitoring (SIM) mode was deployed to detect analytes. The SIM parameters for detecting esters were as follows: i) for ethyl acetate, ions 45.00, and 61.00 detected from 4.15 to 5.70 min, ii) for isoamyl alcohol (internal standard), ions 45.00, and 88.00 detected from 5.70 to 7.20 min, iii) for ethyl butyrate, ions 47.00, and 116.00 detected from 7.20 to 7.75 min, iv) for butyl acetate, ions 61.00, and 116.00 detected from 7.75 to 11.25 min, vi) for butyl butyrate, ions 101.00, and 116.00 detected from 11.25 to 12.50 min.

2.4 Results

2.4.1 Combinatorial modular plasmid assembly for rapid construction of biosynthesis pathways of C4-derived esters

We first designed the biosynthesis pathways of C4-derived esters. Biologically, esters can be synthesized by condensing an acyl-CoA and an alcohol using an alcohol acyltransferase (AAT) (Figure 2-1a). Thus, to establish the biosynthesis pathway of C4-derived esters, three major pathways including i) butyl-CoA synthesis pathway, ii) alcohol synthesis pathway such as ethanol, butanol, and iii) ester synthesis pathway (AAT) are needed. In our previous studies, the production modules for butyl-CoA and alcohols (ethanol, butanol) have been constructed [9] and the substrate

specificity of AATs has been studied [21]. Additionally, it was reported that co-expression of a NAD⁺-dependent formate dehydrogenase (Fdh) improve butanol production by increasing intracellular NADH availability [35, 36]. Thus, using these previously identified genetic parts and gained knowledge, we modulated the ester synthetic pathways into four submodules; submodule1 (SM1) carrying *E. coli* *atoB* (*atoB*_{Ec}), *Clostridium acetobutylicum* *Hbd* (*hbd*_{Ca}), *C. acetobutylicum* *crt* (*crt*_{Ca}), and *Treponema denticola* *ter* (*ter*_{Td}) for butyl-CoA synthesis, submodule2 (SM2) consisted of *Zymomonas mobilis* *pdc* (*pdc*_{Zm}) and *adhB* (*adhB*_{Zm}) or *C. acetobutylicum* *adhE2* (*adhE2*_{Ca}) for alcohol synthesis, submodule3 (SM3) carrying *Candida boidinii* *fdh* (*fdh*_{Cb}) for NADH regeneration, and submodule4 (SM4) carrying *Saccharomyces cerevisiae* *ATF1* (*ATF1*_{Sc}) or *Fragaria x ananassa* (cultivated strawberry) *SAAT* (*SAAT*_{Fa}) for ester synthesis (Figure 2-1b). Then, by using a combinatorial plasmid assembly approach, the submodules were assembled into two parts - plasmid #1 carrying the SM1, a common pathway for C4-derived esters biosynthesis, and plasmid #2 carrying the SM2-SM3-SM4, variable pathways in C4-derived esters using plasmids with various copy numbers for pathway optimization (Figure 2-1c, Table 2-2). Figure 2-2a, 2-3a, and 2-4a show how the combination of each submodule can comprise the biosynthesis pathways of BA, EB, and BB, respectively. Finally, a combination of plasmid #1 and plasmid #2 for each compound were transferred into the modular *E. coli* strain, TCS083 (DE3) Δ *fadE* [9], resulting in six initial strains, EcJWBA1-6; EcJWEB1-6; and EcJWBB1-6 (Table 2-1, Figure 2-2b, Figure 2-3b, and Figure 2-4b).

2.4.2 Endogenous production of C4-derived esters in initial strains

Following the construction of initial strains, we characterized them in conical tubes to validate the constructed biosynthesis pathways of C4-derived esters as well as to identify the best combination of plasmid #1 (SM1) and plasmid #2 (SM2-SM3-SM4). We also confirmed protein

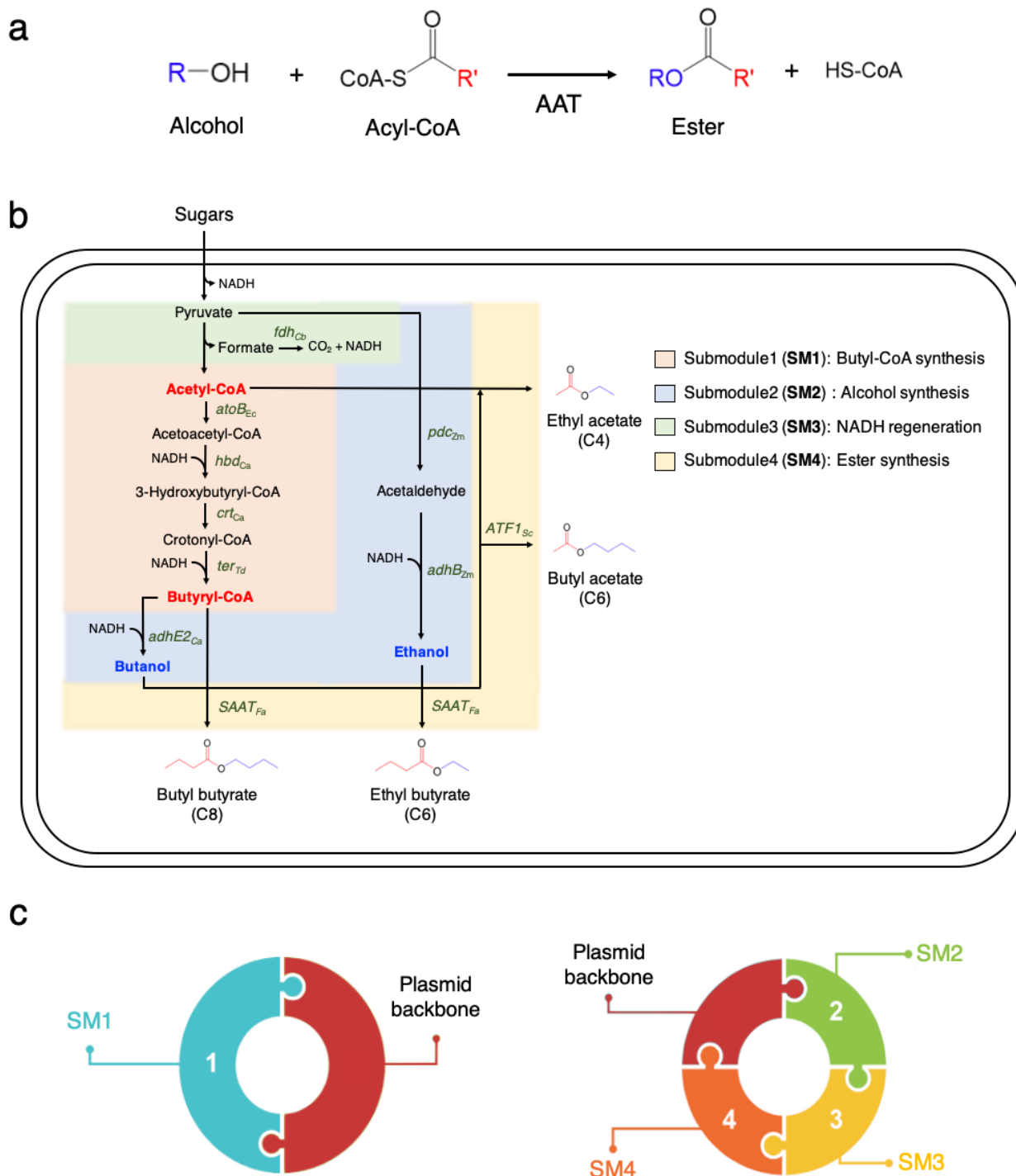


Figure 2-1: Design one-step modular fermentative pathways of C4-derived esters. **(a)** Biosynthesis of esters by an alcohol acyltransferase (AAT). **(b)** Modular biosynthetic pathways of C4-derived esters. **(c)** Schematic representation of modular plasmid assembly.

expressions by SDS-PAGE analysis (Figure 2-6). The results show that EcJWBA2, EcJWEB2, and EcJWBB2 carrying the plasmid #1 with low copy number and the plasmid #2 with high copy number achieved the highest ester production among the characterized six initial strains for each compound. For BA production, EcJWBA2 produced 34.2 ± 6.6 mg/L of BA with the selectivity of 74.27% (Figure 2-2c, Table 2-5, Figure 2-7a). For EB production, EcJWEB2 produced 71.0 ± 6.6 mg/L of EB with the selectivity of 91.97% (Figure 2-3c, Table 2-6, Figure 2-8a). For BB production, EcJWBB2 produced 33.5 ± 2.9 mg/L of BB with the selectivity of 20.75% (Figure 2-4c, Table 2-7, Figure 2-9a).

After validating the synthetic pathways of C4-derived esters, we next optimized induction conditions with the identified best ester producers, EcJWBA2, EcJWEB2, and EcJWBB2. To optimize induction conditions, we tested various induction conditions using a combination of two different temperatures (28/37°C), and three different concentrations of the inducer (0.01/0.1/1.0 mM IPTG). The results show that the titer of BA, EB, and BB were improved by 1.40, 2.82, and 3.80-fold at the optimized induction conditions, respectively. Specifically, for BA production, EcJWBA2 produced 48.0 ± 7.1 mg/L of BA with the selectivity of 83.13% when it induced by 0.1 mM of IPTG at 28°C (Figure 2-2d, Table 2-8, Figure 2-7b). For EB production, EcJWEB2 produced 200.4 ± 9.4 mg/L of EB with the selectivity of 89.57% when it induced by 0.1 mM of IPTG at 28°C (Figure 2-3d, Table 2-9, Figure 2-8b). For BB production, EcJWBB2 produced 127.4 ± 32.5 mg/L of BB with the selectivity of 33.96% when it induced by 0.1 mM of IPTG at 37°C (Figure 2-4c, Table 2-7, Figure 2-9b). Collectively, we found the optimized induction conditions for production of C4-derived esters and we used these conditions for further studies. However, because SDS-PAGE analysis results show that weak protein bands of ATF1 and SAAT compared to the other protein bands (Figure 2-10), we next investigated whether the enhanced sol-

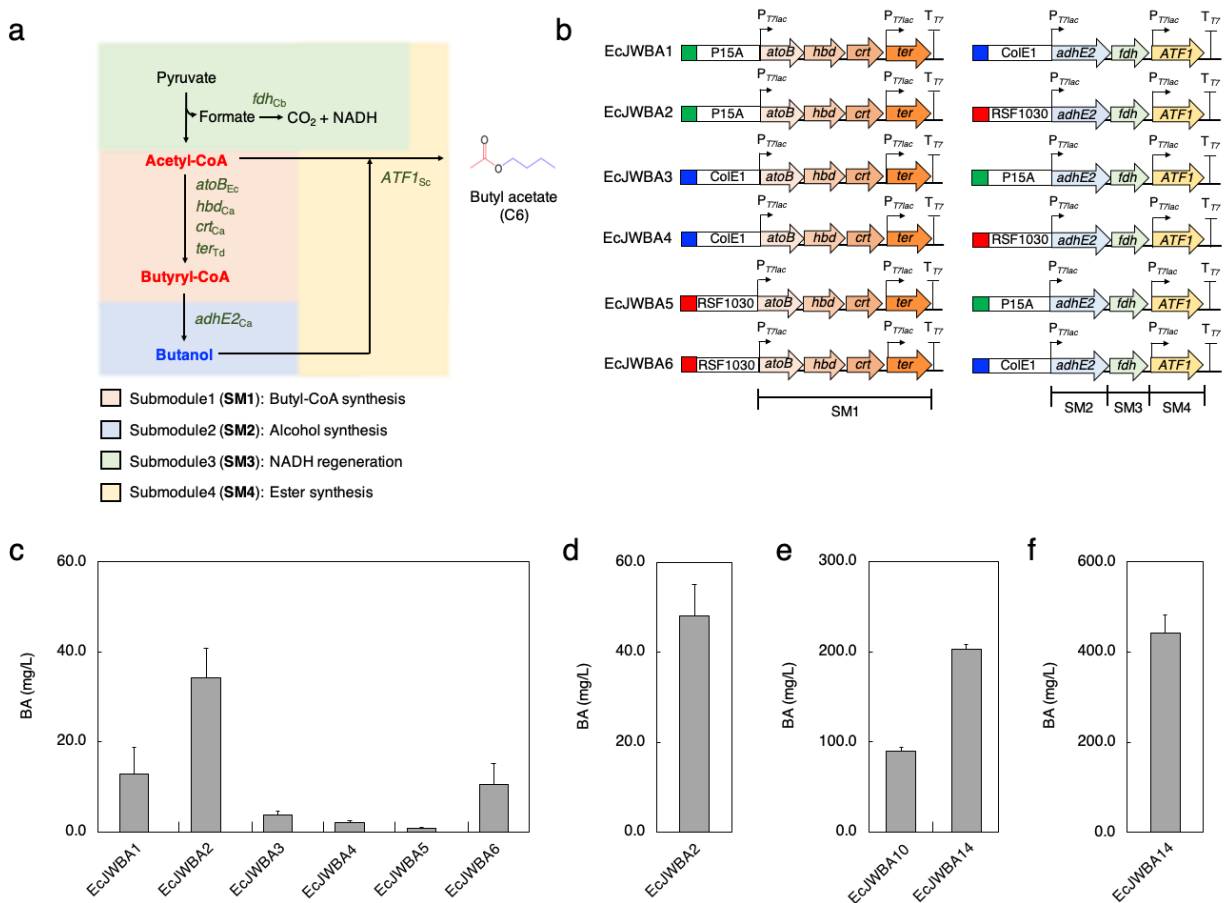


Figure 2-2: One-step fermentative butyl acetate (BA) production from glucose. **(a)** Modular *de novo* biosynthesis pathway of BA. **(b)** Schematic of initial six strains carrying BA production modules with different copy numbers. The plasmid copy numbers are as follows: P15A (green square), ~10; ColE1 (blue square), ~40; RSF1030 (red square), ~100. **(c-f)** *De novo* BA production from glucose. Endogenous BA production by **(c)** six initial strains (EcJWBA1~EcJWBA6); **(d)** EcJWBA2 in the optimized induction conditions (28°C, 0.1 mM IPTG); **(e)** EcJWBA10 and EcJWBA14; **(f)** EcJWBA14 in anaerobic bottles with pH-adjustment.

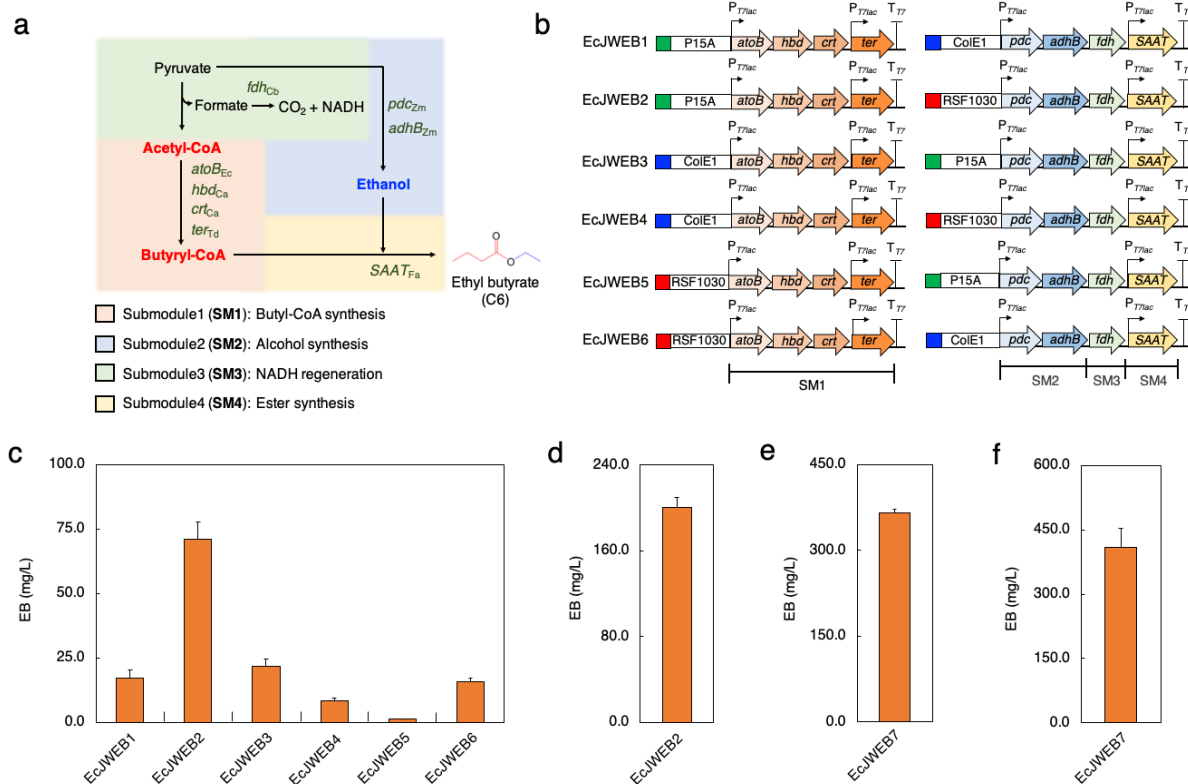


Figure 2-3: One-step fermentative ethyl butyrate (EB) production from glucose. **(a)** Modular *de novo* biosynthesis pathway of EB. **(b)** Schematic of initial six strains carrying EB production modules with different copy numbers. The plasmid copy numbers are as follows: P15A (green square), ~10; ColE1 (blue square), ~40; RSF1030 (red square), ~100. **(c-f)** *De novo* EB production from glucose. Endogenous EB production by **(c)** six initial strains (EcJWEB1~EcJWEB6); **(d)** EcJWEB2 in the optimized induction conditions (28°C, 0.1 mM IPTG); **(e)** EcJWEB7; **(f)** EcJWEB7 in anaerobic bottles with pH-adjustment.

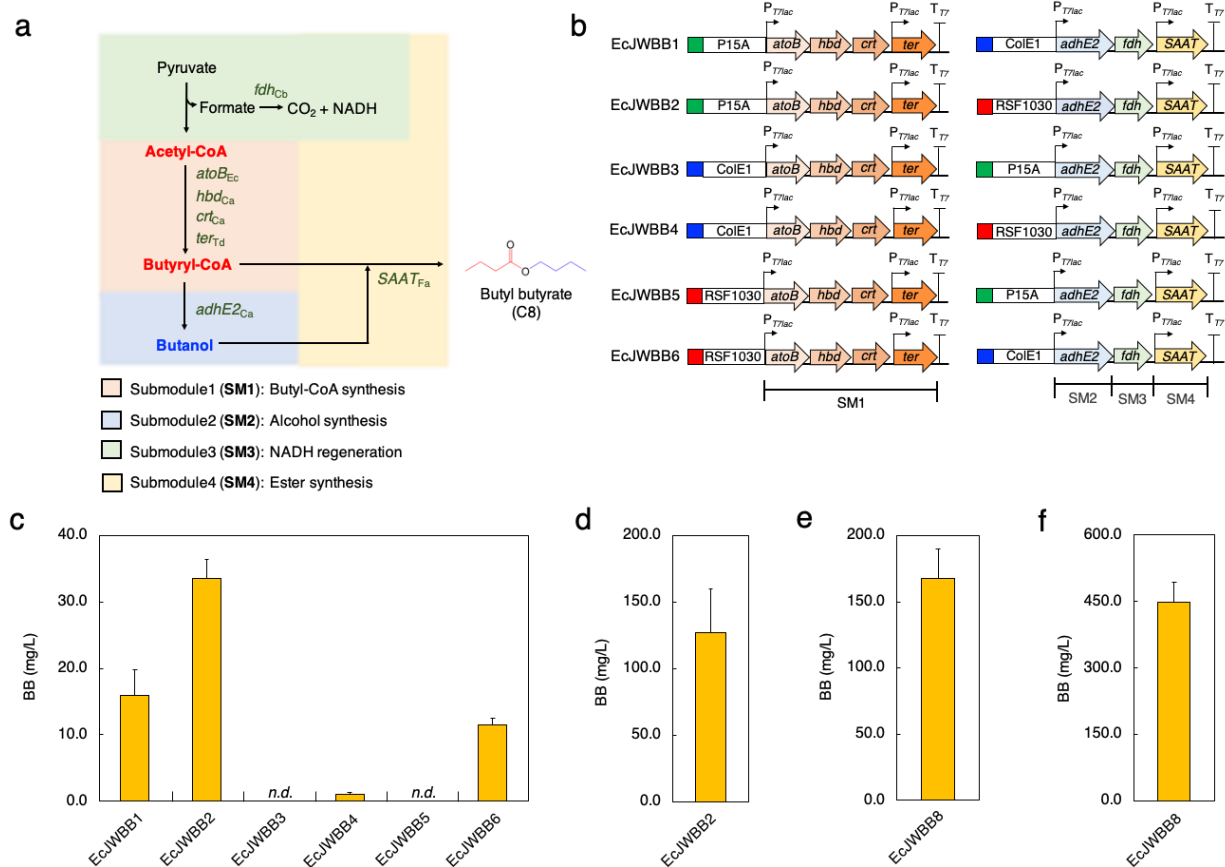


Figure 2-4: One-step fermentative butyl butyrate (BB) production from glucose. **(a)** Modular *de novo* biosynthesis pathway of BB. **(b)** Schematic of initial six strains carrying BB production modules with different copy numbers. The plasmid copy numbers are as follows: P15A (green square), ~10; ColE1 (blue square), ~40; RSF1030 (red square), ~100. **(c-f)** *De novo* BB production from glucose. Endogenous BB production by **(c)** six initial strains (EcJWBB1~EcJWBB6); **(d)** EcJWBB2 in the optimized induction conditions (37°C, 0.1 mM IPTG); **(e)** EcJWBB8; **(f)** EcJWBB8 in anaerobic bottles with pH-adjustment.

uble expression of AATs could improve ester production.

2.4.3 Evaluation of various AATs solubilization strategies for improved ester synthesis in alcohol doping experiments

To examine the effect of AAT solubilization on ester production, we first chose three well-studied strategies including i) codon optimization [37, 38]; ii) the use of fusion partners such as maltose binding protein (MBP) [39], N-utilization substrate A (NusA) [40], or thioredoxin 1 (TrxA) [41]; and iii) co-expression of molecular chaperones (DnaK/DnaJ/GrpE, GroES/GroEL, or Trigger factor (Tf)) [42]. Then, we started to construct plasmids carrying wildtype AATs, codon optimized AATs, fusion partner tagged AATs. Specifically, for BA production, the plasmids carrying wildtype *ATF1_{Sc}*, codon optimized *ATF1_{Sc}* (*ATF1_{Sc}^{opt}*), and N'-terminus MBP-, NusA-, or TrxA-tagged *ATF1_{Sc}* (*malE_ATF1_{Sc}*, *nusA_ATF1_{Sc}*, or *trxATF1_{Sc}*) were constructed and introduced into TCS083 (DE3) $\Delta fadE$ [9], resulting EcJWATF1, EcJWATF1^{opt}, EcJWATF1^{MBP}, EcJWATF1^{NusA}, EcJWATF1^{TrxA}, respectively (Table 2-1).

For EB and BB production, the plasmids carrying wildtype SAAT (*SAAT_{Fa}*), codon optimize *SAAT_{Fa}* (*SAAT_{Fa}^{opt}*), and N'-terminus MBP-, NusA-, or TrxA-tagged *SAAT_{Fa}* (*malE_SAAT_{Fa}*; *nusA_SAAT_{Fa}*; or *trxA_SAAT_{Fa}*, respectively) were constructed and introduced into TCS083 (DE3) $\Delta fadE$ [9] with the pACYCDuet-1 carrying the SM1 (Butyl-CoA pathway), resulting EcJWSAAT, EcJWSAAT^{opt}, EcJWSAAT^{MBP}, EcJWSAAT^{NusA}, EcJWSAAT^{TrxA}, respectively (Table 2-1). To co-express chaperones with AATs, the chaperone plasmid set consisted of five different plasmids carrying various chaperones (Cat. #3340, TaKaRa Bio Inc.) were introduced into EcJWATF1 and EcJWSAAT, resulting EcJWATF1^{Chp1}~EcJWATF1^{Chp5} and EcJWSAAT^{Chp1}~EcJWSAAT^{Chp5}, respectively (Table 2-1). Finally, we characterized them in conical tubes with 2 g/L of alcohol doping including ethanol and butanol to evaluate the conversion

of an alcohol (ethanol/butanol) into an ester (ethyl acetate/butyl acetate) by ATF1_{Sc} (Figure 2-11a) or EB/BB by SAAT_{Fa} (Figure 2-12a), respectively. The protein expressions were confirmed by SDS-PAGE analysis (Figure 2-13). The cultures were induced by 0.1 mM of IPTG, 0.5 mg/ml of L-arabinose (if applicable) and/or 5 ng/ml of tetracycline (if applicable).

The results show that, indeed, the strategies for enhancing soluble expression of AATs improved the conversion of an alcohol into an ester. However, we observed that the different strategies are required for different AATs. For example, while codon optimization and the use of a fusion partner including MBP, NusA, or TrxA improved the EA/BA conversion with ATF1, co-expression of chaperones including GroES/EL, GroES/EL/Tf, or DnaK/DnaJ/GrpE/GroES/EL improved the EB/BB conversion with SAAT. Specifically, for the EA conversion, EcJWATF1^{opt}, EcJWATF1^{MBP}, EcJWATF1^{NusA}, EcJWATF1^{TrxA} achieved 2.30%, 1.01%, 1.31%, and 1.32% of EA conversion, resulting 20.53, 9.04, 11.66, and 11.78-fold improvement compared with the EcJWATF1 (0.11%), respectively (Figure 2-11b, Table 2-11).

For the BA conversion, EcJWATF1^{opt}, EcJWATF1^{MBP}, EcJWATF1^{NusA}, EcJWATF1^{TrxA} achieved 85.90%, 74.55%, 79.53%, and 86.61% of BA conversion, resulting 7.14, 6.20, 6.61, and 7.20-fold improvement as compared to EcJWATF1 (12.03%), respectively (Figure 2-11c, Table 2-11). For EB production, EcJWSAAT^{Chp2}, EcJWSAAT^{Chp3}, and EcJWSAAT^{Chp5} achieved 2.60%, 1.50%, and 1.97% of EB conversion, resulting 8.45, 4.67, and 6.40-fold improvement as compared to EcJWSAAT (0.31%), respectively (Figure 2-12b, Table 2-12). For BB conversion, EcJWSAAT^{Chp2}, EcJWSAAT^{Chp3}, and EcJWSAAT^{Chp5} achieved 36.82%, 19.14%, and 11.86% of BB conversion, resulting 9.04, 4.70, and 2.91-fold improvement as compared to EcJWSAAT (4.07%), respectively (Figure 2-12c, Table 2-12).

After confirming the improved ester conversion via AAT solubilization strategies in the al-

cohol doping experiments, we next evaluated the reproducibility of this result in the endogenous ester production from glucose. Moreover, for BA production, because both strategies, including codon optimization and tagging a fusion partner, improved ester production with ATF1, we also investigated whether the synergistic effect exists when these two strategies are combined.

2.4.4 Combinatorial solubilization of ATF1_{sc} improved BA production

To evaluate the reproducible AAT solubilization results in the endogenous production from glucose, for the BA production, we first constructed the four pRSFDuet-1 carrying SM2(*adhE2*_{Ca})-SM3(*fdh*^{opt})-SM4(ATF1_{sc}^{opt}, *malE*_ATF1_{sc}^{opt}, *nusA*_ATF1_{sc}^{opt}, or *trxA*_ATF1_{sc}^{opt}), respectively (Table 2-2). Then, we introduced them into TCS083 (DE3) Δ *fadE* [9] with the pACYCDuet-1 carrying the SM1 (Butyl-CoA pathway), resulting EcJWBA7~EcJWBA10, respectively (Table 2-1). For the EB/BB production, we additionally introduced the plasmid carrying *groES* and *groEL* into EcJWEB2 and EcJWBB2, resulting EcJWEB7 and EcJWBB7, respectively (Table 2-1). Finally, we characterized them in conical tubes and evaluated the AAT solubilization strategies for improved ester production in the endogenous production from glucose. For the expression of chaperones in EB/BB production, we also tested the three different concentrations of inducer (L-arabinose).

Unfortunately, the characterization results show that while BA and EB production were improved by 1.08~1.86-fold and 1.32~1.82-fold, respectively, BB production was reduced by 0.20~0.40-fold compared with the initial strains. Specifically, for BA production, EcJWBA7~EcJWBA10 produced 51.7 ± 7.1 mg/L, 79.3 ± 9.8 mg/L, 76.1 ± 6.2 mg/L, and 89.5 ± 14.8 mg/L of BA, resulting 1.08, 1.65, 1.59, and 1.86-fold improved BA production compared with the EcJWBA2 (48.0 ± 7.1 mg/L), respectively (Figure 2-7c, Table 2-13). Notably, because EcJWBA8~10 expressing ATF1_{sc}^{opt} with N'-terminus fusion partner such as MBP, NusA, and

TrxA achieved the higher BA production than that of EcJWBA7 expressing ATF1_{Sc}^{opt} alone, we could confirm that there is a synergistic effect between codon optimization and the use of a fusion partner in BA production with ATF1_{Sc}^{opt}.

For EB production, EcJWEB7 produced 263.9 ± 51.8 mg/L, 337.6 ± 46.1 mg/L, 365.7 ± 69.2 mg/L, and 346.2 ± 59.8 mg/L of EB, resulting 1.32, 1.68, 1.82, and 1.73-fold improved EB production compared with the EcJWEB2 (200.4 ± 9.4 mg/L) when the cultures were induced by 0 mg/ml, 0.1 mg/ml, 0.5 mg/ml, and 5.0 mg/ml of L-arabinose, respectively (Figure 2-8c, Table 2-14). For BB production, EcJWBB7 produced 25.6 ± 10.7 mg/L, 30.0 ± 4.8 mg/L, 51.4 ± 3.4 mg/L, and 42.1 ± 4.1 mg/L of BB, achieving 0.20, 0.24, 0.40, and 0.33-fold decreased BB production compared with the EcJWBB2 (127.4 ± 32.5 mg/L) when the cultures were induced by 0 mg/ml, 0.1 mg/ml, 0.5 mg/ml, and 5.0 mg/ml of L-arabinose, respectively (Figure 2-9c, Table 2-15).

2.4.5 Combinatorial solubilization of AdhE2_{Ca} improved BA/BB production

Next, because we observed the low residual butanol in BA/BB production throughout the experiment and the low butanol production, one of the precursors of butyl esters, can limit BA/BB production, we next tested whether the approach used for ATF1_{Sc} could also be applied for solubilizing AdhE2_{Ca} for improved BA/BB production. AdhE2_{Ca} is known for its critical role in butanol production and its insolubility can significantly reduce *in vivo* activities compared with the *in vitro* activities [35]. For BA production, we first constructed four pRSFDuet-1 carrying SM2(*adhE2*_{Ca}^{opt}, *malE*_*adhE2*_{Ca}^{opt}, *nusA*_*adhE2*_{Ca}^{opt}, or *trxA*_*adhE2*_{Ca}^{opt})-SM3(*fdh*^{opt})-SM4(*trxA*_*ATF1*_{Sc}^{opt}), respectively (Table 2-2). Then, we introduced them into TCS083 (DE3) Δ *fadE* [9] with the pACYCDuet-1 carrying the SM1 (Butyl-CoA pathway), resulting EcJWBA11~EcJWBA14, respectively (Table 2-1). Finally, we characterized them in conical tubes and evaluated whether the AdhE2_{Ca} solubilization strategies improved BA production. The protein

expressions were confirmed by SDS-PAGE analysis (Figure 2-14).

Remarkably, the results show that EcJWBA14 achieved 2.27-fold improved BA production (203.0 ± 5.7 mg/L) as compared to EcJWBA10 (89.5 ± 14.8 mg/L), indicating that solubilization of pathway enzymes using a fusion partner can be a simple, but useful extendable pathway optimization strategy in metabolic engineering. Specifically, EcJWBA11~EcJWBA14 produced 80.3 ± 9.0 mg/L, 54.2 ± 4.9 mg/L, 82.8 ± 15.5 mg/L, and 203.0 ± 5.7 mg/L of BA, respectively (Figure 2-7d, Table 2-16). To strengthen this result, we also evaluated whether the use of TrxA fusion partner with $\text{AdhE2}_{\text{Ca}}^{\text{opt}}$ could improve BB production. We first constructed the pRSFDuet-1 carrying SM2(*trxA_adhE2_{Ca}^{opt}*)-SM3(*fdh^{opt}*)-SM4(*SAAT_{Fa}*) (Table 2-3) and introduced it into TCS083 (DE3) ΔfadE [9] with the pACYCDuet-1 carrying the SM1 (Butyl-CoA pathway), resulting EcJWBB8 (Table 2-1). Finally, we characterized EcJWBB8 in conical tubes.

Indeed, the results shows that EcJWBB8 achieved 1.31-fold improved BB production (167.3 ± 18.2 mg/L) as compared to EcJWBB2 (127.4 ± 32.5 mg/L), confirming the potential of combinatorial solubilization as a pathway optimization strategy in metabolic engineering (Figure 2-9d, Table 2-16). Furthermore, notably, EcJWBB8 achieved ~1.49-fold improved BB selectivity (50.63%) as compared to EcBB2 (33.96%), resulting ~1.65-fold improved butanol/ethanol ratio (g/g of butanol to ethanol) (from 0.04 to 0.07) and ~0.63-fold reduced EB production (from 246.2 ± 72.6 mg/L to 156.3 ± 22.4 mg/L) (Table 2-10 and 2-16). This result suggests that there was a substrate competition between ethanol and butanol in the enzymatic reaction of ATF1_{Sc} and hence it requires two potential strategies for improved production of designer esters by AATs including i) engineering alcohol substrate preference of AATs and ii) selective alcohol production.

Although BA and BB production were improved to some extent via combinatorial solubilization of AdhE2_{Ca} , residual butanol titers were still low, at the levels of 0.18 ± 0.00 g/L for

EcJWBB8 and for 0.20 ± 0.01 g/L (EcJWBA14) (Table 2-10 and 2-16). Given that the abundant alcohol production is important for ester synthesis due to the high K_M value of AATs [3, 30], butanol production should be improved for further improvement in butyl esters production. Because anaerobic conditions can improve alcohol production [35, 43], we next characterized the final strains, EcJWBA14, EcJWEB7, and EcJWBB8, in anaerobic bottles with pH-adjustment to evaluate their performance in production of C4-derived esters. The culture pH was adjusted to around 7 with 10 M NaOH every 24 hours to maintain the optimum growth pH of *E. coli* [44].

2.4.6 Production of C4-derived esters in anaerobic bottles with pH-adjustment

The characterization results of the final strains, including EcJWBA14, EcJWEB7, and EcJWBB8, achieved 12.89-, 5.76-, and 13.42-fold improvements in titers, 4.79-, 3.71-, and 4.64-fold improvements in yields, and 6.45, 1.44, and 3.36-fold improvement in productivities as compared to the initial strains, EcJWBA2, EcJWEB2, and EcJWBB2, respectively. Specifically, EcJWBA14 produced 441.4 ± 40.9 mg/L of BA (9.2% of maximum theoretical yield) with 91.74% of selectivity (Fig. 2-2f, Figure 2-15, and Table 2-17), EcJWEB7 produced 408.9 ± 44.3 mg/L of EB (8.5% of maximum theoretical yield) 85.5% of selectivity (Fig. 2-3f, Figure 2-16, and Table 2-17), and EcJWBB8 produced 449.6 ± 43.0 mg/L of BB (10.0% of maximum theoretical yield) 53.52% of selectivity (Fig. 2-4f, Figure 2-17, and Table 2-17). As compared to the best direct fermentative production of C4-derived esters in literature, EcJWBA14 achieved 52.73, 79.27, and 135.76-fold improved TRY (titer, productivity, and yield) in BA production, EcJWEB7 achieved 3.05, 3.05, and 11.02-fold improved TRY in EB production, and EcJWBB8 achieved 8.98, 6.74, and 23.31-fold improved TRY in BB production (Table 2-18).

2.5 Discussion

With the recent shale oil revolution, the cost of raw materials for fuels and chemicals with low carbon numbers such as short-chain linear alcohols are expected to decrease [45]. Thus, to ensure economic feasibility of microbial production of biochemicals from renewable resources, we should focus on production of chemicals with high carbon numbers and/or more complex structures. Esterification reaction, condensing an alcohol and an acyl-CoA into esters, can be used for increasing the carbon numbers as well as the degree of complexity of raw materials. For example, to valorize short-chain linear alcohols such as ethanol (C2), butanol (C4), the alcohols can be condensed with butyl-CoA or benzyl-CoA resulting in ethyl butyrate (C6), butyl butyrate (C8) or ethyl benzoate (C9), butyl benzoate (C11), respectively. Furthermore, this reaction can be used for synthesis of a large array of diverse esters with various industrial applications such as flavors, fragrances, solvents, lubricants, coatings, and drop-in fuels [25].

While the biosynthesis pathway of esters is simple and enzymes consisting of the pathway have been well elucidated, their production is still too low for industrial production. Seeking to develop a platform for rapid design, construction, and validation of various ester synthetic pathways, *E. coli* was chosen as a chassis cell because of its various benefits in biochemical production. However, for the efficient ester production in *E. coli*, the pool (soluble) expression issue of AATs should be addressed [3, 33]. To address this problem, we applied a combinatorial protein solubilization including codon optimization, the use of fusion tags, co-expression of chaperones, and/or the combination thereof and achieved improved C4-derived ester production. To date, while a combinatorial protein solubilization has not been commonly considered as a metabolic engineering strategy, this study suggests it can provide a useful tool for improved microbial production of biochemicals(fuels) with (eukaryotic) aggregate-prone enzymes in the ba-

acterial chassis cells like *E. coli*.

More importantly, our findings can be valuable for efficient strain development with laboratory automation. To date, while the recent advances in laboratory automation have significantly accelerated the Design-Build-Test-Learn (DBTL) cycle [46, 47], still not many successful commercialization cases have been reported. This indicates that there must be other critical factors we overlooked in strain development than the mechanical assembly of genetic parts including promoters, RBS, biosynthesis pathways or introducing the assembled genetic parts into chassis cells. The recent study used machine learning approach for production of dodecanol in *E. coli* emphasized the critical need of tools for accurate prediction of protein expression [47], suggesting the importance of enzyme expression in synthetic pathways design.

Taken together, to facilitate the development of efficient production strains, we need tools for predicting soluble expression of pathway enzymes as well as solubilizing aggregate-prone enzymes. Interestingly, for predicting soluble expression of pathway enzymes, Protein-Sol: a web tool for predicting protein solubility from sequence [48] identified the low soluble enzymes such as AATs and AdhE2_{Ca} which targeted for solubilization in this study (Figure 2-18). To date, to solubilize these enzymes, several approaches have been proposed including codon optimization [37, 38], the use of fusion partners [39-41], co-expression of chaperones [42], and protein solubility engineering [27, 28, 49, 50]. However, considering the simplicity and compatibility with the automated plasmid assembly platform, the use of a combinatorial library of fusion partners can be a cost-effective solution coupled with the laboratory automation. Indeed, a recent study has demonstrated the potential of this approach in construction of efficient synthetic pathways with aggregate-prone enzymes using a combinatorial library of different fusion partners in a systematic way [51].

The presented modular design has a lot of benefits in strain development. First, it enables rapid construction of combinatorial synthetic pathways. For example, Figure 2-1b well represents how the modular pathway design facilitates the construction of C4-esters producing strains in a plug-and-play fashion. Second, it allows quick evaluation of various engineering strategy. For example, the summary of C4-derived ester production in engineered strains shows how the various engineering strategy can be evaluated with the combinatorial modular synthetic pathways to improve biochemical production (Figure 2-19). Lastly, it also allows rapid calculation of theoretical yield of multiple products derived from the same intermediate. For example, the stoichiometric balance for synthesis of C4-derived esters (Figure 2-5, Figure 2-20).

In this study, we reported the development of a microbial platform for efficient production of C4-derived esters from renewable resources by one-step fermentation. Based on rational knowledge of the C4-derived ester pathways, we modularized a set of 11 genes into four parts, Submodule (SM) 1~4, to facilitate construction and validation of each aspect of the design for synthesis of three different target products with minimal experimental effort. Finally, our efforts led to the highest ester production for all targeted compounds including BA, EB, and BB ever reported in an engineered *E. coli*. Furthermore, the modular microbial ester synthesis platform presented is expected to accelerate strain development for biosynthesis of diverse natural esters with various industrial applications. This study also highlights a combinatorial protein solubilization as a metabolic engineering strategy as well as the importance of synthetic pathway-chassis compatibility in microbial production of biochemicals(fuels) by demonstrating the improved biochemical (ester) production via solubilizing pathway enzymes. Therefore, a combination of modular pathway design, a model-driven prediction of protein solubility, and laboratory automation with solubilizing aggregate-prone enzymes would help accelerate the strain

a

	Pathways	Reactions	#Modules
A	Glycolysis	Glucose $\rightarrow$ 2 Pyruvate + 2 NADH	Endogenous
B	Pfl reaction	Pyruvate $\rightarrow$ Acetyl-CoA + Formate	Endogenous
C	Fdh reaction	Formate $\rightarrow$ CO ₂ + NADH	3
D	Butyl-CoA synthesis	2 Acetyl-CoA + 2 NADH $\rightarrow$ Butyl-CoA	1
E	AdhE2 reaction	Butyl-CoA + 2 NADH $\rightarrow$ Butanol	2
F	PDC/adhE reaction	Pyruvate + NADH $\rightarrow$ Ethanol	2
G	BA synthesis	Butanol + Acetyl-CoA $\rightarrow$ Butyl acetate	4
H	EB synthesis	Ethanol + Butyl-CoA $\rightarrow$ Ethyl butyrate	4
I	BB synthesis	Butanol + Butyl-CoA $\rightarrow$ Butyl butyrate	4

b

Products	Stoichiometric equations	Net Reactions	Maximum theoretical yield*	
			Cmol/Cmol	g/g
BA	1.5A + 3B + 3C + D + E + G	1.5 Glucose + 2 NAD ⁺ $\rightarrow$ Butyl acetate + 3 CO ₂ + 2 NADH	0.67	0.43
EB	1.5A + 2B + 2C + D + F + H	1.5 Glucose + 2 NAD ⁺ $\rightarrow$ Ethyl butyrate + 3 CO ₂ + 2 NADH	0.67	0.43
BB	2A + 4B + 4C + 2D + E + I	2 Glucose + 2 NAD ⁺ $\rightarrow$ Butyl butyrate + 4 CO ₂ + 2 NADH	0.50	0.40

Figure 2-5: Stoichiometric balance for synthesis of C4-derived esters. **(a)** Reactions in modular biosynthetic pathways of C4-derived esters from glucose. **(b)** Net reactions for biosynthesis of butyl acetate, ethyl butyrate, or butyl butyrate from glucose. *Ester yield on glucose.

development.

Overall, this work offers a blueprint of efficient strain development for production of various biochemicals with increased carbon numbers and the degree of structural complexity.

2.6 References

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2.7 Appendix

Table 2-2: A list of plasmids used in this study. Abbreviations: MCS, multiple cloning site.

Plasmid	Primers (5'→3')	Cloning strategies
Module 1: Butyl-CoA synthesis		
pACYCDuet-1 P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} - P _{T7lac} ::ter _{Td}	CrCoA_F: CAGCAGCCATCACCATCATCACCACAGCCAGGATCCGATG AAAAATTGTGTCATCGTCAG CrCoA_R: CTATCTATTTTTGAAGCCTTCAATTT MCS1_MCS2 linker_F: GCTTTCATAGAGAAAAAATTGAAGGCTTCAAAAATA GATAGGAATTCGAGCTCGGC MCS1_MCS2 linker_R: ATTGTTCTTAACCATTTGGTTTACAATCATCATATGTATATC TCCTTCTTATACTTAACT ter _{Td} _F: AGTTAAGTATAAGAAGGAGATATACATATGATGATTGTAA AACCAATGGTTAGG ter _{Td} _R: TCAAATTTTCGAGCAGCGGTTTCTTTACCAGACTCGAGTTA AATCCTGTGCGAACCTTTCT BB_Duet_F: CGGATCCTGGCTGTGG BB_Duet_R: CTCGAGTCTGGTAAAGAAACC	Biosynthesis pathway of Butyl-CoA in pDL2 [1] was subcloned into the plasmids with various copy numbers by Gibson Assembly method [2]. Specifically, atoB _{Ec} - hbd _{Ca} - crt _{Ca} and Ter _{Td} were subcloned into the MCS1and MCS2 of the duet vectors, respectively.
pETDuet-1 P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} - P _{T7lac} ::ter _{Td}		
pRSFDuet-1 P _{T7lac} ::atoB _{Ec} ::hbd _{Ca} ::crt _{Ca} - P _{T7lac} ::ter _{Td}		
Module 2/3/4 for BA: Butanol+NADH+ATF1		
pACYCDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::ATF1 _{Sc}	adhE2 _{Ca} _F: GCCATCACCATCATCACCACAGCCAGGATCCGATGAAAGT TACAAATCAAAAAGAACTAA adhE2 _{Ca} _R: TATATCTCCTTTTAAAAATGATTTTATATAGATATCCTTAAGT TCAC fdh _{Cb} ^{opt} _F: AGGATATCTATATAAAATCATTTTAAAGGAGATATAATG AAAATTGTGCTGGTTTGTGA fdh _{Cb} ^{opt} _R: TTGTCGACCTGCAGGCGCGCCGAGCTCGAATTCCTATTTT TATCGTGTTTACCATACGC MCS1_MCS2 linker_BA_F: TAAGAATTCGAGCTCGGC MCS1_MCS2 linker_BA_R: GATTTCATTCATCATATGTATATCTCCTTCTTATACTTAACT ATF1 _{Sc} _F: ATATTAGTTAAGTATAAGAAGGAGATATACATATGATGAA TGAAATCGATGAGAAAAATC ATF1 _{Sc} _R: CGTTCAAATTTTCGAGCAGCGGTTTCTTTACCAGACTCGAG CTAAGGGCCTAAAAGGAGA BB_Duet_F: CGGATCCTGGCTGTGG BB_Duet_R: CTCGAGTCTGGTAAAGAAACC	Five PCR fragments including i) adhE2 _{Ca} from gDNA of <i>C. acetobutylicum</i> , ii) fdh _{Cb} ^{opt} from pET29 P _{T7lac} ::fdh _{Cb} ^{opt} , iii) MCS1_MCS2 linker from pACYCDuet-1, iv) ATF1 _{Sc} from , and v) plasmid backbone with various copy numbers were assembled by Gibson Assembly method [2]. Specifically, adhE2 _{Ca} -fdh _{Cb} and ATF1 _{Sc} were subcloned into the MCS1and MCS2 of the duet vectors, respectively.
pETDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::ATF1 _{Sc}		
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::ATF1 _{Sc}		
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::ATF1 _{Sc} ^{opt}	ATF1 _{Sc} ^{opt} _F: ATGAACGAAATCGACGAAAAAATCAG ATF1 _{Sc} ^{opt} _R: CGGACCCAGTAACAAAGCTTTATA BB_BA_ATF1 _{Sc} ^{opt} _F: GAGCTTTGTAGCATATATAAAGCTTTGTACTGGGTCCGCT CGAGTCTGGTAAAGAAAC BB_BA_ATF1 _{Sc} ^{opt} _R: GCGCCTGATTTTTTCGTCGATTTCGTTTCATCATATGTATAT CTCCTTCTTATACTTAAAC	ATF1 _{Sc} in pRSFDuet-1 P _{T7} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7} ::ATF1 _{Sc} was replaced with ATF1 _{Sc} ^{opt} from pET29 P _{T7} ::ATF1 _{Sc} ^{opt} by Gibson Assembly method [2].

Table 2-2: Continued

Plasmid	Primers (5'→3')	Cloning strategies
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::malE_ATF1 _{Sc} ^{opt}	malE_ATF1 _{Sc} ^{opt} _F; ATATTAGTTAAGTATAAGAAGGAGATATACATATGAT GAAAATCGAAGAAGGTAAGTGG malE_ATF1 _{Sc} ^{opt} _R; CCTGCTGAAGTGGCGCCTGATTTTTTCGTCGATTTCG TTCTTGGTGATACGAGTCTGC BB_BA_FP_ATF1 _{Sc} ^{opt} _F; AACGAAATCGACGAAAAAATCAG BB_BA_FP_ATF1 _{Sc} ^{opt} _R; CATATGTATATCTCCTTCTTATACTTAACTAATATACT	The start codon of ATF1 _{Sc} ^{opt} in pRSFDuet-1 P _{T7} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7} ::ATF1 _{Sc} ^{opt} was replaced with the <i>malE</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::nusA_ATF1 _{Sc} ^{opt}	nusA_ATF1 _{Sc} ^{opt} _F; ATATTAGTTAAGTATAAGAAGGAGATATACATATGAT GAACAAAGAAATTTTGGCTGTAG nusA_ATF1 _{Sc} ^{opt} _R; CACTCTGCTGAAGTGGCGCCTGATTTTTTCGTCGAT TTCGTTTCGCTTCGTCACCGAAC BB_BA_FP_ATF1 _{Sc} ^{opt} _F; AACGAAATCGACGAAAAAATCAG BB_BA_FP_ATF1 _{Sc} ^{opt} _R; CATATGTATATCTCCTTCTTATACTTAACTAATATACT	The start codon of ATF1 _{Sc} ^{opt} in pRSFDuet-1 P _{T7} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7} ::ATF1 _{Sc} ^{opt} was replaced with the <i>nusA</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt}	trxA_ATF1 _{Sc} ^{opt} _F; TATTAGTTAAGTATAAGAAGGAGATATACATATGATG AGCGATAAAATTATTCACCTGAC trxA_ATF1 _{Sc} ^{opt} _R; CTGCTGAAGTGGCGCCTGATTTTTTCGTCGATTTCGT TCGCCAGGTTAGCGTC BB_BA_FP_ATF1 _{Sc} ^{opt} _F; AACGAAATCGACGAAAAAATCAG BB_BA_FP_ATF1 _{Sc} ^{opt} _R; CATATGTATATCTCCTTCTTATACTTAACTAATATACT	The start codon of ATF1 _{Sc} ^{opt} in pRSFDuet-1 P _{T7} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7} ::ATF1 _{Sc} ^{opt} was replaced with the <i>trxA</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt}	adhE2 _{Ca} ^{opt} _F; ATGAAAGTGACCAATCAAAAAGAGC adhE2 _{Ca} ^{opt} _R; TTAGAAACTCTTTATGTAATGTCTTTGAGTTTCG BB_BA_adhE2 _{Ca} ^{opt} _F; CAAAGACATTTACATAAAGAGTTTCTAAAAGGAGATA TAATGAAAAATTGTGCTGGTTTTG BB_BA_adhE2 _{Ca} ^{opt} _R; GTTTCAGTTTCTGTTTCAGCTCTTTTTGATTGGTCACTT CATCGGATCCTGGCTGTGG	adhE2 _{Ca} in pRSFDuet-1 P _{T7} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7} ::trxA_ATF1 _{Sc} was replaced with adhE2 _{Ca} ^{opt} from pET29 P _{T7} :: adhE2 _{Ca} ^{opt} by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::malE_adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt}	malE_adhE2 _{Ca} ^{opt} _F; GCAGCCATCACCATCATCACACAGCCAGGATCCGAT GAAAATCGAAGAAGGTAAGTGG malE_adhE2 _{Ca} ^{opt} _R; CGTTTCAGTTTCTGTTTCAGCTCTTTTTGATTGGTCACTT TCTTGGTGATACGAGTCTGC BB_BA_FP_adhE2 _{Ca} ^{opt} _F; CGGATCCTGGCTGTGG BB_BA_FP_adhE2 _{Ca} ^{opt} _R; AAAGTGACCAATCAAAAAGAGCTG	The start codon of adhE2 _{Ca} ^{opt} in pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt} was replaced with the <i>malE</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::nusA_adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt}	nusA_adhE2 _{Ca} ^{opt} _F; GCAGCCATCACCATCATCACACAGCCAGGATCCGAT GAACAAAGAAATTTTGGCTGTAG nusA_adhE2 _{Ca} ^{opt} _R; CGTTTCAGTTTCTGTTTCAGCTCTTTTTGATTGGTCACTT TCGCTTCGTCACCGAAC BB_BA_FP_adhE2 _{Ca} ^{opt} _F; CGGATCCTGGCTGTGG BB_BA_FP_adhE2 _{Ca} ^{opt} _R; AAAGTGACCAATCAAAAAGAGCTG	The start codon of adhE2 _{Ca} ^{opt} in pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt} was replaced with the <i>nusA</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pRSFDuet-1 P _{T7lac} ::trxA_adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt}	trxA_adhE2 _{Ca} ^{opt} _F; CAGCCATCACCATCATCACACAGCCAGGATCCGATG AGCGATAAAATTATTCACCTGAC trxA_adhE2 _{Ca} ^{opt} _R; CGTTTCAGTTTCTGTTTCAGCTCTTTTTGATTGGTCACTT TCGCCAGGTTAGCGTC BB_BA_FP_adhE2 _{Ca} ^{opt} _F; CGGATCCTGGCTGTGG BB_BA_FP_adhE2 _{Ca} ^{opt} _R; AAAGTGACCAATCAAAAAGAGCTG	The start codon of adhE2 _{Ca} ^{opt} in pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt} was replaced with the <i>trxA</i> from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].

Table 2-2: Continued

Plasmid	Primers (5'→3')	Cloning strategies
Module 2/3/4 for EB: Ethanol+NADH+SAAT		
pACYCDuet-1 P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}	EtOH_F: GGCAGCAGCCATCACCATCATCACCACAGCCAGGATC CGATGAGTTATACTGTCGGTACC EtOH_R: CGCATCATACAAAACCAGCACAAATTTTCATTATATCTC CTTTTAGAAAGCGCTCAGGAAG fdh _{Cb} ^{opt} _F: ATGAAAATTGTGCTGGTTTTGTAATGAAAATTGTGCTG GTTTTGTA fdh _{Cb} ^{opt} _R: TTGTCGACCTGCAGGCGCGCCGAGCTCGAATTCTTATT TTTTATCGTGTTTACCATACGC MCS1_MCS2 linker_F: AAAATAAGAATTTCGAGCTCGGC MCS1_MCS2 linker_R: GGAATTTTACTGACCTCAATTTTCTCCATCATATGTA TATCTCCTTCTTATACTTAACT SAAT _{Fa} _F: AGTTAAGTATAAGAAGGAGATATACATATGATGGAGA AAATTGAGGTCAGTATA SAAT _{Fa} _R: AAATTTTCGAGCAGCGGTTTTCTTTACCAGACTCGAGTT AAATTAAGGTCTTTGGAGATGC BB_Duet_F: CGGATCCTGGCTGTGG BB_Duet_R: CTCGAGTCTGGTAAAGAAACC	Five PCR fragments including i) pdc _{Zm} -adhB _{Zm} from pCT24 [1], ii) fdh _{Cb} ^{opt} from pET29 P _{T7lac} ::fdh _{Cb} ^{opt} , iii) MCS1_MCS2 linker from pACYCDuet-1, iv) SAAT _{Fa} from pDL1 [1], and v) plasmid backbone with various copy numbers were assembled by Gibson Assembly method [2]. Specifically, pdc _{Zm} -adhB _{Zm} -fdh _{Cb} and SAAT _{Fa} were subcloned into the MCS1 and MCS2 of the duet vectors, respectively.
pETDuet-1 P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}		
pRSFDuet-1 P _{T7lac} ::pdc _{Zm} ::adhB _{Zm} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}		
Module 2/3/4 for BB: Butanol+NADH+SAAT		
pACYCDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}	adhE2 _{Ca} _F: GCCATCACCATCATCACCACAGCCAGGATCCGATGAA AGTTACAAATCAAAAAGAACTAA adhE2 _{Ca} _R: TATATCTCCTTTTAAAAATGATTTTATATAGATATCCTTA AGTTCAC BB_Duet_F: CGGATCCTGGCTGTGG BB_BB_R: AGGATATCTATATAAAATCATTTTAAAAGGAGATATA ATGAAAATTGTGCTGGTTTTGTA	pdc _{Zm} -adhB _{Zm} in EB production modules were replaced with adhE2 _{Ca} from gDNA of <i>C. acetobutylicum</i> by Gibson Assembly method [2].
pETDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}		
pRSFDuet-1 P _{T7lac} ::adhE2 _{Ca} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}		
pRSFDuet-1 P _{T7lac} ::trxA_adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} - P _{T7lac} ::SAAT _{Fa}	SAAT _{Fa} _F: AGTTAAGTATAAGAAGGAGATATACATATGATGGAGA AAATTGAGGTCAGTATA SAAT _{Fa} _R: AAATTTTCGAGCAGCGGTTTTCTTTACCAGACTCGAGTT AAATTAAGGTCTTTGGAGATGC BB_trxA_adhE2 _{Ca} ^{opt} _F: ATGTATATCTCCTTCTTATACTTAATAATACTAAG ATGG BB_Duet_R: CTCGAGTCTGGTAAAGAAACC	trxA_ATF1 _{Sc} ^{opt} in pRSFDuet-1 P _{T7lac} ::trxA_adhE2 _{Ca} ^{opt} ::fdh _{Cb} ^{opt} -P _{T7lac} ::trxA_ATF1 _{Sc} ^{opt} was replaced with the SAAT _{Fa} from pET29 P _{T7lac} ::SAAT _{Fa} by Gibson Assembly method [2].
Wildtype AATs		
pET29 P _{T7lac} ::ATF1 _{Sc}	-	Chapter 5

Table 2-2: Continued

Plasmid	Primers (5'→3')	Cloning strategies
pET29 P _{T7lac} ::SAAT _{Fa}	SAAT _{Fa} _F: TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAGATATGGAGAAAATTGAGGTCAG SAAT _{Fa} _R: GTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGGATAATTAAGGTCTTTGGAGATGC BB_pET29_F: ATCCACCACCACCACC BB_pET29_R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTATTCTAG	SAAT _{Fa} in pDL001 [3] was subcloned into pET29 by Gibson Assembly method [2].
Codon optimized genes from JGI		
pET29 P _{T7lac} ::ATF1 _{Sc} ^{opt}	univ_F: CCTCTAGAAATAATTTGTTTAACTTTAAGAAGGAG A univ_R: CGGATCTCAGTGGTGGTGGT	The plasmids were constructed by JGI. The synthesized genes were amplified and cloned into pET29 plasmid using the EcoRV restriction site. The genes were codon optimized to <i>E. coli</i> .
pET29 P _{T7lac} ::SAAT _{Fa} ^{opt}		
pET29 P _{T7lac} ::fdh _{Cb} ^{opt}		
pET29 P _{T7lac} ::adhE2 _{Ca} ^{opt}		
AATs with fusion partners		
pET29 P _{T7lac} ::malE_ATF1 _{Sc}	malE_ATF1 _{Sc} _F: TTTGTTTAACTTTAAGAAGGAGATATAGATATGAAAATCGAAGAAGGTAAACTGGTAATC malE_ATF1 _{Sc} _R: CTTGTTGCACGGGGCCTGATTTTCTCATCGATTTCATTCTGGTGATACGAGTCTGCG BB_pET29 ATF1 _{Sc} _F: AATGAAATCGATGAGAAAAATCAGGC BB_pET29 ATF1 _{Sc} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTATTCTAG	The start codon of ATF1 _{Sc} in pET29 P _{T7} ::ATF1 _{Sc} was replaced with malE from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pET29 P _{T7lac} ::nusA_ATF1 _{Sc}	nusA_ATF1 _{Sc} _F: ATTTTGTTTAACTTTAAGAAGGAGATATAGATATGACAAAGAAATTTGGCTGTAGTTG nusA_ATF1 _{Sc} _R: ATTCTTGTTGCACGGGGCCTGATTTTCTCATCGATTTCATTGCTTCGTCACCGAACC BB_pET29 ATF1 _{Sc} _F: AATGAAATCGATGAGAAAAATCAGGC BB_pET29 ATF1 _{Sc} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTATTCTAG	The start codon of ATF1 _{Sc} in pET29 P _{T7} ::ATF1 _{Sc} was replaced with nusA from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pET29 P _{T7lac} ::trxA_ATF1 _{Sc}	trxA_ATF1 _{Sc} _F: ATTTTGTTTAACTTTAAGAAGGAGATATAGATATGAGCGATAAAATTATTCACCTGACTG trxA_ATF1 _{Sc} _R: CATTCTTGTTGCACGGGGCCTGATTTTCTCATCGATTTCATTGCGCAGGTTAGCGTCG BB_pET29 ATF1 _{Sc} _F: AATGAAATCGATGAGAAAAATCAGGC BB_pET29 ATF1 _{Sc} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTATTCTAG	The start codon of ATF1 _{Sc} in pET29 P _{T7} ::ATF1 _{Sc} was replaced with trxA from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].

Table 2-2: Continued

Plasmid	Primers (5'→3')	Cloning strategies
pET29 P _{T7lac} ::malE_SAAT _{Fa}	malE_ATF1 _{Sc} _F: TTTGTTTAACTTTAAGAAGGAGATATAGATATGAA AATCGAAGAAGGTAAACTGGTAATC malE_ATF1 _{Sc} _R: TGATGGTGTGTTTGAATTTATACTGACCTCAATT TTCTCCTTGGTGATACGAGTCTGCG BB_pET29 SAAT _{Fa} _F: GAGAAAATTGAGGTCAGTATAAAATTCCAAAC BB_pET29 SAAT _{Fa} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTAT TTCTAG	The start codon of SAAT _{Fa} in pET29 P _{T7} ::SAAT _{Fa} was replaced with malE from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pET29 P _{T7lac} ::nusA_SAAT _{Fa}	nusA_ATF1 _{Sc} _F: ATTTTGTTTAACTTTAAGAAGGAGATATAGATATG AACAAAGAAATTTTGGCTGTAGTTG nusA_ATF1 _{Sc} _R: GTTTGATGGTGTGTTTGAATTTATACTGACCTCA ATTTTCTCCGCTTCGTCACCGAACC BB_pET29 SAAT _{Fa} _F: GAGAAAATTGAGGTCAGTATAAAATTCCAAAC BB_pET29 SAAT _{Fa} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTAT TTCTAG	The start codon of SAAT _{Fa} in pET29 P _{T7} ::SAAT _{Fa} was replaced with nusA from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
pET29 P _{T7lac} ::trxA_SAAT _{Fa}	trxA_ATF1 _{Sc} _F: ATTTTGTTTAACTTTAAGAAGGAGATATAGATATG AGCGATAAAATTATTCACCTGACTG trxA_ATF1 _{Sc} _R: GGTTTGATGGTGTGTTTGAATTTATACTGACCTC AATTTTCTCCGCCAGGTTAGCGTCG BB_pET29 SAAT _{Fa} _F: GAGAAAATTGAGGTCAGTATAAAATTCCAAAC BB_pET29 SAAT _{Fa} _R: ATCTATATCTCCTTCTTAAAGTTAAACAAAATTAT TTCTAG	The start codon of SAAT _{Fa} in pET29 P _{T7} ::SAAT _{Fa} was replaced with trxA from gDNA of <i>E. coli</i> MG1655 by Gibson Assembly method [2].
Co-expression of chaperones		
pACYC P _{araB} ::tig	-	The chaperone plasmid kit was purchased from TaKaRa Bio Inc. (Cat. #3340).
pACYC P _{araB} ::groES::groEL	-	
pACYC P _{pzt-1} ::groES::groEL::tig	-	
pACYC P _{araB} ::dnaK::dnaJ::grpE	-	
pACYC P _{araB} ::dnaK::dnaJ::grpE-P _{pzt-1} ::groES::groEL	-	
pACYC P _{araB} ::groES::groEL (Amp ^R)	Amp ^R _F: ATTAGTTAAGTATAAGAAGGAGATATACATATGA TGAACGAAATCGACGAAAAAATCAG Amp ^R _R: TTTCGCAGCAGCGGTTTCTTTACCAGACTCGAGCG GACCCAGTAACAAAGCTTTATATAT BB_groES/EL_F: CTCGAGTCTGGTAAAGAAACC BB_groES/EL_R: CATATGTATATCTCCTTCTTATACTTAACTAATATA CT	The chloramphenicol resistance marker of pACYC P _{araB} ::groES::groEL was replaced with the ampicillin resistance marker from pETDuet-1 by Gibson Assembly method [2].

Table 2-3: A list of enzymes used in this study.

#Module		Gene	Enzyme	Organism	Assigned function	#Accession (UniProt)
1	Butyl-CoA synthesis	AtoB _{Ec}	Acetyl-CoA acetyltransferase	<i>E. coli</i>	Synthesis of acetoacetyl-CoA from two acetyl-CoA	P76461
		Hbd _{Ca}	3-hydroxybutyryl-CoA dehydrogenase	<i>C. acetobutylicum</i>	Synthesis of 3-hydroxybutyryl-CoA from acetoacetyl-CoA	P52041
		Crt _{Ca}	Short-chain-enoil-CoA hydratase	<i>C. acetobutylicum</i>	Synthesis of crotonyl-CoA from 3-hydroxybutyryl-CoA	P52046
		Ter _{Td}	Trans-2-enoil-CoA reductase	<i>T. denticola</i>	Synthesis of butyryl-CoA from crotonyl-CoA	Q73Q47
2	Alcohol synthesis	AdhE2 _{Ca}	Aldehyde-alcohol dehydrogenase	<i>C. acetobutylicum</i>	Synthesis of butanol from butyryl-CoA	Q9ANR5
		AdhE2 _{Ca} ^{opt}	Codon optimized adhE2 _{Ca} for expression in <i>E. coli</i>	-	Improving soluble expression of adhE2 _{Ca}	-
		TrxA_AdhE2 _{Ca} ^{opt}	adhE2 _{Ca} ^{opt} tagged with TrxA	-		-
		PdC _{Zm}	Pyruvate decarboxylase	<i>Z. mobilis</i>	Synthesis of acetaldehyde from pyruvate	P06672
		AdhB _{Zm}	Alcohol dehydrogenase	<i>Z. mobilis</i>	Synthesis of ethanol from acetaldehyde	P0DJA2
3	NADH synthesis	Fdh _{Ch} ^{opt}	Formate dehydrogenase	<i>C. boidinii</i>	Synthesis of NADH from NAD ⁺	O13437
4	Ester synthesis	ATF1 _{Sc}	Alcohol O-acetyltransferase	<i>S. cerevisiae</i>	Synthesis of an acetate ester from an alcohol and acetyl-CoA.	P40353
		ATF1 _{Sc} ^{opt}	Codon optimized ATF1 _{Sc} for expression in <i>E. coli</i>	-	Improving soluble expression of ATF1 _{Sc}	-
		MBP_ATF1 _{Sc}	ATF1 _{Sc} tagged with MBP without the N-terminus signal sequence	-		-
		NusA_ATF1 _{Sc}	ATF1 _{Sc} tagged with NusA	-		-
		TrxA_ATF1 _{Sc}	ATF1 _{Sc} tagged with TrxA	-		-
		TrxA_ATF1 _{Sc} ^{opt}	ATF1 _{Sc} ^{opt} tagged with TrxA	-		-
		SAAT _{Fa}	Alcohol O-acetyltransferase	<i>F. ananassa</i>	Synthesis of a butyrate ester from an alcohol and butyl-CoA.	Q9FVF1
		SAAT _{Fa} ^{opt}	Codon optimized SAAT _{Fa} for expression in <i>E. coli</i>	-	Improving soluble expression of SAAT _{Fa}	-
		MBP_SAAT _{Fa}	SAAT _{Fa} tagged with MBP	-		-
		NusA_SAAT _{Fa}	SAAT _{Fa} tagged with NusA	-		-
		TrxA_SAAT _{Fa}	SAAT _{Fa} tagged with TrxA	-		-
etc.	Fusion tags	MBP	Maltose binding protein without the N-terminus signal sequence (KIKTGARILALSALTTM MFSASALA)	<i>E. coli</i>	Improving the solubility of heterologous proteins[4]	P0AEX9
		NusA	N-Utilization substrate A	<i>E. coli</i>		P0AFF6
		TrxA	Thioredoxin 1	<i>E. coli</i>		P0AA25
	Chaperones	Tf	Trigger factor	<i>E. coli</i>	Protein folding. Acts as a mechanical foldase by interacting directly with the newly synthesized proteins[5]	P0A850
		DnaK	Chaperone protein	<i>E. coli</i>	Unfolding, disaggregation, stabilization of extended chains[6].	P0A6Y8
		DnaJ	Chaperone protein	<i>E. coli</i>		P08622
		GrpE	Protein GrpE	<i>E. coli</i>		P09372
		GroES	10 kDa chaperonin	<i>E. coli</i>	Protein folding, prevention of aggregation[6].	P0A6F9
		GroEL	60 kDa chaperonin	<i>E. coli</i>		P0A6F5

Table 2-4: A list of codon optimized gene sequences.

Gene	Sequences
ATF1 ^{opt}	ATGAACGAAATCGACGAAAAAATCAGGCGCCAGTTCAGCAGGAGTGTCTGAAAGAAATGATTCAAATGGTCATGCGCGTAGA ATGGGTTTCAGTGGAGGATTTGTACGTTGCGCTGAATCGCCAAAAACCTTTACCGTAACCTTTTGACGATGTTGGTGTAGCTGTGAGATT ACTGCACTCGTGATCAGTTGACGCTGGCGCTCCGTGAAATCTGCTTAAAAAACCCGACTCTTCTGCATATTTGTGTTACCGGACGCG CTGGCCGAACCCAGAAAACTATTATAGAAGCAGTGAATATTACAGCCGTCCTCACCTGTGCACGACTACATATCGGTCTTACAG GAATTAAGAGCTGTGCGGCGTGGTGTGAATGAACAACTGAATACTCAGCGGTTATGAAACAGATTCTGGAAGAATTCAAAAATA GCAAGGGCAGCTATACGGCTAAGATCTTTAACTTACCACCACCCTGACAATCCCATACTTCGGCCCCACAGGCCCATCTTGGCG CTTGATTTGTTTGGCCGAAGAGCACACTGAAAAATGGAAGAAATTCATATTTGTGAGCAATCATTGCATGTGAGATGGCGGCTCC TCCATTCTTTCTCCAGATCTGCGTGACGAACTTAACAATATTAATACTCCGCCGAAGAAGCTAGATTACATCTTCAAAATACGA AGAAGACTACCAGTCTGCGTAACTGCCGAGCCGATCGAAAAAGTGATTGATTTTCGTCGCCGCTATCTTTTATTCCTAAA TCCCTCTTATCGGGATTTATATACAATCACCTTCGATTTTCACTAAAAAGGGGTATGTATGCGCATGGATGATGTGGAAGAGACGG ACGACGTCGTTTACCGAAATCATTAATATTAGCCGAGCTGAATTTCAAGCGATAAAAGCAAACATCAAAATCGAACATCCAGGGCAA ATGTACCATTAATCTCCCTTTCTCCATGTTTGTGGTTCGTGTCTGCTGATAAATGGGGCAAGTCTTTAAACCTCTGAACCTTTGAAT GGCTGACTGATATTTTATCCCTGCGGACTGCCGCTCTCAGTTGCCTGATGACGATGAAATGCGCCGAGATGTACCGGTATGGTGC GAATGTGGGCTTCATTGACTTCACTCGGTGGATCAGCGAATTTGACATGAATGATAACAAAGAAATTTTGGCCAGGATTGAG CACTAATCATGAGGTGATCAGTGAGGCGCTTCGTAACAAAAAAGTATGCTGATGGTTTGGGTTTCAACATCAGGGTTCTGTACAA AGTACGTGAACATAGATAAAGTCAATGTGTGACGGGCTATTGTGTAACGTCGTGGCGGTACTCTACTCAGCAATGTTGGTCTGTT CAATCAACTTGAAGAACCTGACGCCAAATATTCTATTTGCGACTTAGCTTTTGGCCAATTCCAGGGCAGCTGGCACCAGGCTTTT AGTCTCGGTGTGTCTACGAATGTTAAAGGTATGAATATGCTGCTGCGCTGCGACAAAAACGTCGTAGGTTACAGGAGTCCC TGGAAGAGCTTTGTAGCATATATAAAGCTTTGTACTGGGTCG
SAAT ^{opt}	ATGGAAAAATCGAAGTCTCAATTAACCTCAAGCATACCATCAACCCCTCTACCTCTAGTACCCCGCTACAACCCCTATAAACTGA CCTTACTGGATCAGCTGACGCCACCAGCCTATGTCCCGATCGTGTGTTTTTACCCGATCACCGATCACGATTTTAATCTGCCGCA GAGCTGCGGCACTTACGTCAAGCTCTGTGCGGAGACTCTCAGCGTGTATTATCCCTGAGCGGACGTGTAAACAAATTTGTTCTAAA ATTGATGACTTTGAGGAAGGCGTGCCGTACTTAGAAGCGCGGTGTAATTTGCGATATGACCGATTTTCTGCGTCTGCGCAAAATG AGTGCTTGAACGAATTTGTACCGATTAAACCTTTTCAATGATGAAGCAATTAGCGATGAACGTTACCCGCTGTGGGCGTCCAGGT TAATGTTTGTGATTCCGGCATCGCGATCGGAGTGTGACGTAGTCAATAAATGATAGATGGTGGCAGCGGACGATTTTCTCTAAA TCTTGGGGCGCGTGTTCGTGGCTGCCGTGAAAAATATTACACCCGAGTCTGAGTGAGGCTGCGCTGTTGTCCCCACAGC GATGACCTGCCAGAAAAATACGTTGACCAATGAGGCGCTTTCGCTGAGTAAAGTCTGCGGGGAAAAAAGTCGCCACGCTGTTGTTT GGTGTGAAGCGATTAGCAGCATTCAAGACGAAGCAAAATCTGAGAGCGTCCCAAAACCGTCTCGCTTCATGCCGTGACAGGC TTCCTATGGAAGCAGCTGATAGCTGCGTCCCGCGCATTGACAAGTGGGACTACATCGACCCGCTCTGCAATTGCCGCGCAAGCA GTAAACCTGCGTACTGCGCATGAACATGGAACCCGTTTATGATAACGCTACGGGTAACCTGTCTGTTGGTGGGCCAGGCGATCCTA GAACTGTGCGATACCAACCCCGGAGATTTCTGACCTTAACTGTGTGATCTGGTTAACTTGCTGAACGGATCCGTCAGCAGTGCA ATGGCGACTATTTCCAGACCTTTAAAGTAAAGAAGGCTATGCGCGGATGTGTGAATACTTGGATTTCCAGCGAACAATGTGCTC CATGGAACCGGCTCCGACATTTATCTGTTTCTATCCTGACTAATCTTTTAAACCGTGGATTTCCGGCTGGGGCCGACCTCC TGGATTGGAGTTGACAGTAAATGAGAGTGCCTCTGCAAAATTTAATCCTCTGTCGCCACACAGTGCGGTTCCGGGTATCGAG GCGTGGTCAACTTGGAGGAAGAAAAATGGCCATGCTCGAACAGGATCCGCAATTTCTAGCGCTGGCGTCACTAAAACCGTG ATC
adhE2Ca ^{opt}	ATGAAAGTGACCAATCAAAAAGAGCTGAAACAGAAATGAACGAACCTTCGTGAAGCGCAAAAAAATTCGCAACCTACACGCAG GAGCAGGTGGATAAAATTTTCAAGCAGTGTGCGATTGCGGCGCGGAAAGAGCGGATTAACCTGGCGAAGCTGGCGGTGGAAGA AACCGGGATTGGGCTGGTGGAGGACAAAATCATCAAGAACCATTTTCCCGCGGAATACATTTATAACAAGTACAAAAACGAAAA GAGCTGTGGTATTATTGATCAGCATGACTCCCTGGGCATCAGGAAGGTGGCAGAACCGATCCGTATCGCTCGCTGCGATCGTCT TAGACCAATCCACGTCAACCGCGCATCTTTAAAGTTTGAATGAGCTGAAAAACTCGTAACGCCATCTTCTTTTCCCGCCACCC CGTGCGAAAAAGAGCACCATCGCGGCCGCGAAACCTATCTTGGATGCTGCGGTGAAGGCGGGGGCCCGAAGAATCATTTGGC TGGATCGACGAGCCGTCAATTGAGCTCAGCCAAGACCTGATGAGCGAAGCGGATATTATCTGGCGACAGGCGGTCCGAGCATG GTTAAAGCGGCTACTCGTCTGGCAACCCAGCCATTGGTGTGAGCGCGCAACACTCGCGCAATTTATGATGAGAGCGCCGAT ATCGATATGCGCGTTAGTAGCATCATCTGTCTAAAACCTACGACAATGGTGTGATCTGCGCGAGCGCAACAAAGTATTCTCGTCA TGAACCTCTATGCTAATAAGGTGAAAGAGAGTTTGTGAAGCGTGTGAGCTATATCTGAAACGAAACGAAATGCAAAATG AGAAACAATGTTTAAAGATGAGCTATCAATGCCGATATCTGTTGTAAGTGCCTATATTATCGCGAAATGGCTGGCATCGAA GTCCCGCAAACTACCAAAATTTTAAATGGTGAAGTTCAGAGCGTGAAAAAATCGGAGTGTGTTTCCACGAAAAAATGTCGCGG TATTAGCAATGTATAAAGTAAAGATTTTGACGAAGCCCTGAAAAAGGCCAGCGCTTAATTTAGGTAGGCGGCTGTCGCTGATAC CTCATCGCTGATCATGATAGCCAGAACATAAAGATAAAGTTAAAGAAATTTGGCCTGGCCATGAAGACAAGTCGACGTTATC AATATGCCCTCAAGCCAGGCGCCTCGGGTATCTTTAATTTGCAATCGCGCGAGCTTCACTCTGGGTTGTGGACGTGGG GTGGCAATAGCTCTCACAGAATGCGAACCGGACATTACTGAAACATCAATCAGTCCGCGAAGCCGCTGAAACGATCGTGT GGTTCAAAGTCCGCGCAAAAAATCTATTTCAAGTATGGTTCGCTGCGATTGCGACTGAAGGAATGAAAGATATGAACAAAAACG TGCGTTTATTGTTACCGGATAAGGACCTGTTTAAACTGGGCTATGTGAACAAAAATTAATAAGTCTGGAATTTGATTAAT ACTCGATCTTACCGATATTAAGAGTGACCTACGATCGATAGCGTGAAGAAAGGCGCGAAGAAATGCTTAACCTTTGAACCGGA TACCATTATCAGTATTGGGGGTGGTCTCCGATGGATGCGGCCAAAGTTATGATCTGTTGATGAGTATCCGGAAGCCGAGATT GAGAACCTGGCTATCAATTTTATGACATCCGAAGCGGATCTGCAACTTCCCAAGTTAGGTACGAAAGCCATTAGCGTAGCCA TTCCTACCACGCGGGTACCGGTAGTGAGGCCACGCGCTTCCGCGGTGATTACCAATGATGAGACCGGTATGAAATACCGGTGA CCAGCTATGAATTAACACCAACATGGCAATTATCGATACGGAATGATGCTGAACATGCCTCGTAACTTACAGCGCGGACTGG GATTGATGCCCTGGTTACGCGATTGAAGCATACGTGTAGTGTGATGGCAACCGATTATACCGATGAGCTGCGCGCTAT CAAAATGATCTTCAATATCTGCGCGTGCCCTACAAAACCGCACCAATGATATCGAAGCTCGAGAAAAATGGCGCATGCCTCA AACATCGCAGGGATGGCATTTCGCGAACGCGTTTCTGGGCGTGTGCCACTCTATGGCACACAAACTGGGTGCGATGCAACATGTG CCACACGGCATTCATGTGTGTGTTAATTGAAGAAGTTATTAATATAATGCGACCGACTGCCCAACCAACAAACCGCGTTTC CGCAATACAAAAGCCGAATGCTAAGCGTAAATACGCTGAAATTTGGGAATATCTGAATCTGAAAGGCACCTCTGATACCGAGAA AGTGACCGCCCTGATTGAAGCTATCTCTAAGTTAAAAATGATCTGAGCATCCCGCAGAACATCTCGGCAGCTGGGATCAATAAA AAAGATTTTACAATACCTTGGATAAAATGCTGAACTCGCGTTCGACGATCAATGCACAACCGCAACCCACGCTATCCGCTGA TCTCGAATCAAGACATTTACATAAAGAGTTTC
fdh _{Cb} ^{opt}	ATGAAAATTGTGCTGTTTTGTATGATGTCGGGAAAGCATGCGCGGACGAAAGAAAGCTTTATGGGTGTACCGAAAAACAACTG GGCATTGTCTAACTGGCTGAAAGACAGGGTTCATGAACCTATACCACGAGCGATAAAGAAGGCGAAACCTCCGAATTAGCAAG CATATTTCCGATCGCGACATTATTACCACTCCGTTTACCCCGGCATACATTACCAAGAAGCTTTAGATGAATGAAGTCAAAAC TGAAACTGGTCTGCTGCGCGGTGTCGGCAGTGATCACATTGATTTAGATTATATTAACAGACGGGCAAAAAATCTCCGTGTT AGAGGTAACAGGTAGCAACGTCGTCTCAGTGGCGGAACACGTGGTAATGACCATGCTAGTTTGGTCCGTAACCTTTGTTCCGG ACATGAACAAATATCAATCATGACTGGGAGTTCGCGGCTGATGCGAAAGATGCTACGATATTGAGGCAAAACCATTCGCGAC TATTGGTGGCGGCGCATCGGTTATCTGTACTTGAACGCTTCTTCCGTTTAAACCGAAGGAGCTGTTGTACTACGACTACCA GCGTTTACCAAAAGAGGCTGAAGAAAAAGTGGGCGCGCGCGTGTGAGAATATTGAGGAACCTGTTGACAGGCGAGATTTGTT ACCGTGAATGCGCGCTGATGACGCGGGAACCAAGGTTTGTGACCAAAAGAACTACTGAGTAAATTTAAAAAGCGCTGGCT GTGAATACAGCTCGTGGTGCATTTGCGTAGCTGAAGATGTGGCGCGACACTGGAGAGCGGTGACGTGCGCGGTATGGGG CGACGTGTGTTTCCCAACCGCACCTAAAGATCACTCTGCGGTGATATGCGTAACAAATACCGCGGACCAATGCTATGAC CCGCGATTACTCCGCGCACACCTTGGACGCAAACTCGCTACGCGGAAGGTAAGAACATTCTGGAGTCTGTTTTTACGGGC AAGTTTGATTACCGACCGCAGGATATAATCTTCTGAACGGTGAATACGTGACCAAGGCGTATGTTAAACACGATAAAAAA

Table 2-5: Summary of validation of biosynthesis pathway of BA. Key strain is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strains		EcJWBA1	EcJWBA2	EcJWBA3	EcJWBA4	EcJWBA5	EcJWBA6
O.D. ₆₀₀		2.13 ± 0.28	2.06 ± 0.05	2.34 ± 0.21	1.94 ± 0.02	1.20 ± 0.08	2.50 ± 0.13
Consumed glucose, g/L (mM)		5.41 ± 0.43 (30.05 ± 2.37)	4.20 ± 0.25 (23.31 ± 1.39)	6.03 ± 0.13 (33.49 ± 0.72)	5.26 ± 0.10 (29.20 ± 0.56)	2.57 ± 0.16 (14.28 ± 0.91)	6.63 ± 0.05 (36.83 ± 0.30)
Lactate, g/L (mM)		0.30 ± 0.07 (3.37 ± 0.75)	0.18 ± 0.02 (2.00 ± 0.20)	0.26 ± 0.06 (2.84 ± 0.62)	0.18 ± 0.04 (2.03 ± 0.40)	0.18 ± 0.01 (2.01 ± 0.08)	0.23 ± 0.06 (2.51 ± 0.63)
Formate, g/L (mM)		0.51 ± 0.02 (11.10 ± 0.52)	0.38 ± 0.01 (8.23 ± 0.20)	0.50 ± 0.02 (10.77 ± 0.33)	0.54 ± 0.02 (11.77 ± 0.36)	0.49 ± 0.01 (10.61 ± 0.12)	0.66 ± 0.04 (14.42 ± 0.94)
Acetate, g/L (mM)		0.62 ± 0.02 (10.37 ± 0.39)	0.65 ± 0.00 (10.87 ± 0.02)	0.54 ± 0.00 (9.02 ± 0.06)	0.52 ± 0.00 (8.68 ± 0.03)	0.40 ± 0.00 (6.74 ± 0.02)	0.59 ± 0.02 (9.90 ± 0.32)
Ethanol, g/L (mM)		2.46 ± 0.12 (53.45 ± 2.70)	2.41 ± 0.03 (52.38 ± 0.69)	3.00 ± 0.08 (65.17 ± 1.82)	2.54 ± 0.08 (55.21 ± 1.63)	2.38 ± 0.03 (51.65 ± 0.68)	2.46 ± 0.02 (53.34 ± 0.54)
Butanol, g/L (mM)		0.04 ± 0.01 (0.58 ± 0.12)	0.06 ± 0.00 (0.84 ± 0.01)	0.03 ± 0.00 (0.45 ± 0.01)	0.03 ± 0.00 (0.42 ± 0.01)	0.11 ± 0.01 (1.43 ± 0.13)	0.05 ± 0.01 (0.67 ± 0.08)
Ester titers, mg/L (mM)	EA	5.7 ± 1.3 (0.06 ± 0.01)	11.9 ± 1.9 (0.13 ± 0.02)	3.7 ± 0.5 (0.04 ± 0.01)	0.8 ± 0.1 (0.01 ± 0.00)	0.2 ± 0.0 (0.00 ± 0.00)	1.3 ± 0.5 (0.01 ± 0.01)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	12.9 ± 5.9 (0.11 ± 0.05)	34.2 ± 6.6 (0.29 ± 0.06)	3.8 ± 0.8 (0.03 ± 0.01)	2.1 ± 0.3 (0.02 ± 0.00)	0.7 ± 0.12 (0.01 ± 0.00)	10.5 ± 4.6 (0.09 ± 0.04)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	18.6 ± 7.2 (0.18 ± 0.06)	46.1 ± 8.5 (0.43 ± 0.08)	7.5 ± 1.3 (0.07 ± 0.01)	2.9 ± 0.4 (0.03 ± 0.00)	0.9 ± 0.2 (0.01 ± 0.00)	11.8 ± 5.0 (0.11 ± 0.04)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.23 ± 0.02 (2.59 ± 0.27)	0.50 ± 0.07 (5.62 ± 0.82)	0.14 ± 0.03 (1.57 ± 0.33)	0.04 ± 0.00 (0.40 ± 0.04)	0.01 ± 0.00 (0.16 ± 0.01)	0.05 ± 0.02 (0.51 ± 0.18)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	0.51 ± 0.16 (4.37 ± 1.36)	1.43 ± 0.26 (12.31 ± 2.21)	0.14 ± 0.04 (1.22 ± 0.37)	0.09 ± 0.01 (0.78 ± 0.12)	0.05 ± 0.01 (0.42 ± 0.08)	0.36 ± 0.15 (3.12 ± 1.33)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	0.74 ± 0.18 (6.95 ± 1.60)	1.93 ± 0.33 (17.93 ± 3.04)	0.28 ± 0.07 (2.79 ± 0.69)	0.13 ± 0.02 (1.18 ± 0.16)	0.06 ± 0.01 (0.58 ± 0.08)	0.41 ± 0.17 (3.63 ± 1.50)
Ester yields, mg/g glucose (mM/M)	EA	1.04 ± 0.16 (2.13 ± 0.32)	2.84 ± 0.60 (5.81 ± 1.23)	0.62 ± 0.09 (1.26 ± 0.18)	0.15 ± 0.02 (0.31 ± 0.04)	0.08 ± 0.01 (0.16 ± 0.03)	0.20 ± 0.07 (0.40 ± 0.14)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	2.33 ± 0.86 (3.62 ± 1.33)	8.22 ± 2.02 (12.75 ± 3.13)	0.63 ± 0.15 (0.98 ± 0.24)	0.39 ± 0.07 (0.60 ± 0.11)	0.27 ± 0.08 (0.42 ± 0.13)	1.59 ± 0.70 (2.46 ± 1.08)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	3.38 ± 1.01 (5.75 ± 1.63)	11.06 ± 2.62 (18.56 ± 4.36)	1.25 ± 0.24 (2.24 ± 0.41)	0.54 ± 0.09 (0.92 ± 0.15)	0.35 ± 0.10 (0.58 ± 0.15)	1.78 ± 0.77 (2.86 ± 1.23)

Table 2-6: Summary of validation of biosynthesis pathway of EB. Key strain is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strains		EcJWEB1	EcJWEB2	EcJWEB3	EcJWEB4	EcJWEB5	EcJWEB6
O.D. ₆₀₀		2.12 ± 0.03	3.39 ± 0.26	3.86 ± 0.24	3.02 ± 0.08	0.93 ± 0.02	2.79 ± 0.06
Consumed glucose, g/L (mM)		7.53 ± 0.13 (41.79 ± 0.72)	7.21 ± 0.39 (40.02 ± 2.19)	19.61 ± 0.20 (108.86 ± 1.10)	13.40 ± 0.22 (74.40 ± 1.24)	2.77 ± 0.37 (15.37 ± 2.05)	9.30 ± 0.39 (51.62 ± 2.16)
Lactate, g/L (mM)		0.02 ± 0.03 (0.22 ± 0.34)	<i>n.d.</i>	0.19 ± 0.01 (2.10 ± 0.16)	0.18 ± 0.00 (1.95 ± 0.05)	<i>n.d.</i>	0.06 ± 0.00 (0.62 ± 0.01)
Formate, g/L (mM)		0.44 ± 0.01 (9.63 ± 0.16)	0.17 ± 0.01 (3.79 ± 0.12)	0.61 ± 0.03 (13.32 ± 0.57)	0.69 ± 0.02 (14.96 ± 0.39)	0.18 ± 0.02 (3.93 ± 0.46)	0.56 ± 0.01 (12.23 ± 0.32)
Acetate, g/L (mM)		0.48 ± 0.01 (7.94 ± 0.12)	0.37 ± 0.00 (6.10 ± 0.04)	0.56 ± 0.01 (9.25 ± 0.09)	0.53 ± 0.00 (8.80 ± 0.07)	0.36 ± 0.00 (6.03 ± 0.06)	0.49 ± 0.01 (8.16 ± 0.11)
Ethanol, g/L (mM)		4.92 ± 0.11 (106.72 ± 2.32)	5.16 ± 0.14 (111.92 ± 2.96)	10.89 ± 0.10 (236.41 ± 2.13)	7.25 ± 0.04 (157.34 ± 0.90)	2.84 ± 0.04 (61.66 ± 0.89)	5.29 ± 0.15 (114.85 ± 3.27)
Butanol, g/L (mM)		<i>n.d.</i>	<i>n.d.</i>	0.02 ± 0.00 (0.25 ± 0.02)	<i>n.d.</i>	0.03 ± 0.00 (0.40 ± 0.02)	<i>n.d.</i>
Ester titers, mg/L (mM)	EA	0.5 ± 0.2 (0.01 ± 0.00)	4.9 ± 0.5 (0.06 ± 0.01)	7.4 ± 1.7 (0.08 ± 0.02)	2.5 ± 0.4 (0.03 ± 0.00)	0.1 ± 0.1 (0.00 ± 0.00)	1.8 ± 0.3 (0.02 ± 0.00)
	EB	17.1 ± 3.4 (0.15 ± 0.03)	71.0 ± 6.6 (0.61 ± 0.06)	21.7 ± 2.9 (0.19 ± 0.03)	8.4 ± 1.0 (0.07 ± 0.01)	1.4 ± 0.2 (0.01 ± 0.00)	15.8 ± 1.3 (0.14 ± 0.01)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	<i>n.d.</i>	1.3 ± 0.7 (0.01 ± 0.00)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	17.6 ± 3.2 (0.15 ± 0.03)	77.2 ± 7.7 (0.68 ± 0.07)	29.1 ± 4.7 (0.27 ± 0.04)	10.9 ± 1.3 (0.10 ± 0.01)	1.5 ± 0.1 (0.01 ± 0.00)	17.6 ± 1.6 (0.16 ± 0.01)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.02 ± 0.01 (0.22 ± 0.10)	0.12 ± 0.02 (1.42 ± 0.17)	0.17 ± 0.05 (1.88 ± 0.57)	0.07 ± 0.01 (0.82 ± 0.14)	0.01 ± 0.01 (0.07 ± 0.07)	0.05 ± 0.01 (0.61 ± 0.11)
	EB	0.69 ± 0.13 (5.98 ± 1.10)	1.81 ± 0.23 (15.60 ± 1.95)	0.49 ± 0.10 (4.20 ± 0.85)	0.24 ± 0.02 (2.05 ± 0.20)	0.13 ± 0.01 (1.09 ± 0.12)	0.49 ± 0.05 (4.20 ± 0.43)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	<i>n.d.</i>	0.03 ± 0.02 (0.23 ± 0.12)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	0.71 ± 0.12 (6.19 ± 1.00)	1.97 ± 0.26 (17.25 ± 2.24)	0.65 ± 0.15 (6.08 ± 1.41)	0.31 ± 0.03 (2.87 ± 0.31)	0.13 ± 0.01 (1.16 ± 0.05)	0.54 ± 0.06 (4.81 ± 0.53)
Ester yields, mg/g glucose (mM/M)	EA	0.06 ± 0.03 (0.13 ± 0.06)	0.68 ± 0.03 (1.39 ± 0.06)	0.38 ± 0.09 (0.77 ± 0.19)	0.19 ± 0.04 (0.39 ± 0.07)	0.02 ± 0.02 (0.05 ± 0.04)	0.19 ± 0.04 (0.39 ± 0.07)
	EB	2.27 ± 0.43 (3.53 ± 0.66)	9.84 ± 0.42 (15.26 ± 0.65)	1.11 ± 0.16 (1.72 ± 0.25)	0.62 ± 0.07 (0.97 ± 0.12)	0.51 ± 0.14 (0.79 ± 0.21)	1.71 ± 0.21 (2.65 ± 0.33)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	<i>n.d.</i>	0.18 ± 0.08 (0.22 ± 0.10)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	2.34 ± 0.40 (3.65 ± 0.60)	10.70 ± 0.53 (16.87 ± 0.81)	1.48 ± 0.25 (2.49 ± 0.44)	0.81 ± 0.10 (1.35 ± 0.18)	0.53 ± 0.11 (0.84 ± 0.17)	1.90 ± 0.24 (3.03 ± 0.40)

Table 2-7: Summary of validation of biosynthesis pathway of BB. Key strain is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strains		EcJWBB1	EcJWBB2	EcJWBB3	EcJWBB4	EcJWBB5	EcJWBB6
O.D. ₆₀₀		2.31 ± 0.10	1.96 ± 0.02	2.61 ± 0.08	2.31 ± 0.06	1.24 ± 0.05	2.01 ± 0.07
Consumed glucose, g/L (mM)		6.98 ± 0.17 (38.72 ± 0.96)	3.90 ± 0.26 (21.63 ± 1.43)	10.46 ± 0.15 (58.04 ± 0.86)	8.90 ± 1.05 (49.42 ± 5.82)	1.75 ± 0.04 (9.71 ± 0.20)	5.15 ± 0.21 (28.60 ± 1.18)
Lactate, g/L (mM)		0.21 ± 0.01 (2.32 ± 0.14)	0.09 ± 0.01 (1.05 ± 0.06)	0.17 ± 0.01 (1.87 ± 0.15)	0.16 ± 0.02 (1.76 ± 0.17)	0.10 ± 0.01 (1.15 ± 0.13)	0.17 ± 0.01 (1.87 ± 0.11)
Formate, g/L (mM)		0.49 ± 0.01 (10.56 ± 0.14)	0.36 ± 0.01 (7.82 ± 0.17)	0.56 ± 0.02 (12.25 ± 0.50)	0.63 ± 0.02 (13.70 ± 0.40)	0.40 ± 0.05 (8.67 ± 1.19)	0.61 ± 0.01 (13.27 ± 0.15)
Acetate, g/L (mM)		0.54 ± 0.00 (8.93 ± 0.03)	0.46 ± 0.00 (7.71 ± 0.05)	0.53 ± 0.01 (8.87 ± 0.10)	0.52 ± 0.02 (8.59 ± 0.32)	0.40 ± 0.01 (6.64 ± 0.09)	0.49 ± 0.00 (8.15 ± 0.04)
Ethanol, g/L (mM)		3.04 ± 0.07 (65.93 ± 1.53)	2.66 ± 0.09 (57.82 ± 1.88)	3.68 ± 0.08 (79.96 ± 1.74)	2.91 ± 0.16 (63.23 ± 3.44)	2.47 ± 0.30 (53.58 ± 6.42)	2.50 ± 0.03 (54.21 ± 0.71)
Butanol, g/L (mM)		0.11 ± 0.01 (1.54 ± 0.15)	0.13 ± 0.00 (1.71 ± 0.06)	0.02 ± 0.00 (0.24 ± 0.00)	0.04 ± 0.03 (0.54 ± 0.43)	0.09 ± 0.02 (1.27 ± 0.29)	0.13 ± 0.01 (1.76 ± 0.12)
Ester titers, mg/L (mM)	EA	0.7 ± 0.1 (0.01 ± 0.00)	1.5 ± 0.3 (0.02 ± 0.00)	4.6 ± 0.5 (0.05 ± 0.01)	1.1 ± 0.2 (0.01 ± 0.00)	<i>n.d.</i>	0.4 ± 0.6 (0.00 ± 0.01)
	EB	43.6 ± 6.1 (0.38 ± 0.05)	126.2 ± 11.5 (1.09 ± 0.10)	5.3 ± 0.1 (0.05 ± 0.00)	1.6 ± 0.2 (0.01 ± 0.00)	0.4 ± 0.7 (0.00 ± 0.01)	4.6 ± 3.1 (0.04 ± 0.03)
	BA	0.3 ± 0.1 (0.00 ± 0.00)	0.3 ± 0.0 (0.00 ± 0.00)	0.2 ± 0.1 (0.00 ± 0.00)	0.2 ± 0.1 (0.00 ± 0.00)	<i>n.d.</i>	0.0 ± 0.1 (0.00 ± 0.00)
	BB	16.0 ± 3.8 (0.11 ± 0.03)	33.5 ± 2.9 (0.23 ± 0.02)	<i>n.d.</i>	1.0 ± 0.4 (0.01 ± 0.00)	<i>n.d.</i>	11.5 ± 1.0 (0.08 ± 0.01)
	Ester total	60.5 ± 9.9 (0.50 ± 0.08)	161.4 ± 14.4 (1.34 ± 0.12)	10.0 ± 0.6 (0.10 ± 0.01)	4.0 ± 0.4 (0.04 ± 0.00)	0.4 ± 0.7 (0.00 ± 0.01)	16.5 ± 1.7 (0.12 ± 0.01)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.03 ± 0.00 (0.29 ± 0.03)	0.07 ± 0.01 (0.75 ± 0.14)	0.15 ± 0.02 (1.71 ± 0.24)	0.04 ± 0.01 (0.48 ± 0.09)	<i>n.d.</i>	0.02 ± 0.03 (0.19 ± 0.32)
	EB	1.62 ± 0.20 (13.95 ± 1.71)	5.54 ± 0.48 (47.66 ± 4.16)	0.17 ± 0.01 (1.50 ± 0.07)	0.06 ± 0.01 (0.52 ± 0.05)	0.03 ± 0.05 (0.24 ± 0.42)	0.20 ± 0.13 (1.68 ± 1.10)
	BA	0.01 ± 0.00 (0.08 ± 0.03)	0.01 ± 0.00 (0.11 ± 0.01)	0.01 ± 0.00 (0.05 ± 0.02)	0.01 ± 0.00 (0.07 ± 0.03)	<i>n.d.</i>	0.00 ± 0.00 (0.02 ± 0.03)
	BB	0.59 ± 0.12 (4.11 ± 0.86)	1.47 ± 0.13 (10.19 ± 0.89)	<i>n.d.</i>	0.04 ± 0.01 (0.26 ± 0.09)	<i>n.d.</i>	0.49 ± 0.05 (3.42 ± 0.37)
	Ester total	2.25 ± 0.32 (18.43 ± 2.57)	7.08 ± 0.61 (58.71 ± 5.10)	0.33 ± 0.03 (3.26 ± 0.32)	0.15 ± 0.01 (1.33 ± 0.07)	0.03 ± 0.05 (0.24 ± 0.42)	0.71 ± 0.05 (5.30 ± 0.44)
Ester yields, mg/g glucose (mM/M)	EA	0.10 ± 0.01 (0.20 ± 0.01)	0.39 ± 0.10 (0.80 ± 0.21)	0.44 ± 0.05 (0.89 ± 0.10)	0.13 ± 0.02 (0.26 ± 0.03)	<i>n.d.</i>	0.07 ± 0.12 (0.14 ± 0.25)
	EB	6.24 ± 0.75 (9.68 ± 1.16)	32.61 ± 5.19 (50.57 ± 8.06)	0.51 ± 0.01 (0.78 ± 0.02)	0.19 ± 0.04 (0.29 ± 0.06)	0.23 ± 0.40 (0.36 ± 0.62)	0.90 ± 0.61 (1.40 ± 0.95)
	BA	0.04 ± 0.01 (0.06 ± 0.02)	0.07 ± 0.01 (0.11 ± 0.02)	0.02 ± 0.01 (0.03 ± 0.01)	0.02 ± 0.01 (0.04 ± 0.01)	<i>n.d.</i>	0.01 ± 0.02 (0.01 ± 0.02)
	BB	2.29 ± 0.50 (2.86 ± 0.63)	8.65 ± 1.32 (10.81 ± 1.65)	<i>n.d.</i>	0.12 ± 0.06 (0.15 ± 0.07)	<i>n.d.</i>	2.23 ± 0.22 (2.79 ± 0.28)
	Ester total	8.66 ± 1.26 (12.80 ± 1.80)	41.72 ± 6.59 (62.29 ± 9.88)	0.96 ± 0.07 (1.71 ± 0.13)	0.46 ± 0.10 (0.74 ± 0.14)	0.23 ± 0.40 (0.36 ± 0.62)	3.21 ± 0.44 (4.34 ± 0.64)

Table 2-8: Summary of optimization of biosynthetic pathway of BA. The best culture condition is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strain		EcJWBA2					
Temperature		28°C			37°C		
IPTG concentration, mM		0.01	0.1	1.0	0.01	0.1	1.0
O.D. ₆₀₀		6.55 ± 0.12	3.80 ± 0.02	1.62 ± 0.08	8.95 ± 0.23	2.50 ± 0.07	1.78 ± 0.06
Consumed glucose, g/L (mM)		7.77 ± 0.16 (43.14 ± 0.90)	4.39 ± 0.19 (24.35 ± 1.03)	2.02 ± 0.02 (11.23 ± 0.11)	12.17 ± 0.40 (67.53 ± 2.23)	3.82 ± 0.36 (21.21 ± 2.00)	3.54 ± 0.51 (19.63 ± 2.84)
Lactate, g/L (mM)		0.26 ± 0.01 (2.88 ± 0.15)	0.27 ± 0.06 (2.95 ± 0.64)	0.01 ± 0.02 (0.15 ± 0.18)	0.84 ± 0.02 (9.38 ± 0.17)	0.11 ± 0.00 (1.22 ± 0.02)	0.08 ± 0.01 (0.91 ± 0.10)
Formate, g/L (mM)		2.32 ± 0.06 (50.34 ± 1.27)	0.86 ± 0.35 (18.68 ± 7.50)	0.23 ± 0.02 (4.95 ± 0.37)	2.35 ± 0.07 (51.15 ± 1.42)	0.51 ± 0.01 (11.17 ± 0.17)	0.61 ± 0.02 (13.18 ± 0.49)
Acetate, g/L (mM)		0.94 ± 0.02 (15.62 ± 0.26)	0.76 ± 0.01 (12.68 ± 0.09)	0.41 ± 0.00 (6.80 ± 0.07)	0.87 ± 0.01 (14.42 ± 0.16)	0.55 ± 0.00 (9.15 ± 0.04)	0.43 ± 0.01 (7.09 ± 0.17)
Ethanol, g/L (mM)		3.56 ± 0.11 (77.24 ± 2.28)	2.30 ± 0.23 (49.97 ± 5.07)	1.69 ± 0.02 (36.62 ± 0.44)	3.06 ± 0.08 (66.42 ± 1.65)	2.39 ± 0.04 (51.82 ± 0.79)	2.69 ± 0.17 (58.31 ± 3.71)
Butanol, g/L (mM)		0.17 ± 0.03 (2.33 ± 0.43)	0.09 ± 0.04 (1.19 ± 0.58)	0.06 ± 0.01 (0.86 ± 0.07)	0.07 ± 0.00 (0.88 ± 0.05)	0.10 ± 0.01 (1.32 ± 0.07)	0.11 ± 0.00 (1.45 ± 0.06)
Ester titers, mg/L (μM)	EA	10.1 ± 2.4 (0.11 ± 0.03)	8.6 ± 2.6 (0.10 ± 0.03)	2.4 ± 0.9 (0.03 ± 0.01)	13.4 ± 5.1 (0.15 ± 0.06)	5.3 ± 1.1 (0.06 ± 0.01)	2.1 ± 0.8 (0.02 ± 0.01)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	35.9 ± 5.5 (0.31 ± 0.05)	48.0 ± 7.1 (0.41 ± 0.06)	8.0 ± 3.8 (0.07 ± 0.03)	19.5 ± 7.3 (0.17 ± 0.06)	15.0 ± 1.8 (0.13 ± 0.02)	8.9 ± 1.5 (0.08 ± 0.01)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	46.0 ± 5.6 (0.42 ± 0.05)	56.6 ± 9.7 (0.51 ± 0.09)	10.3 ± 4.7 (0.10 ± 0.04)	32.8 ± 12.4 (0.32 ± 0.12)	20.3 ± 2.4 (0.19 ± 0.02)	11.0 ± 2.2 (0.10 ± 0.02)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.13 ± 0.03 (1.51 ± 0.33)	0.19 ± 0.06 (2.21 ± 0.69)	0.13 ± 0.05 (1.43 ± 0.58)	0.13 ± 0.05 (1.47 ± 0.59)	0.18 ± 0.03 (2.06 ± 0.39)	0.10 ± 0.03 (1.15 ± 0.37)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	0.47 ± 0.07 (4.06 ± 0.61)	1.09 ± 0.17 (9.35 ± 1.46)	0.43 ± 0.21 (3.68 ± 1.80)	0.19 ± 0.07 (1.62 ± 0.64)	0.52 ± 0.05 (4.44 ± 0.41)	0.43 ± 0.06 (3.70 ± 0.54)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	0.60 ± 0.07 (5.57 ± 0.59)	1.28 ± 0.23 (11.57 ± 2.14)	0.55 ± 0.26 (5.11 ± 2.37)	0.32 ± 0.13 (3.09 ± 1.23)	0.70 ± 0.06 (6.50 ± 0.63)	0.53 ± 0.09 (4.85 ± 0.85)
Ester yields, mg/g glucose (mM/M)	EA	1.31 ± 0.33 (2.67 ± 0.67)	1.94 ± 0.48 (3.97 ± 0.98)	1.12 ± 0.61 (2.29 ± 1.26)	1.09 ± 0.39 (2.24 ± 0.80)	1.37 ± 0.18 (2.81 ± 0.37)	0.59 ± 0.12 (1.20 ± 0.25)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	4.61 ± 0.62 (7.16 ± 0.97)	10.88 ± 0.98 (16.88 ± 1.51)	4.02 ± 2.62 (6.23 ± 4.07)	1.59 ± 0.57 (2.47 ± 0.88)	3.95 ± 0.64 (6.13 ± 0.99)	2.52 ± 0.27 (3.91 ± 0.42)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	5.92 ± 0.65 (9.83 ± 1.08)	9.01 ± 0.55 (14.72 ± 0.92)	5.13 ± 3.24 (8.52 ± 5.32)	2.70 ± 0.95 (4.73 ± 1.66)	5.33 ± 0.68 (8.94 ± 1.08)	3.10 ± 0.29 (5.10 ± 0.47)

Table 2-9: Summary of optimization of biosynthetic pathway of EB. The best culture condition is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strain		EcJWEB2					
Temperature		28°C			37°C		
IPTG concentration, mM		0.01	0.1	1.0	0.01	0.1	1.0
O.D. ₆₀₀		5.90 ± 0.21	3.88 ± 0.10	2.07 ± 0.14	17.37 ± 0.74	3.03 ± 0.03	3.50 ± 0.51
Consumed glucose, g/L (mM)		18.69 ± 0.26 (103.73 ± 1.46)	9.84 ± 0.24 (54.63 ± 1.35)	3.32 ± 0.46 (18.44 ± 2.54)	40.80 ± 2.00 (226.47 ± 11.10)	8.14 ± 0.23 (45.19 ± 1.26)	7.71 ± 1.38 (42.82 ± 7.64)
Lactate, g/L (mM)		0.02 ± 0.00 (0.17 ± 0.00)	<i>n.d.</i>	0.03 ± 0.00 (0.29 ± 0.00)	1.37 ± 0.08 (15.17 ± 0.86)	0.03 ± 0.00 (0.39 ± 0.02)	<i>n.d.</i>
Formate, g/L (mM)		0.11 ± 0.01 (2.44 ± 0.16)	0.12 ± 0.00 (2.60 ± 0.11)	0.04 ± 0.00 (0.80 ± 0.06)	0.42 ± 0.15 (9.23 ± 3.28)	0.38 ± 0.01 (8.33 ± 0.25)	0.31 ± 0.10 (6.77 ± 2.09)
Acetate, g/L (mM)		0.37 ± 0.00 (6.16 ± 0.03)	0.27 ± 0.00 (4.48 ± 0.03)	0.25 ± 0.00 (4.09 ± 0.04)	0.63 ± 0.00 (10.44 ± 0.07)	0.28 ± 0.00 (4.66 ± 0.00)	0.26 ± 0.02 (4.40 ± 0.26)
Ethanol, g/L (mM)		9.52 ± 0.21 (206.66 ± 4.61)	5.64 ± 0.09 (122.33 ± 1.88)	2.35 ± 0.19 (50.91 ± 4.10)	18.55 ± 0.30 (402.67 ± 6.44)	5.14 ± 0.08 (111.56 ± 1.67)	5.18 ± 0.39 (112.35 ± 8.41)
Butanol, g/L (mM)		0.05 ± 0.02 (0.69 ± 0.22)	<i>n.d.</i>	<i>n.d.</i>	0.23 ± 0.00 (3.11 ± 0.02)	<i>n.d.</i>	<i>n.d.</i>
Ester titers, mg/L (μM)	EA	14.5 ± 4.6 (0.17 ± 0.05)	18.2 ± 1.6 (0.21 ± 0.02)	2.3 ± 1.1 (0.03 ± 0.01)	8.3 ± 1.0 (0.09 ± 0.01)	4.5 ± 0.3 (0.05 ± 0.00)	5.8 ± 1.6 (0.07 ± 0.02)
	EB	123.4 ± 37.2 (1.06 ± 0.32)	200.4 ± 9.4 (1.73 ± 0.08)	23.9 ± 20.0 (0.21 ± 0.17)	131.5 ± 12.5 (1.13 ± 0.11)	113.4 ± 34.1 (0.98 ± 0.29)	48.3 ± 19.6 (0.42 ± 0.17)
	BA	0.1 ± 0.2 (0.00 ± 0.00)	<i>n.d.</i>	<i>n.d.</i>	0.5 ± 0.1 (0.00 ± 0.00)	<i>n.d.</i>	<i>n.d.</i>
	BB	6.7 ± 1.7 (0.05 ± 0.01)	5.1 ± 0.3 (0.04 ± 0.00)	<i>n.d.</i>	14.3 ± 2.5 (0.10 ± 0.02)	1.7 ± 1.5 (0.01 ± 0.01)	0.9 ± 1.3 (0.01 ± 0.01)
	Ester total	144.8 ± 42.7 (1.27 ± 0.38)	223.7 ± 10.7 (1.97 ± 0.10)	26.2 ± 21.1 (0.23 ± 0.18)	154.5 ± 15.9 (1.33 ± 0.14)	119.6 ± 32.7 (1.04 ± 0.28)	55.0 ± 19.3 (0.49 ± 0.16)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.21 ± 0.08 (2.43 ± 0.85)	0.40 ± 0.02 (4.58 ± 0.25)	0.10 ± 0.05 (1.10 ± 0.57)	0.04 ± 0.00 (0.46 ± 0.03)	0.13 ± 0.01 (1.45 ± 0.08)	0.09 ± 0.09 (1.07 ± 1.06)
	EB	1.81 ± 0.59 (15.58 ± 5.06)	4.44 ± 0.06 (38.19 ± 0.48)	1.01 ± 0.86 (8.68 ± 7.42)	0.64 ± 0.03 (5.51 ± 0.28)	3.23 ± 1.00 (27.80 ± 8.63)	0.75 ± 0.67 (6.43 ± 5.80)
	BA	0.00 ± 0.00 (0.01 ± 0.02)	<i>n.d.</i>	<i>n.d.</i>	0.00 ± 0.00 (0.02 ± 0.00)	<i>n.d.</i>	<i>n.d.</i>
	BB	0.10 ± 0.03 (0.68 ± 0.18)	0.11 ± 0.01 (0.78 ± 0.08)	<i>n.d.</i>	0.07 ± 0.01 (0.48 ± 0.06)	0.05 ± 0.04 (0.33 ± 0.29)	0.01 ± 0.02 (0.09 ± 0.16)
	Ester total	2.12 ± 0.68 (18.70 ± 6.00)	4.95 ± 0.07 (43.55 ± 0.65)	1.11 ± 0.91 (9.78 ± 7.98)	0.75 ± 0.04 (6.47 ± 0.38)	3.40 ± 0.96 (29.57 ± 8.31)	0.86 ± 0.76 (7.60 ± 6.69)
Ester yields, mg/g glucose (mM/M)	EA	0.78 ± 0.24 (1.59 ± 0.49)	1.87 ± 0.13 (3.83 ± 0.27)	0.69 ± 0.32 (1.41 ± 0.65)	0.14 ± 0.12 (0.28 ± 0.24)	0.55 ± 0.05 (1.13 ± 0.10)	0.69 ± 0.07 (1.41 ± 0.15)
	EB	6.61 ± 2.00 (10.25 ± 3.10)	20.61 ± 0.59 (31.96 ± 0.91)	7.11 ± 5.72 (11.03 ± 8.87)	2.17 ± 1.88 (3.37 ± 2.92)	13.88 ± 3.92 (21.52 ± 6.08)	6.16 ± 3.45 (9.55 ± 5.35)
	BA	0.00 ± 0.01 (0.01 ± 0.01)	<i>n.d.</i>	<i>n.d.</i>	0.01 ± 0.01 (0.01 ± 0.01)	<i>n.d.</i>	<i>n.d.</i>
	BB	0.36 ± 0.09 (0.45 ± 0.11)	0.53 ± 0.04 (0.66 ± 0.06)	<i>n.d.</i>	0.24 ± 0.21 (0.29 ± 0.26)	0.21 ± 0.18 (0.26 ± 0.23)	0.13 ± 0.18 (0.16 ± 0.23)
	Ester total	7.75 ± 2.29 (12.30 ± 3.63)	23.01 ± 0.67 (36.45 ± 1.12)	7.80 ± 6.04 (12.44 ± 9.52)	2.55 ± 2.21 (3.96 ± 3.43)	14.63 ± 3.72 (22.91 ± 5.79)	6.98 ± 3.57 (11.13 ± 5.44)

Table 2-10: Summary of optimization of biosynthetic pathway of BB. The best culture condition is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strain		EcJWBB2					
Temperature		28°C			37°C		
IPTG concentration, mM		0.01	0.1	1.0	0.01	0.1	1.0
O.D. ₆₀₀		6.65 ± 0.23	4.37 ± 0.22	2.20 ± 0.18	8.47 ± 0.24	3.29 ± 0.11	1.78 ± 0.05
Consumed glucose, g/L (mM)		9.02 ± 0.19 (50.05 ± 1.03)	6.89 ± 0.14 (38.23 ± 0.76)	3.75 ± 0.07 (20.80 ± 0.37)	13.07 ± 0.16 (72.56 ± 0.90)	4.89 ± 0.31 (27.14 ± 1.72)	3.99 ± 0.21 (22.15 ± 1.18)
Lactate, g/L (mM)		0.31 ± 0.05 (3.46 ± 0.61)	0.44 ± 0.03 (4.86 ± 0.32)	0.10 ± 0.02 (1.14 ± 0.18)	1.01 ± 0.02 (11.25 ± 0.20)	0.14 ± 0.00 (1.52 ± 0.02)	0.05 ± 0.00 (0.52 ± 0.02)
Formate, g/L (mM)		2.33 ± 0.12 (50.66 ± 2.60)	1.66 ± 0.03 (36.17 ± 0.71)	0.55 ± 0.06 (11.94 ± 1.32)	2.54 ± 0.03 (55.28 ± 0.70)	1.16 ± 0.01 (25.11 ± 0.16)	0.72 ± 0.01 (15.71 ± 0.13)
Acetate, g/L (mM)		0.53 ± 0.01 (8.79 ± 0.11)	0.41 ± 0.00 (6.75 ± 0.04)	0.31 ± 0.00 (5.21 ± 0.01)	0.62 ± 0.01 (10.35 ± 0.08)	0.50 ± 0.00 (8.40 ± 0.07)	0.34 ± 0.00 (5.66 ± 0.02)
Ethanol, g/L (mM)		3.83 ± 0.21 (83.05 ± 4.53)	3.65 ± 0.11 (79.19 ± 2.48)	2.63 ± 0.07 (57.00 ± 1.56)	3.18 ± 0.01 (69.12 ± 0.17)	3.48 ± 0.03 (75.63 ± 0.65)	3.07 ± 0.05 (66.64 ± 1.11)
Butanol, g/L (mM)		0.36 ± 0.04 (4.92 ± 0.53)	0.14 ± 0.05 (1.89 ± 0.62)	0.09 ± 0.04 (1.24 ± 0.52)	0.22 ± 0.00 (2.97 ± 0.04)	0.15 ± 0.00 (1.99 ± 0.04)	0.14 ± 0.00 (1.89 ± 0.02)
Ester titers, mg/L (μM)	EA	2.7 ± 1.1 (0.03 ± 0.01)	2.4 ± 0.0 (0.03 ± 0.00)	1.1 ± 1.0 (0.01 ± 0.01)	1.8 ± 0.5 (0.02 ± 0.01)	1.3 ± 0.6 (0.01 ± 0.01)	0.59 ± 0.18 (0.01 ± 0.00)
	EB	77.9 ± 24.3 (0.67 ± 0.21)	229.8 ± 72.7 (1.98 ± 0.63)	175.6 ± 12.8 (1.51 ± 0.11)	43.7 ± 4.6 (0.38 ± 0.04)	246.2 ± 72.6 (2.12 ± 0.63)	121.1 ± 4.4 (1.04 ± 0.04)
	BA	0.8 ± 0.2 (0.01 ± 0.00)	0.5 ± 0.1 (0.00 ± 0.00)	<i>n.d.</i>	0.4 ± 0.6 (0.00 ± 0.00)	0.2 ± 0.3 (0.00 ± 0.00)	<i>n.d.</i>
	BB	36.2 ± 12.7 (0.25 ± 0.09)	89.1 ± 28.7 (0.62 ± 0.20)	47.0 ± 10.0 (0.33 ± 0.07)	21.4 ± 2.5 (0.15 ± 0.02)	127.4 ± 32.5 (0.88 ± 0.23)	57.9 ± 1.6 (0.40 ± 0.01)
	Ester total	117.6 ± 38.3 (0.96 ± 0.31)	321.8 ± 101.6 (2.63 ± 0.83)	223.7 ± 16.9 (1.85 ± 0.13)	67.2 ± 6.0 (0.55 ± 0.05)	375.1 ± 105.5 (3.02 ± 0.86)	179.6 ± 4.9 (1.45 ± 0.04)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.03 ± 0.01 (0.39 ± 0.17)	0.05 ± 0.00 (0.52 ± 0.01)	0.04 ± 0.04 (0.50 ± 0.45)	0.02 ± 0.01 (0.21 ± 0.06)	0.03 ± 0.02 (0.39 ± 0.17)	0.03 ± 0.01 (0.32 ± 0.10)
	EB	1.01 ± 0.31 (8.66 ± 2.69)	4.40 ± 1.44 (37.92 ± 12.40)	6.92 ± 0.89 (59.55 ± 7.68)	0.45 ± 0.05 (3.88 ± 0.41)	6.44 ± 1.92 (55.42 ± 16.55)	5.85 ± 0.34 (50.34 ± 2.96)
	BA	0.01 ± 0.00 (0.09 ± 0.03)	0.01 ± 0.00 (0.09 ± 0.02)	<i>n.d.</i>	0.00 ± 0.01 (0.04 ± 0.05)	0.00 ± 0.01 (0.04 ± 0.06)	<i>n.d.</i>
	BB	0.47 ± 0.17 (3.25 ± 1.15)	1.71 ± 0.57 (11.84 ± 3.94)	1.83 ± 0.25 (12.68 ± 1.72)	0.22 ± 0.03 (1.53 ± 0.18)	3.33 ± 0.85 (23.08 ± 5.89)	2.80 ± 0.15 (19.38 ± 1.02)
	Ester total	1.52 ± 0.50 (12.40 ± 4.02)	6.17 ± 2.01 (50.37 ± 16.37)	8.79 ± 0.88 (72.73 ± 7.68)	0.69 ± 0.06 (5.65 ± 0.48)	9.80 ± 2.78 (78.93 ± 22.58)	8.67 ± 0.46 (70.04 ± 3.82)
Ester yields, mg/g glucose (mM/M)	EA	0.30 ± 0.13 (0.61 ± 0.26)	0.35 ± 0.00 (0.71 ± 0.00)	0.29 ± 0.26 (0.59 ± 0.52)	0.14 ± 0.04 (0.28 ± 0.08)	0.27 ± 0.13 (0.55 ± 0.27)	0.15 ± 0.04 (0.30 ± 0.08)
	EB	8.68 ± 2.84 (13.45 ± 4.41)	32.97 ± 9.94 (51.13 ± 15.42)	46.86 ± 2.91 (72.68 ± 4.51)	3.34 ± 0.29 (5.18 ± 0.46)	50.96 ± 17.85 (79.03 ± 27.69)	30.39 ± 1.31 (47.13 ± 2.03)
	BA	0.09 ± 0.03 (0.14 ± 0.04)	0.08 ± 0.02 (0.12 ± 0.03)	<i>n.d.</i>	0.03 ± 0.04 (0.05 ± 0.07)	0.03 ± 0.06 (0.05 ± 0.09)	<i>n.d.</i>
	BB	4.04 ± 1.48 (5.05 ± 1.85)	12.78 ± 3.92 (15.96 ± 4.90)	12.51 ± 2.46 (15.63 ± 3.08)	1.64 ± 0.16 (2.05 ± 0.20)	26.31 ± 8.04 (32.87 ± 10.04)	14.55 ± 1.15 (18.18 ± 1.43)
	Ester total	13.11 ± 4.47 (19.25 ± 6.55)	46.17 ± 13.88 (67.93 ± 20.34)	59.66 ± 3.51 (88.90 ± 5.05)	5.15 ± 0.37 (7.55 ± 0.51)	77.57 ± 26.00 (112.50 ± 37.96)	45.09 ± 2.38 (65.61 ± 3.37)

Table 2-11: Summary of solubility engineering of ATF1_{sc}. Key strategies are in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.a.*: not applicable.

Target products	Expressed enzymes/pathways	Ester titer (mg/L (mM))			Conversion yield (%)
		EA	BA	Total	
EA (Ethanol doping)	ATF1 _{sc}	10.4 ± 0.9 (0.12 ± 0.01)	<i>n.a.</i>	10.4 ± 0.9 (0.12 ± 0.01)	0.11 ^a
	ATF1 _{sc} ^{opt}	188.1 ± 25.0 (2.13 ± 0.28)	<i>n.a.</i>	188.1 ± 25.0 (2.13 ± 0.28)	2.30 ^a
	MAP_ATF1 _{sc}	84.5 ± 11.5 (0.96 ± 0.13)	<i>n.a.</i>	84.5 ± 11.5 (0.96 ± 0.13)	1.01 ^a
	NusA_ATF1 _{sc}	104.4 ± 3.0 (1.19 ± 0.03)	<i>n.a.</i>	104.4 ± 3.0 (1.19 ± 0.03)	1.31 ^a
	TrxA_ATF1 _{sc}	110.6 ± 8.1 (1.25 ± 0.09)	<i>n.a.</i>	110.6 ± 8.1 (1.25 ± 0.09)	1.32 ^a
	ATF1 _{sc} +Tf	14.6 ± 0.8 (0.17 ± 0.01)	<i>n.a.</i>	14.6 ± 0.8 (0.17 ± 0.01)	0.15 ^a
	ATF1 _{sc} +GroES/GroEL	17.6 ± 5.3 (0.20 ± 0.06)	<i>n.a.</i>	17.6 ± 5.3 (0.20 ± 0.06)	0.17 ^a
	ATF1 _{sc} +GroES/GroEL/Tf	18.9 ± 4.1 (0.21 ± 0.05)	<i>n.a.</i>	18.9 ± 4.1 (0.21 ± 0.05)	0.19 ^a
	ATF1 _{sc} +DnaK/DnaJ/GrpE	12.5 ± 2.4 (0.14 ± 0.03)	<i>n.a.</i>	12.5 ± 2.4 (0.14 ± 0.03)	0.12 ^a
	ATF1 _{sc} +DnaK/DnaJ/GrpE+ /GroES/GroEL	12.4 ± 1.9 (0.14 ± 0.02)	<i>n.a.</i>	12.4 ± 1.9 (0.14 ± 0.02)	0.13 ^a
BA (Butanol doping)	ATF1 _{sc}	3.2 ± 1.4 (0.04 ± 0.02)	370.7 ± 42.7 (3.19 ± 0.37)	373.9 ± 44.0 (3.23 ± 0.38)	12.03 ^b
	ATF1 _{sc} ^{opt}	44.7 ± 12.3 (0.51 ± 0.14)	2,256.1 ± 222.8 (19.42 ± 1.92)	2,300.8 ± 228.3 (19.93 ± 1.98)	85.90 ^b
	MBP_ATF1 _{sc}	25.7 ± 8.3 (0.29 ± 0.09)	2,062.3 ± 165.3 (17.75 ± 1.42)	2,088.0 ± 173.5 (18.05 ± 1.52)	74.55 ^b
	NusA_ATF1 _{sc}	31.5 ± 8.2 (0.36 ± 0.09)	2,081.2 ± 97.5 (17.92 ± 0.84)	2,112.6 ± 95.6 (18.27 ± 0.82)	79.53 ^b
	TrxA_ATF1 _{sc}	33.1 ± 12.5 (0.38 ± 0.14)	2,095.3 ± 192.0 (18.04 ± 1.65)	2,128.3 ± 187.9 (18.41 ± 1.61)	86.61 ^b
	ATF1 _{sc} +Tf	7.1 ± 1.2 (0.08 ± 0.01)	480.4 ± 73.8 (4.14 ± 0.64)	487.5 ± 74.8 (4.22 ± 0.65)	15.19 ^b
	ATF1 _{sc} +GroES/GroEL	8.0 ± 0.7 (0.09 ± 0.01)	635.1 ± 165.3 (5.47 ± 1.42)	643.0 ± 164.6 (5.56 ± 1.42)	19.65 ^b
	ATF1 _{sc} +GroES/GroEL/Tf	6.3 ± 0.6 (0.07 ± 0.01)	380.3 ± 67.0 (3.27 ± 0.58)	386.6 ± 66.4 (3.35 ± 0.57)	10.99 ^b
	ATF1 _{sc} +DnaK/DnaJ/GrpE	4.5 ± 1.1 (0.05 ± 0.01)	500.2 ± 178.3 (4.31 ± 1.53)	504.7 ± 177.3 (4.36 ± 1.52)	14.21 ^b
	ATF1 _{sc} +DnaK/DnaJ/GrpE+ /GroES/GroEL	4.1 ± 1.5 (0.05 ± 0.02)	484.1 ± 17.1 (4.17 ± 0.15)	488.2 ± 18.6 (4.21 ± 0.16)	13.48 ^b

^a(EA produced) / (EA produced + Ethanol remained)*100, (mole/mole).

^b(BA produced) / (BA produced + Butanol remained)*100, (mole/mole).

Table 2-12: Summary of solubility engineering of SAAT_{Fa}. Key strategy is in bold. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate.

Target products	Expressed enzymes/pathways	Ester titer (mg/L (mM))					Conversion yield (%)
		EA	EB	BA	BB	Total	
EB (Ethanol doping)	BCoA+SAAT _{Fa}	1.8 ± 0.2 (0.02 ± 0.00)	46.5 ± 5.3 (0.40 ± 0.05)	0.3 ± 0.1 (0.00 ± 0.00)	11.4 ± 1.9 (0.08 ± 0.01)	59.9 ± 7.3 (0.50 ± 0.06)	0.32 ^a
	BCoA+SAAT _{Fa} ^{opt}	1.0 ± 0.7 (0.01 ± 0.01)	55.2 ± 16.3 (0.47 ± 0.14)	0.2 ± 0.0 (0.00 ± 0.00)	14.0 ± 5.4 (0.10 ± 0.04)	70.3 ± 21.1 (0.58 ± 0.17)	0.34 ^a
	BCoA+MBP_SAAT _{Fa}	1.4 ± 0.1 (0.02 ± 0.00)	24.0 ± 2.0 (0.21 ± 0.01)	0.1 ± 0.0 (0.00 ± 0.00)	6.5 ± 1.0 (0.05 ± 0.01)	32.0 ± 2.6 (0.27 ± 0.02)	0.16 ^a
	BCoA+NusA_SAAT _{Fa}	1.5 ± 0.1 (0.02 ± 0.00)	13.0 ± 1.0 (0.11 ± 0.01)	0.2 ± 0.1 (0.00 ± 0.00)	3.0 ± 0.5 (0.02 ± 0.00)	17.7 ± 1.5 (0.15 ± 0.01)	0.09 ^a
	BCoA+TrxA_SAAT _{Fa}	1.8 ± 0.3 (0.02 ± 0.00)	30.8 ± 2.9 (0.27 ± 0.02)	0.2 ± 0.0 (0.00 ± 0.00)	5.8 ± 0.8 (0.04 ± 0.01)	38.6 ± 3.9 (0.33 ± 0.03)	0.21 ^a
	BCoA+SAAT _{Fa} +Tf	4.1 ± 0.8 (0.05 ± 0.01)	25.2 ± 4.7 (0.22 ± 0.04)	0.3 ± 0.1 (0.00 ± 0.00)	3.0 ± 0.0 (0.02 ± 0.00)	32.6 ± 5.4 (0.29 ± 0.05)	0.17 ^a
	BCoA+SAAT _{Fa} +GroES/GroEL	6.3 ± 1.3 (0.07 ± 0.01)	464.2 ± 8.9 (4.00 ± 0.08)	0.4 ± 0.0 (0.00 ± 0.00)	66.7 ± 1.6 (0.46 ± 0.01)	537.6 ± 7.4 (4.53 ± 0.07)	2.64 ^a
	BCoA+SAAT _{Fa} +GroES/GroEL/Tf	9.0 ± 1.2 (0.10 ± 0.01)	250.2 ± 35.8 (2.15 ± 0.31)	0.7 ± 0.1 (0.01 ± 0.00)	39.1 ± 5.6 (0.27 ± 0.04)	299.0 ± 42.6 (2.53 ± 0.36)	1.57 ^a
	BCoA+SAAT _{Fa} +DnaK/DnaJ/GrpE	2.7 ± 0.9 (0.03 ± 0.01)	10.3 ± 1.1 (0.09 ± 0.01)	0.3 ± 0.0 (0.00 ± 0.00)	1.5 ± 0.2 (0.01 ± 0.00)	14.8 ± 2.1 (0.13 ± 0.02)	0.10 ^a
	BCoA+SAAT _{Fa} +DnaK/DnaJ/GrpE+GroES/GroEL	4.8 ± 1.2 (0.05 ± 0.01)	330.5 ± 44.4 (2.85 ± 0.38)	0.3 ± 0.0 (0.00 ± 0.00)	44.8 ± 6.1 (0.31 ± 0.04)	380.4 ± 48.6 (3.21 ± 0.41)	2.01 ^a
BB (Butanol doping)	BCoA+SAAT _{Fa}	0.2 ± 0.0 (0.00 ± 0.00)	10.5 ± 0.9 (0.09 ± 0.01)	17.9 ± 1.3 (0.15 ± 0.01)	207.8 ± 5.1 (1.44 ± 0.04)	236.5 ± 5.4 (1.69 ± 0.04)	4.49 ^b
	BCoA+SAAT _{Fa} ^{opt}	0.0 ± 0.1 (0.00 ± 0.00)	7.2 ± 2.7 (0.06 ± 0.02)	21.1 ± 9.3 (0.18 ± 0.08)	344.2 ± 171.6 (2.39 ± 1.19)	372.5 ± 183.6 (2.63 ± 1.29)	6.97 ^b
	BCoA+MBP_SAAT _{Fa}	0.2 ± 0.0 (0.00 ± 0.00)	6.4 ± 0.4 (0.06 ± 0.00)	16.5 ± 0.7 (0.14 ± 0.01)	147.1 ± 14.6 (1.02 ± 0.10)	170.2 ± 14.9 (1.22 ± 0.10)	3.39 ^b
	BCoA+NusA_SAAT _{Fa}	0.1 ± 0.0 (0.00 ± 0.00)	2.9 ± 0.2 (0.02 ± 0.00)	14.2 ± 0.2 (0.12 ± 0.00)	81.5 ± 7.2 (0.57 ± 0.05)	98.7 ± 7.0 (0.71 ± 0.05)	1.97 ^b
	BCoA+TrxA_SAAT _{Fa}	0.2 ± 0.1 (0.00 ± 0.00)	10.8 ± 4.4 (0.09 ± 0.04)	20.2 ± 4.5 (0.17 ± 0.04)	189.2 ± 64.4 (1.31 ± 0.45)	220.4 ± 73.0 (1.58 ± 0.52)	4.65 ^b
	BCoA+SAAT _{Fa} +Tf	0.9 ± 0.4 (0.01 ± 0.00)	11.0 ± 0.9 (0.09 ± 0.01)	59.1 ± 14.8 (0.51 ± 0.13)	152.0 ± 28.8 (1.05 ± 0.20)	223.0 ± 44.3 (1.67 ± 0.33)	4.40 ^b
	BCoA+SAAT _{Fa} +GroES/GroEL	10.5 ± 3.1 (0.12 ± 0.04)	195.7 ± 15.4 (1.69 ± 0.13)	244.0 ± 101.4 (2.10 ± 0.87)	1,710.1 ± 256.6 (11.86 ± 1.78)	2,160.4 ± 349.7 (15.76 ± 2.59)	40.68 ^b
	BCoA+SAAT _{Fa} +GroES/GroEL/Tf	3.1 ± 0.3 (0.04 ± 0.00)	81.3 ± 6.2 (0.70 ± 0.05)	120.3 ± 16.9 (1.04 ± 0.15)	822.1 ± 109.0 (5.70 ± 0.76)	1,026.9 ± 131.0 (7.47 ± 0.95)	21.85 ^b
	BCoA+SAAT _{Fa} +DnaK/DnaJ/GrpE	0.6 ± 0.1 (0.01 ± 0.00)	3.5 ± 0.4 (0.03 ± 0.00)	39.5 ± 2.4 (0.34 ± 0.02)	48.3 ± 5.3 (0.34 ± 0.04)	91.9 ± 7.6 (0.71 ± 0.06)	2.04 ^b
	BCoA+SAAT _{Fa} +DnaK/DnaJ/GrpE+GroES/GroEL	1.6 ± 0.1 (0.02 ± 0.00)	52.0 ± 8.0 (0.45 ± 0.07)	56.6 ± 7.5 (0.49 ± 0.06)	565.0 ± 50.4 (3.92 ± 0.35)	675.1 ± 52.7 (4.87 ± 0.37)	13.14 ^b

^a(Ethyl ester produced) / (Ethyl ester produced + Ethanol remained)*100, (mole/mole).

^b(Butyl ester produced) / (Butyl ester produced + Butanol remained)*100, (mole/mole).

Table 2-13: Summary of BA production with strategies for improving solubility of ATF1_{Sc}. Key strain is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Target product		BA			
Strains		EcJWBA7	EcJWBA8	EcJWBA9	EcJWBA10
Strategies for improving solubility of ATF1 _{Sc}		Codon optimization	Codon opt. +MBP-tag (N'-terminus)	Codon opt. +NusA-tag (N'-terminus)	Codon opt. +TrxA-tag (N'-terminus)
O.D. ₆₀₀		5.35 ± 0.18	5.57 ± 0.05	4.45 ± 0.11	5.87 ± 0.23
Consumed glucose, g/L (mM)		5.68 ± 0.14 (31.51 ± 0.76)	6.54 ± 0.12 (36.29 ± 0.67)	5.31 ± 0.18 (29.50 ± 1.01)	5.36 ± 0.06 (29.75 ± 0.35)
Lactate, g/L (mM)		0.14 ± 0.01 (1.56 ± 0.06)	0.21 ± 0.03 (2.28 ± 0.36)	0.06 ± 0.00 (0.62 ± 0.03)	0.14 ± 0.05 (1.51 ± 0.51)
Formate, g/L (mM)		1.60 ± 0.05 (34.72 ± 0.99)	1.78 ± 0.09 (38.75 ± 1.86)	1.81 ± 0.04 (39.38 ± 0.88)	1.51 ± 0.56 (32.89 ± 12.25)
Acetate, g/L (mM)		1.09 ± 0.02 (18.11 ± 0.28)	0.79 ± 0.00 (13.21 ± 0.04)	0.66 ± 0.01 (11.00 ± 0.10)	0.88 ± 0.13 (14.62 ± 2.21)
Ethanol, g/L (mM)		2.17 ± 0.06 (47.08 ± 1.32)	3.09 ± 0.06 (67.12 ± 1.37)	3.23 ± 0.06 (70.22 ± 1.40)	2.49 ± 0.26 (54.07 ± 5.54)
Butanol, g/L (mM)		0.06 ± 0.00 (0.82 ± 0.02)	0.17 ± 0.01 (2.27 ± 0.11)	0.24 ± 0.01 (3.24 ± 0.20)	0.11 ± 0.02 (1.48 ± 0.20)
Ester titers, mg/L (mM)	EA	18.7 ± 1.8 (0.21 ± 0.02)	11.6 ± 2.0 (0.13 ± 0.02)	6.4 ± 0.6 (0.07 ± 0.01)	16.7 ± 1.8 (0.19 ± 0.02)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	51.7 ± 7.1 (0.44 ± 0.06)	79.3 ± 9.8 (0.68 ± 0.08)	76.1 ± 6.2 (0.66 ± 0.05)	89.5 ± 14.8 (0.77 ± 0.13)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	70.4 ± 8.8 (0.66 ± 0.08)	90.9 ± 10.8 (0.81 ± 0.10)	82.5 ± 6.3 (0.73 ± 0.05)	106.1 ± 16.0 (0.96 ± 0.14)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.30 ± 0.04 (0.00 ± 0.00)	0.18 ± 0.03 (0.00 ± 0.00)	0.12 ± 0.01 (0.00 ± 0.00)	0.24 ± 0.02 (0.00 ± 0.00)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	0.83 ± 0.13 (0.01 ± 0.00)	1.22 ± 0.14 (0.01 ± 0.00)	1.47 ± 0.09 (0.01 ± 0.00)	1.31 ± 0.18 (0.01 ± 0.00)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	1.13 ± 0.17 (0.01 ± 0.00)	1.40 ± 0.16 (0.01 ± 0.00)	1.59 ± 0.09 (0.01 ± 0.00)	1.55 ± 0.18 (0.01 ± 0.00)
Ester yields, mg/g glucose (mM/M)	EA	3.29 ± 0.34 (6.74 ± 0.69)	1.77 ± 0.27 (3.62 ± 0.55)	1.20 ± 0.12 (2.46 ± 0.24)	3.11 ± 0.31 (6.35 ± 0.63)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BA	9.11 ± 1.25 (14.12 ± 1.94)	12.12 ± 1.44 (18.80 ± 2.23)	14.31 ± 0.71 (22.19 ± 1.10)	16.68 ± 2.75 (25.88 ± 4.26)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	Ester total	12.40 ± 1.57 (20.86 ± 2.59)	13.89 ± 1.55 (22.42 ± 2.46)	15.51 ± 0.66 (24.65 ± 1.01)	19.79 ± 2.95 (32.23 ± 4.68)

Table 2-14: Summary of production of EB with strategy for improving solubility of SAAT_{Fa}. The best culture condition is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Target product		EB			
Strains		EcJWEB7			
Strategy for improving solubility of SAAT _{Fa}		Co-expression of GroES/EL			
Arabinose conc. for inducing expression of GroES/EL		0 mg/ml	1 mg/ml	5 mg/ml	50 mg/ml
O.D. ₆₀₀		3.85 ± 0.13	3.86 ± 0.34	3.47 ± 0.23	3.69 ± 0.10
Consumed glucose, g/L (mM)		9.83 ± 0.10 (54.57 ± 0.54)	9.78 ± 0.08 (54.28 ± 0.45)	9.49 ± 0.19 (52.68 ± 1.07)	7.71 ± 0.16 (42.81 ± 0.91)
Lactate, g/L (mM)		0.08 ± 0.00 (0.87 ± 0.01)	0.08 ± 0.00 (0.88 ± 0.01)	0.08 ± 0.00 (0.87 ± 0.01)	0.09 ± 0.00 (0.97 ± 0.02)
Formate, g/L (mM)		0.26 ± 0.01 (5.74 ± 0.16)	0.25 ± 0.01 (5.42 ± 0.15)	0.25 ± 0.01 (5.41 ± 0.23)	0.24 ± 0.00 (5.24 ± 0.05)
Acetate, g/L (mM)		0.24 ± 0.00 (4.03 ± 0.01)	0.24 ± 0.00 (4.04 ± 0.01)	0.24 ± 0.00 (4.01 ± 0.01)	0.25 ± 0.00 (4.14 ± 0.04)
Ethanol, g/L (mM)		5.17 ± 0.03 (112.22 ± 0.60)	5.12 ± 0.05 (111.15 ± 0.99)	5.04 ± 0.09 (109.31 ± 2.05)	5.45 ± 0.07 (118.26 ± 1.52)
Butanol, g/L (mM)		0.03 ± 0.00 (0.47 ± 0.04)	0.03 ± 0.00 (0.40 ± 0.06)	0.03 ± 0.00 (0.34 ± 0.01)	0.03 ± 0.01 (0.43 ± 0.07)
Ester titers, mg/L (mM)	EA	15.4 ± 1.1 (0.18 ± 0.01)	29.8 ± 6.4 (0.34 ± 0.07)	19.6 ± 11.2 (0.22 ± 0.13)	41.0 ± 4.5 (0.47 ± 0.05)
	EB	263.9 ± 51.8 (2.27 ± 0.45)	337.6 ± 46.1 (2.91 ± 0.40)	365.7 ± 69.2 (3.15 ± 0.60)	346.2 ± 59.8 (2.98 ± 0.51)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	6.4 ± 1.9 (0.04 ± 0.01)	7.5 ± 1.3 (0.05 ± 0.01)	8.4 ± 2.4 (0.06 ± 0.02)	7.6 ± 1.2 (0.05 ± 0.01)
	Ester total	285.7 ± 53.8 (2.49 ± 0.46)	374.9 ± 51.1 (3.30 ± 0.45)	393.6 ± 72.7 (3.43 ± 0.63)	394.7 ± 62.0 (3.50 ± 0.54)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.34 ± 0.01 (3.83 ± 0.11)	0.66 ± 0.18 (6.15 ± 2.02)	0.48 ± 0.26 (5.45 ± 2.90)	0.96 ± 0.14 (9.46 ± 1.63)
	EB	5.73 ± 1.52 (49.30 ± 13.11)	6.70 ± 0.49 (57.64 ± 4.25)	8.79 ± 1.63 (75.70 ± 14.01)	8.80 ± 1.40 (75.74 ± 12.07)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	0.13 ± 0.05 (0.91 ± 0.33)	0.16 ± 0.01 (1.08 ± 0.07)	0.20 ± 0.06 (1.41 ± 0.44)	0.20 ± 0.02 (1.37 ± 0.13)
	Ester total	6.19 ± 1.56 (54.04 ± 13.33)	7.39 ± 0.59 (64.86 ± 5.48)	9.48 ± 1.54 (82.56 ± 12.81)	9.83 ± 1.44 (86.56 ± 12.44)
Ester yields, mg/g glucose (mM/M)	EA	1.57 ± 0.12 (3.21 ± 0.25)	3.05 ± 0.63 (6.23 ± 1.29)	2.06 ± 1.15 (4.20 ± 2.35)	5.32 ± 0.68 (10.88 ± 1.39)
	EB	26.81 ± 5.05 (41.58 ± 7.83)	34.53 ± 4.72 (53.55 ± 7.32)	38.46 ± 6.72 (59.64 ± 10.42)	44.87 ± 7.47 (69.58 ± 11.59)
	BA	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
	BB	0.65 ± 0.18 (0.81 ± 0.23)	0.76 ± 0.13 (0.95 ± 0.17)	0.88 ± 0.25 (1.10 ± 0.31)	0.98 ± 0.15 (1.22 ± 0.18)
	Ester total	29.03 ± 5.24 (45.60 ± 8.08)	38.34 ± 5.20 (60.73 ± 8.22)	41.39 ± 6.95 (64.94 ± 10.78)	51.17 ± 7.78 (81.69 ± 12.14)

Table 2-15: Summary of production of BB with strategies for improving solubility of SAAT_{Fa}. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Target product		BB			
Strains		EcJWBB7			
Strategy for improving solubility of SAAT _{Fa}		Co-expression of GroES/EL			
Arabinose conc. for inducing expression of GroES/EL		0 mg/ml	1 mg/ml	5 mg/ml	50 mg/ml
O.D. ₆₀₀		2.97 ± 0.47	2.09 ± 0.08	2.27 ± 0.10	3.14 ± 0.16
Consumed glucose, g/L (mM)		3.72 ± 2.80 (20.67 ± 15.52)	1.58 ± 0.98 (8.77 ± 5.43)	0.95 ± 0.87 (5.26 ± 4.83)	1.10 ± 0.61 (6.08 ± 3.36)
Lactate, g/L (mM)		0.19 ± 0.02 (2.08 ± 0.23)	0.12 ± 0.00 (1.29 ± 0.05)	0.08 ± 0.00 (0.85 ± 0.02)	0.14 ± 0.00 (1.60 ± 0.03)
Formate, g/L (mM)		1.03 ± 0.35 (22.47 ± 7.54)	0.76 ± 0.02 (16.49 ± 0.36)	0.81 ± 0.01 (17.58 ± 0.26)	0.93 ± 0.03 (20.30 ± 0.59)
Acetate, g/L (mM)		0.48 ± 0.03 (8.05 ± 0.55)	0.41 ± 0.01 (6.75 ± 0.17)	0.44 ± 0.01 (7.40 ± 0.15)	0.49 ± 0.01 (8.18 ± 0.24)
Ethanol, g/L (mM)		2.71 ± 0.23 (58.92 ± 4.99)	1.99 ± 0.02 (43.14 ± 0.54)	2.11 ± 0.08 (45.70 ± 1.67)	2.85 ± 0.09 (61.81 ± 1.90)
Butanol, g/L (mM)		0.02 ± 0.01 (0.31 ± 0.13)	0.06 ± 0.04 (0.75 ± 0.48)	0.07 ± 0.01 (0.90 ± 0.12)	0.04 ± 0.04 (0.53 ± 0.52)
Ester titers, mg/L (mM)	EA	6.0 ± 1.6 (0.07 ± 0.02)	6.1 ± 1.0 (0.07 ± 0.01)	8.2 ± 1.6 (0.09 ± 0.02)	6.9 ± 2.6 (0.08 ± 0.03)
	EB	155.8 ± 7.7 (1.34 ± 0.07)	147.3 ± 15.1 (1.27 ± 0.13)	240.4 ± 14.4 (2.07 ± 0.12)	224.2 ± 14.0 (1.93 ± 0.12)
	BA	0.3 ± 0.1 (0.00 ± 0.00)	0.6 ± 0.1 (0.01 ± 0.00)	1.9 ± 0.1 (0.02 ± 0.00)	1.5 ± 0.1 (0.01 ± 0.00)
	BB	25.6 ± 10.7 (0.18 ± 0.07)	30.0 ± 4.8 (0.21 ± 0.03)	51.4 ± 3.4 (0.36 ± 0.02)	42.1 ± 4.1 (0.29 ± 0.03)
	Ester total	187.7 ± 16.2 (1.59 ± 0.12)	184.0 ± 20.3 (1.55 ± 0.17)	302.0 ± 18.7 (2.54 ± 0.16)	274.7 ± 15.8 (2.31 ± 0.13)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.18 ± 0.06 (2.02 ± 0.66)	0.25 ± 0.05 (2.84 ± 0.54)	0.31 ± 0.05 (3.53 ± 0.61)	0.19 ± 0.08 (2.17 ± 0.89)
	EB	4.57 ± 0.50 (39.32 ± 4.32)	6.07 ± 0.82 (52.28 ± 7.06)	9.12 ± 0.21 (78.52 ± 1.82)	6.17 ± 0.63 (53.10 ± 5.45)
	BA	0.01 ± 0.00 (0.07 ± 0.02)	0.03 ± 0.00 (0.22 ± 0.03)	0.07 ± 0.00 (0.61 ± 0.03)	0.04 ± 0.00 (0.35 ± 0.01)
	BB	0.74 ± 0.29 (5.13 ± 2.01)	1.24 ± 0.24 (8.58 ± 1.63)	1.95 ± 0.05 (13.53 ± 0.35)	1.16 ± 0.13 (8.02 ± 0.90)
	Ester total	5.49 ± 0.62 (46.54 ± 5.15)	7.59 ± 1.08 (63.92 ± 9.00)	11.45 ± 0.25 (96.19 ± 2.04)	7.56 ± 0.74 (63.64 ± 6.20)
Ester yields, mg/g glucose (mM/M)	EA	1.20 ± 0.21 (2.46 ± 0.42)	2.59 ± 0.26 (5.29 ± 0.54)	5.41 ± 0.87 (11.06 ± 1.77)	6.49 ± 3.77 (13.26 ± 7.70)
	EB	28.85 ± 4.50 (44.74 ± 6.98)	67.66 ± 2.00 (104.94 ± 3.10)	161.52 ± 16.97 (250.51 ± 26.32)	166.53 ± 62.14 (258.27 ± 96.38)
	BA	0.04 ± 0.02 (0.07 ± 0.03)	0.29 ± 0.00 (0.45 ± 0.01)	1.25 ± 0.17 (1.94 ± 0.27)	1.13 ± 0.27 (1.76 ± 0.41)
	BB	4.22 ± 2.70 (5.28 ± 3.37)	13.54 ± 1.48 (16.91 ± 1.85)	34.44 ± 3.39 (43.02 ± 4.24)	30.14 ± 9.03 (37.66 ± 11.29)
	Ester total	34.32 ± 7.01 (52.54 ± 9.96)	84.08 ± 3.22 (127.59 ± 4.42)	202.62 ± 19.67 (306.54 ± 29.06)	204.29 ± 75.21 (310.95 ± 115.78)

Table 2-16: Summary of BA and BB production with strategies for improving solubility of AdhE2_{Ca}. Key strain is in bold; Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Target product		BA				BB
Strains		EcJWBA11	EcJWBA12	EcJWBA13	EcJWBA14	EcJWBB8
Strategies for improving solubility of AdhE2 _{Ca}		Codon optimization	Codon opt. +MBP-tag (N'-terminus)	Codon opt. +NusA-tag (N'-terminus)	Codon opt. +TrxA-tag (N'-terminus)	Codon opt. +TrxA-tag (N'-terminus)
O.D. ₆₀₀		4.19 ± 0.14	3.70 ± 0.19	3.85 ± 0.20	4.28 ± 0.19	4.97 ± 0.26
Consumed glucose, g/L (mM)		8.23 ± 0.71 (45.67 ± 3.95)	2.13 ± 1.55 (11.82 ± 8.62)	2.21 ± 0.04 (12.25 ± 0.19)	2.34 ± 0.06 (13.00 ± 0.35)	4.00 ± 2.37 (22.20 ± 13.13)
Lactate, g/L (mM)		0.04 ± 0.00 (0.42 ± 0.02)	0.06 ± 0.01 (0.65 ± 0.09)	0.03 ± 0.00 (0.37 ± 0.03)	0.05 ± 0.00 (0.51 ± 0.04)	0.10 ± 0.01 (1.15 ± 0.07)
Formate, g/L (mM)		0.60 ± 0.12 (12.98 ± 2.63)	0.67 ± 0.07 (14.62 ± 1.63)	0.63 ± 0.03 (13.78 ± 0.62)	0.69 ± 0.02 (15.08 ± 0.34)	0.92 ± 0.09 (19.89 ± 1.96)
Acetate, g/L (mM)		0.69 ± 0.05 (11.43 ± 0.75)	0.75 ± 0.01 (12.57 ± 0.16)	0.80 ± 0.01 (13.24 ± 0.24)	0.78 ± 0.01 (12.99 ± 0.13)	0.47 ± 0.03 (7.77 ± 0.54)
Ethanol, g/L (mM)		1.15 ± 0.03 (24.90 ± 0.71)	1.60 ± 0.09 (34.83 ± 1.86)	1.33 ± 0.02 (28.81 ± 0.37)	1.40 ± 0.02 (30.33 ± 0.42)	2.53 ± 0.05 (54.84 ± 1.02)
Butanol, g/L (mM)		0.10 ± 0.00 (1.30 ± 0.05)	0.14 ± 0.00 (1.93 ± 0.06)	0.16 ± 0.01 (2.11 ± 0.15)	0.20 ± 0.01 (2.76 ± 0.13)	0.18 ± 0.00 (2.40 ± 0.02)
Ester titers, mg/L (mM)	EA	12.4 ± 1.5 (0.14 ± 0.02)	9.2 ± 0.5 (0.10 ± 0.01)	9.7 ± 4.1 (0.11 ± 0.05)	2.0 ± 0.4 (0.02 ± 0.00)	2.7 ± 1.0 (0.03 ± 0.01)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	156.3 ± 22.4 (1.35 ± 0.19)
	BA	80.3 ± 9.0 (0.69 ± 0.08)	54.2 ± 4.9 (0.47 ± 0.04)	82.8 ± 15.5 (0.71 ± 0.13)	203.0 ± 5.7 (1.75 ± 0.05)	4.1 ± 0.5 (0.04 ± 0.00)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	167.3 ± 18.2 (1.16 ± 0.13)
	Ester total	92.8 ± 10.5 (0.83 ± 0.09)	63.3 ± 4.8 (0.57 ± 0.04)	92.5 ± 19.6 (0.82 ± 0.18)	205.0 ± 6.1 (1.77 ± 0.05)	330.5 ± 16.3 (2.57 ± 0.14)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.26 ± 0.04 (0.00 ± 0.00)	0.21 ± 0.02 (0.00 ± 0.00)	0.22 ± 0.09 (0.00 ± 0.00)	0.04 ± 0.01 (0.00 ± 0.00)	0.05 ± 0.02 (0.54 ± 0.21)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	2.72 ± 0.49 (23.40 ± 4.24)
	BA	1.65 ± 0.24 (0.01 ± 0.00)	1.26 ± 0.07 (0.01 ± 0.00)	1.90 ± 0.30 (0.02 ± 0.00)	4.18 ± 0.11 (0.04 ± 0.00)	0.07 ± 0.01 (0.62 ± 0.08)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	2.90 ± 0.35 (20.10 ± 2.42)
	Ester total	1.91 ± 0.28 (0.02 ± 0.00)	1.47 ± 0.08 (0.01 ± 0.00)	2.12 ± 0.38 (0.02 ± 0.00)	4.22 ± 0.12 (0.04 ± 0.00)	5.74 ± 0.57 (44.65 ± 4.70)
Ester yields, mg/g glucose (mM/M)	EA	1.53 ± 0.32 (3.13 ± 0.66)	8.85 ± 9.69 (18.09 ± 19.82)	4.33 ± 1.77 (8.85 ± 3.62)	0.88 ± 0.20 (1.79 ± 0.41)	0.64 ± 0.34 (1.32 ± 0.70)
	EB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	42.54 ± 21.00 (65.97 ± 32.56)
	BA	9.88 ± 2.04 (15.32 ± 3.16)	56.91 ± 67.39 (88.27 ± 104.51)	37.22 ± 6.42 (57.72 ± 9.96)	86.79 ± 5.78 (134.61 ± 8.96)	1.17 ± 0.68 (1.81 ± 1.05)
	BB	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	53.29 ± 30.08 (66.57 ± 37.58)
	Ester total	11.41 ± 2.36 (18.45 ± 3.82)	65.76 ± 77.08 (106.36 ± 124.32)	41.54 ± 8.19 (66.57 ± 13.58)	87.67 ± 5.98 (136.40 ± 9.38)	97.64 ± 52.10 (135.68 ± 71.90)

Table 2-17: Summary of production of C4-derived esters in anaerobic bottles with pH-adjustment. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate; *n.d.*: not detected.

Strains		EcJWBA14	EcJWEB7	EcJWBB8
O.D. ₆₀₀		3.76 ± 0.20	4.36 ± 0.26	4.22 ± 0.20
Consumed glucose, g/L (mM)		11.85 ± 0.91 (65.80 ± 5.04)	29.71 ± 0.23 (164.90 ± 1.27)	41.40 ± 1.50 (229.78 ± 8.32)
Lactate, g/L (mM)		0.06 ± 0.00 (0.68 ± 0.02)	0.17 ± 0.01 (1.93 ± 0.15)	1.05 ± 0.17 (11.71 ± 1.85)
Formate, g/L (mM)		2.14 ± 0.06 (46.55 ± 1.20)	0.18 ± 0.01 (3.89 ± 0.18)	3.58 ± 0.57 (77.81 ± 12.33)
Acetate, g/L (mM)		1.03 ± 0.01 (17.17 ± 0.23)	0.22 ± 0.00 (3.61 ± 0.04)	0.40 ± 0.02 (6.65 ± 0.38)
Ethanol, g/L (mM)		2.05 ± 0.02 (44.56 ± 0.51)	12.52 ± 0.10 (271.74 ± 2.25)	9.88 ± 0.28 (214.41 ± 5.98)
Butanol, g/L (mM)		0.16 ± 0.01 (2.11 ± 0.17)	0.03 ± 0.00 (0.46 ± 0.03)	0.20 ± 0.02 (2.76 ± 0.33)
Ester titers, mg/L (mM)	EA	39.8 ± 7.6 (0.45 ± 0.09)	58.3 ± 9.3 (0.66 ± 0.11)	1.2 ± 0.5 (0.01 ± 0.01)
	EB	<i>n.d.</i>	408.9 ± 44.3 (3.52 ± 0.38)	381.9 ± 24.7 (3.29 ± 0.21)
	BA	441.4 ± 40.9 (3.80 ± 0.35)	0.9 ± 0.1 (0.01 ± 0.00)	7.4 ± 1.2 (0.06 ± 0.01)
	BB	<i>n.d.</i>	10.1 ± 1.0 (0.07 ± 0.01)	449.6 ± 43.0 (3.12 ± 0.30)
	Ester total	481.1 ± 48.4 (4.25 ± 0.44)	478.2 ± 54.8 (4.26 ± 0.50)	839.9 ± 68.4 (6.48 ± 0.52)
Specific ester productivity, mg/gDCW/h (μM/gDCW/h)	EA	0.45 ± 0.09 (0.01 ± 0.00)	0.31 ± 0.05 (0.00 ± 0.00)	0.01 ± 0.00 (0.00 ± 0.00)
	EB	<i>n.d.</i>	2.17 ± 0.23 (0.02 ± 0.00)	2.02 ± 0.13 (0.02 ± 0.00)
	BA	5.04 ± 0.47 (0.04 ± 0.00)	0.00 ± 0.00 (0.00 ± 0.00)	0.04 ± 0.01 (0.00 ± 0.00)
	BB	<i>n.d.</i>	0.05 ± 0.01 (0.00 ± 0.00)	2.38 ± 0.23 (0.02 ± 0.00)
	Ester total	5.49 ± 0.55 (0.05 ± 0.00)	2.53 ± 0.29 (0.02 ± 0.00)	4.45 ± 0.36 (0.03 ± 0.00)
Ester yields, mg/g glucose (mM/M)	EA	3.55 ± 0.68 (7.25 ± 1.38)	5.20 ± 0.83 (10.63 ± 1.70)	0.10 ± 0.04 (0.21 ± 0.08)
	EB	<i>n.d.</i>	36.47 ± 3.95 (56.57 ± 6.13)	34.06 ± 2.20 (52.82 ± 3.41)
	BA	39.37 ± 3.64 (61.06 ± 5.65)	0.08 ± 0.01 (0.13 ± 0.02)	0.66 ± 0.11 (1.02 ± 0.17)
	BB	<i>n.d.</i>	0.90 ± 0.09 (1.12 ± 0.11)	40.10 ± 3.84 (50.09 ± 4.79)
	Ester total	42.91 ± 4.32 (68.31 ± 7.03)	42.65 ± 4.88 (68.45 ± 7.96)	74.91 ± 6.10 (104.14 ± 8.29)
Culture time (h)		48	96	96

Table 2-18: Summary of direct fermentative production of C4-derived esters from sugar. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; BB, butyl butyrate.

Products	Strains	Strategies	Titers (mg/L)	Volumetric productivity (mg/L/h)	Yields (mg ester/g glucose)	Ester Selectivity (%)	*Maximum theoretical yield (%)	Ref.
BA	<i>E. coli</i>	SAAT _{Fa}	0.5	0.005	0.01	0.28	0.002	[1]
	<i>C. acetobutylicum</i>	SAAT _{Fa} ^{opt}	8.4	0.116	0.29	14.17	0.07	[7]
	<i>E. coli</i>	ATF1 _{Sc}	34.2	1.426	8.22	74.27	1.91	This study
	<i>E. coli</i>	ATF1 _{Sc}	48.0	1.999	10.88	83.13	2.53	
	<i>E. coli</i>	TrxA_ATF1 _{Sc} ^{opt}	89.5	3.727	16.68	84.30	3.88	
	<i>E. coli</i>	TrxA_ATF1 _{Sc} ^{opt} +TrxA_adhE2 ^{opt}	203.0	8.457	86.79	99.00	20.18	
	<i>E. coli</i>	TrxA_ATF1 _{Sc} ^{opt} +TrxA_adhE2 ^{opt} +Anaerobic culture with pH adjustment	441.4	9.195	39.37	91.74	9.16	
EB	<i>E. coli</i>	SAAT _{Fa}	134.0	1.396	3.31	78.22	0.77	[1]
	<i>C. acetobutylicum</i>	SAAT _{Fa} ^{opt}	0.6	0.008	0.02	1.03	0.005	[7]
	<i>E. coli</i>	SAAT _{Fa}	71.0	2.960	9.84	91.97	2.29	This study
	<i>E. coli</i>	SAAT _{Fa}	200.4	8.350	20.61	89.57	4.79	
	<i>E. coli</i>	SAAT _{Fa} +GroES/EL	365.7	15.236	38.46	92.90	8.94	
	<i>E. coli</i>	SAAT _{Fa} +GroES/EL +Anaerobic culture with pH adjustment	408.9	4.260	36.47	85.51	8.48	
BB	<i>E. coli</i>	SAAT _{Fa}	36.8	0.384	0.91	21.50	0.23	[1]
	<i>C. acetobutylicum</i>	SAAT _{Fa} ^{opt}	50.1	0.695	1.72	84.79	0.43	[7]
	<i>E. coli</i>	SAAT _{Fa}	33.5	1.395	8.65	20.75	2.16	This study
	<i>E. coli</i>	SAAT _{Fa}	127.4	5.308	26.31	33.96	6.58	
	<i>E. coli</i>	SAAT _{Fa} +TrxA_adhE2 ^{opt}	167.3	6.971	53.29	50.63	13.32	
	<i>E. coli</i>	SAAT _{Fa} +TrxA_adhE2 ^{opt} +Anaerobic culture with pH adjustment	449.6	4.683	40.10	53.52	10.03	

*Actual molar yield / theoretical molar yield from glucose *100.

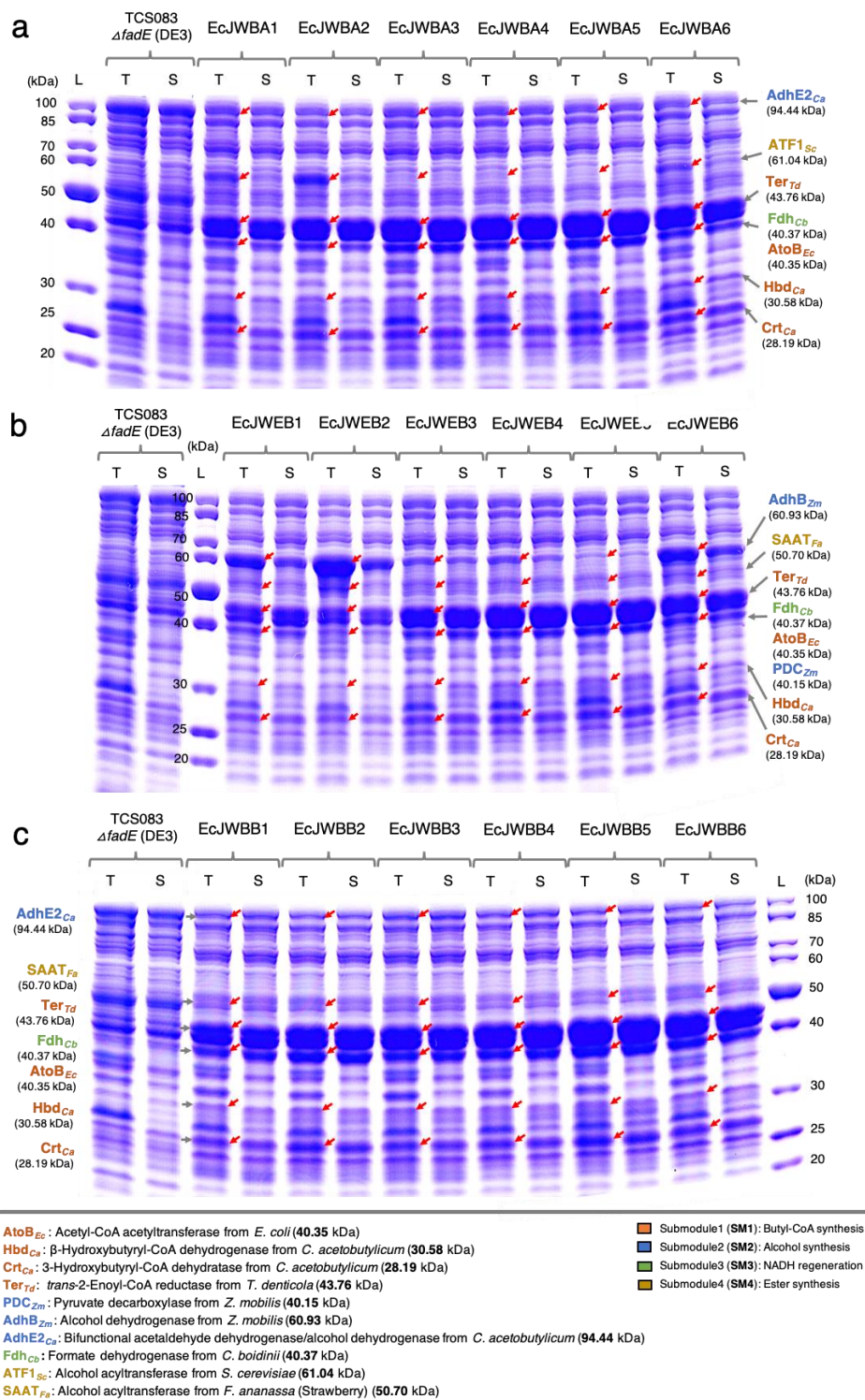


Figure 2-6: SDS-PAGE analysis for confirming expression of heterologous enzymes in EcJWBA2, EcJWEB2, and EcJWBB2. (a) EcJWBA1-6. (b) EcJWEB1-6. (c) EcJWBB1-6. Red arrows indicate the expected size of overexpressed enzymes. Abbreviations: L, protein ladder; T, total fractions; S, Soluble fractions. (Induction conditions: 37°C, 0.5 mM IPTG)

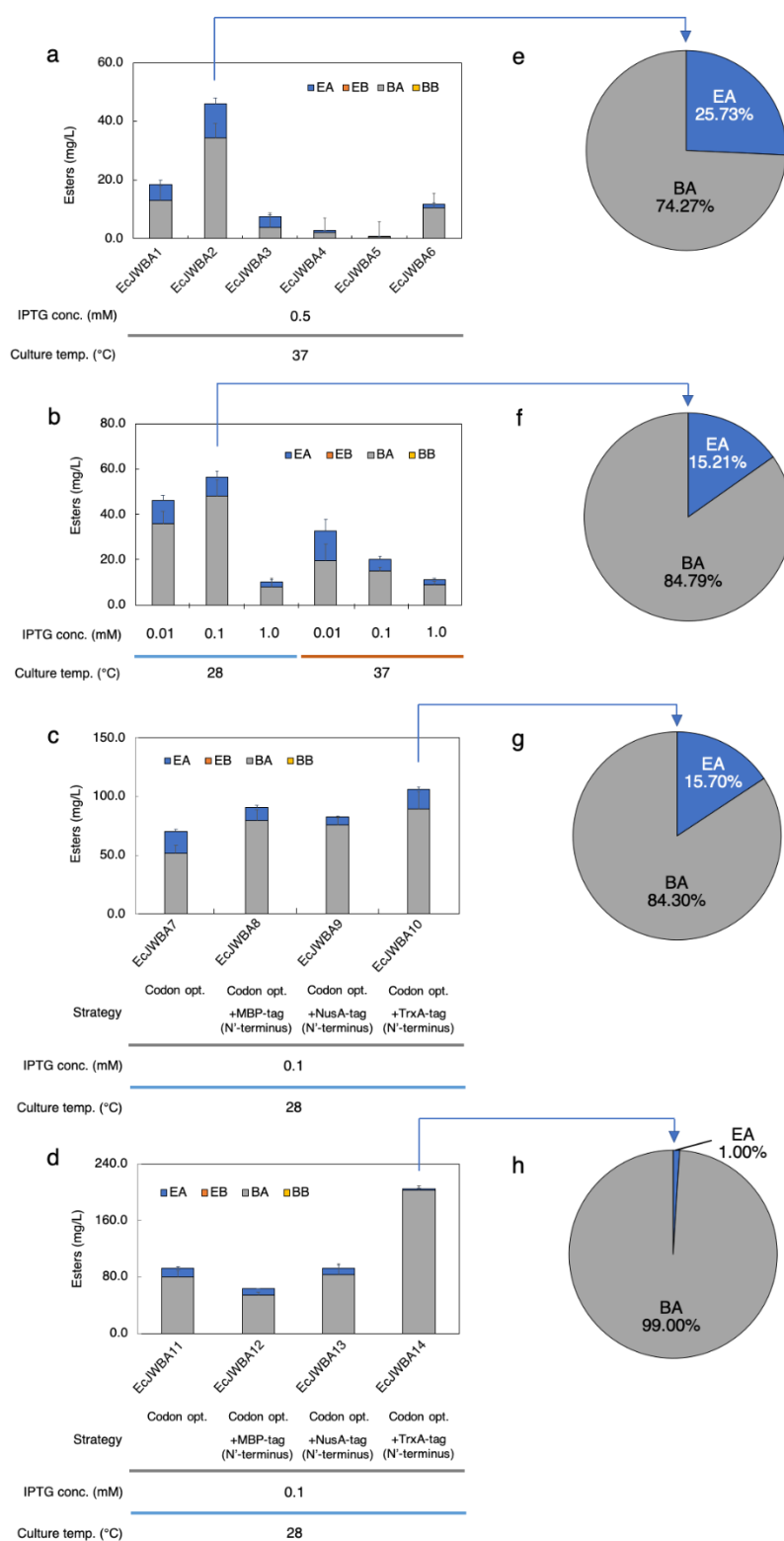


Figure 2-7: Summary of BA production optimization. **(a)** Pathway validation. **(b)** Optimization of induction conditions. **(c)** Improving solubility of ATF1_{Sc}. **(d)** Improving solubility of adhE2_{Ca}. **(e-h)** Ester selectivity.

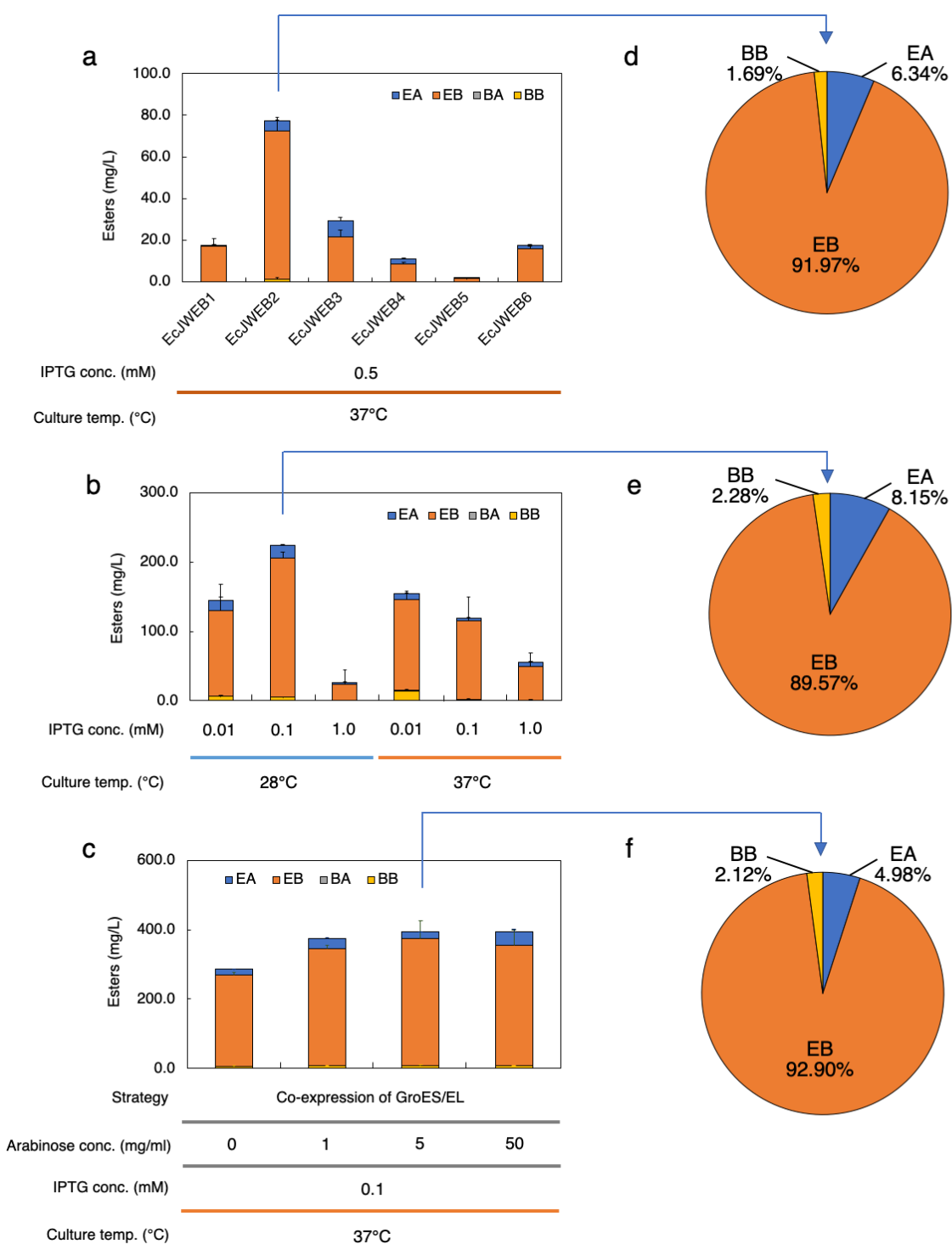


Figure 2-8: Summary of EB production optimization. **(a)** Pathway validation. **(b)** Optimization of induction conditions. **(c)** Improving solubility of SAAT_{Fa}. **(d-f)** Ester selectivity.

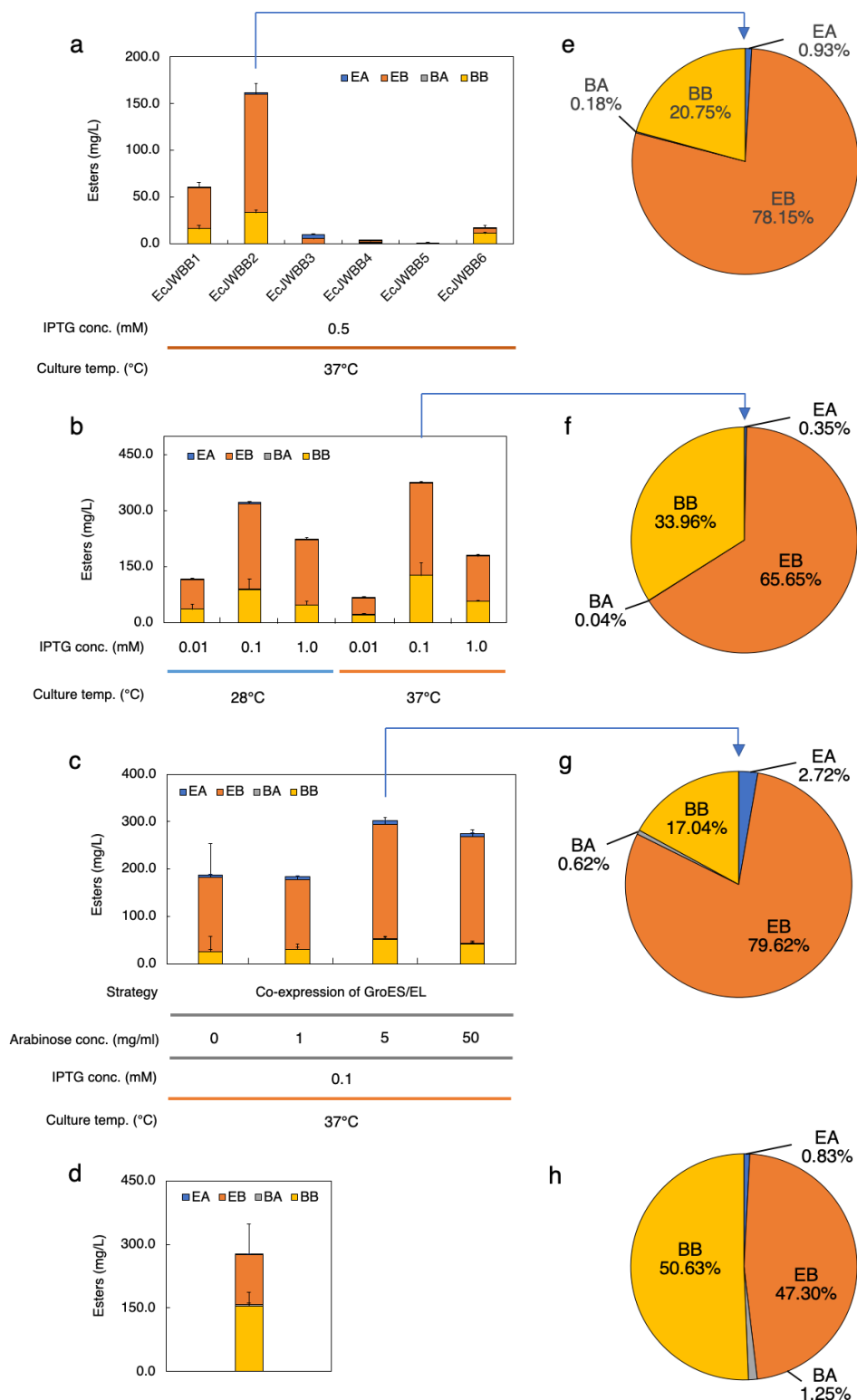


Figure 2-9: Summary of BB production optimization. **(a)** Pathway validation. **(b)** Optimization of induction conditions. **(c)** Improving solubility of SAAT_{Fa}. **(d)** Improving solubility of AdhE2_{Ca}. **(e-h)** Ester selectivity.

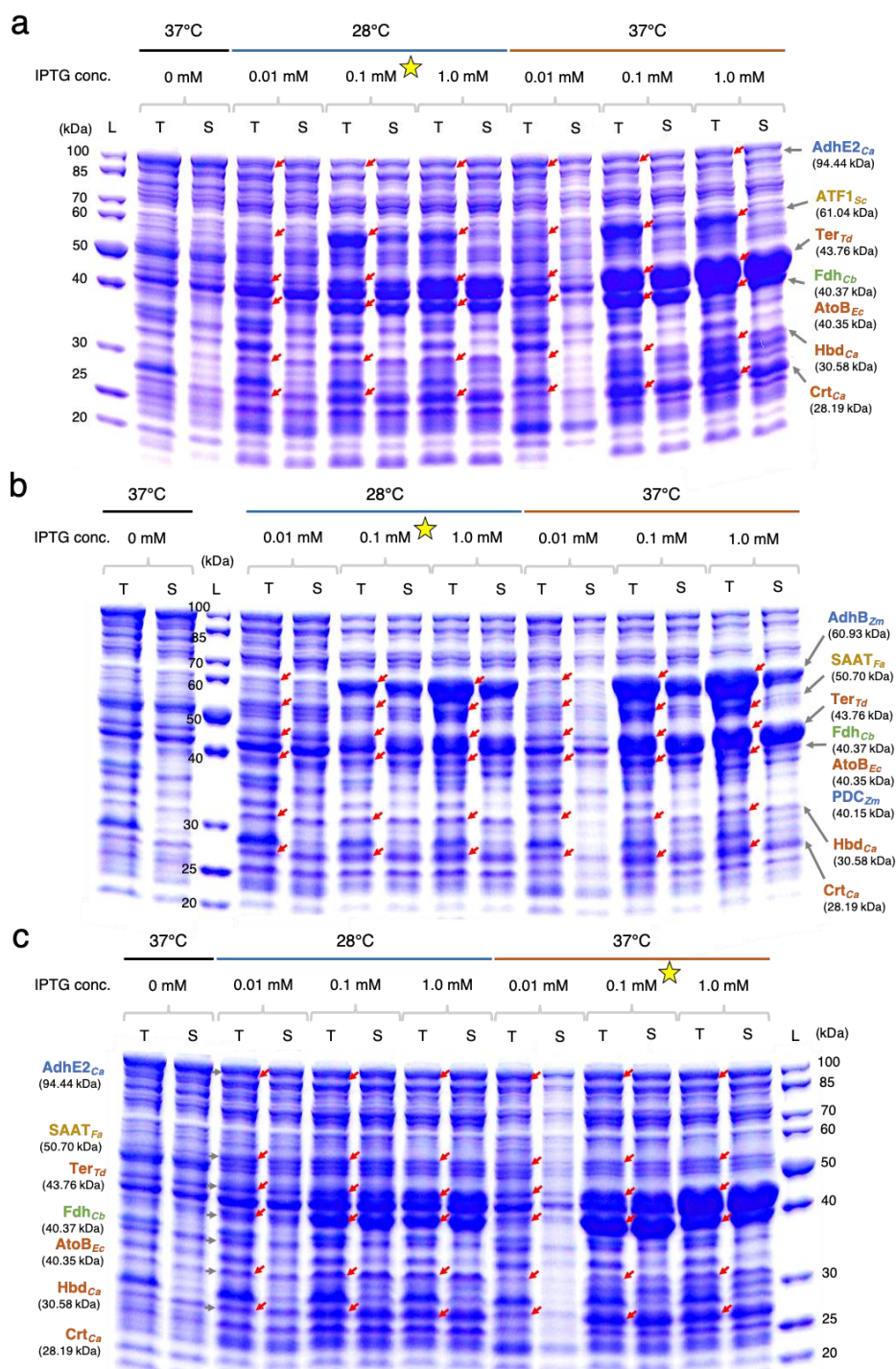


Figure 2-10: SDS-PAGE analysis for confirming expression of heterologous enzymes in EcJWBA2, EcJWEB2, and EcJWBB2 with various induction conditions. (a) EcJWBA2. (b) EcJWEB2. (c) EcJWBB2. Red arrows indicate the expected size of overexpressed enzymes. Yellow stars indicate the conditions result in the highest esters production among the evaluated conditions. Abbreviations: L, protein ladder; T, total fractions; S, Soluble fractions. (Induction conditions: 28/37°C, 0.01/0.1/1.0 mM IPTG)

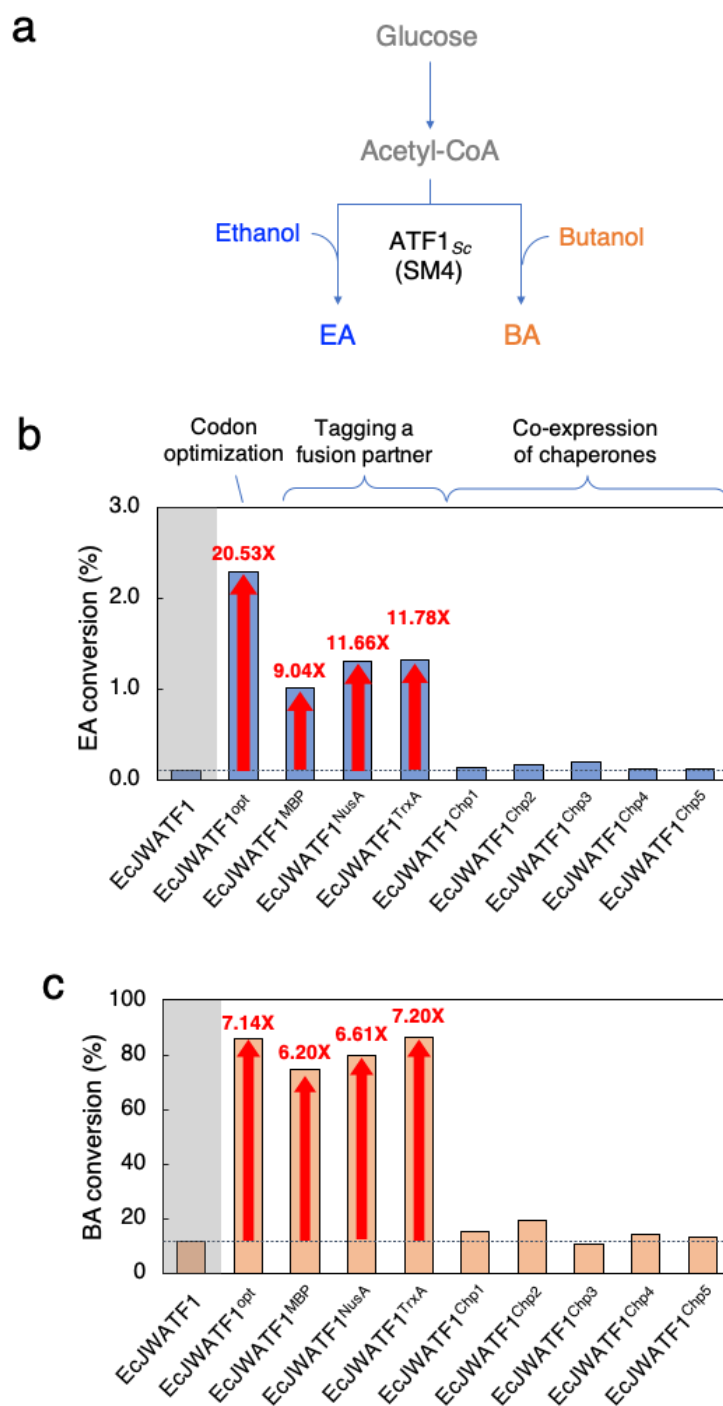


Figure 2-11: Summary of solubility engineering of ATF1_{Sc}. **(a)** Schematic representation of the conversion of an alcohol (ethanol/butanol) into an ester (ethyl acetate (EA)/butyl acetate (BA)) by ATF1_{Sc} in *E. coli*. Evaluation of various soluble protein expression strategies in the conversion of **(b)** ethanol into EA, and **(c)** butanol into BA. Grey box indicates a negative control.

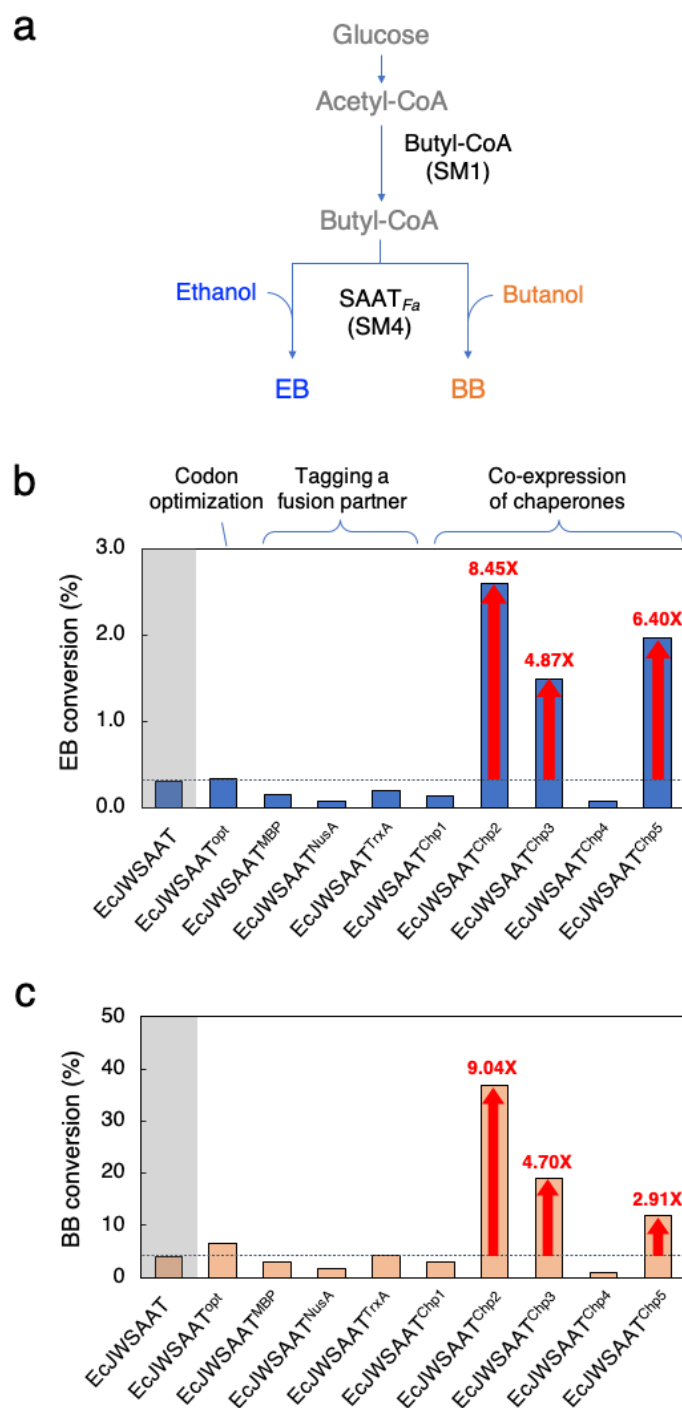


Figure 2-12: Summary of solubility engineering of SAAT_{Fa}. (a) Schematic representation of the conversion of an alcohol (ethanol/butanol) into an ester (ethyl butyrate (EB)/butyl butyrate (BB)) by SAAT_{Fa} in *E. coli*. Evaluation of various soluble protein expression strategies in the conversion (b) ethanol into EB and (c) butanol into BB. Grey box indicates a negative control.

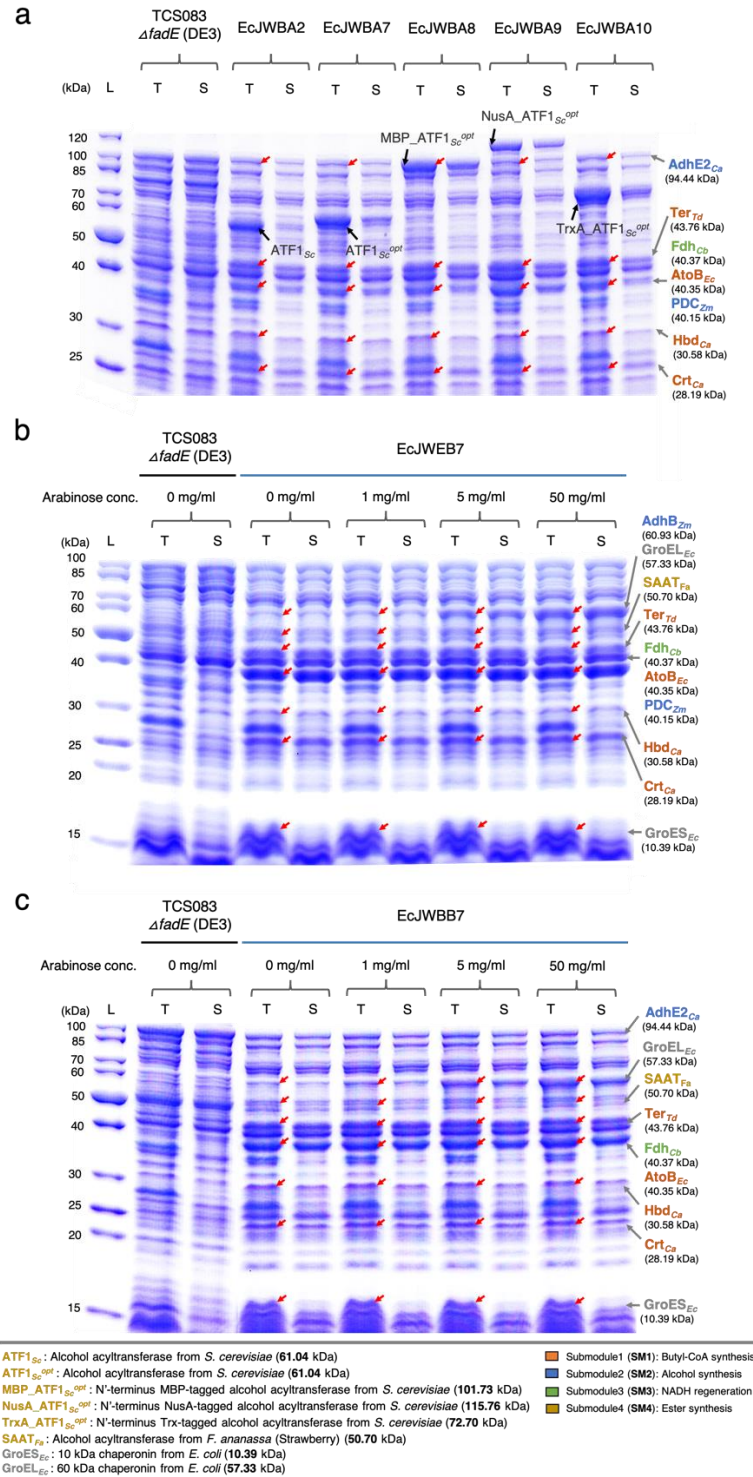


Figure 2-13: SDS-PAGE analysis for confirming expression of heterologous enzymes in (a) EcJWBA2 and EcJWBA7-10, (b) EcJWEB7, and (c) EcJWBB7 with various induction conditions. Red arrows indicate the expected size of overexpressed enzymes. Abbreviations: L, protein ladder; T, total fractions; S, Soluble fractions. (Induction conditions: EcJWBA7-10 and EcJWEB7: 28°C, 0.1 mM IPTG; EcJWBB7: 37°C, 0.1 mM IPTG).

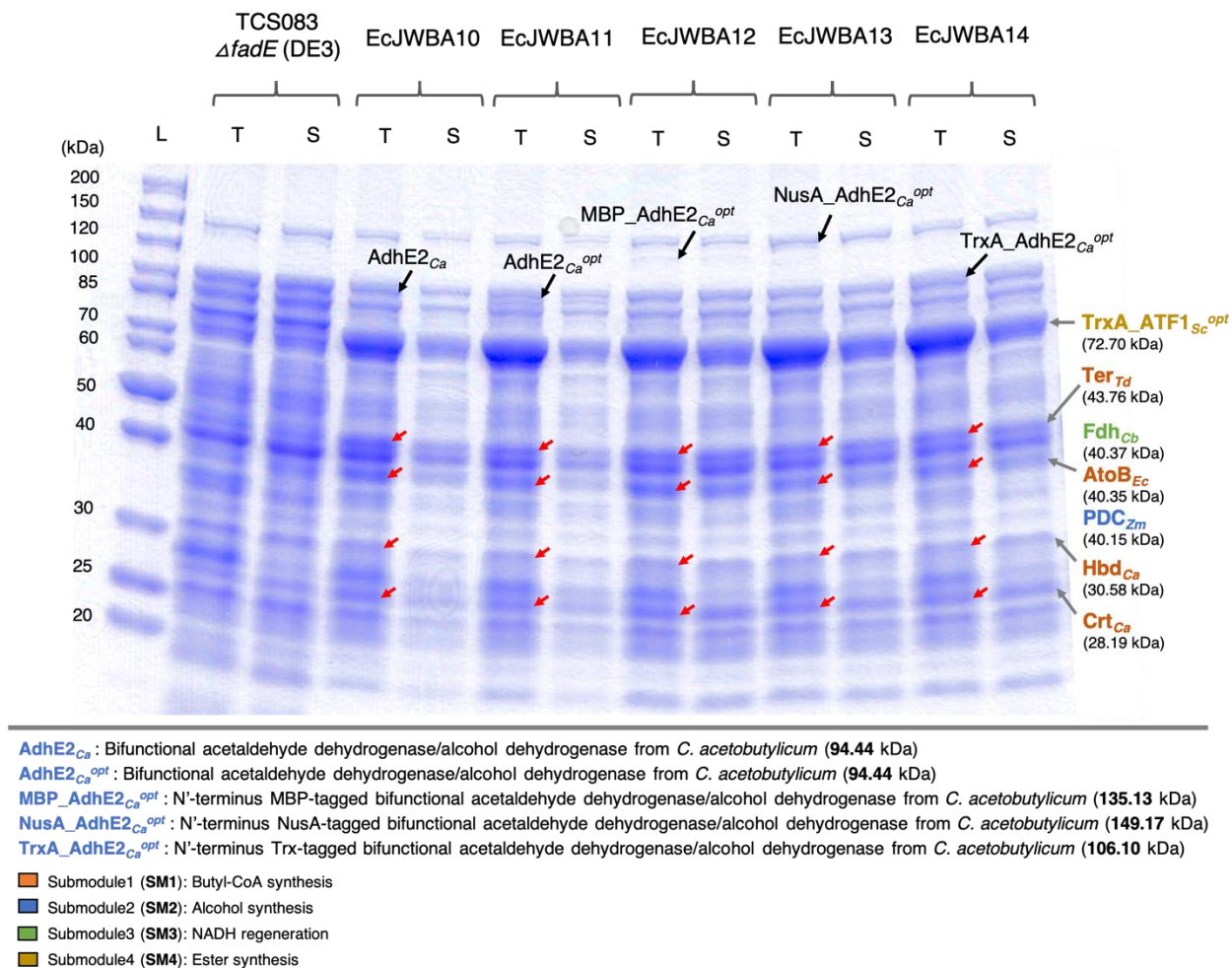


Figure 2-14: SDS-PAGE analysis for confirming expression of heterologous enzymes in EcJWBA10-14. Red arrows indicate the expected size of overexpressed enzymes. Abbreviations: L, protein ladder; T, total fractions; S, Soluble fractions. (Induction conditions: 28°C, 0.1 mM IPTG).

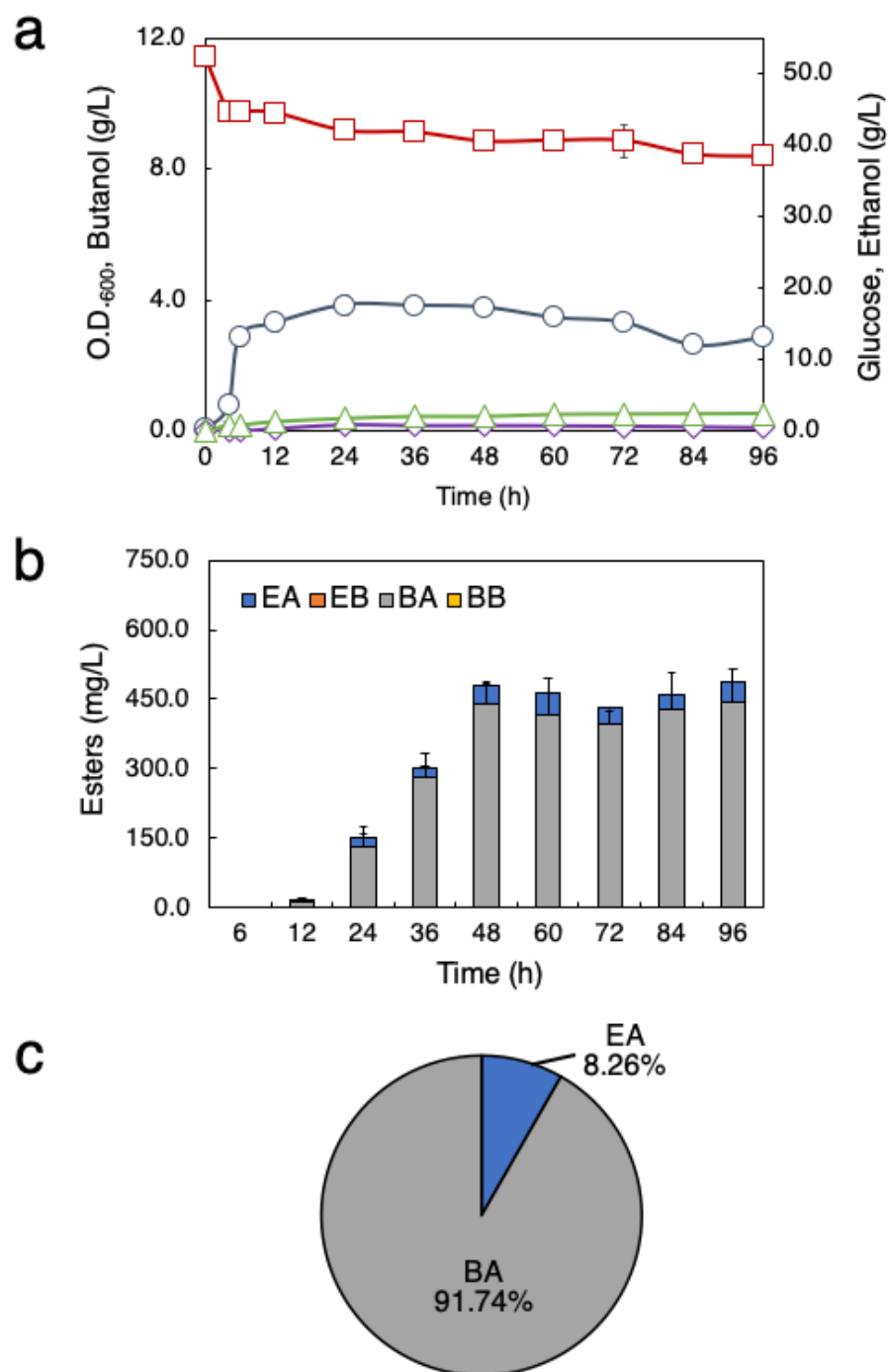


Figure 2-15: BA production by EcJWBA14 in anaerobic bottles with pH-adjustment to pH ~7 every 24 h. **(a)** Fermentation profile of cell growth (O.D.₆₀₀), glucose consumption, and alcohol production. Consumed glucose (in red squares); O.D.₆₀₀ values (in blue circles); Ethanol (in green triangles); Butanol, (in purple diamonds). **(b)** Ester production. **(c)** Ester selectivity at 48 h. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; and BB, butyl butyrate.

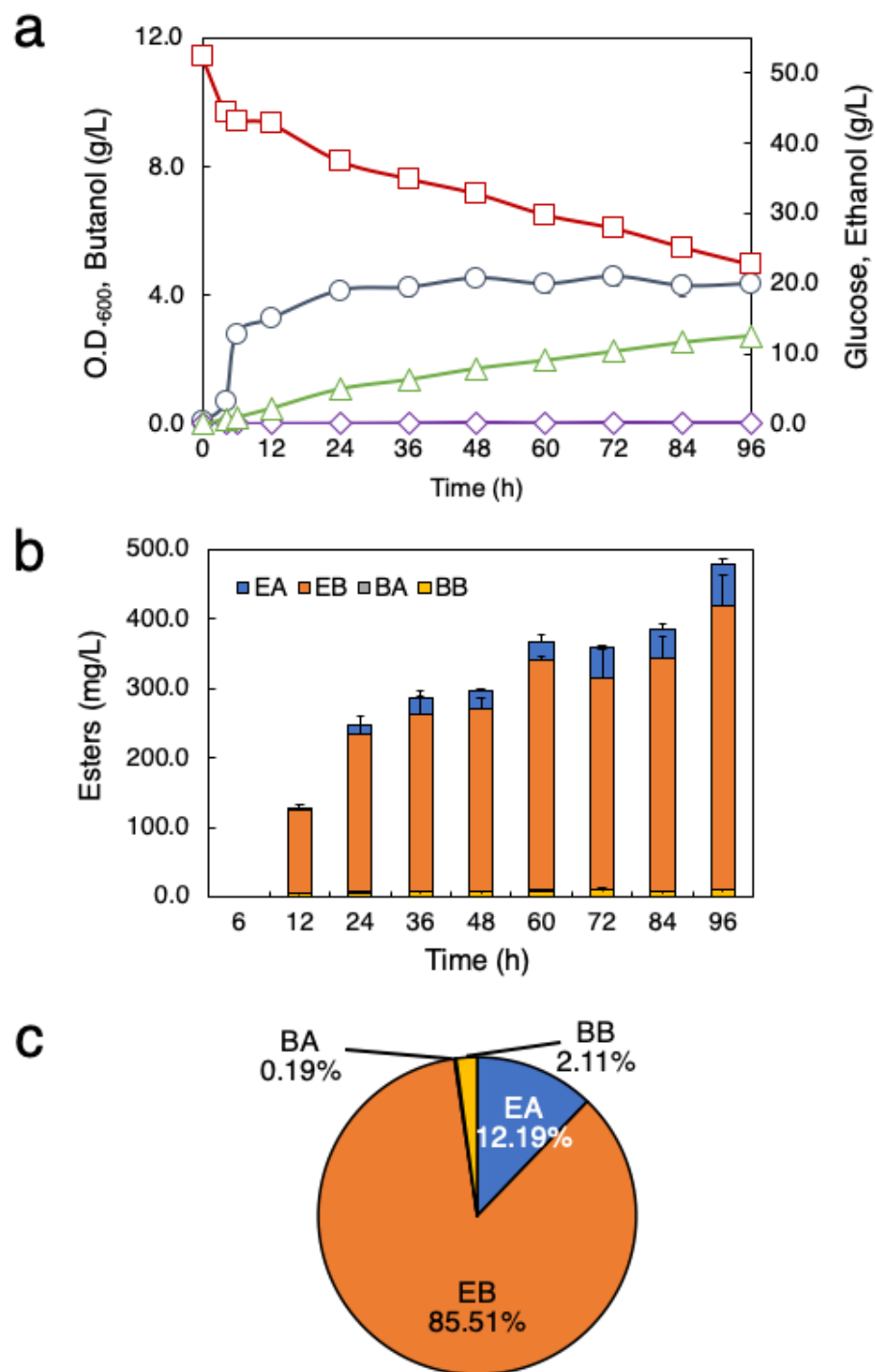


Figure 2-16: EB production by EcJWEB7 in anaerobic bottles with pH-adjustment to pH ~7 every 24 h. **(a)** Fermentation profile of cell growth (O.D.₆₀₀), glucose consumption, and alcohol production. Consumed glucose (in red squares); O.D.₆₀₀ values (in blue circles); Ethanol (in green triangles); Butanol, (in purple diamonds). **(b)** Ester production. **(c)** Ester selectivity at 96 h. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; and BB, butyl butyrate.

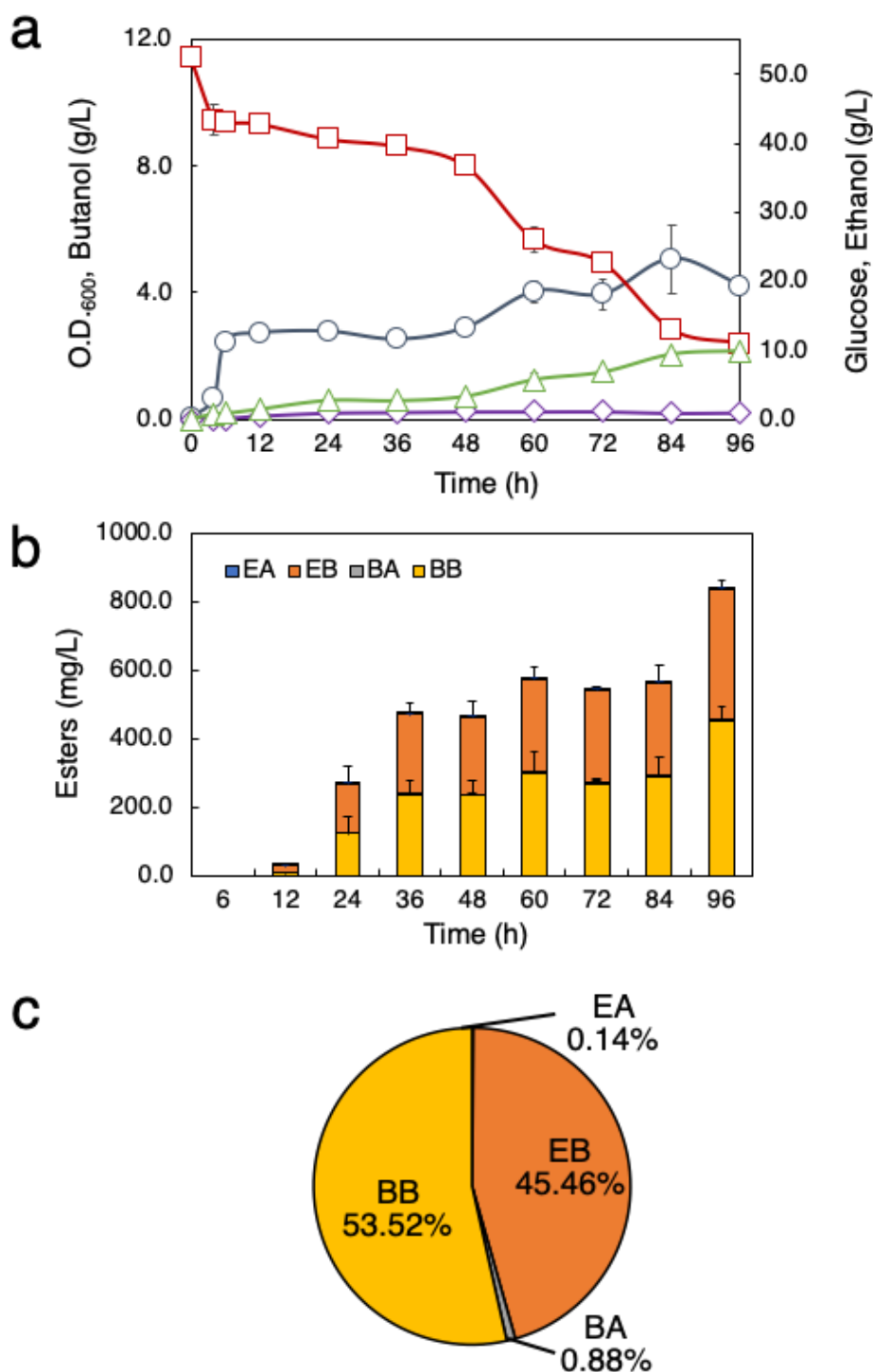


Figure 2-17: BB production by EcJWBB8 in anaerobic bottles with pH-adjustment to pH ~7 every 24 h. **(a)** Fermentation profile of cell growth (O.D.₆₀₀), glucose consumption, and alcohol production. Consumed glucose (in red squares); O.D.₆₀₀ values (in blue circles); Ethanol (in green triangles); Butanol, (in purple diamonds). **(b)** Ester production. **(c)** Ester selectivity at 96 h. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; and BB, butyl butyrate.

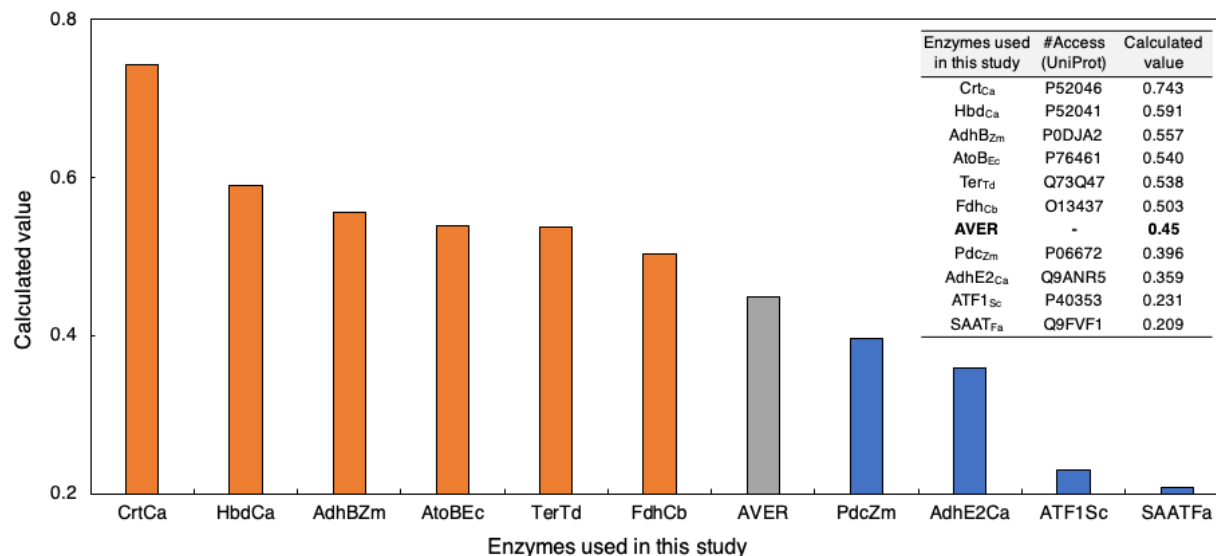


Figure 2-18: Predicted solubility of enzymes used in this study. The solubility of enzymes were predicted with Protein-Sol [8] (<https://protein-sol.manchester.ac.uk>) from the amino acid sequences. The AVER value (0.45) indicates the population average for the experimental dataset[9] (in grey). Thus, while the solubility values greater than 0.45 is predicted to have a higher solubility than the average soluble *E. coli* protein (in orange), the values lower than 0.45 is predicted to be less soluble (in blue).

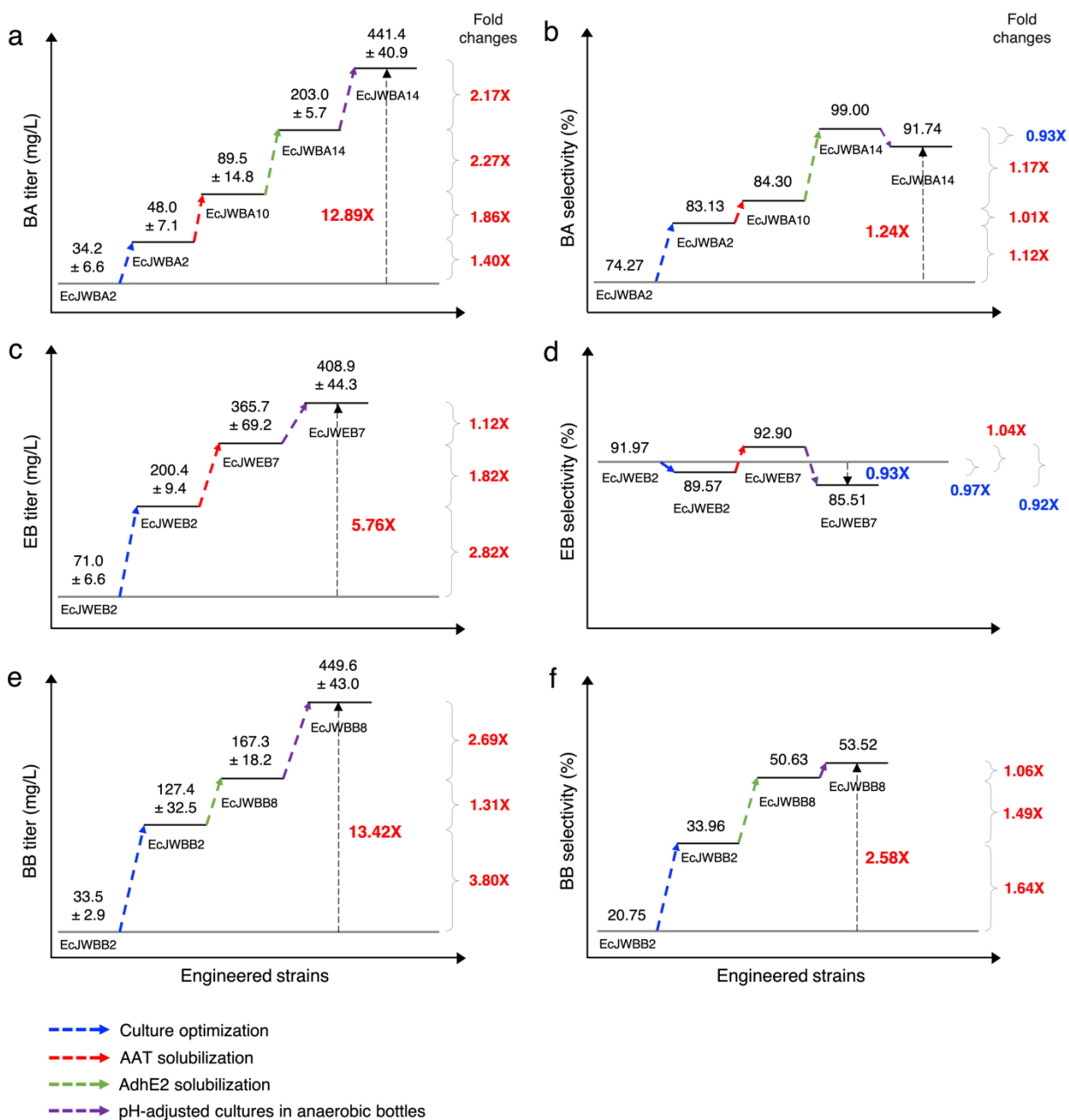


Figure 2-19: Summary of C4-derived esters production in engineered strains. (a-b) BA production; (a) BA titer, (b) BA selectivity. (c-d) EB production; (c) EB titer, (d) EB selectivity; (e-f) BB production (e) BB titer, (f) BB selectivity. Abbreviations: EA, ethyl acetate; EB, ethyl butyrate; BA, butyl acetate; and BB, butyl butyrate.

	Pathways	Stoichiometric balance	Reactions	#Modules
A	Glycolysis	1.5A	1.5 Glucose + 3 NAD ⁺ → 3 Pyruvate + 3 NADH	Endogenous
B	Pfl reaction	3B	3 Pyruvate → 3 Acetyl-CoA + 3 Formate	Endogenous
C	Fdh reaction	3C	3 Formate + 3 NAD ⁺ → 3 CO ₂ + 3 NADH	3
D	Butyl-CoA synthesis	D	2 Acetyl-CoA + 2 NADH → Butyl-CoA + 2 NAD ⁺	1
E	AdhE2 reaction	E	Butyl-CoA + 2 NADH → Butanol + 2 NAD ⁺	2
G	BA synthesis	G	Butanol + Acetyl-CoA → Butyl acetate	4
Net Reactions for BA production			1.5 Glucose + 2 NAD ⁺ → Butyl acetate + 3 CO ₂ + 2 NADH	

	Pathways	Stoichiometric balance	Reactions	#Modules
A	Glycolysis	1.5A	1.5 Glucose + 3 NAD ⁺ → 3 Pyruvate + 3 NADH	Endogenous
B	Pfl reaction	2B	2 Pyruvate → 2 Acetyl-CoA + 2 Formate	Endogenous
C	Fdh reaction	2C	2 Formate + 2 NAD ⁺ → 2 CO ₂ + 2 NADH	3
D	Butyl-CoA synthesis	D	2 Acetyl-CoA + 2 NADH → Butyl-CoA + 2 NAD ⁺	1
F	PDC/adhE reaction	F	Pyruvate + NADH → CO ₂ + Ethanol + NAD ⁺	2
G	EB synthesis	H	Ethanol + Butyl-CoA → Ethyl butyrate	4
Net Reactions for EB production			1.5 Glucose + 2 NAD ⁺ → Ethyl butyrate + 3 CO ₂ + 2 NADH	

	Pathways	Stoichiometric balance	Reactions	#Modules
A	Glycolysis	2A	2 Glucose + 4 NAD ⁺ → 4 Pyruvate + 4 NADH	Endogenous
B	Pfl reaction	4B	4 Pyruvate → 4 Acetyl-CoA + 4 Formate	Endogenous
C	Fdh reaction	4C	4 Formate + 4 NAD ⁺ → 4 CO ₂ + 4 NADH	3
D	Butyl-CoA synthesis	2D	4 Acetyl-CoA + 4 NADH → 2 Butyl-CoA + 4 NAD ⁺	1
E	AdhE2 reaction	E	Butyl-CoA + 2 NADH → Butanol + 2 NAD ⁺	2
I	BB synthesis	I	Butanol + Butyl-CoA → Butyl butyrate	4
Net Reactions for BB production			2 Glucose + 2 NAD ⁺ → Butyl butyrate + 4 CO ₂ + 2 NADH	

Figure 2-20: Summary of stoichiometric balance for synthesis of C4-derived esters.

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3 Microbial Production of Lactate Esters

This chapter was published by:

Lee, J.W., and Trinh, C.T., 2019. Microbial Biosynthesis of Lactate Esters, *Biotechnol. Biofuels*, **12**:226.

JWL and CTT designed the experiments, analyzed the data, and drafted the manuscript. **JWL** performed the experiments. CTT conceived and supervised this study.

3.1 Abstract

Green organic solvents such as lactate esters have broad industrial applications and favorable environmental profiles. Thus, manufacturing and use of these biodegradable solvents from renewable feedstocks help benefit the environment. However, to date, the direct microbial biosynthesis of lactate esters from fermentable sugars has not yet been demonstrated.

In this work, we present a microbial conversion platform for direct biosynthesis of lactate esters from fermentable sugars. First, we designed a pyruvate-to-lactate ester module, consisting of a lactate dehydrogenase (*ldhA*) to convert pyruvate to lactate, a propionate CoA-transferase (*pct*) to convert lactate to lactyl-CoA, and an alcohol acyltransferase (*AAT*) to condense lactyl-CoA and alcohol(s) to make lactate ester(s). By generating a library of five pyruvate-to-lactate ester modules with divergent *AAT*s, we screened for the best module(s) capable of producing a wide range of linear, branched, and aromatic lactate esters with an external alcohol supply. By co-introducing a pyruvate-to-lactate ester module and an alcohol (i.e., ethanol, isobutanol) module into a modular *Escherichia coli* (chassis) cell, we demonstrated for the first time the microbial biosynthesis of ethyl and isobutyl lactate esters directly from glucose. In an attempt to enhance ethyl lactate

production as a proof-of-study, we re-modularized the pathway into 1) the upstream module to generate the ethanol and lactate precursors and 2) the downstream module to generate lactyl-CoA and condense it with ethanol to produce the target ethyl lactate. By manipulating the metabolic fluxes of the upstream and downstream modules through plasmid copy numbers, promoters, ribosome binding sites, and environmental perturbation, we were able to probe and alleviate the metabolic bottlenecks by improving ethyl lactate production by 4.96-fold. We found that AAT is the most rate limiting step in biosynthesis of lactate esters likely due to its low activity and specificity towards the non-natural substrate lactyl-CoA and alcohols.

Here, we have successfully established the biosynthesis pathway of lactate esters from fermentable sugars and demonstrated for the first time the direct fermentative production of lactate esters from glucose using an *E. coli* modular cell. This study defines a cornerstone for the microbial production of lactate esters as green solvents from renewable resources with novel industrial applications.

3.2 Introduction

Solvents are widely used as primary components of cleaning agents, adhesives, and coatings and in assisting mass and heat transfer, separation and purification of chemical processes [1]. However, these solvents are volatile organic compounds (VOCs) that contribute to ozone depletion and photochemical smog via free radical air oxidation and hence cause many public health problems such as eye irritation, headache, allergic skin reaction, and cancer [1, 2]. Thus, recent interest in use of alternative green solvents is increasing due to environmental regulation and compelling demand for the eco-friendly solvents derived from renewable sources [3, 4].

Lactate esters are platform chemicals that have a broad range of industrial applications in

flavor, fragrance, and pharmaceutical industries [5]. These esters are generally considered as green solvents because of their favorable toxicological and environmental profiles. For instance, ethyl lactate is 100% biodegradable, non-carcinogenic, non-corrosive, low volatile, and unhazardous to human health and the environment [6]. Due to the unique beneficial properties of ethyl lactate, it has been approved as a Significant New Alternatives Policy (SNAP) solvent by the U.S. Environmental Protection Agency (EPA) and as food additives by the U.S. Food and Drug Administration (FDA) [6]. Recent technical and economic analysis conducted by the National Renewable Energy Laboratory (NREL) considers ethyl lactate to be one of the top twelve bioproducts [7].

In industrial chemical processes, lactate esters are currently produced by esterification of lactic acid with alcohols using homogenous catalysts (e.g., sulfuric acid, hydrogen chloride, and/or phosphoric acid) under high temperature reaction conditions [8]. However, use of strong acids as catalysts cause corrosive problems and often require more costly equipment for process operation and safety. Furthermore, the esterification reactions are thermodynamically unfavorable ($\Delta G = +5$ kcal/mol) in aqueous solutions and often encounter significant challenge due to self-polymerization of lactate [9]. Alternatively, microbial catalysts can be harnessed to produce these esters from renewable and sustainable feedstocks in a thermodynamically favorable reaction ($\Delta G = -7.5$ kcal/mol) in an aqueous phase environment at room temperature and atmospheric pressure [10-16]. This reaction uses an alcohol acyltransferase (AAT) to generate an ester by condensing an alcohol and an acyl-CoA. AAT can catalyze a broad substrate range including i) linear or branched short-to-long chain fatty alcohols [10, 11, 17], ii) aromatic alcohols [18], and iii) terpenols [19-22] as well as various fatty acyl-CoAs [11, 13]. To date, while microbial biosynthesis of the precursor metabolites for lactate esters have been well established such as lactate [13, 16,

23-27], lactyl-CoA [28-30], ethanol [31, 32], propanol [33], isopropanol [34], butanol [35], isobutanol [36], amyl alcohol [37], isoamyl alcohol [38], benzyl alcohol [39], 2-phenylethanol [40, 41], and terpenols [19-22], the direct microbial biosynthesis of lactate esters from fermentable sugars has not yet been demonstrated.

In this work, we aimed to demonstrate the feasibility of microbial production of lactate esters as green organic solvents from renewable resources. To enable the direct microbial biosynthesis of lactate esters from fermentable sugars, we first screened for an efficient AAT suitable for lactate ester production using a library of five pyruvate-to-lactate ester modules with divergent AATs. We next demonstrated direct fermentative biosynthesis of ethyl and isobutyl lactate esters from glucose by co-introducing a pyruvate-to-lactate ester module and an alcohol module (i.e., ethanol and isobutanol) into an engineered *Escherichia coli* modular cell. As a proof-of-study to improve ethyl lactate production, we employed a combination of metabolic engineering and synthetic biology approaches to dissect the pathway to probe and alleviate the potential metabolic bottlenecks.

3.3 Materials and Methods

3.3.1 Strain construction

The list of strains used in this study are presented in Table 3-1. For molecular cloning, *E. coli* TOP10 strain was used. To generate the lactate ester production strains, the modules, including i) the pyruvate-to-lactate ester modules (pJW002-006), ii) the upstream and/or downstream modules (pJW007-pJW028), and iii) the alcohol modules (pCT24 or pCT13), were transformed into the engineered modular *E. coli* chassis cell, EcDL002 [10] via electroporation [42].

Table 3-1: A list of strains used in this study.

Strains	Genotypes	Sources
<i>E. coli</i> TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>recA1</i> <i>araD</i> 139 Δ (<i>ara leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i> MG1655	F ⁻ λ ⁻	ATCC 47076
<i>Clostridium propionicum</i>	Wildtype	ATCC 25522
EcDL002	TCS083 (λ DE3) Δ <i>fadE</i>	[10]
EcJW101	EcDL002/pJW002; amp ^R	This study
EcJW102	EcDL002/pJW003; amp ^R	This study
EcJW103	EcDL002/pJW004; amp ^R	This study
EcJW104	EcDL002/pJW005; amp ^R	This study
EcJW105	EcDL002/pJW006; amp ^R	This study
EcJW201	EcDL002/pJW005 pCT24; amp ^R kan ^R	This study
EcJW202	EcDL002/pJW005 pCT13; amp ^R kan ^R	This study
EcJW106	EcDL002/pJW013; cm ^R	This study
EcJW203	EcDL002/pJW007 pJW011; cm ^R amp ^R	This study
EcJW204	EcDL002/pJW007 pJW012; cm ^R kan ^R	This study
EcJW205	EcDL002/pJW008 pJW010; cm ^R amp ^R	This study
EcJW107	EcDL002/pJW014; amp ^R	This study
EcJW206	EcDL002/pJW008 pJW012; amp ^R kan ^R	This study
EcJW207	EcDL002/pJW009 pJW010; cm ^R kan ^R	This study
EcJW208	EcDL002/pJW009 pJW011; amp ^R kan ^R	This study
EcJW108	EcDL002/pJW015; kan ^R	This study
EcJW209	EcDL002/pJW019 pJW012; cm ^R kan ^R	This study
EcJW210	EcDL002/pJW020 pJW012; cm ^R kan ^R	This study
EcJW211	EcDL002/pJW021 pJW012; cm ^R kan ^R	This study
EcJW212	EcDL002/pJW022 pJW012; cm ^R kan ^R	This study
EcJW213	EcDL002/pJW007 pJW027; cm ^R kan ^R	This study
EcJW214	EcDL002/pJW007 pJW028; cm ^R kan ^R	This study
EcJW215	EcDL002/pJW007 pJW029; cm ^R kan ^R	This study
EcJW216	EcDL002/pJW007 pJW030; cm ^R kan ^R	This study
EcJW217	EcDL002/pJW007 pJW031; cm ^R kan ^R	This study
EcJW218	EcDL002/pJW007 pJW032; cm ^R kan ^R	This study
EcJW219	EcDL002/pJW007 pJW033; cm ^R kan ^R	This study
EcJW220	EcDL002/pJW007 pJW034; cm ^R kan ^R	This study
EcJW221	EcDL002/pJW007 pJW035; cm ^R kan ^R	This study
EcJW109	EcDL002/pJW027; kan ^R	This study
EcJW110	EcDL002/pJW028; kan ^R	This study
EcJW111	EcDL002/pJW029; kan ^R	This study
EcJW112	EcDL002/pJW030; kan ^R	This study
EcJW113	EcDL002/pJW031; kan ^R	This study
EcJW114	EcDL002/pJW032; kan ^R	This study
EcJW115	EcDL002/pJW033; kan ^R	This study
EcJW116	EcDL002/pJW034; kan ^R	This study
EcJW117	EcDL002/pJW035; kan ^R	This study

Table 3-2: A list of plasmids used in this study.

Plasmids	Genotypes	Sources
pACYCDuet-1	Two sets of MCS, T ₇ promoter, P15A ori; cm ^R	Novagen
pETDuet-1	Two sets of MCS, T ₇ promoter, ColE1 ori; amp ^R	Novagen
pRSFDuet-1	Two sets of MCS, T ₇ promoter, RSF1030 ori; kan ^R	Novagen
pETite*	T ₇ promoter, pBR322 ori; kan ^R	[10]
pCT24	pETite* P _{T7} :: <i>pdC::adhB</i> ::T _{T7} ; kan ^R	[10]
pCT13	pCOLA P _{T7} :: <i>alsS::ilvC::ilvD</i> -P _{T7} :: <i>kivd::adhE</i> ::T _{T7} ; kan ^R	[43]
pDL004	pETite* <i>ATF1</i> ; kan ^R	[13]
pDL005	pETite* <i>ATF2</i> ; kan ^R	[13]
pDL001	pETite* <i>SAAT</i> ; kan ^R	[13]
pDL006	pETite* <i>VAAT</i> ; kan ^R	[13]
pCT16	pETite* <i>atfA</i> ; kan ^R	[44]
pJW001	pETite* P _{T7} :: <i>ldhA::pct</i> ::T _{T7} ; amp ^R	This study
pJW002	pJW001 P _{T7} :: <i>ldhA::pct</i> -P _{T7} :: <i>ATF1</i> ::T _{T7} ; amp ^R	This study
pJW003	pJW001 P _{T7} :: <i>ldhA::pct</i> -P _{T7} :: <i>ATF2</i> ::T _{T7} ; amp ^R	This study
pJW004	pJW001 P _{T7} :: <i>ldhA::pct</i> -P _{T7} :: <i>SAAT</i> ::T _{T7} ; amp ^R	This study
pJW005	pJW001 P _{T7} :: <i>ldhA::pct</i> -P _{T7} :: <i>VAAT</i> ::T _{T7} ; amp ^R	This study
pJW006	pJW001 P _{T7} :: <i>ldhA::pct</i> -P _{T7} :: <i>atfA</i> ::T _{T7} ; amp ^R	This study
pJW007	pACYCDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> ::T _{T7} ; cm ^R	This study
pJW008	pETDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> ::T _{T7} ; amp ^R	This study
pJW009	pRSFDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> ::T _{T7} ; kan ^R	This study
pJW010	pACYCDuet-1 P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; cm ^R	This study
pJW011	pETDuet-1 P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; amp ^R	This study
pJW012	pRSFDuet-1 P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; kan ^R	This study
pJW013	pACYCDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> -P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; cm ^R	This study
pJW014	pETDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> -P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; amp ^R	This study
pJW015	pRSFDuet-1 P _{T7} :: <i>ldhA::pdC::adhB</i> -P _{T7} :: <i>pct::VAAT</i> ::T _{T7} ; kan ^R	This study
pJW016	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{T7} ::T _{T7} ; cm ^R	This study
pJW017	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY1} ::T _{T7} ; cm ^R	This study
pJW018	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY3} ::T _{T7} ; cm ^R	This study
pJW019	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY1} ::synRBS _{pdC#1} :: <i>pdC::adhB</i> ::T _{T7} ; cm ^R	This study
pJW020	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY1} ::synRBS _{pdC#2} :: <i>pdC::adhB</i> ::T _{T7} ; cm ^R	This study
pJW021	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY3} ::synRBS _{pdC#3} :: <i>pdC::adhB</i> ::T _{T7} ; cm ^R	This study
pJW022	pACYCDuet-1 P _{T7} :: <i>ldhA</i> ::T _{T7} -P _{AY3} ::synRBS _{pdC#4} :: <i>pdC::adhB</i> ::T _{T7} ; cm ^R	This study
pJW023	pRSFDuet-1 P _{T7} :: <i>pct</i> ::T _{T7} -P _{T7} ::T _{T7} ; kan ^R	This study
pJW024	pRSFDuet-1 P _{T7} ::synRBS _{pct#1} :: <i>pct</i> ::T _{T7} -P _{T7} ::T _{T7} ; kan ^R	This study
pJW025	pRSFDuet-1 P _{T7} ::synRBS _{pct#2} :: <i>pct</i> ::T _{T7} -P _{T7} ::T _{T7} ; kan ^R	This study
pJW026	pRSFDuet-1 P _{T7} ::synRBS _{pct#3} :: <i>pct</i> ::T _{T7} -P _{T7} ::T _{T7} ; kan ^R	This study
pJW027	pRSFDuet-1 P _{T7} ::synRBS _{pct#1} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#1} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW028	pRSFDuet-1 P _{T7} ::synRBS _{pct#1} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#2} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW029	pRSFDuet-1 P _{T7} ::synRBS _{pct#1} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#3} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW030	pRSFDuet-1 P _{T7} ::synRBS _{pct#2} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#1} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW031	pRSFDuet-1 P _{T7} ::synRBS _{pct#2} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#2} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW032	pRSFDuet-1 P _{T7} ::synRBS _{pct#2} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#3} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW033	pRSFDuet-1 P _{T7} ::synRBS _{pct#3} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#1} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW034	pRSFDuet-1 P _{T7} ::synRBS _{pct#3} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#2} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study
pJW035	pRSFDuet-1 P _{T7} ::synRBS _{pct#3} :: <i>pct</i> ::T _{T7} -P _{T7} ::synRBS _{VAAT#3} :: <i>VAAT</i> ::T _{T7} ; kan ^R	This study

Table 3-3: A list of primers used in this study.

Primers	Sequences (5'→3')
<i>Pyruvate-to-lactyl-CoA module</i>	
DL_0001	CATCATCACCACCATCACTAA
DL_0002	ATGTATATCTCCTTCTTATAGTTAAAC
DL_0032	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATGAAACTCGCCGTTTATAG
DL_0033	GGGAACCTTTCTCATTATATCTCCTTTTAAACCAGTTCGTTCCGGC
DL_0034	ACGAACTGGTTTTAAAGGAGATATAATGAGAAAGTTCCATTAT
DL_0035	GCCGCTCTATTAGTGATGGTGGTGATGATGTCAGGACTTCATTTCTTCAG
<i>Pyruvate-to-lactate ester module</i>	
DL_0013	GAGCCTCAGACTCCAGCGTA
DL_0014	ATATCAAGCTTGAATTCGTTACCCGG
DL_0015	GGAGGAACCTATATCCGGGTAAACGAATTCAGCTTGATATTAATACGACTCACTATAGGG
DL_0016	GTCCAGTTACGCTGGAGTCTGAGGCTC
<i>Upstream module</i>	
JW_0001	GGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCATGAAACTCGCCGTTTATAGC
JW_0002	CTAAATAGGTACCGACAGTATAACTCATTATATCTCCTTTTAAACCAGTTCGTTCCGGC
JW_0003	CGAAACCTGCCCCAACGAACCTGGTTTAAAGGAGATATAATGAGTTATACTGTCGGTACC
JW_0004	CGCAAGCTTGTGACCTGCAGGCGCGCCGAGCTCGAATTCTTAGAAAGCGCTCAGGAAG
JW_0005	GGATCCTGGCTGTGGTGATGA
JW_0006	GAATTCGAGCTCGGCGCG
<i>Downstream module</i>	
JW_0007	GTATATTAGTTAAGTATAAGAAGGAGATATACATATGATGAGAAAGGTTCCCATTTATTAC
JW_0008	GAAATTATACTGACCTCAATTTTCTCCATTATATCTCCTTTCAGGACTTCATTTCTCTC
JW_0009	AATGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATAATGGAGAAAATTGAGGTCAG
JW_0010	CAAATTTTCGACGAGCGGTTTCTTTACCAGACTCGAGTCAATATCTTGAAATTAGCGTCT
JW_0011	CATATGTATATCTCCTTCTTATACTTAACT
JW_0012	CTCGAGTCTGGTAAAGAAAC
<i>Synthetic operons for upstream module</i>	
JW_0013	GGGAATTGTGAGCGGATAACAATTTCCCAAGGAGATATAATGAAACTCGCCGTTTATAGC
JW_0014	TTATGCTAGTTATTGCTCAGCGGTGGCGGCCGCTCTATTATTAAACCAGTTCGTTCCGG
JW_0015	TCTGGAAAAAGGCGAAACCTGCCCCAACGAACCTGGTTTAATAATAGAGCGGCCGC
JW_0016	GATTATGCGGCCGTGTACAATACGATTACTTTCTGTTTCGATTTCTACCGAAGAAAGGC
JW_0017	CATTATATCTCCTTGGGGAATTGTTATCCGC
JW_0018	TCGAACAGAAAGTAATCGTATTG
JW_0019	AAATTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCATGAGTTATACTGTCCGTACC
JW_0020	GCGTTCAAATTTTCGACGAGCGGTTTCTTTACCAGACTCGAGTTAGAAAGCGCTCAGGAA
JW_0021	AAATCTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCATGAGTTATACTGTCCGTACC
JW_0022	CATGCTAGCACTGTACCTAGGACTGAGCTAGCCGTCAAATTCGATTATGCGGCC
JW_0023	CATGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAGATTTTCGATTATGCGGCC
JW_0024	TACAGTGCTAGCAGCTTAGCGACAACCTAGGCGCTCGCATGAGTTATACTGTCCGTACC
JW_0025	GTATAATGCTAGCTTAGCAGTACCAGGACGTACCGGAGTATGAGTTATACTGTCCGTACC
JW_0026	TAGGTACAGTGCTAGCACTAGGCCTAGCGATTCCGCTAAATGAGTTATACTGTCCGTACC
JW_0027	TATAATGCTAGCAGTTTACCTAGGGCAATAGCGTACCGAATGAGTTATACTGTCCGTACC
JW_0028	CATGCGAGCGCTAGGGTTGTCGCTAAGCTGCTAGCACTGTACCTAGG

Table 3-3: Continued

Primers	Sequences (5'→3')
JW_0029	CATTTAGCGGAATCGCTAGGCCTAGTGCTAGCACTGTACCTAGG
JW_0030	CATACTCCGGTACGTCCTGGTACTGCTAAGCTAGCATTATACCTAGG
JW_0031	CATTCGGTACGCTATTGCCCTAGGTAACTGCTAGCATTATACCTAGG
<i>Synthetic operons for downstream module</i>	
JW_0032	TTATGCTAGTTATTGCTCAGCGGTGGCGGCCGCTCTATTATCAGGACTTCATTTCCTTCA
JW_0033	TGCAGAAGGCTTAATGGGTCTGAAGGAAATGAAGTCCTGATAATAGAGCGGCCGC
JW_0034	GATTATGCGGCCGTGTACAATACGATTACTTTCTGTTTCGATTTCTACCGAAGAAAGGC
JW_0035	GATATAGCTCGAACGCGGAAAGAGATGAGAAAGGTTCCCATTATTAC
JW_0036	TCAGGACTTCATTTCCTTCA
JW_0037	GCAACCTATTTTAATCCAAGGAAGATCTAATGAGAAAGGTTCCCATTATTAC
JW_0038	GCAATAACAACCTAGGAGAGACGACATGAGAAAGGTTCCCATTATTAC
JW_0039	TAATGGGAACCTTTCTCATCTCTTTCCGCGTTCGAGCTATATCGGGGAATTGTTATCCGC
JW_0040	TGCAGAAGGCTTAATGG
JW_0041	GGAACCTTTCTCATTAGATCTTCCTTGGATTAAAATAGGTTGCGGGGAATTGTTATCCGC
JW_0042	TAATGGGAACCTTTCTCATGTCGTCTCTCCTAGTTGTTATTGCGGGGAATTGTTATCCGC
JW_0043	TAACCAAAACACTAACGCAAGATGGAGAAAATTGAGGTCAGT
JW_0044	AGGGCACGAGGAGGAACCACTAGATAATGGAGAAAATTGAGGTCAGT
JW_0045	GCAACCAACACAACGAGGAGGCATTTAATGGAGAAAATTGAGGTCAGT
JW_0046	TACTGACCTCAATTTTCTCCATCTTGCGTTAGTGTTTTGGTTAGGGGAATTGTTATCCGC
JW_0047	CTCAATTTTCTCCATTCTACTGGTTCCTCCTCGTGCCCTGGGGGAATTGTTATCCGC
JW_0048	CTCAATTTTCTCCATTAAATGCCTCCTCGTTGTGTTGGTTGCGGGGAATTGTTATCCGC

3.3.2 Plasmid construction

The list of plasmids and primers used in this study are presented in Table 3-2 and Table 3-3, respectively. Pathway construction includes pyruvate-to-lactate ester modules and a library of upstream and downstream modules with various plasmid copy numbers, promoters, and ribosome binding sites (RBSs).

3.3.2.1 Construction of pyruvate-to-lactate ester modules

A library of pyruvate-to-lactate ester modules with five divergent AATs were constructed to screen for an efficient AAT for production of lactate esters via two rounds of cloning. First, the pyruvate-to-lactyl-CoA module (pJW001) was constructed by assembling three DNA fragments: i) the *ldhA* gene, encoding D-lactate dehydrogenase, amplified from *E. coli* MG1655 genomic DNA using the primer pair DL_0032/DL_0033, ii) the *pct* gene, encoding propionate CoA-transferase, amplified from *Clostridium propionicum* genomic DNA using the primer pair DL_0034/DL_0035, and iii) the backbone amplified from pETite* using the primer pair DL_0001/DL_0002 [45]. Then, the pyruvate-to-lactate ester modules (pJW002-006) were constructed by assembling three DNA fragments: i) the pyruvate-to-lactyl-CoA module amplified from pJW001 using the primer pair DL_0032/DL_0014, ii) the *ATF1* gene amplified from pDL004 for pJW002, the *ATF2* gene amplified from pDL005 for pJW003, the *SAAT* gene amplified from pDL001 for pJW004, the *VAAT* gene amplified from pDL006 for pJW005, or the *atfA* gene amplified from pCT16 for pJW006, using the primer pair DL_0015/DL_0016, and iii) the backbone amplified from pETite* using the primer pair DL_0013/ DL_0002. The genes *ATF1* and *ATF2* are originated from *Saccharomyces cerevisiae* [46], whereas the genes *SAAT*, *VAAT* and *atfA* are derived from *Fragaria ananassa* [47], *F. vesca* [48], and *Acinetobacter* sp. ADP1 [49], respectively.

3.3.2.2 Construction of a library of upstream and downstream modules with various plasmid copy numbers

A library of upstream and downstream modules were constructed to improve ethyl lactate biosynthesis through a combinatorial pathway optimization strategy using three different plasmids: i) pACYCDuet-1 (P15A origin of replication), ii) pETDuet-1 (ColE1 origin), and iii) pRSFDuet-1 (RSF1030 origin), having the plasmid copy numbers of 10, 40, and 100, respectively [50].

The upstream modules (pJW007-009) were constructed by assembling three DNA fragments: i) the *ldhA* gene amplified from pJW001 using the primer pair JW_0001/JW_0002, ii) the ethanol module containing *pdh* and *adhB* genes amplified from pCT24 using the primer pair JW_0003/JW_0004, and iii) the backbone amplified from pACYCDuet-1 for pJW007, from pETDuet-1 for pJW008, or from pRSFDuet-1 for pJW009 using the primer pair JW_0005/JW_0006.

The downstream modules (pJW010-012) were constructed by assembling three DNA fragments: i) the *pct* gene amplified from pJW001 using the primer pair JW_0007/JW_0008, ii) the *VAAT* gene amplified from pJW005 using the primer pair JW_0009/JW_0010, and iii) the backbone amplified from pACYCDuet-1 for pJW010, pETDuet-1 for pJW011, or pRSFDuet-1 for pJW012 using the primer pair JW_0011/JW_0012.

The combined upstream and downstream modules (pJW013-015) were constructed by assembling two DNA fragments: i) the upstream module amplified from pJW007 using the primer pair JW_0001/JW_0004 and ii) the backbone containing the downstream module amplified from pJW010 for pJW013, pJW011 for pJW014, or pJW012 for pJW015 using the primer pair JW_0005/JW_0006.

3.3.2.3 Construction of a library of upstream and downstream modules with various promoters and RBSs

For tighter regulation of biosynthetic pathway of ethyl lactate, we constructed the upstream and downstream modules with tunable promoters and RBSs. The upstream modules (pJW019-022) were constructed via three rounds of cloning. First, the T7 terminator (T_{T7}) was added between the multiple cloning site 1 (MCS1) and MCS2 of the pACYCDuet-1 backbone to create the first intermediate plasmid, pJW016, by assembling three DNA fragments: i) the *ldhA* gene amplified from pJW001 using the primer pair JW_0013/JW_0014, ii) the linker containing T_{T7} terminator amplified from pETite* using the primer pair JW_0015/JW_0016, and iii) the backbone amplified from pACYCDuet-1 using the primer pair JW_0017/JW_0018. Next, the original T7 promoter (P_{T7}) in MCS2 of pJW016 was replaced with the P_{AY1} (BBa_J23100) promoter and P_{AY3} (BBaJ23108) promoter to generate two second intermediate plasmids, pJW017 and pJW018, respectively, by assembling two DNA fragments: i) the ethanol module amplified from pCT24 under the P_{AY1} promoter for pJW017 or P_{AY3} promoter for pJW018 using the primer pair JW_0019/JW_0020 or JW_0021/JW_0020, respectively, and ii) the backbone amplified from pJW016 using the primer pair JW_0022/JW_0012 or JW_0023/JW_0012, respectively. Lastly, the final four synthetic operons (pJW019-022) were constructed by assembling two DNA fragments: i) the ethanol module amplified from pCT24 with the synthetic RBS sequences with predicted translation initiation rates of 0.33au for pJW019 and pJW021 and 0.03au for pJW020 and pJW022 using the primer pairs JW_0024/JW_0020, JW_0025/JW_0020, JW_0026/JW_0020, and JW_0027/JW_0020, respectively, and ii) the backbone amplified from pJW017 for pJW019, pJW017 for pJW020, pJW018 for pJW021, and pJW018 for pJW022 using the primer pairs JW_0028/JW_0012, JW_0029/JW_0012, JW_0030/JW_0012, and JW_0031/JW_0012,

respectively. The P_{AY1} and P_{AY3} promoter sequences were obtained from the iGEM Anderson promoter library (<http://parts.igem.org/Promoters/Catalog/Anderson>) and the strength of promoters were assigned as $P_{AY3} = 0.5 \times P_{AY1}$. The RBS Calculator v2.0 [51, 52] was used to generate four synthetic RBS sequences with predicted translation initiation rates of 0.33 and 0.03 between the P_{AY1} (or P_{AY3}) promoter and *pdv* start codon (Figure 3-8).

The downstream modules (pJW027-035) were constructed via three rounds of cloning. First, the T_{T7} terminator was added between MCS1 and MCS2 of the pRSFDuet-1 backbone to generate the first intermediate plasmid, pJW023, by assembling three DNA fragments: i) the *pct* gene amplified from pJW001 using the primer pair JW_0013/JW_0032, ii) the linker containing T_{T7} terminator from pETite* using the primer pair JW_0033/JW_0034, and iii) the backbone from pRSFDuet-1 using the primer pair JW_0017/JW_0018. Then, the original RBS in MCS1 of pJW023 was replaced with synthetic RBSs of various strengths to generate the second intermediate plasmids, pJW024-026, by assembling two DNA fragments: i) the *pct* gene amplified from pJW001 with the synthetic RBS sequences with predicted translation initiation rates at 90, 9000, or 90000au for pJW024, pJW025 or pJW026 using the primer pair JW_0035/JW_0036, JW_0037/JW_0036, or JW_0038/JW_0036, respectively, and ii) the backbone amplified from pJW023 using the primer pair JW_0039/JW_0040 for pJW024, JW_0041/JW_0040 for pJW025, or JW_0042/JW_0040 for pJW026, respectively. Lastly, the final nine downstream modules (pJW027-035) were constructed by assembling a combination of two DNA fragments: i) the *VAAT* gene amplified from pDL006 with the synthetic RBS sequences predicted with translation initiation rates of 90, 9000, or 90000au for pJW027/pJW030/pJW033, pJW028/pJW031/pJW034, or pJW029/pJW032/pJW035 using the primer pair JW_0043/JW_0010, JW_0044/JW_0010, or JW_0045/JW_0010, respectively, and ii) the backbone amplified from pJW024, pJW025, or

pJW026 for pJW027-029, pJW030-032, or pJW033-035 using the primer pair JW_0046/JW_0012, JW_0047/JW_0012 or JW_0048/JW_0012, respectively. The RBS Calculator v2.0 [51, 52] was used to generate six synthetic RBS sequences with predicted translation initiation rates of 90, 9000, and 90000au between the P_{T7} promoter and *pct* (or *VAAT*) start codon (Figure 3-8).

3.3.3 Culture media and conditions

3.3.3.1 Culture media

For molecular cloning, seed cultures, and protein expression analysis, the Luria-Bertani (LB) medium, comprising of 10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl, was used. For high-cell density cultures, pre-cultures of bioreactor batch fermentations, and growth inhibition analysis of lactate esters, the M9 hybrid medium [10] with 20 g/L glucose was used. For bioreactor batch fermentations, the M9 hybrid medium with 50 g/L glucose and 100 μ L of antifoam (Antifoam 204, Sigma-Aldrich, MO, USA) was used. 30 μ g/mL chloramphenicol (cm), 50 μ g/mL kanamycin (kan), and/or 50 μ g/mL ampicillin (amp) was added to the media for selection where applicable.

3.3.3.2 High-cell density cultures

For seed cultures, 2% (v/v) of stock cells were grown overnight in 5 mL of LB with appropriate antibiotics. For pre-cultures, 1% (v/v) of seed cultures were transferred into 100 mL of LB medium in 500 mL baffled flasks. For main cultures, pre-cultures were aerobically grown overnight (at 37°C, 200 rpm), centrifuged (4700 rpm, 10 min), and resuspended to yield an optical density measured at 600nm (OD_{600nm}) of 3 in M9 hybrid medium containing appropriate concentration of isopropyl-beta-D-thiogalactopyranoside (IPTG) and antibiotics. The resuspended cultures were distributed into 15 mL polypropylene centrifuge tubes (Thermo Scientific, IL, USA)

with a working volume of 5 mL and grown for 24 hour (h) on a 75° angled platform in a New Brunswick Excella E25 at 37°C, 200 rpm. The tubes were capped to generate anaerobic condition. All high-cell density culture studies were performed in biological triplicates.

3.3.3.3 pH-Controlled bioreactor batch fermentations

pH-Controlled bioreactor batch fermentations were conducted with a Biostat B+ (Sartorius Stedim, NY, USA) dual 1.5 L fermentation system at a working volume of 1 L M9 hybrid medium. The seed and pre-cultures were prepared as described in high-cell density cultures in LB and M9 hybrid media, respectively. For main cultures, 10% (v/v) of pre-cultures were inoculated into fermentation cultures. During the fermentation, to achieve high cell density, dual-phase fermentation approach [25, 53], aerobic cell growth phase followed by anaerobic production phase, was applied. For the first aerobic phase, the temperature, agitation, and air flow rate were maintained at 37°C, 1000 rpm, and 1 volume/volume/min (vvm) for 4 h, respectively. Then, the oxygen in the medium was purged by sparing nitrogen gas at 2 vvm for 2 h to generate anaerobic condition. For the subsequent anaerobic phase, 0.5 mM of IPTG was added to induce the protein expression, and the culture temperature and nitrogen flow rate were maintained at 30°C and 0.2 vvm, respectively. During the fermentation, the pH was maintained at 7.0 with 5 M KOH and 40% H₃PO₄. Bioreactor batch fermentation studies were performed in biological duplicates.

3.3.3.4 Growth inhibition analysis of lactate esters

Seed cultures of EcDL002 were prepared as described in high-cell density cultures. 4 % (v/v) of seed cultures were inoculated into 100 µL of the M9 hybrid media, containing various concentrations (0.5~40 g/L) of lactate esters including ethyl-, propyl-, butyl-, isobutyl-, isoamyl-, or benzyl lactate, in a 96-well microplate. Then, the microplate was sealed with a plastic adhesive

sealing film, SealPlate® (EXCEL Scientific, Inc., CA, USA) to prevent evaporation of lactate esters and incubated at 37°C with continuous shaking using a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., VT, USA). OD_{600nm} was measured at 20 min intervals. Growth inhibition studies of lactate esters were performed twice in biological triplicates ($n = 6$).

3.3.4 Protein expression and SDS-PAGE analysis

Seed cultures were prepared as described in high-cell density cultures. 1% (v/v) of seed cultures subsequently inoculated in 500 mL baffled flasks containing 100 ml of LB medium. Cells were aerobically grown at 37°C and 200 rpm and induced at an OD_{600nm} of 0.6~0.8 with 0.5 mM of IPTG. After 4 h of induction, cells were collected by centrifugation and resuspended in 100 mM of sodium phosphate buffer (pH 7.0) at the final OD_{600nm} of 10. Cell pellets were disrupted using a probe-type sonicator (Model 120, Fisher Scientific, NH, USA) on ice-water mixture. The resulting crude extracts were mixed with 6x sodium dodecyl sulfate (SDS) sample buffer, heated at 100°C for 5 min, and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 14% polyacrylamide gel). Protein bands were visualized with Coomassie Brilliant Blue staining.

3.3.5 Analytical methods

3.3.5.1 Determination of cell concentrations

The optical density was measured at 600 nm using a spectrophotometer (GENESYS 30, Thermo Scientific, IL, USA). The dry cell mass was obtained by multiplication of the optical density of culture broth with a pre-determined conversion factor, 0.48 g/L/OD.

3.3.5.2 High performance liquid chromatography (HPLC)

Glucose, lactate, acetate, ethanol, isobutanol, isoamyl alcohol, and benzyl alcohol were quantified by using the Shimadzu HPLC system (Shimadzu Inc., MD, USA) equipped with the

Aminex HPX-87H cation exchange column (BioRad Inc., CA, USA) heated at 50°C. A mobile phase of 10 mN H₂SO₄ was used at a flow rate of 0.6 mL/min. Detection was made with the reflective index detector (RID) and UV detector (UVD) at 220 nm.

3.3.5.3 Gas chromatography coupled with mass spectroscopy (GC/MS)

All esters were quantified by GC/MS. For GC/MS analysis, analytes in the supernatants were extracted with dichloromethane (DCM), containing pentanol as an internal standard, in a 1:1 (v/v) ratio for 1 h at 37°C, 200 rpm in 15 mL polypropylene centrifuge tubes. After extraction, supernatant-DCM mixtures were centrifuged and 5 µL of DCM extracts were injected into a gas chromatograph (GC) HP 6890 equipped with the mass selective detector (MS) HP 5973. For the GC system, helium was used as the carrier gas at a flow rate of 0.5 mL/min and the analytes were separated on a Phenomenex ZB-5 capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C. Next a 25°C/min ramp was deployed to 235°C and then finally held a temperature of 300°C for 2 minutes to elute any residual non-desired analytes. The injection was performed using a splitless mode with an initial injector temperature of 280°C. For the MS system, a selected ion monitoring (SIM) mode was deployed to detect analytes.

The SIM parameters for detecting lactate esters were as follows: i) for pentanol, ions 53.00, 60.00, and 69.00 detected from 5.00 to 7.70 min, ii) for ethyl lactate, ions 46.00, 47.00, and 75.00 detected from 7.70 to 10.10 min, iii) for propyl lactate, ions 59.00, 88.00, and 89.00 detected from 10.10 to 11.00 min, iv) for isobutyl lactate, ions 56.00, 57.00, and 59.00 detected from 11.00 to 11.60 min, v) for butyl lactate, ions 75.00, 91.00, and 101.00 detected from 11.60 to 12.30 min, vi) for isoamyl lactate, ions 46.00, 73.00, 75.00 from 12.30 to 14.50 min, and vii) for benzyl lactate, ions 45.00, 91.00, and 180.00 from 14.50 to 15.08 min. The SIM parameters for detecting acetate

esters were as follows: i) for ethyl acetate, ions 45.00, 61.00, and 70.00 detected from 4.22 to 5.35 min, ii) for propyl acetate, ions 57.00, 59.00, and 73.00 detected from 5.35 to 6.40 min, iii) for pentanol, ions 53.00, 60.00, and 69.00 detected from 6.40 to 6.60 min, iv) for isobutyl acetate, ions 56.00, 61.00, and 73.00 detected from 6.60 to 7.70 min, v) for butyl acetate, ions 57.00, 71.00, and 87.00 detected from 7.70 to 9.45 min, vi) for isoamyl acetate, ions 58.00, 70.00, and 88.00 detected from 9.45 to 13.10 min, and vii) for benzyl acetate, ions 63.00, 107.00, and 150.00 from 13.10 to 15.82 min.

3.3.6 Statistics

Statistical analysis was performed with SigmaPlot v.14 using the two-tailed unpaired Student's t-test.

3.4 Results and Discussion

3.4.1 *In vivo* screening of efficient AATs critical for lactate ester biosynthesis

The substrate specificity of AATs is critical to produce target esters [13]. For example, ATF1 exhibits substrate preference for biosynthesis of acyl (C4-C6) acetates while SAAT and VAAT prefer biosynthesis of ethyl (C2-C6) acylates. Even though both SAAT and VAAT are derived from the same strawberry genus, they also show very distinct substrate preferences; specifically, SAAT prefers longer (C4-C6) acyl-CoAs whereas VAAT prefers shorter (C2-C4) acyl-CoAs. To date, none of AATs have been tested for lactate ester biosynthesis. Thus, to enable lactate ester biosynthesis, we began with identification of the best AAT candidate. We designed, constructed, and characterized a library of five pyruvate-to-lactate ester modules (pJW002-006) carrying five divergent AATs including ATF1, ATF2, SAAT, VAAT, and AtfA, respectively. AtfA was used as a negative control because it prefers long-chain acyl-CoAs (C14-C18) and

alcohols (C14-C18) [54]. For characterization, 2 g/L of ethanol, propanol, butanol, isobutanol, isoamyl alcohol, and benzyl alcohol were added to culture media with 0.5 mM of IPTG for pathway induction to evaluate biosynthesis of six different lactate esters including ethyl lactate, propyl lactate, butyl lactate, isobutyl lactate, isoamyl lactate, and benzyl lactate, respectively, in high-cell density cultures (Figure 3-1A). The results show that most of the strains could produce different types of lactate esters with external supply of alcohols (Figure 3-1B and C). EcJW104 achieved the highest titer of lactate esters in all cases, producing 1.6 ± 0.0 mg/L of ethyl lactate, 5.5 ± 0.3 mg/L of propyl lactate, 11.8 ± 0.4 mg/L of butyl lactate, 9.9 ± 0.1 mg/L of isobutyl lactate, 24.7 ± 0.6 mg/L of isoamyl lactate, and 51.6 ± 2.1 mg/L of benzyl lactate in ethanol, propanol, butanol, isobutanol, isoamyl alcohol, and benzyl alcohol doping, respectively. The lactate ester biosynthesis of EcJW104 exhibited different alcohol substrate preference in the following order: benzyl alcohol > isoamyl alcohol > butanol > isobutanol > propanol > ethanol (Figure 3-1B, Table 3-5).

Due to the presence of endogenous acetyl-CoA, we also produced acetate esters in addition to lactate esters (Figure 3-1). Among the strains, EcJW101 achieved the highest titers of acetate esters in all cases, producing 115.5 ± 4.8 mg/L of ethyl acetate, 801.6 ± 33.5 mg/L of propyl acetate, $1,017.9 \pm 20.2$ mg/L of butyl acetate, $1,210.4 \pm 24.8$ mg/L of isobutyl acetate, 692.7 ± 7.7 mg/L of isoamyl acetate, and $1,178.0 \pm 45.7$ mg/L of benzyl acetate in ethanol, propanol, butanol, isobutanol, isoamyl alcohol, and benzyl alcohol doping, respectively. EcJW101 showed different alcohol substrate preference for the acetate ester biosynthesis in the following order: isobutanol > benzyl alcohol > butanol > propanol > isoamyl alcohol > ethanol (Table 3-5).

Taken altogether, VAAT and ATF1 are the most suitable AATs for biosynthesis of lactate esters and acetate esters, respectively. Among the library of 12 esters (Figure 3-1C), seven of these

esters, including ethyl lactate, propyl lactate, butyl lactate, isobutyl lactate, isoamyl lactate, benzyl lactate, and benzyl acetate, were demonstrated for *in vivo* production in microbes for the first time. EcJW104 that harbors the pyruvate-to-lactate module with VAAT could produce 6 out of 6 target lactate esters including ethyl, propyl, butyl, isobutyl, isoamyl, and benzyl lactate. Since EcJW104 achieved the highest titer of lactate esters in all cases, it was selected for establishing the biosynthesis pathway of lactate esters from glucose.

3.4.2 Establishing the lactate ester biosynthesis pathways

We next demonstrated direct fermentative production of lactate esters from glucose using the best VAAT candidate. We focused on the biosynthesis of ethyl and isobutyl lactate esters. We designed the biosynthesis pathways for ethyl and isobutyl lactate by combining the pyruvate-to-lactate ester module (pJW005) with the ethanol (pCT24) and isobutanol (pCT13) modules, respectively. By co-transforming pJW005/pCT24 and pJW005/pCT13 into the modular cell EcDL002, we generated the production strains, EcJW201 and EcJW202, for evaluating direct conversion of glucose to ethyl and isobutyl lactate esters. We characterized EcJW201 and EcJW202 together with the parent strain, EcDL002, as a negative control in high-cell density cultures. The results show EcJW201 and EcJW202 produced ethyl (Figure 3-2A) and isobutyl (Figure 3-2B) lactate from glucose, respectively, while the negative control strain EcDL002 could not.

Consistently, the expressions of metabolic enzymes of the ethyl and isobutyl lactate pathways were confirmed in EcJW201 and EcJW202, respectively, by SDS-PAGE analysis (Figure 3-6). During 24 h fermentation, EcJW201 produced 2.2 ± 0.3 mg/L of ethyl lactate with a specific productivity of 0.04 ± 0.00 mg/gDCW/h while EcJW202 produced 0.3 ± 0.0 mg/L of isobutyl lactate with a specific productivity of 0.01 ± 0.00 mg/gDCW/h. In addition to ethyl or

isobutyl lactate biosynthesis, EcJW201 also produced 92.3 ± 9.2 mg/L of ethyl acetate while EcJW202 generated 1.4 ± 0.7 mg/L of ethyl acetate and 0.3 ± 0.1 mg/L of isobutyl acetate (Table 3-6A). Taken altogether, the direct microbial synthesis of lactate esters from fermentable sugar was successfully demonstrated. Since the lactate ester production was low, the next logical step was to identify and alleviate the key pathway bottlenecks for enhanced lactate ester biosynthesis. As proof-of-principle, we focused on optimization of the ethyl lactate production as presented in the subsequent sections.

3.4.3 Identifying and alleviating key bottlenecks of the ethyl lactate biosynthesis pathway

3.4.3.1 Evaluating the biosynthesis of ethyl lactate in pH-controlled fermentation as a basis to identify potential pathway bottlenecks

To identify the key bottlenecks of the ethyl lactate biosynthesis pathway, we characterized EcJW201 in pH-controlled bioreactors. The results show that EcJW201 produced 9.2 ± 0.1 mg/L of ethyl lactate with a specific productivity of 0.15 ± 0.02 mg/gDCW/h and a yield of 0.19 ± 0.00 mg/g glucose (Figure 3-2C, Table 3-6B) in 18 h. Under pH-controlled fermentation, EcJW201 achieved 4.09-fold (from 2.2 ± 0.3 to 9.2 ± 0.1 mg/L), 3.75-fold (from 0.04 ± 0.00 to 0.15 ± 0.02 mg/gDCW/h), and 19-fold (from 0.01 ± 0.00 to 0.19 ± 0.00 mg/g glucose) improvement in titer, specific productivity, and yield, respectively, as compared to the strain performance in the high cell density culture. It is interesting to observe that ethyl acetate was first produced then consumed after 10 h, which is likely due to the endogenous esterase of *E. coli* as observed in a recent study [55]. Different from ethyl acetate, we did not observe ethyl lactate degradation during fermentation, especially after glucose was depleted. Even though the strain performance in pH-controlled bioreactors was enhanced by increased supply of precursor metabolites (19.35 ± 0.29 g/L of lactate and 10.31 ± 0.41 g/L of ethanol, Table 3-6B) from higher concentration of carbon source, the titer

of ethyl lactate did not increase during the fermentation. This result suggests that (i) rate-limiting conversion of lactate into lactyl-CoA by Pct and/or condensation of lactyl-CoA with an ethanol by VAAT and/or (ii) toxicity of ethyl lactate on *E. coli* health might have limited lactate ester biosynthesis. Therefore, to enhance ethyl lactate production, it is important to elucidate and alleviate these identified potential bottlenecks.

3.4.3.2 Ethyl lactate exhibited minimal toxicity on cell growth among lactate esters

To determine whether lactate esters inhibited cell growth and hence contributed to low lactate ester production, we cultured the parent strain, EcDL002, in a microplate reader with or without supply of various concentrations of lactate esters including ethyl, propyl, butyl, isobutyl, isoamyl, or benzyl lactate. The results show that ethyl lactate was the least toxic among the six lactate esters characterized where the growth rate (0.47 ± 0.04 1/h) and cell titer ($OD = 0.42 \pm 0.03$) decreased by 6% and 10%, respectively, upon cell exposure to 5 g/L ethyl lactate. On the other hand, isoamyl lactate was the most toxic among the lactate esters, where cell exposure to only 0.5 g/L ester resulted in 18% and 15% reduction in the growth rate (0.41 ± 0.02 1/h) and OD (0.40 ± 0.03), respectively (Figure 3-7A). This result was consistent with literature, illustrating that increasing toxicity of esters is highly correlated with increasing chain length of alcohol moieties that can severely disrupt cell membrane [56]. The toxicity of lactate esters can be ranked in the following order: isoamyl lactate > benzyl lactate > butyl lactate > isobutyl lactate > propyl lactate > ethyl lactate. There existed a positive correlation between the logP values of lactate esters and their degrees of toxicity (Figure 3-7B).

It should be noted that since *E. coli* can effectively secrete short-chain esters [10], external exposure of cells to lactate esters in our experiment design is sufficient to probe the potential toxicity caused by endogenous production of these esters. Taken altogether, ethyl lactate is the

least toxic and was not likely the main reason for the low production of ethyl lactate observed. It was likely the downstream pathway, responsible for conversion of lactate into lactyl-CoA by Pct and/or condensation of lactyl-CoA with ethanol by VAAT, might have been contributed to the inefficient ethyl lactate biosynthesis.

3.4.3.3 Downstream pathway of the lactate ester biosynthesis is the key bottleneck

To identify and alleviate the ethyl lactate biosynthesis pathway, we re-modularized it with two new parts: i) the upstream module carrying *ldhA*, *pdc*, and *adhB* for production of lactate and ethanol from sugar and ii) the downstream module carrying *pct* and *VAAT* for converting lactate into lactyl-CoA and condensing lactyl-CoA and ethanol (Figure 3-3A). We controlled metabolic fluxes of these modules by manipulating their plasmid copy numbers and levels of promoter induction with IPTG. By introducing the plasmids pJW007-015 into EcDL002, we generated the strains EcJW106-108 and EcJW203-208, respectively (Figure 3-3B). To evaluate the performance of these constructed strains for ethyl lactate production, we characterized them in high cell density cultures induced with various concentrations of IPTG (0.01, 0.1, and 1.0 mM).

The results show that EcJW204, carrying the upstream module with a low copy number plasmid (P15A origin) and the downstream module with a high copy number plasmid (RSF1030 origin) induced by 0.01 mM of IPTG, achieved the highest titer of ethyl lactate. As compared to EcJW201, EcJW204 achieved 4.96-fold (an increase from 2.2 to 11.1 ± 0.6 mg/L), 5.50-fold (from 0.04 ± 0.00 to 0.22 ± 0.02 mg/gDCW/h), and 54.0-fold (from 0.01 ± 0.00 to 0.54 ± 0.04 mg/g glucose) improvement in titer, specific productivity, and yield of ethyl lactate, respectively (Figure 3-3B, Table 3-8). Upon IPTG induction at 24 h, we observed the reduced cell growth of the host strains with use of high concentration of IPTG (Figure 3-3C, Table 3-7), suggesting that they suffered from metabolic burden due to overexpression of multiple enzymes [57] and also explain-

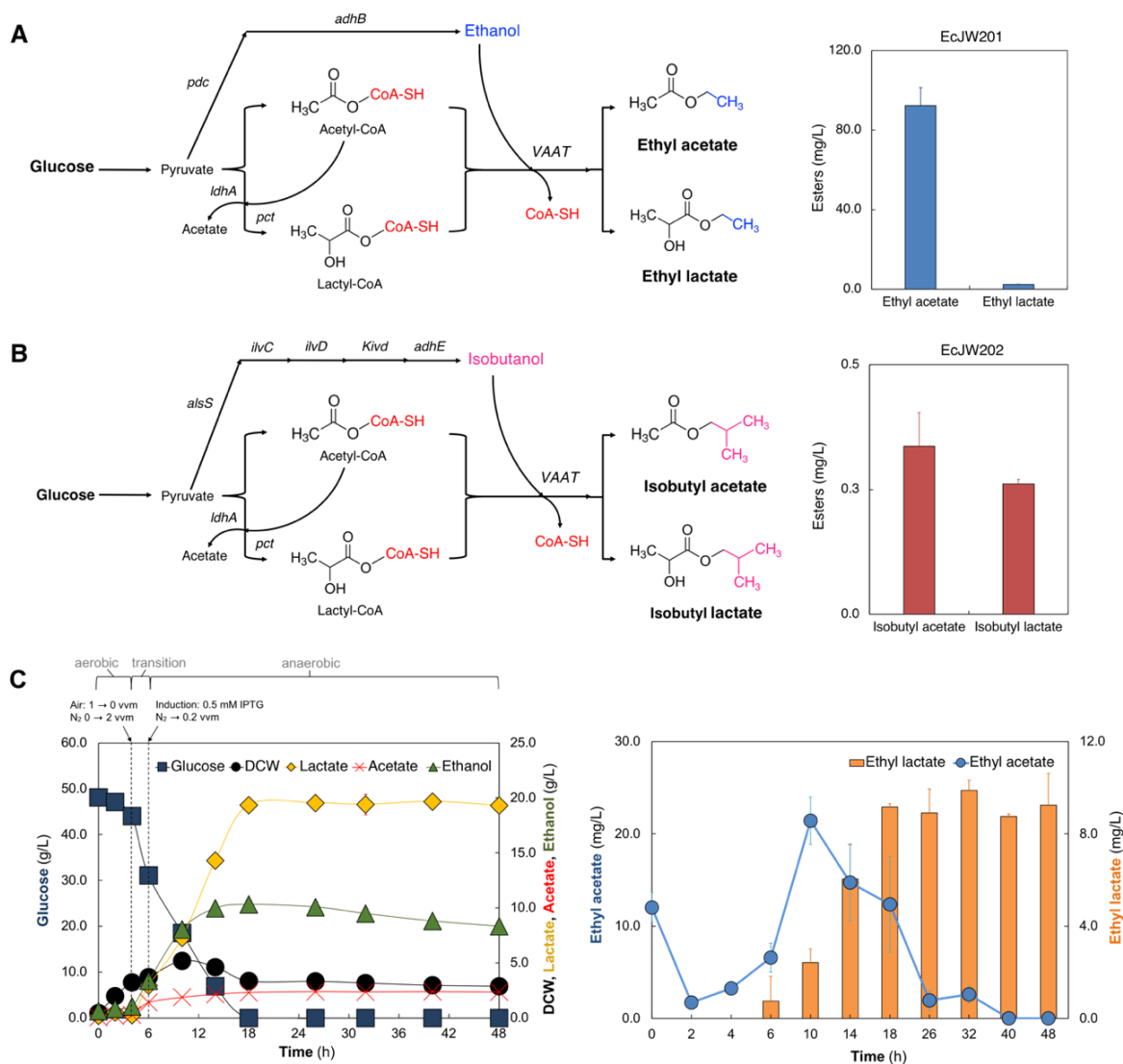


Figure 3-2: Design, construction, and validation of the lactate ester biosynthesis pathways in *E. coli*. **(A)** Engineered biosynthesis pathway of ethyl lactate from glucose and its production in high cell density culture of EcJW201. **(B)** Engineered biosynthesis pathway of isobutyl lactate from glucose and its production in high cell density culture of EcJW202. In Figure 2A and 2B, all the strains were induced at 0 h with 0.5 mM IPTG. Each error bar represents 1 s.d. ($n=3$). **(C)** Production of ethyl lactate from glucose in pH-controlled batch fermentation of EcJW201. The strain was induced at 6 h with 0.5 mM IPTG. Each error bar represents 1 s.d. ($n=2$).

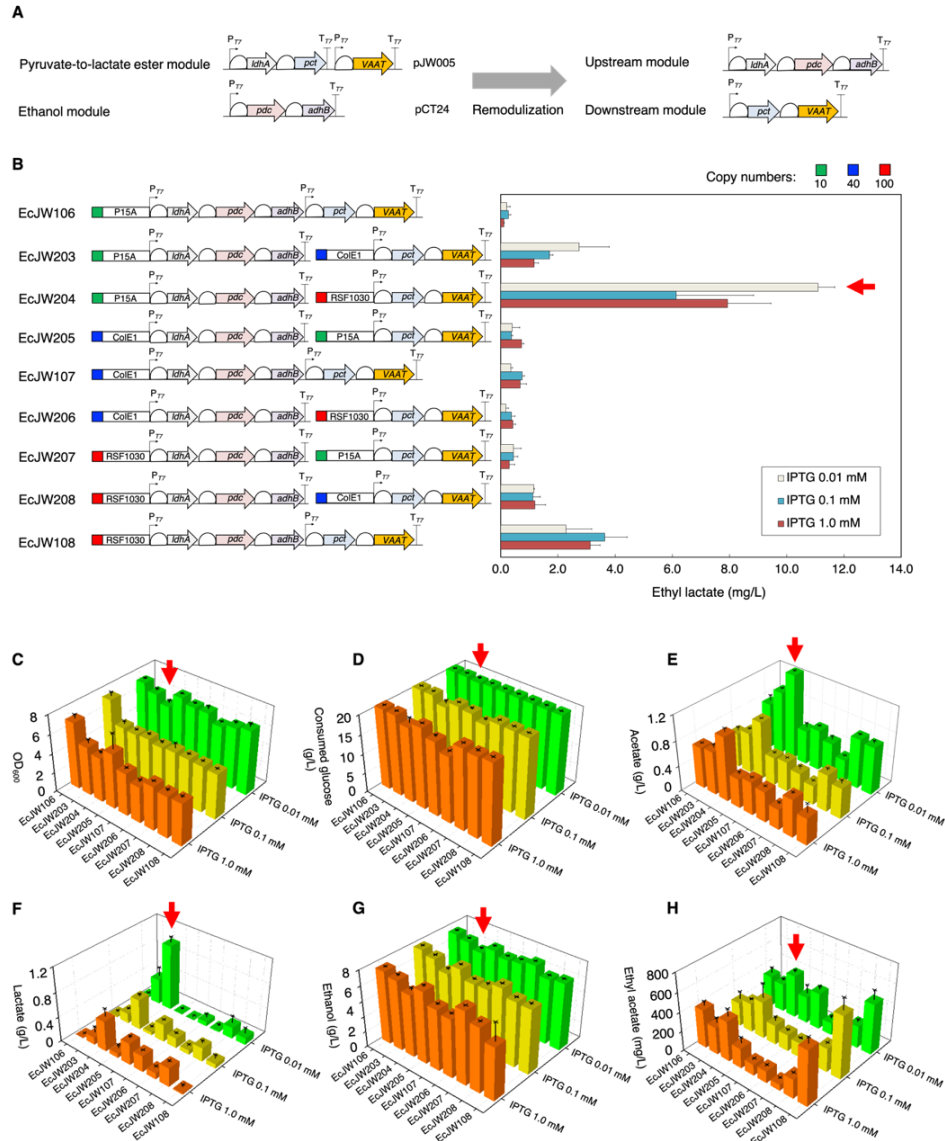


Figure 3-3: Combinatorial modular pathway optimization of enhanced ethyl lactate biosynthesis by varying plasmid copy number. **(A)** Re-modularization of the ethyl lactate biosynthesis pathway. Pyruvate-to-lactate ester and ethanol modules were re-modulated into upstream and downstream modules using plasmids with different copy numbers. **(B)** Ethyl lactate production, **(C)** OD₆₀₀, **(D)** consumed glucose, **(E)** acetate, **(F)** lactate, **(G)** ethanol, and **(H)** ethyl acetate of EcJW106-108 and EcJW203-208 in high cell density cultures induced with various concentrations of IPTG. Green rectangle: low copy number plasmid (10); P15A: origin of pACYCDuet-1; Blue rectangle: medium copy number plasmid (40); Cole1: origin of pETDuet-1; Red rectangle: high copy number plasmid (100); RSF1030: origin of pRSFDuet-1; P_{T7}: T7 promoter; T_{T7}: T7 terminator. All the strains were induced at 0 h with 0.01, 0.1, or 1.0 mM IPTG, respectively. Each error bar represents 1 s.d. ($n=3$). Red arrows indicate the selected strain with an optimum concentration of IPTG for the further studies.

ing why the use of low concentration of IPTG can help yield better production of ethyl lactate. Although EcJW204 showed better performance in ethyl lactate production than EcJW201, the accumulation of lactate and ethanol was still observed (Figure 3-3F and G, Table 3-9), indicating the pathway bottleneck remained. In particular, the downstream module flux was outcompeted by the upstream module flux and hence failed to turn over the precursor metabolites quickly enough.

This result helps explain why a combination of the upstream module (for producing lactate and ethanol from sugar) with a low copy number plasmid and the downstream module (for converting lactate into lactyl-CoA and condensing lactyl-CoA and ethanol) with a high copy number plasmid outperformed eight other combinations. Notably, the best ethyl lactate producer EcJW204 achieved the highest lactate and lowest ethanol production among the nine characterized strains (Figure 3-3F and G, Table 3-7), suggesting re-distribution of the carbon flux from ethanol to lactate likely helped improve ethyl lactate production. Thus, we hypothesized that redistribution of the carbon source from ethanol to lactate would help to improve ethyl lactate production. To test this hypothesis, we first examined whether i) downregulation of the ethanol flux of the upstream module enabled redistribution of the carbon flow from ethanol to lactate and ii) this redistribution could improve ethyl lactate production before proceeding to investigate the potential bottleneck of downstream module.

3.4.3.4 High ethanol synthesis of the upstream module was critical for ethyl lactate biosynthesis due to low specificity and activity of AAT.

To downregulate the ethanol flux of the upstream module, we first reconfigured pJW007, the upstream module of the best performer EcJW204, with a library of two weaker promoters and four weaker synthetic RBSs (Figure 3-4A, Figure 3-8A), resulting in four new upstream modules (pJW019-022). By introducing each newly constructed upstream module into EcDL002 together

with the downstream module pJW012 used in EcJW204, we next generated the strains EcJW209-212 and characterized them in high cell density cultures induced with 0.01 mM IPTG. The results show that while the carbon flux was successfully redistributed from ethanol to lactate, resulting in 5.97~6.92-fold decrease in ethanol production (from 8.30 ± 0.17 to $1.39 \pm 0.10 \sim 1.20 \pm 0.01$ g/L) and 1.67~2.59-fold increase in lactate production (from 1.06 ± 0.09 to 1.77 ± 0.37 g/L~ 2.75 ± 0.09 g/L) (Table 3-9A), the ethyl lactate production was reduced by 7.99~11.81-fold in ethyl lactate production (from 11.1 ± 0.6 to $1.4 \pm 0.4 \sim 0.9 \pm 0.2$ mg/L) in all four characterized strains as compared to that of EcJW204 (Figure 3-4B, Table 3-9B). This result suggests that a high level of ethanol is critical for VAAT to produce ethyl lactate.

To support this conclusion, we evaluated the effect of external ethanol supply on production of ethyl esters in high cell density cultures of EcJW209-212 induced with 0.01 mM IPTG. Indeed, with external ethanol supply, we observed enhanced production of both ethyl lactate and ethyl acetate in EcJW209-212. Specifically, with addition of 2 g/L of ethanol, the ethyl lactate and ethyl acetate production increased by 2.27 ~ 3.33-fold (from 1.4 ± 0.4 to 3.2 ± 0.2 mg/L ~ from 1.0 ± 0.2 to 3.2 ± 0.3 mg/L) and 1.27~2.07-fold (from 36.5 ± 3.9 to 46.2 ± 1.3 mg/L ~ from 22.0 ± 0.8 to 45.4 ± 1.2 mg/L), respectively (Table 3-9). Further addition of ethanol up to 10 g/L improved the ethyl lactate and ethyl acetate production by 3.78~5.26-fold (from 1.4 ± 0.4 to 5.3 ± 0.3 mg/L ~ from 0.9 ± 0.2 mg/L to 4.5 ± 0.4 mg/L) and 4.09~6.92-fold (from 36.5 ± 3.9 to 149.0 ± 3.8 mg/L ~ from 22.0 ± 0.8 mg/L to 151.9 ± 2.3 mg/L), respectively (Table 3-9).

Interestingly, while the total titer of ethyl esters increased with the increasing addition of ethanol (Figure 3-5A), the proportion of ethyl lactate in the total ester slightly increased in the range of 3.2~7.0% (Figure 3-5B), suggesting that VAAT prefers acetyl-CoA over lactyl-CoA with ethanol as a co-substrate. Notably, we observed a strong linear correlation between ethyl esters

production and the amount of added ethanol (i.e., for ethyl lactate, $R^2 = 0.85\sim 0.94$; for ethyl acetate, $R^2 = 0.99\sim 1.00$) (Supplementary Figure S4A). The results revealed that abundant availability of ethanol is essential to achieve high production of ethyl esters, indicating the main reason for the improved ethyl lactate production in EcJW204 was most likely due to the upregulation of downstream module with a high copy number plasmid.

3.4.3.1 AAT was the most rate limiting step of the downstream module

To determine whether Pct for conversion of lactate to lactyl-CoA or VAAT for condensation of lactyl-CoA and an alcohol was the most rate limiting step of the downstream module, we constructed nine downstream modules (pJW027-035) using a combination of synthetic RBSs for expression of Pct (synRBS_{pct#1-3}) and VAAT (synRBS_{VAAT#1-3}) with pJW012 from the best performer EcJW204, respectively (Figure 3-4A, Figure 3-8B). We introduced each newly constructed downstream module into EcDL002 together with the original upstream module (pJW007) used in EcJW204 to generate EcJW213-221. Then, we characterized the constructed strains in high cell density cultures induced with 0.01 mM IPTG.

The results show that the strains harboring the stronger RBSs for VAAT expression achieved the higher titers of ethyl lactate and ethyl acetate regardless of the RBS strengths for Pct expression (Figure 3-4C, Table 3-10). There is a strong linear correlation between ethyl ester production and the strength of RBS for VAAT expression (Figure 3-9B). To further validate these results without the influence of the upstream module, we additionally constructed the strains EcJW109-117 by introducing nine individual downstream modules (pJW027-035) into EcDL002 and then characterized these strains in high cell density cultures with addition of 2 g/L of lactate, 2 g/L of ethanol, and 0.01 mM of IPTG. We could observe the same strong linear correlation between ethyl ester production and high VAAT expression without the upstream module (Figure

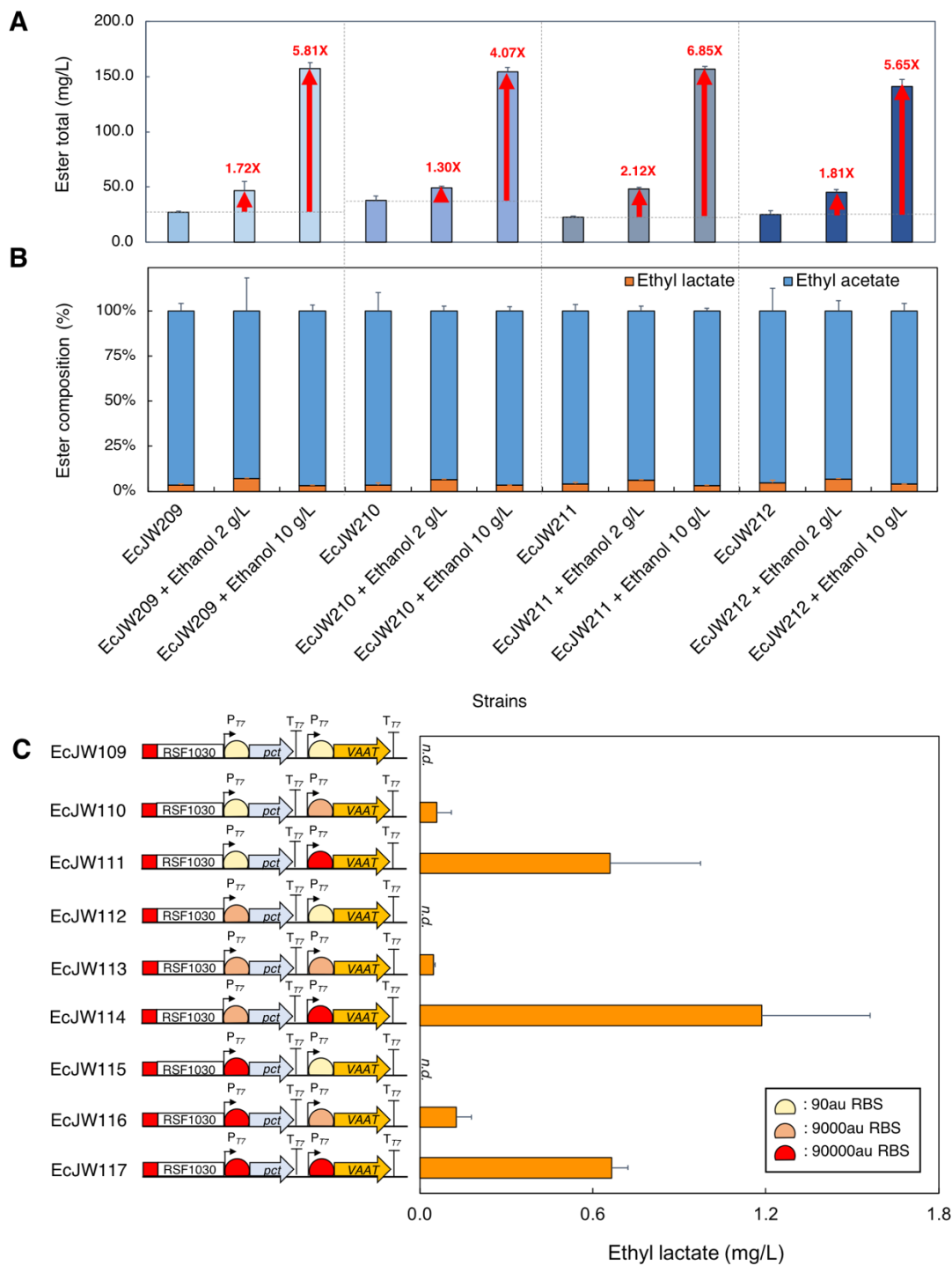


Figure 3-5: (A) Total esters and (B) composition of total esters produced in high cell density cultures of EcJW209-212 with or without addition of ethanol. (C) Ethyl lactate production of EcJW109-117 with addition of 2 g/L of lactate and ethanol. Red rectangle: high copy number plasmid (100); RSF1030: origin of pRSFDuet-1; P_{T7}: T7 promoter; T_{T7}: T7 terminator. All the strains were induced at 0 h with 0.01 mM IPTG. Each error bar represents 1 s.d. ($n=3$).

3-5C, Table 3-11). Taken altogether, these results suggest that VAAT not Pct was the most rate limiting step of the downstream module of the ethyl lactate biosynthesis pathway. Specifically, a combination of low affinity towards lactyl-CoA and ethanol of VAAT contributed to low ethyl lactate biosynthesis. Further studies on discovery of novel AATs, exhibiting high activity towards lactyl-CoA and alcohols but not acetyl-CoA, together with rational protein engineering of these enzymes would be warranted for improving lactate ester production. In principle, the lactate ester platform can be controlled to produce enantiomers with broad industrial applications. Since the endogenous *E. coli* D-lactate dehydrogenase (LdhA) was overexpressed in the *ldhA*-deficient modular cell of our study, it is anticipated that D-(-)-lactate and the associated D-(-)-lactate esters were mainly produced.

To date, production of optically pure D-(-)-[23] and L-(+)-form [26] of lactate from glucose in *E. coli* [25] has been well established. In addition, *pct* from *C. propionicum* [28] and *Megasphaera elsdenii* [29, 30] have been used for converting D-(-)-lactate into D-(-)-lactyl-CoA in polylactic acid (PLA) production in *E. coli* and their catalytic activity towards L-(+)-lactate has also been demonstrated [58, 59]. Thus, by combining stereospecific Ldh and Pct enzymes together with AATs, it is highly feasible to extend our lactate ester platform for microbial production of stereospecific lactate esters from renewable resources.

3.5 Conclusions

In this study, we have successfully developed a microbial lactate ester production platform and demonstrated for the first time the microbial biosynthesis of lactate esters directly from fermentable sugars in an *E. coli* modular cell. This study defines a cornerstone for the microbial

production of lactate esters as green solvents from renewable resources with novel industrial applications.

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3.7 Appendix

Table 3-4: Summary of high cell density cultures of EcJWL101, EcJWL102, EcJWL103, EcJWL104, and EcJWL105 with addition of glucose and various alcohols after 24 h. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respective. Abbreviations: *n.a.*: not applicable.

Doped alcohols	Strains	OD _i	OD _f	Glucose _i , g/L	Glucose _f , g/L	Lactate, g/L	Acetate, g/L	Added alcohol _i , g/L	Added alcohol _f , g/L
None	EcJWL101	2.96 ± 0.00	4.82 ± 0.15	23.65 ± 0.00	17.66 ± 0.12	0.25 ± 0.01	1.13 ± 1.34	<i>n.a.</i>	<i>n.a.</i>
	EcJWL102	2.91 ± 0.00	4.77 ± 0.05	23.65 ± 0.00	17.35 ± 0.03	5.75 ± 0.31	0.52 ± 0.11	<i>n.a.</i>	<i>n.a.</i>
	EcJWL103	2.93 ± 0.00	4.16 ± 0.09	23.65 ± 0.00	17.44 ± 0.04	5.62 ± 0.45	0.74 ± 0.30	<i>n.a.</i>	<i>n.a.</i>
	EcJWL104	2.84 ± 0.00	4.05 ± 0.03	23.65 ± 0.00	17.56 ± 0.03	5.49 ± 0.38	0.49 ± 0.10	<i>n.a.</i>	<i>n.a.</i>
	EcJWL105	3.01 ± 0.00	4.94 ± 0.05	23.65 ± 0.00	17.84 ± 0.02	4.81 ± 0.14	0.66 ± 0.25	<i>n.a.</i>	<i>n.a.</i>
Ethanol	EcJWL101	2.96 ± 0.00	4.70 ± 0.11	23.65 ± 0.00	17.97 ± 0.05	0.39 ± 0.01	0.78 ± 0.01	2.74 ± 0.00	2.74 ± 0.07
	EcJWL102	2.91 ± 0.00	4.64 ± 0.14	23.65 ± 0.00	17.61 ± 0.02	5.93 ± 0.01	1.00 ± 0.00	2.46 ± 0.00	2.18 ± 0.00
	EcJWL103	2.93 ± 0.00	4.07 ± 0.03	23.65 ± 0.00	17.76 ± 0.02	5.81 ± 0.01	0.97 ± 0.00	2.32 ± 0.00	2.18 ± 0.00
	EcJWL104	2.84 ± 0.00	4.04 ± 0.04	23.65 ± 0.00	17.61 ± 0.20	5.34 ± 0.71	0.98 ± 0.04	2.31 ± 0.00	2.12 ± 0.01
	EcJWL105	3.01 ± 0.00	4.87 ± 0.01	23.65 ± 0.00	18.02 ± 0.06	5.40 ± 0.02	1.02 ± 0.02	2.39 ± 0.00	2.14 ± 0.00
Propanol	EcJWL101	2.96 ± 0.00	4.65 ± 0.04	23.65 ± 0.00	17.85 ± 0.25	0.24 ± 0.01	0.31 ± 0.00	2.13 ± 0.00	2.02 ± 0.01
	EcJWL102	2.91 ± 0.00	4.74 ± 0.66	23.65 ± 0.00	17.49 ± 0.15	4.66 ± 0.85	0.65 ± 0.08	2.13 ± 0.00	1.99 ± 0.01
	EcJWL103	2.93 ± 0.00	4.07 ± 0.03	23.65 ± 0.00	17.70 ± 0.02	5.75 ± 0.02	0.86 ± 0.01	2.32 ± 0.00	2.22 ± 0.00
	EcJWL104	2.84 ± 0.00	4.12 ± 0.02	23.65 ± 0.00	17.43 ± 0.20	5.42 ± 0.20	0.53 ± 0.01	2.09 ± 0.00	1.96 ± 0.00
	EcJWL105	3.01 ± 0.00	4.71 ± 0.01	23.65 ± 0.00	17.95 ± 0.05	4.86 ± 0.04	0.50 ± 0.02	2.15 ± 0.00	1.99 ± 0.00
Butanol	EcJWL101	2.96 ± 0.00	3.92 ± 0.04	23.65 ± 0.00	18.23 ± 0.03	0.31 ± 0.00	0.54 ± 0.00	1.87 ± 0.00	1.12 ± 0.02
	EcJWL102	2.91 ± 0.00	4.73 ± 0.10	23.65 ± 0.00	17.42 ± 0.07	5.24 ± 0.91	0.69 ± 0.14	1.97 ± 0.00	1.84 ± 0.02
	EcJWL103	2.93 ± 0.00	4.14 ± 0.03	23.65 ± 0.00	17.72 ± 0.01	5.68 ± 0.00	0.90 ± 0.22	2.00 ± 0.00	1.88 ± 0.01
	EcJWL104	2.84 ± 0.00	4.04 ± 0.03	23.65 ± 0.00	17.58 ± 0.17	5.32 ± 0.46	1.02 ± 0.04	2.20 ± 0.00	2.04 ± 0.01
	EcJWL105	3.01 ± 0.00	4.80 ± 0.01	23.65 ± 0.00	18.29 ± 0.03	4.48 ± 0.01	0.62 ± 0.02	2.00 ± 0.00	1.88 ± 0.00
Isobutanol	EcJWL101	2.96 ± 0.00	4.09 ± 0.09	23.65 ± 0.00	18.06 ± 0.04	0.32 ± 0.01	0.59 ± 0.01	1.98 ± 0.00	1.44 ± 0.01
	EcJWL102	2.91 ± 0.00	4.74 ± 0.12	23.65 ± 0.00	17.42 ± 0.01	5.65 ± 0.27	0.66 ± 0.02	1.88 ± 0.00	1.83 ± 0.01
	EcJWL103	2.93 ± 0.00	4.17 ± 0.08	23.65 ± 0.00	17.67 ± 0.01	5.63 ± 0.01	1.04 ± 0.01	2.05 ± 0.00	2.01 ± 0.01
	EcJWL104	2.84 ± 0.00	4.11 ± 0.06	23.65 ± 0.00	17.45 ± 0.17	5.45 ± 0.19	1.03 ± 0.01	2.04 ± 0.00	1.99 ± 0.00
	EcJWL105	3.01 ± 0.00	4.79 ± 0.19	23.65 ± 0.00	17.98 ± 0.01	4.68 ± 0.01	0.65 ± 0.01	1.98 ± 0.00	1.93 ± 0.01
Isoamyl alcohol	EcJWL101	2.96 ± 0.00	2.79 ± 0.12	23.65 ± 0.00	20.24 ± 0.28	0.16 ± 0.03	0.56 ± 0.03	1.44 ± 0.00	0.57 ± 0.06
	EcJWL102	2.91 ± 0.00	4.36 ± 0.07	23.65 ± 0.00	17.73 ± 0.28	4.85 ± 0.08	0.60 ± 0.05	1.59 ± 0.00	1.49 ± 0.01
	EcJWL103	2.93 ± 0.00	4.22 ± 0.10	23.65 ± 0.00	17.95 ± 0.06	5.17 ± 0.09	1.16 ± 0.01	1.78 ± 0.00	1.73 ± 0.00
	EcJWL104	2.84 ± 0.00	3.96 ± 0.04	23.65 ± 0.00	17.99 ± 0.01	5.11 ± 0.03	1.12 ± 0.00	1.95 ± 0.00	1.89 ± 0.00
	EcJWL105	3.01 ± 0.00	4.48 ± 0.13	23.65 ± 0.00	17.90 ± 0.33	4.75 ± 0.27	1.03 ± 0.27	1.92 ± 0.00	1.88 ± 0.01
Benzyl alcohol	EcJWL101	2.96 ± 0.00	2.85 ± 0.03	23.65 ± 0.00	20.58 ± 0.56	0.14 ± 0.02	0.59 ± 0.05	2.60 ± 0.00	2.02 ± 0.31
	EcJWL102	2.91 ± 0.00	4.21 ± 0.08	23.65 ± 0.00	17.66 ± 0.11	4.45 ± 1.08	0.61 ± 0.11	2.56 ± 0.00	2.36 ± 0.09
	EcJWL103	2.93 ± 0.00	4.12 ± 0.02	23.65 ± 0.00	17.71 ± 0.22	4.38 ± 0.17	1.88 ± 0.60	2.66 ± 0.00	2.62 ± 0.00
	EcJWL104	2.84 ± 0.00	4.06 ± 0.06	23.65 ± 0.00	17.79 ± 0.23	4.46 ± 0.82	1.75 ± 0.56	2.62 ± 0.00	2.58 ± 0.00
	EcJWL105	3.01 ± 0.00	4.62 ± 0.12	23.65 ± 0.00	18.05 ± 0.02	4.61 ± 0.45	0.62 ± 0.12	2.65 ± 0.00	2.62 ± 0.00

Table 3-5: Summary of titer, specific productivity, and yield of esters in high cell density cultures of EcJW101, EcJW102, EcJW103, EcJW104, and EcJW105 with addition of glucose and various alcohols after 24 h. The acyl acetate, and acyl lactate columns correspond to the acyl alcohols added. For example, with the exogenous addition of ethanol, the acyl acetate and acyl lactate columns represent ethyl acetate, and ethyl lactate, respectively. Abbreviations: *n.a.*: not applicable, *n.d.*: not detected.

Doped alcohols	Strains	Ester titer (mg/L)			Specific ester productivity (mg/gDCW/h)			Ester yield (mg/g glucose)		
		Acyl acetate	Acyl lactate	Total	Acyl acetate	Acyl lactate	Total	Acyl acetate	Acyl lactate	Total
None	EcJWL101	11.4 ± 0.4	<i>n.d.</i>	11.4 ± 0.4	0.25 ± 0.01	<i>n.a.</i>	0.25 ± 0.01	1.89 ± 0.05	<i>n.a.</i>	1.89 ± 0.05
	EcJWL102	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcJWL103	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcJWL104	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcJWL105	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Ethanol	EcJWL101	115.5 ± 4.8	<i>n.d.</i>	115.5 ± 4.8	2.60 ± 0.14	<i>n.a.</i>	2.60 ± 0.14	20.33 ± 0.99	<i>n.a.</i>	20.33 ± 0.99
	EcJWL102	0.7 ± 0.0	<i>n.d.</i>	0.7 ± 0.0	0.02 ± 0.00	<i>n.a.</i>	0.02 ± 0.00	0.12 ± 0.00	<i>n.a.</i>	0.12 ± 0.00
	EcJWL103	5.8 ± 0.2	1.4 ± 0.1	7.2 ± 0.3	0.14 ± 0.01	0.03 ± 0.00	0.18 ± 0.01	0.99 ± 0.04	0.24 ± 0.01	1.23 ± 0.04
	EcJWL104	5.4 ± 0.2	1.6 ± 0.0	6.9 ± 0.3	0.13 ± 0.01	0.04 ± 0.00	0.17 ± 0.01	0.89 ± 0.05	0.26 ± 0.01	1.15 ± 0.05
	EcJWL105	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Propanol	EcJWL101	801.6 ± 33.5	<i>n.d.</i>	801.6 ± 33.5	18.13 ± 0.73	<i>n.a.</i>	18.13 ± 0.73	138.22 ± 4.09	<i>n.a.</i>	138.22 ± 4.09
	EcJWL102	18.8 ± 0.8	<i>n.d.</i>	18.8 ± 0.8	0.43 ± 0.05	<i>n.a.</i>	0.43 ± 0.05	3.05 ± 0.20	<i>n.a.</i>	3.05 ± 0.20
	EcJWL103	19.9 ± 0.8	4.9 ± 0.3	24.8 ± 1.1	0.49 ± 0.02	0.12 ± 0.01	0.61 ± 0.02	3.35 ± 0.14	0.82 ± 0.06	4.18 ± 0.19
	EcJWL104	18.8 ± 0.9	5.5 ± 0.3	24.3 ± 1.1	0.47 ± 0.02	0.14 ± 0.01	0.60 ± 0.02	3.03 ± 0.19	0.88 ± 0.01	3.91 ± 0.18
	EcJWL105	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Butanol	EcJWL101	1017.9 ± 20.2	<i>n.d.</i>	1017.9 ± 20.2	25.45 ± 0.38	<i>n.a.</i>	25.45 ± 0.38	187.85 ± 2.75	<i>n.a.</i>	187.85 ± 2.75
	EcJWL102	60.6 ± 6.1	<i>n.d.</i>	60.6 ± 6.1	1.37 ± 0.15	<i>n.a.</i>	1.37 ± 0.15	9.73 ± 1.03	<i>n.a.</i>	9.73 ± 1.03
	EcJWL103	16.2 ± 0.7	10.4 ± 0.3	26.6 ± 1.0	0.39 ± 0.02	0.25 ± 0.01	0.65 ± 0.02	2.73 ± 0.12	1.75 ± 0.05	4.48 ± 0.16
	EcJWL104	16.6 ± 0.6	11.8 ± 0.4	28.3 ± 1.0	0.41 ± 0.01	0.29 ± 0.01	0.71 ± 0.01	2.73 ± 0.16	1.94 ± 0.02	4.67 ± 0.15
	EcJWL105	<i>n.d.</i>	0.9 ± 0.1	0.9 ± 0.1	<i>n.a.</i>	0.02 ± 0.00	0.02 ± 0.00	<i>n.a.</i>	0.17 ± 0.02	0.17 ± 0.02
Isobutanol	EcJWL101	1210.4 ± 24.8	<i>n.d.</i>	1210.4 ± 24.8	29.54 ± 0.71	<i>n.a.</i>	29.54 ± 0.71	216.69 ± 2.92	<i>n.a.</i>	216.69 ± 2.92
	EcJWL102	102.4 ± 0.9	<i>n.d.</i>	102.4 ± 0.9	2.30 ± 0.05	<i>n.a.</i>	2.30 ± 0.05	16.44 ± 0.18	<i>n.a.</i>	16.44 ± 0.18
	EcJWL103	19.4 ± 0.3	9.4 ± 0.2	28.8 ± 0.4	0.47 ± 0.01	0.23 ± 0.01	0.70 ± 0.02	3.25 ± 0.04	1.57 ± 0.03	4.82 ± 0.06
	EcJWL104	19.1 ± 0.7	9.9 ± 0.1	29.1 ± 0.8	0.48 ± 0.02	0.25 ± 0.00	0.72 ± 0.03	3.09 ± 0.20	1.60 ± 0.05	4.69 ± 0.25
	EcJWL105	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Isoamyl alcohol	EcJWL101	692.7 ± 7.7	<i>n.d.</i>	692.7 ± 7.7	20.72 ± 0.31	<i>n.a.</i>	20.72 ± 0.31	204.48 ± 18.70	<i>n.a.</i>	204.48 ± 18.70
	EcJWL102	170.9 ± 10.2	0.9 ± 0.1	171.8 ± 10.3	4.04 ± 0.21	0.02 ± 0.00	4.07 ± 0.21	28.94 ± 2.52	0.15 ± 0.02	29.10 ± 2.54
	EcJWL103	21.4 ± 1.2	23.8 ± 0.9	45.3 ± 2.2	0.52 ± 0.03	0.57 ± 0.02	1.09 ± 0.05	3.76 ± 0.21	4.18 ± 0.20	7.94 ± 0.39
	EcJWL104	21.0 ± 0.8	24.7 ± 0.6	45.8 ± 1.3	0.53 ± 0.03	0.62 ± 0.01	1.15 ± 0.03	3.72 ± 0.13	4.37 ± 0.11	8.09 ± 0.09
	EcJWL105	0.6 ± 0.0	0.4 ± 0.1	1.0 ± 0.1	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.10 ± 0.01	0.07 ± 0.02	0.17 ± 0.03
Benzyl alcohol	EcJWL101	1178.0 ± 45.7	<i>n.d.</i>	1178.0 ± 45.7	34.86 ± 1.30	<i>n.a.</i>	34.86 ± 1.30	393.18 ± 78.03	<i>n.a.</i>	393.18 ± 78.03
	EcJWL102	734.3 ± 120.3	7.5 ± 1.8	741.7 ± 122.0	17.76 ± 3.13	0.18 ± 0.04	17.94 ± 3.11	122.79 ± 20.81	1.25 ± 0.31	124.04 ± 20.72
	EcJWL103	108.2 ± 5.4	49.0 ± 2.4	157.3 ± 7.7	2.64 ± 0.13	1.20 ± 0.06	3.84 ± 0.11	18.25 ± 1.49	8.26 ± 0.51	26.51 ± 1.71
	EcJWL104	108.5 ± 4.5	51.6 ± 2.1	160.1 ± 6.6	4.06 ± 2.44	1.91 ± 1.10	5.98 ± 3.54	18.53 ± 1.25	8.82 ± 0.68	27.35 ± 1.80
	EcJWL105	<i>n.d.</i>	5.2 ± 0.4	5.2 ± 0.4	<i>n.a.</i>	0.12 ± 0.01	0.12 ± 0.01	<i>n.a.</i>	0.93 ± 0.07	0.93 ± 0.07

Table 3-6: (A) Summary of high cell density cultures of EcDL002, EcJW201, and EcJW202 after 24 h. **(B)** Summary of bioreactor batch fermentation of EcJW201 after 18 h. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respectively. Abbreviations: *n.a.*: not applicable, *n.d.*: not detected.

A

		Strains		
		EcDL002	EcJW201	EcJW202
OD _i		3.10 ± 0.00	3.01 ± 0.00	2.97 ± 0.00
OD _f		5.92 ± 0.19	7.20 ± 0.08	5.07 ± 0.05
pH _i		7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
pH _f		4.96 ± 0.03	6.10 ± 0.03	4.99 ± 0.03
Glucose _i (g/L)		22.6 ± 0.00	22.6 ± 0.00	22.6 ± 0.00
Glucose _f (g/L)		18.16 ± 0.02	0.00 ± 0.00	14.89 ± 0.09
Lactate (g/L)		0.38 ± 0.00	0.39 ± 0.03	5.36 ± 0.01
Acetate (g/L)		0.47 ± 0.01	0.50 ± 0.01	0.26 ± 0.00
Ethanol (g/L)		0.95 ± 0.01	10.33 ± 0.02	0.22 ± 0.00
Isobutanol (g/L)		<i>n.d.</i>	<i>n.d.</i>	0.26 ± 0.01
Ester titer (mg/L)	Ethyl acetate	1.2 ± 0.9	92.3 ± 9.2	1.4 ± 0.7
	Ethyl lactate	<i>n.d.</i>	2.2 ± 0.3	<i>n.a.</i>
	Isobutyl acetate	<i>n.d.</i>	<i>n.a.</i>	0.3 ± 0.1
	Isobutyl lactate	<i>n.d.</i>	<i>n.a.</i>	0.3 ± 0.0
	Ester total	1.2 ± 0.9	94.5 ± 8.9	2.0 ± 0.8
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.02 ± 0.02	1.55 ± 0.16	0.03 ± 0.02
	Ethyl lactate	<i>n.a.</i>	0.04 ± 0.00	<i>n.a.</i>
	Isobutyl acetate	<i>n.a.</i>	<i>n.a.</i>	0.01 ± 0.00
	Isobutyl lactate	<i>n.a.</i>	<i>n.a.</i>	0.01 ± 0.00
	Ester total	0.02 ± 0.02	1.59 ± 0.16	0.04 ± 0.02
Ester yield (mg/g glucose)	Ethyl acetate	0.27 ± 0.21	4.08 ± 0.41	0.18 ± 0.09
	Ethyl lactate	<i>n.a.</i>	0.10 ± 0.01	<i>n.a.</i>
	Isobutyl acetate	<i>n.a.</i>	<i>n.a.</i>	0.04 ± 0.01
	Isobutyl lactate	<i>n.a.</i>	<i>n.a.</i>	0.03 ± 0.00
	Ester total	0.27 ± 0.21	4.18 ± 0.40	0.25 ± 0.10

B

OD		6.92 ± 0.74
Consumed glucose (g/L)		48.11 ± 0.82
Lactate (g/L)		19.35 ± 0.29
Acetate (g/L)		2.32 ± 0.01
Ethanol (g/L)		10.31 ± 0.41
Ester titer (mg/L)	Ethyl acetate	12.4 ± 5.2
	Ethyl lactate	9.4 ± 0.1
	Ester total	21.5 ± 5.3
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.21 ± 0.11
	Ethyl lactate	0.15 ± 0.02
	Ester total	0.28 ± 0.01
Ester yield (mg/g glucose)	Ethyl acetate	0.26 ± 0.10
	Ethyl lactate	0.19 ± 0.00
	Ester total	0.35 ± 0.03

Table 3-7: Summary of high cell density cultures of EcJW106-108 and EcJW203-208 with different concentrations of IPTG (0.01, 0.1, or 1.0 mM) after 24 h. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respectively. Abbreviations: *n.a.*: not applicable.

	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM
	EcJW106			EcJW203			EcJW204		
OD _i	3.02 ± 0.00	3.02 ± 0.00	3.02 ± 0.00	2.86 ± 0.00	2.86 ± 0.00	2.86 ± 0.00	3.12 ± 0.00	3.12 ± 0.00	3.12 ± 0.00
OD _f	7.07 ± 0.18	7.21 ± 0.39	6.91 ± 0.38	6.42 ± 0.24	4.86 ± 0.30	5.02 ± 0.37	5.64 ± 0.29	4.79 ± 0.12	4.58 ± 0.21
pH _i	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
pH _f	6.02 ± 0.05	6.14 ± 0.01	6.16 ± 0.03	5.74 ± 0.11	6.01 ± 0.02	6.13 ± 0.10	5.25 ± 0.03	5.85 ± 0.01	5.93 ± 0.08
Glucose _i (g/L)	20.74 ± 0.00	20.74 ± 0.01	20.74 ± 0.02	20.74 ± 0.03	20.74 ± 0.04	20.74 ± 0.05	20.74 ± 0.06	20.74 ± 0.07	20.74 ± 0.08
Glucose _f (g/L)	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.34 ± 0.47	1.72 ± 0.68	1.54 ± 1.29
Lactate (g/L)	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00	0.41 ± 0.15	0.16 ± 0.02	0.12 ± 0.10	1.06 ± 0.09	0.49 ± 0.03	0.49 ± 0.13
Acetate (g/L)	0.72 ± 0.09	0.54 ± 0.05	0.65 ± 0.01	0.99 ± 0.02	0.64 ± 0.02	0.68 ± 0.05	1.34 ± 0.02	0.91 ± 0.00	1.01 ± 0.06
Ethanol (g/L)	9.38 ± 0.10	9.72 ± 0.08	9.47 ± 0.10	9.02 ± 0.06	9.24 ± 0.03	9.08 ± 0.22	8.30 ± 0.17	8.07 ± 0.22	8.05 ± 0.35
	EcJW205			EcJW107			EcJW206		
OD _i	3.18 ± 0.00	3.18 ± 0.00	3.18 ± 0.00	2.94 ± 0.00	2.94 ± 0.00	2.94 ± 0.00	2.84 ± 0.00	2.84 ± 0.00	2.84 ± 0.00
OD _f	7.14 ± 0.08	4.94 ± 0.13	5.67 ± 1.58	6.68 ± 0.11	4.70 ± 0.19	4.39 ± 0.11	6.69 ± 0.32	4.86 ± 0.49	3.74 ± 0.24
pH _i	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
pH _f	6.03 ± 0.04	6.06 ± 0.01	6.18 ± 0.09	6.03 ± 0.04	6.08 ± 0.01	6.04 ± 0.01	6.01 ± 0.01	6.19 ± 0.01	6.20 ± 0.07
Glucose _i (g/L)	20.74 ± 0.00	20.74 ± 0.01	20.74 ± 0.02	20.74 ± 0.03	20.74 ± 0.04	20.74 ± 0.05	20.74 ± 0.06	20.74 ± 0.07	20.74 ± 0.08
Glucose _f (g/L)	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.38 ± 0.40	1.84 ± 0.51	0.07 ± 0.00	1.66 ± 0.50	3.73 ± 0.19
Lactate (g/L)	0.01 ± 0.02	0.05 ± 0.02	0.07 ± 0.06	0.00 ± 0.00	0.28 ± 0.01	0.35 ± 0.04	0.07 ± 0.06	0.16 ± 0.01	0.26 ± 0.01
Acetate (g/L)	0.54 ± 0.07	0.44 ± 0.02	0.46 ± 0.04	0.64 ± 0.02	0.47 ± 0.00	0.52 ± 0.00	0.52 ± 0.06	0.39 ± 0.05	0.48 ± 0.01
Ethanol (g/L)	9.26 ± 0.17	9.39 ± 0.10	9.29 ± 0.23	8.74 ± 0.04	8.27 ± 0.16	7.88 ± 0.16	8.96 ± 0.09	8.36 ± 0.23	7.55 ± 0.13
	EcJW207			EcJW208			EcJW108		
OD _i	3.19 ± 0.00	3.19 ± 0.00	3.19 ± 0.00	2.88 ± 0.00	2.88 ± 0.00	2.88 ± 0.00	3.14 ± 0.00	3.14 ± 0.00	3.14 ± 0.00
OD _f	5.88 ± 0.17	4.82 ± 0.12	4.53 ± 0.05	6.34 ± 0.25	4.78 ± 0.09	4.29 ± 0.18	6.70 ± 0.32	4.70 ± 0.07	4.36 ± 0.20
pH _i	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
pH _f	6.09 ± 0.03	6.10 ± 0.02	6.19 ± 0.06	5.90 ± 0.02	6.03 ± 0.02	6.10 ± 0.06	6.09 ± 0.04	6.23 ± 0.11	6.30 ± 0.11
Glucose _i (g/L)	20.74 ± 0.00	20.74 ± 0.01	20.74 ± 0.02	20.74 ± 0.03	20.74 ± 0.04	20.74 ± 0.05	20.74 ± 0.06	20.74 ± 0.07	20.74 ± 0.08
Glucose _f (g/L)	0.07 ± 0.00	0.05 ± 0.00	0.08 ± 0.05	0.07 ± 0.00	0.05 ± 0.00	0.38 ± 0.32	0.07 ± 0.00	0.05 ± 0.00	0.03 ± 0.02
Lactate (g/L)	0.02 ± 0.03	0.07 ± 0.01	0.10 ± 0.02	0.18 ± 0.12	0.22 ± 0.04	0.30 ± 0.03	0.11 ± 0.09	0.07 ± 0.06	0.02 ± 0.04
Acetate (g/L)	0.35 ± 0.01	0.30 ± 0.01	0.32 ± 0.03	0.78 ± 0.02	0.58 ± 0.03	0.59 ± 0.03	0.75 ± 0.04	0.48 ± 0.05	0.42 ± 0.14
Ethanol (g/L)	9.49 ± 0.04	9.33 ± 0.03	9.43 ± 0.03	8.46 ± 0.02	8.43 ± 0.04	8.33 ± 0.11	8.71 ± 0.07	8.35 ± 0.15	6.86 ± 2.31

Table 3-8: Summary of titer, specific productivity, and yield of esters in high cell density cultures of EcJW106-108 and EcJW203-208 with different concentrations of IPTG (0.01, 0.1, or 1.0 mM) after 24 h.

	Esters	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM	IPTG 0.01 mM	IPTG 0.1 mM	IPTG 1.0 mM
		EcJW106			EcJW203			EcJW204		
Ester titer (mg/L)	Ethyl acetate	315.9 ± 56.9	243.0 ± 75.7	393.6 ± 44.1	291.4 ± 17.8	300.8 ± 4.6	280.8 ± 50.5	439.8 ± 22.0	380.2 ± 146.8	411.7 ± 76.9
	Ethyl lactate	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	2.7 ± 1.1	1.7 ± 0.1	1.2 ± 0.2	11.1 ± 0.6	6.1 ± 2.7	7.9 ± 1.5
	Ester total	316.1 ± 57.0	243.2 ± 75.8	393.7 ± 44.1	294.2 ± 18.9	302.5 ± 4.7	281.9 ± 50.6	450.9 ± 22.6	386.4 ± 149.5	419.6 ± 78.4
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	5.39 ± 0.97	4.09 ± 1.31	6.84 ± 1.01	5.40 ± 0.31	6.71 ± 0.16	6.14 ± 1.19	8.63 ± 0.27	8.25 ± 3.14	9.19 ± 1.57
	Ethyl lactate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.02	0.04 ± 0.00	0.03 ± 0.00	0.22 ± 0.02	0.13 ± 0.06	0.18 ± 0.03
	Ester total	5.39 ± 0.97	4.10 ± 1.30	6.85 ± 1.01	5.45 ± 0.30	6.75 ± 0.16	6.17 ± 1.19	8.85 ± 0.26	8.38 ± 3.19	9.37 ± 1.61
Ester yield (mg/g glucose)	Ethyl acetate	15.28 ± 2.75	11.76 ± 3.66	19.04 ± 2.13	14.10 ± 0.86	14.55 ± 0.22	13.57 ± 2.44	21.56 ± 0.99	19.89 ± 7.43	21.34 ± 2.53
	Ethyl lactate	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.13 ± 0.05	0.08 ± 0.01	0.06 ± 0.01	0.54 ± 0.04	0.32 ± 0.13	0.41 ± 0.05
	Ester total	15.29 ± 2.75	11.77 ± 3.66	19.04 ± 2.13	14.23 ± 0.84	14.63 ± 0.22	13.63 ± 2.43	22.10 ± 0.97	20.21 ± 7.54	21.75 ± 2.58
		EcJW205			EcJW107			EcJW206		
Ester titer (mg/L)	Ethyl acetate	274.0 ± 86.2	201.1 ± 41.0	203.6 ± 61.4	375.2 ± 34.7	168.8 ± 6.0	112.3 ± 6.7	124.2 ± 29.6	149.8 ± 5.6	119.7 ± 8.3
	Ethyl lactate	0.4 ± 0.3	0.4 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
	Ester total	274.4 ± 86.5	201.5 ± 41.0	204.3 ± 61.5	375.5 ± 34.7	169.5 ± 6.0	112.9 ± 6.9	124.4 ± 29.7	150.2 ± 5.7	120.2 ± 8.4
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	4.56 ± 1.42	4.27 ± 0.94	4.07 ± 1.58	6.71 ± 0.63	3.80 ± 0.05	2.63 ± 0.17	2.23 ± 0.45	3.36 ± 0.29	3.13 ± 0.15
	Ethyl lactate	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Ester total	4.57 ± 1.42	4.28 ± 0.94	4.09 ± 1.58	6.72 ± 0.63	3.82 ± 0.04	2.65 ± 0.18	2.23 ± 0.45	3.37 ± 0.29	3.14 ± 0.15
Ester yield (mg/g glucose)	Ethyl acetate	13.26 ± 4.18	9.72 ± 1.98	9.84 ± 2.97	18.15 ± 1.68	8.29 ± 0.29	5.94 ± 0.29	6.01 ± 1.43	7.85 ± 0.28	7.04 ± 0.45
	Ethyl lactate	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
	Ester total	13.28 ± 4.18	9.74 ± 1.98	9.88 ± 2.97	18.17 ± 1.68	8.33 ± 0.29	5.98 ± 0.30	6.02 ± 1.43	7.87 ± 0.28	7.06 ± 0.45
		EcJW207			EcJW208			EcJW108		
Ester titer (mg/L)	Ethyl acetate	143.0 ± 12.7	60.5 ± 9.4	62.5 ± 10.1	225.5 ± 22.7	243.8 ± 7.3	196.9 ± 9.3	517.6 ± 96.7	640.9 ± 121.5	563.7 ± 64.7
	Ethyl lactate	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	1.2 ± 0.0	1.1 ± 0.3	1.2 ± 0.4	2.3 ± 0.9	3.6 ± 0.8	3.1 ± 0.4
	Ester total	143.4 ± 13.0	61.0 ± 9.5	62.8 ± 10.3	226.6 ± 22.7	244.9 ± 7.6	198.1 ± 9.7	519.9 ± 97.6	644.5 ± 122.3	566.8 ± 65.1
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	2.71 ± 0.22	1.30 ± 0.20	1.39 ± 0.22	4.22 ± 0.53	5.47 ± 0.10	4.73 ± 0.33	9.04 ± 1.56	14.09 ± 2.80	12.96 ± 1.74
	Ethyl lactate	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.08 ± 0.02	0.07 ± 0.01
	Ester total	2.72 ± 0.21	1.31 ± 0.19	1.40 ± 0.23	4.24 ± 0.53	5.50 ± 0.10	4.76 ± 0.33	9.08 ± 1.55	14.17 ± 2.82	13.03 ± 1.74
Ester yield (mg/g glucose)	Ethyl acetate	6.92 ± 0.61	2.92 ± 0.45	3.03 ± 0.49	10.91 ± 1.10	11.78 ± 0.35	9.68 ± 0.57	25.04 ± 4.68	30.97 ± 5.87	27.23 ± 3.15
	Ethyl lactate	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.06 ± 0.00	0.05 ± 0.01	0.06 ± 0.02	0.11 ± 0.04	0.18 ± 0.04	0.15 ± 0.02
	Ester total	6.94 ± 0.60	2.95 ± 0.45	3.04 ± 0.50	10.96 ± 1.10	11.84 ± 0.34	9.73 ± 0.55	25.15 ± 4.65	31.15 ± 5.91	27.38 ± 3.16

Table 3-9: Summary of high cell density cultures of EcJW209-212 with or without addition of ethanol (2 or 10 g/L) after 24 h. **(A)** OD₆₀₀, glucose, lactate, acetate, and ethanol. **(B)** Titer, specific productivity, and yield of esters. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respectively.

A

Strains	OD _i	OD _f	Glucose _i (g/L)	Glucose _f (g/L)	Lactate (g/L)	Acetate (g/L)	Ethanol (g/L)
EcJW209	2.93 ± 0.00	5.23 ± 0.11	22.60 ± 0.00	18.84 ± 1.08	2.17 ± 0.76	1.02 ± 0.17	1.39 ± 0.10
EcJW209 + Ethanol 2 g/L	3.06 ± 0.00	5.44 ± 0.05	21.64 ± 0.00	15.76 ± 0.02	2.03 ± 0.04	1.84 ± 0.00	2.34 ± 0.33
EcJW209 + Ethanol 10 g/L	2.98 ± 0.00	5.64 ± 0.19	21.64 ± 0.04	15.85 ± 0.07	1.65 ± 0.02	1.79 ± 0.00	10.16 ± 0.35
EcJW210	2.83 ± 0.00	5.29 ± 0.07	22.60 ± 0.00	17.73 ± 1.19	1.77 ± 0.37	1.14 ± 0.10	1.39 ± 0.02
EcJW210 + Ethanol 2 g/L	3.01 ± 0.00	5.63 ± 0.07	21.64 ± 0.01	15.85 ± 0.05	1.85 ± 0.01	1.81 ± 0.01	2.64 ± 0.02
EcJW210 + Ethanol 10 g/L	2.91 ± 0.00	5.11 ± 0.41	21.64 ± 0.05	15.72 ± 0.13	1.54 ± 0.03	1.80 ± 0.02	10.23 ± 0.41
EcJW211	2.96 ± 0.00	5.27 ± 0.02	22.60 ± 0.00	18.07 ± 0.69	2.75 ± 0.09	1.19 ± 0.09	1.20 ± 0.01
EcJW211 + Ethanol 2 g/L	3.10 ± 0.00	5.55 ± 0.12	21.64 ± 0.02	15.57 ± 0.48	2.16 ± 0.03	1.71 ± 0.28	2.57 ± 0.15
EcJW211 + Ethanol 10 g/L	2.97 ± 0.00	5.63 ± 0.20	21.64 ± 0.06	15.74 ± 0.16	1.78 ± 0.04	1.82 ± 0.05	10.45 ± 0.03
EcJW212	3.04 ± 0.00	5.36 ± 0.18	22.60 ± 0.00	17.70 ± 1.03	2.28 ± 0.83	1.22 ± 0.14	1.21 ± 0.02
EcJW212 + Ethanol 2 g/L	3.01 ± 0.00	5.43 ± 0.14	21.64 ± 0.03	15.67 ± 0.03	2.53 ± 0.01	1.89 ± 0.01	2.59 ± 0.13
EcJW212 + Ethanol 10 g/L	3.17 ± 0.00	5.32 ± 0.12	21.64 ± 0.07	15.74 ± 0.01	2.35 ± 0.03	1.84 ± 0.02	10.10 ± 0.32

B

	Esters	EcJW209	EcJW209 + Ethanol 2 g/L	EcJW209 + Ethanol 10 g/L
Ester titer (mg/L)	Ethyl acetate	26.1 ± 1.1	43.3 ± 8.5	152.1 ± 5.2
	Ethyl lactate	1.0 ± 0.1	3.3 ± 0.3	5.1 ± 0.3
	Ester total	27.0 ± 1.1	46.6 ± 8.7	157.2 ± 5.4
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.55 ± 0.02	0.88 ± 0.17	3.04 ± 0.15
	Ethyl lactate	0.02 ± 0.00	0.07 ± 0.01	0.10 ± 0.01
	Ester total	0.57 ± 0.02	0.94 ± 0.17	3.14 ± 0.15
Ester yield (mg/g glucose)	Ethyl acetate	7.43 ± 2.64	4.45 ± 0.88	15.76 ± 0.66
	Ethyl lactate	0.27 ± 0.05	0.33 ± 0.03	0.52 ± 0.03
	Ester total	7.70 ± 2.68	4.79 ± 0.89	16.29 ± 0.68
		EcJW210	EcJW210 + Ethanol 2 g/L	EcJW210 + Ethanol 10 g/L
Ester titer (mg/L)	Ethyl acetate	36.5 ± 3.9	46.2 ± 1.3	149.0 ± 3.8
	Ethyl lactate	1.4 ± 0.4	3.2 ± 0.2	5.3 ± 0.3
	Ester total	37.9 ± 4.2	49.4 ± 1.4	154.2 ± 4.1
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.77 ± 0.09	0.92 ± 0.03	3.20 ± 0.13
	Ethyl lactate	0.03 ± 0.01	0.06 ± 0.00	0.11 ± 0.01
	Ester total	0.80 ± 0.10	0.98 ± 0.03	3.31 ± 0.14
Ester yield (mg/g glucose)	Ethyl acetate	7.79 ± 2.08	4.79 ± 0.14	15.23 ± 0.49
	Ethyl lactate	0.30 ± 0.14	0.33 ± 0.02	0.54 ± 0.03
	Ester total	8.09 ± 2.21	5.12 ± 0.15	15.77 ± 0.52
		EcJW211	EcJW211 + Ethanol 2 g/L	EcJW211 + Ethanol 10 g/L
Ester titer (mg/L)	Ethyl acetate	22.0 ± 0.8	45.4 ± 1.2	151.9 ± 2.3
	Ethyl lactate	0.9 ± 0.2	3.0 ± 0.1	4.9 ± 0.4
	Ester total	22.9 ± 1.0	48.4 ± 1.3	156.8 ± 2.2
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.46 ± 0.02	0.90 ± 0.03	3.04 ± 0.06
	Ethyl lactate	0.02 ± 0.00	0.06 ± 0.00	0.10 ± 0.01
	Ester total	0.48 ± 0.02	0.96 ± 0.03	3.14 ± 0.05
Ester yield (mg/g glucose)	Ethyl acetate	4.91 ± 0.69	4.58 ± 0.30	15.57 ± 0.45
	Ethyl lactate	0.21 ± 0.07	0.31 ± 0.02	0.51 ± 0.05
	Ester total	5.12 ± 0.75	4.89 ± 0.32	16.08 ± 0.46
		EcJW212	EcJW212 + Ethanol 2 g/L	EcJW212 + Ethanol 10 g/L
Ester titer (mg/L)	Ethyl acetate	23.8 ± 3.1	42.3 ± 2.5	135.6 ± 5.8
	Ethyl lactate	1.2 ± 0.4	3.1 ± 0.2	5.6 ± 0.3
	Ester total	25.0 ± 3.5	45.3 ± 2.6	141.2 ± 6.1
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.49 ± 0.07	0.86 ± 0.04	2.75 ± 0.08
	Ethyl lactate	0.02 ± 0.01	0.06 ± 0.00	0.11 ± 0.00
	Ester total	0.51 ± 0.08	0.92 ± 0.04	2.86 ± 0.08
Ester yield (mg/g glucose)	Ethyl acetate	5.03 ± 1.40	4.30 ± 0.25	13.90 ± 0.59
	Ethyl lactate	0.26 ± 0.11	0.31 ± 0.02	0.58 ± 0.03
	Ester total	5.29 ± 1.51	4.62 ± 0.26	14.48 ± 0.62

Table 3-10: Summary of high cell density cultures of EcJW213-221 after 24 h. **(A)** OD₆₀₀, glucose, lactate, acetate, and ethanol. **(B)** Titer, specific productivity, and yield of esters. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respectively.

A

Strains	OD _i	OD _f	Glucose _i (g/L)	Glucose _f (g/L)	Lactate (g/L)	Acetate (g/L)	Ethanol (g/L)
EcJW213	3.01 ± 0.00	5.45 ± 0.14	22.60 ± 0.00	0.00 ± 0.00	1.44 ± 0.08	0.56 ± 0.03	9.81 ± 0.06
EcJW214	2.94 ± 0.00	5.27 ± 0.06	22.60 ± 0.00	2.78 ± 1.44	1.43 ± 0.05	0.92 ± 0.01	8.65 ± 0.52
EcJW215	2.90 ± 0.00	4.98 ± 0.09	22.60 ± 0.00	8.17 ± 0.06	1.58 ± 0.01	0.80 ± 0.00	6.42 ± 0.02
EcJW216	2.94 ± 0.00	5.25 ± 0.13	22.60 ± 0.00	5.29 ± 0.39	1.35 ± 0.03	0.94 ± 0.01	7.68 ± 0.12
EcJW217	3.05 ± 0.00	5.29 ± 0.20	22.60 ± 0.00	9.39 ± 4.03	1.41 ± 0.38	1.13 ± 0.23	5.60 ± 1.26
EcJW218	2.92 ± 0.00	5.02 ± 0.05	22.60 ± 0.00	5.58 ± 0.62	1.24 ± 0.06	0.91 ± 0.01	7.70 ± 0.23
EcJW219	3.03 ± 0.00	5.23 ± 0.13	22.60 ± 0.00	8.81 ± 2.00	0.95 ± 0.15	1.44 ± 0.11	6.29 ± 0.73
EcJW220	2.89 ± 0.00	4.94 ± 0.08	22.60 ± 0.00	10.04 ± 0.23	1.00 ± 0.01	1.37 ± 0.01	5.93 ± 0.09
EcJW221	2.95 ± 0.00	5.18 ± 0.05	22.60 ± 0.00	7.83 ± 0.22	1.14 ± 0.02	1.28 ± 0.01	6.85 ± 0.07
EcJW213	3.01 ± 0.00	5.45 ± 0.14	22.60 ± 0.00	0.00 ± 0.00	1.44 ± 0.08	0.56 ± 0.03	9.81 ± 0.06
EcJW214	2.94 ± 0.00	5.27 ± 0.06	22.60 ± 0.00	2.78 ± 1.44	1.43 ± 0.05	0.92 ± 0.01	8.65 ± 0.52
EcJW215	2.90 ± 0.00	4.98 ± 0.09	22.60 ± 0.00	8.17 ± 0.06	1.58 ± 0.01	0.80 ± 0.00	6.42 ± 0.02

B

	Esters	EcJW213	EcJW214	EcJW215
Ester titer (mg/L)	Ethyl acetate	28.9 ± 7.7	56.0 ± 7.3	169.2 ± 9.3
	Ethyl lactate	0.8 ± 0.2	3.4 ± 0.9	6.1 ± 0.4
	Ester total	29.7 ± 7.9	59.4 ± 8.2	175.4 ± 9.7
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.59 ± 0.17	1.17 ± 0.15	3.70 ± 0.21
	Ethyl lactate	0.02 ± 0.00	0.07 ± 0.02	0.13 ± 0.01
	Ester total	0.61 ± 0.17	1.24 ± 0.16	3.83 ± 0.22
Ester yield (mg/g glucose)	Ethyl acetate	1.28 ± 0.34	2.84 ± 0.48	11.73 ± 0.70
	Ethyl lactate	0.04 ± 0.01	0.17 ± 0.05	0.43 ± 0.03
	Ester total	1.31 ± 0.35	3.02 ± 0.53	12.16 ± 0.73
		EcJW216	EcJW217	EcJW218
Ester titer (mg/L)	Ethyl acetate	9.5 ± 0.5	26.4 ± 14.3	145.5 ± 17.9
	Ethyl lactate	0.4 ± 0.2	1.1 ± 0.5	7.0 ± 1.1
	Ester total	9.9 ± 0.5	27.5 ± 14.8	152.5 ± 18.6
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.20 ± 0.01	0.54 ± 0.29	3.15 ± 0.40
	Ethyl lactate	0.01 ± 0.00	0.02 ± 0.01	0.15 ± 0.02
	Ester total	0.21 ± 0.01	0.57 ± 0.30	3.30 ± 0.42
Ester yield (mg/g glucose)	Ethyl acetate	0.55 ± 0.02	2.20 ± 1.52	8.57 ± 1.26
	Ethyl lactate	0.02 ± 0.01	0.09 ± 0.06	0.41 ± 0.08
	Ester total	0.57 ± 0.02	2.29 ± 1.58	8.98 ± 1.32
		EcJW219	EcJW220	EcJW221
Ester titer (mg/L)	Ethyl acetate	7.5 ± 2.7	15.7 ± 0.6	65.2 ± 3.3
	Ethyl lactate	0.5 ± 0.1	0.7 ± 0.1	2.5 ± 0.3
	Ester total	8.0 ± 2.8	16.4 ± 0.6	67.7 ± 3.3
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.15 ± 0.05	0.34 ± 0.02	1.38 ± 0.07
	Ethyl lactate	0.01 ± 0.00	0.02 ± 0.00	0.05 ± 0.01
	Ester total	0.17 ± 0.06	0.36 ± 0.02	1.43 ± 0.07
Ester yield (mg/g glucose)	Ethyl acetate	0.53 ± 0.11	1.25 ± 0.02	4.41 ± 0.20
	Ethyl lactate	0.03 ± 0.00	0.06 ± 0.01	0.17 ± 0.02
	Ester total	0.57 ± 0.12	1.30 ± 0.02	4.59 ± 0.21
		EcJW213	EcJW214	EcJW215
Ester titer (mg/L)	Ethyl acetate	28.9 ± 7.7	56.0 ± 7.3	169.2 ± 9.3
	Ethyl lactate	0.8 ± 0.2	3.4 ± 0.9	6.1 ± 0.4
	Ester total	29.7 ± 7.9	59.4 ± 8.2	175.4 ± 9.7
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.59 ± 0.17	1.17 ± 0.15	3.70 ± 0.21
	Ethyl lactate	0.02 ± 0.00	0.07 ± 0.02	0.13 ± 0.01
	Ester total	0.61 ± 0.17	1.24 ± 0.16	3.83 ± 0.22
Ester yield (mg/g glucose)	Ethyl acetate	1.28 ± 0.34	2.84 ± 0.48	11.73 ± 0.70
	Ethyl lactate	0.04 ± 0.01	0.17 ± 0.05	0.43 ± 0.03
	Ester total	1.31 ± 0.35	3.02 ± 0.53	12.16 ± 0.73

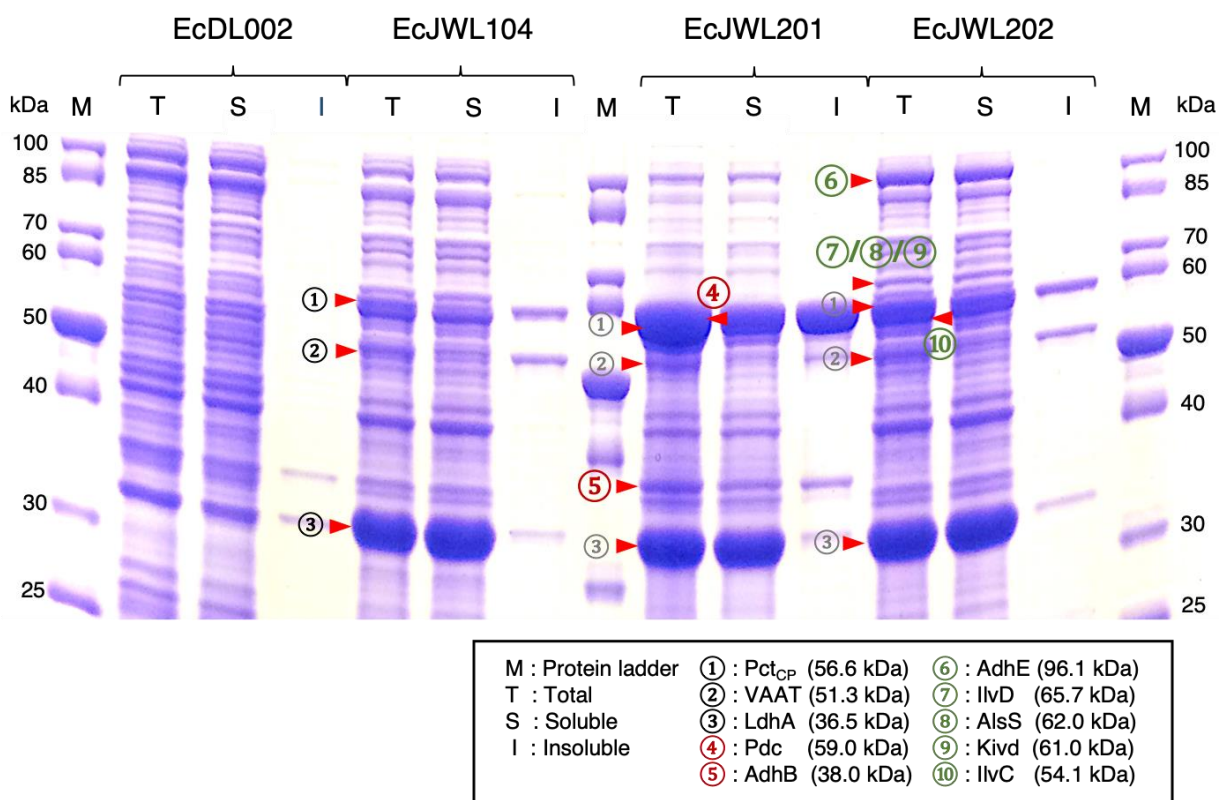
Table 3-11: Summary of high cell density cultures of EcJW109-117 after 24 h. **(A)** OD₆₀₀, glucose, lactate, acetate, and ethanol. **(B)** Titer, specific productivity, and yield of esters. The subscripts i and f are referred to the initial (0 h) and final (24 h) time of the culture, respectively. Abbreviations: *n.a.*: not applicable, *n.d.*: not detected.

A

Strains	OD _i	OD _f	Glucose _i (g/L)	Glucose _f (g/L)	Lactate (g/L)	Acetate (g/L)	Ethanol (g/L)
EcJW109	3.12 ± 0.00	4.93 ± 0.10	19.24 ± 0.00	14.10 ± 0.62	2.08 ± 0.00	1.84 ± 0.31	1.91 ± 0.00
EcJW110	2.98 ± 0.00	4.99 ± 0.40	19.24 ± 0.00	13.56 ± 0.95	2.08 ± 0.00	1.37 ± 1.05	1.91 ± 0.00
EcJW111	3.23 ± 0.00	5.04 ± 0.37	19.24 ± 0.00	13.58 ± 0.89	2.08 ± 0.00	1.43 ± 0.96	1.91 ± 0.00
EcJW112	3.11 ± 0.00	4.83 ± 0.06	19.24 ± 0.00	14.37 ± 0.25	2.08 ± 0.00	2.07 ± 0.01	1.91 ± 0.00
EcJW113	2.99 ± 0.00	4.78 ± 0.13	19.24 ± 0.00	14.46 ± 0.19	2.08 ± 0.00	2.07 ± 0.04	1.91 ± 0.00
EcJW114	2.94 ± 0.00	5.09 ± 0.46	19.24 ± 0.00	13.18 ± 1.34	2.08 ± 0.00	0.82 ± 1.07	1.91 ± 0.00
EcJW115	2.87 ± 0.00	4.74 ± 0.25	19.24 ± 0.00	14.89 ± 0.05	2.08 ± 0.00	1.98 ± 0.02	1.91 ± 0.00
EcJW116	2.82 ± 0.00	4.57 ± 0.09	19.24 ± 0.00	14.99 ± 0.03	2.08 ± 0.00	1.98 ± 0.01	1.91 ± 0.00
EcJW117	2.90 ± 0.00	4.49 ± 0.17	19.24 ± 0.00	14.60 ± 1.11	2.08 ± 0.00	1.78 ± 0.18	1.91 ± 0.00
EcJW109	3.12 ± 0.00	4.93 ± 0.10	19.24 ± 0.00	14.10 ± 0.62	2.08 ± 0.00	1.84 ± 0.31	1.91 ± 0.00
EcJW110	2.98 ± 0.00	4.99 ± 0.40	19.24 ± 0.00	13.56 ± 0.95	2.08 ± 0.00	1.37 ± 1.05	1.91 ± 0.00
EcJW111	3.23 ± 0.00	5.04 ± 0.37	19.24 ± 0.00	13.58 ± 0.89	2.08 ± 0.00	1.43 ± 0.96	1.91 ± 0.00

B

	Esters	EcJW109	EcJW110	EcJW111
Ester titer (mg/L)	Ethyl acetate	0.2 ± 0.0	5.9 ± 1.9	46.7 ± 8.9
	Ethyl lactate	<i>n.d.</i>	0.1 ± 0.1	0.7 ± 0.3
	Ester total	0.2 ± 0.0	6.0 ± 2.0	47.4 ± 9.2
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.00 ± 0.00	0.13 ± 0.03	0.97 ± 0.19
	Ethyl lactate	<i>n.a.</i>	0.00 ± 0.00	0.01 ± 0.01
	Ester total	0.00 ± 0.00	0.13 ± 0.04	0.99 ± 0.20
Ester yield (mg/g glucose)	Ethyl acetate	0.03 ± 0.01	1.07 ± 0.42	8.53 ± 2.89
	Ethyl lactate	<i>n.a.</i>	0.01 ± 0.01	0.12 ± 0.08
	Ester total	0.03 ± 0.01	1.08 ± 0.43	8.65 ± 2.97
		EcJW112	EcJW113	EcJW114
Ester titer (mg/L)	Ethyl acetate	0.2 ± 0.0	7.8 ± 0.5	76.1 ± 21.8
	Ethyl lactate	<i>n.d.</i>	0.1 ± 0.0	1.2 ± 0.4
	Ester total	0.2 ± 0.0	7.9 ± 0.5	77.2 ± 22.1
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.00 ± 0.00	0.17 ± 0.01	1.62 ± 0.44
	Ethyl lactate	<i>n.a.</i>	0.00 ± 0.00	0.03 ± 0.01
	Ester total	0.00 ± 0.00	0.17 ± 0.01	1.65 ± 0.44
Ester yield (mg/g glucose)	Ethyl acetate	0.05 ± 0.00	1.64 ± 0.16	12.42 ± 1.02
	Ethyl lactate	<i>n.a.</i>	0.01 ± 0.00	0.19 ± 0.02
	Ester total	0.05 ± 0.00	1.65 ± 0.16	12.61 ± 1.03
		EcJW115	EcJW116	EcJW117
Ester titer (mg/L)	Ethyl acetate	0.2 ± 0.0	4.7 ± 0.2	26.4 ± 1.3
	Ethyl lactate	<i>n.d.</i>	0.1 ± 0.1	0.7 ± 0.1
	Ester total	0.2 ± 0.0	4.8 ± 0.2	27.1 ± 1.3
Specific ester productivity (mg/gDCW/h)	Ethyl acetate	0.00 ± 0.00	0.11 ± 0.00	0.62 ± 0.04
	Ethyl lactate	<i>n.a.</i>	0.00 ± 0.00	0.02 ± 0.00
	Ester total	0.00 ± 0.00	0.11 ± 0.00	0.63 ± 0.04
Ester yield (mg/g glucose)	Ethyl acetate	0.04 ± 0.01	1.10 ± 0.04	5.93 ± 1.47
	Ethyl lactate	<i>n.a.</i>	0.03 ± 0.01	0.15 ± 0.03
	Ester total	0.04 ± 0.01	1.13 ± 0.05	6.08 ± 1.50



①~③: Pyruvate-to-lactate ester module, ④~⑤: Ethanol module, ⑥~⑩: Isobutanol module

Figure 3-6: Expression of the recombinant enzymes in engineered *E. coli* strains. The positions corresponding to the overexpressed proteins are indicated by arrowheads. Lane M represents protein ladder while lanes T, S, and I are referred to total, soluble, and insoluble proteins, respectively. ①~③, Pyruvate-to-lactate ester module; ④~⑤, Ethanol module; ⑥~⑩, Isobutanol module. Protein sizes were predicted with their amino acid sequences.

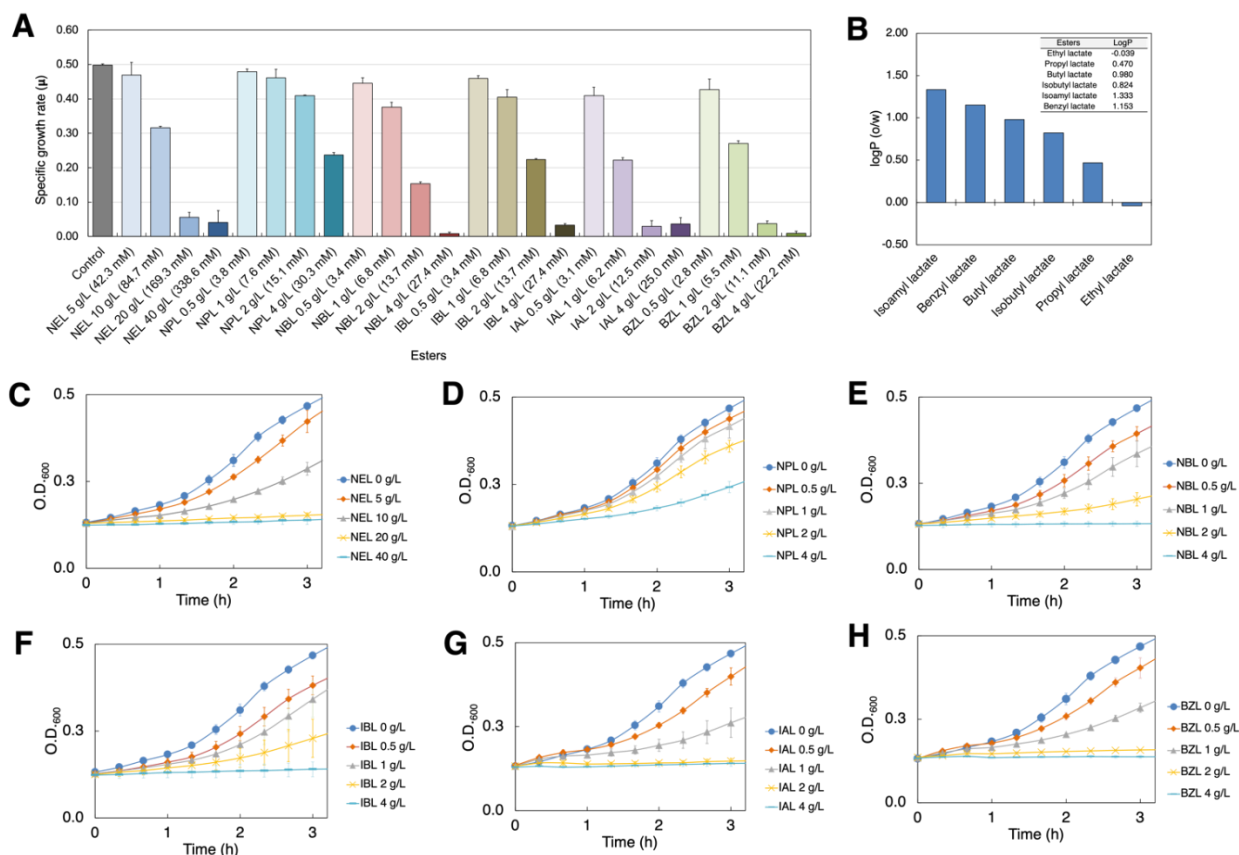
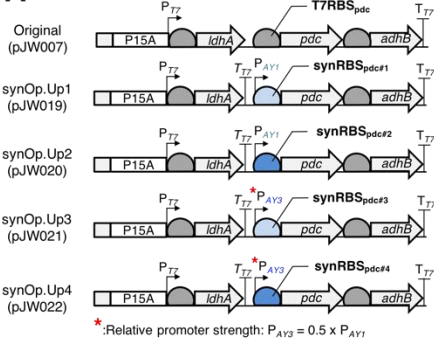
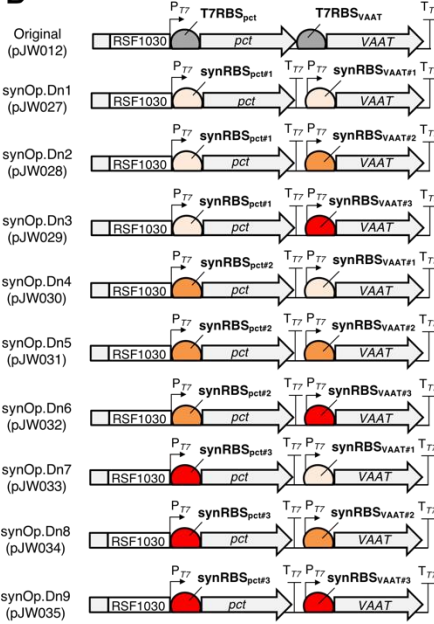


Figure 3-7: Effect of lactate esters on cell growth. **(A)** Specific growth rates of EcDL002 with or without addition of lactate esters. **(B)** logP values of characterized lactate esters. The values were obtained from <http://www.thegoodscentcompany.com>. **(C-H)** Growth curves of EcDL002 with or without addition of **(C)** *n*-ethyl lactate (NEL), **(D)** *n*-propyl lactate (NPL), **(E)** *n*-butyl lactate (NBL), **(F)** *i*-butyl lactate (IBL), **(G)** *i*-amyl lactate (IAL), and **(H)** benzyl lactate (BZL).

A

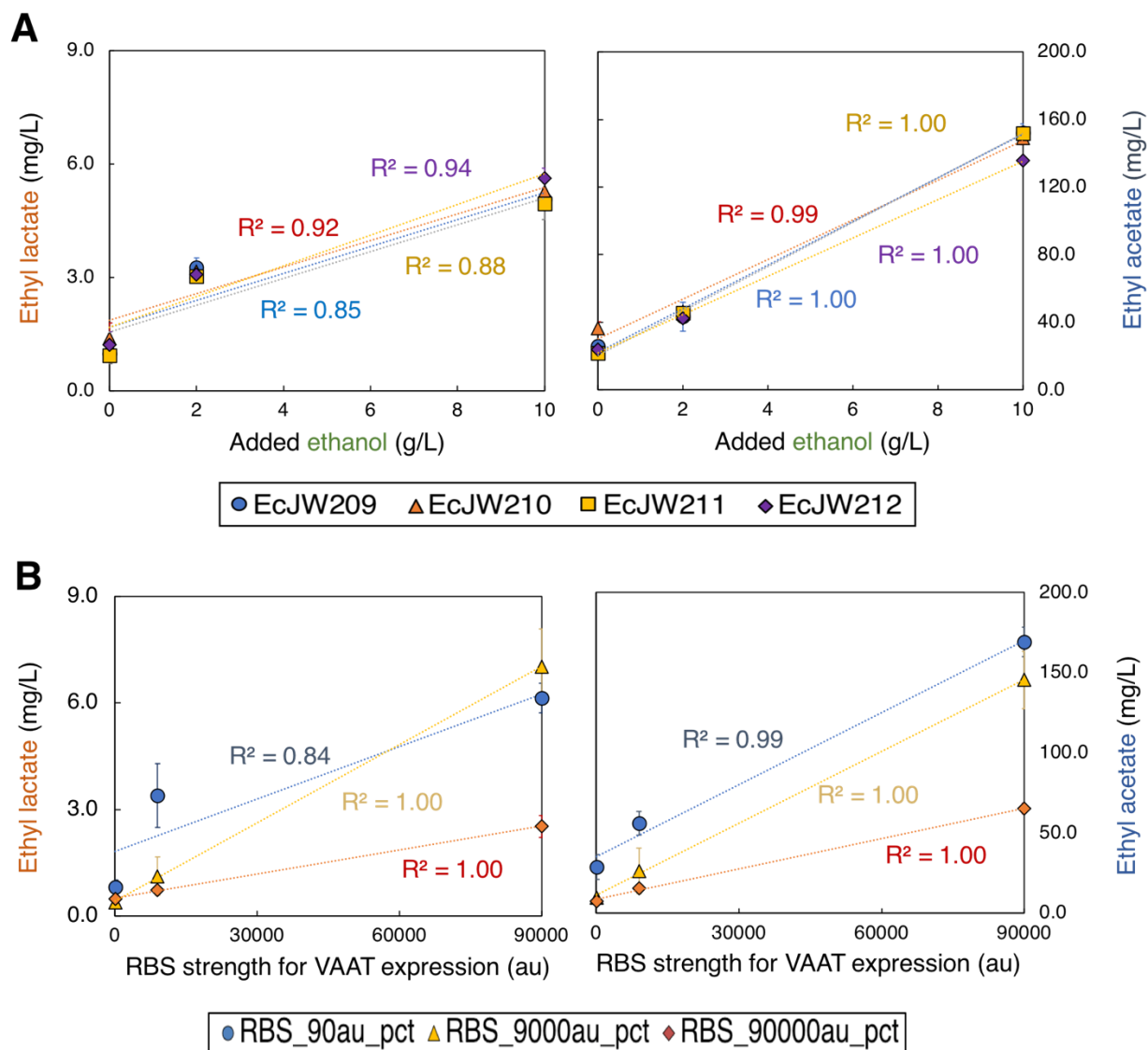
RBS ID	Sequences	Predicted translation initiation rate (au)	Relative translation initiation rate (au)	Length (bp)
T7RBS _{pdc}	AAGGAGATATA	33.11	1	11
synRBS _{pdc#1}	TTAGCAGTACCAGGACGTACCGGAGT	0.33	10^{-2}	27
synRBS _{pdc#2}	AGTTTACCTAGGGCAATAGCGTACCGA	0.03	10^{-3}	27
synRBS _{pdc#3}	AGCTTAGCGACAACCTAGGCGCTCGC	0.35	10^{-2}	27
synRBS _{pdc#4}	ACTAGGCCTAGCGATTCCGCTAA	0.03	10^{-3}	23

P_{AY1} (BBa_J23100) and P_{AY3} (BBa_J23108) were obtained from iGEM Andersen promoter library

B

RBS ID	Sequences	Predicted translation initiation rate (au)	Relative translation initiation rate (au)	Length (bp)
T7RBS _{pct}	AAGGAGATATA	9417.53	10^2	11
synRBS _{pct#1}	GATATAGCTCGAACGCGGAAGAG	84.56	10^0	24
synRBS _{pct#2}	GCAACCTATTTTAATCCAAGGAAGATCT A	8695.18	10^2	29
synRBS _{pct#3}	GCAATAACAACCTAGGAGAGACGAC	95382.72	10^3	24
T7RBS _{VAAT}	AAGGAGATATA	87.01	1	11
synRBS _{VAAT#1}	TAACCAAAACACTAACGCAAG	93.02	10^0	22
synRBS _{VAAT#2}	AGGGCACGAGGAGGAACAGTAGA	8228.14	10^2	24
synRBS _{VAAT#3}	GCAACCAACACAACGAGGAGGCATTTA	93714.75	10^3	27

Figure 3-8: Design of (A) upstream module and (B) downstream module of the ethyl lactate pathway. The RBS Calculator v2.0 software was used to generate synthetic RBS sequences. For the upstream, four synthetic RBS sequences were generated with predicted translation initiation rates at 0.33 and 0.03 between the P_{AY1} or P_{AY3} promoter and *pdc* start codon. For the downstream, six synthetic RBS sequences were generated with predicted translation initiation rates at 90, 9000, and 90000 au between the P_{T7} promoter and *pct* or *VAAT* start codon.



4 Development of a Computer-Guided Rational Protein Engineering Protocol for Improved Biosynthesis of Designer Esters

This chapter was published by:

Seo, H.[#], **Lee, J.W.**[#], Sergio G., and Trinh, C.T., 2019. Single mutation at a highly conserved region of chloramphenicol acetyltransferase enables isobutyl acetate production directly from cellulose by *Clostridium thermocellum* at elevated temperatures, ***Biotechnol. Biofuels***, **12**:245.

HS and **JWL** have contributed equally to this work. HS, **JWL** and CTT designed the experiments, analyzed the data, and drafted the manuscript. HS and **JWL** performed the experiments. SG calculated enzyme kinetic parameters and edited the manuscript. CTT initiated and supervised the project.

4.1 Abstract

Esters are versatile chemicals and potential drop-in biofuels. To develop a sustainable production platform, microbial ester biosynthesis using alcohol acetyltransferases (AATs) has been studied for decades. Volatility of esters endows high-temperature fermentation with advantageous downstream product separation. However, due to the limited thermal stability of AATs known, the ester biosynthesis has largely relied on use of mesophilic microbes. Therefore, developing thermostable AATs is important for ester production directly from lignocellulosic biomass by the thermophilic consolidated bioprocessing (CBP) microbes, e.g., *Clostridium thermocellum*.

In this study, we engineered a thermostable chloramphenicol acetyltransferase from *Staphylococcus aureus* (CAT_{Sa}) for enhanced isobutyl acetate production at elevated temperature. We first analyzed the broad alcohol substrate range of CAT_{Sa}. Then, we targeted a highly

conserved region in the binding pocket of CAT_{Sa} for mutagenesis. The mutagenesis revealed that F97W significantly increased conversion of isobutanol to isobutyl acetate. Using CAT_{Sa} F97W, we demonstrated the engineered *C. thermocellum* could produce isobutyl acetate directly from cellulose.

This study highlights that CAT is a potential thermostable AAT that can be harnessed to develop the thermophilic CBP microbial platform for biosynthesis of designer bioesters directly from lignocellulosic biomass.

4.2 Introduction

Esters are versatile chemicals which have been used as lubricants, solvents, food additives, fragrances and potential drop-in fuels [1]. Currently, ester production largely relies on synthesis from petroleum or extraction from plants, which makes it neither sustainable nor economically feasible. Therefore, microbial production of esters has been studied for decades [2-7]. Most studies have employed an alcohol acetyltransferase (E.C. 2.3.1.84, AAT), belonging to a broad acetyltransferase class, that can synthesize a carboxylic ester by condensing an alcohol and an acyl-CoA in a thermodynamically favorable aqueous environment [5]. For example, an *Escherichia coli*, engineered to use this biosynthetic pathway, could achieve high titer of isobutyl acetate [6, 7]. With appropriate expression of AATs and availability of alcohol and acyl-CoA moieties, various types of esters can be produced [2, 4].

Due to high volatility of esters, ester production at elevated temperatures can benefit downstream product separation and hence reduce the process cost. Interestingly, it has recently been shown that for the same total carbon chain length, short-chain esters are less toxic to microbial health than alcohols, which is potentially beneficial for ester fermentation [8]. However, most of

the AATs known to date are isolated from mesophilic microbes or plants [9-12], and none of them has been reported to be active at elevated temperatures (> 50°C). The highest temperature reported for ester production is 42°C in a thermotolerant yeast [13]. Hence, finding and developing a thermostable AAT is crucial to produce esters at elevated temperature.

Chloramphenicol acetyltransferase (E.C. 2.3.1.28, CAT) is another acetyltransferase class that has been found in various microbes [14]. This enzyme acetylates chloramphenicol, a protein synthesis inhibitor, by transferring the acetyl group from acetyl-CoA. The acetylation of chloramphenicol detoxifies the antibiotic compound and confers chloramphenicol resistance in bacteria. Recent studies have implied that CATs likely recognize a broad substrate range for alcohols and acyl-CoAs [7]. In addition, high thermal stability of some CATs enables them to be used as selection markers in thermophiles [15-17]. Therefore, CAT can function or be repurposed as a thermostable AAT suitable for ester biosynthesis at elevated temperature.

In this study, we engineered a CAT from *Staphylococcus aureus* (CAT_{Sa}) for isobutyl acetate production at elevated temperatures. First, we investigated a broad alcohol substrate range of CAT_{Sa}. Protein homology modeling along with sequence alignment were performed to identify the binding pocket of CAT_{Sa} as a potential target for protein engineering to enhance condensation of isobutanol and acetyl-CoA. *In silico* mutagenesis successfully discovered a variant (F97W) of CAT_{Sa} that was then experimentally validated for improved catalytic activity towards isobutanol. As a proof of concept, the engineered CAT_{Sa} was successfully expressed in *Clostridium thermocellum*. We demonstrated the F97W CAT_{Sa}-overexpressing for consolidated bioprocessing (CBP) to produce isobutyl acetate directly from cellulose without a need for external supply of cellulases. To our knowledge, this study presents the first demonstration of CAT engineering to enable ester production directly from cellulose at elevated temperatures.

4.3 Materials and Methods

4.3.1 Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 4-1. *Clostridium thermocellum* DSM1313 Δhpt (M1354) strain was used as a host for the ester production at elevated temperatures. It should be noted that the deletion of hypoxanthine phosphoribosyltransferase gene (*hpt*, Clo1313_2927) in the wildtype DSM1313 allows genetic engineering by 8-Azahypoxanthine (8-AZH) counter selection; this deletion does not have any known adverse effect on cell growth and metabolism [18, 19]. The plasmid pNW33N, containing CAT_{Sa}, is thermostable and was used to express various CATs in *C. thermocellum*. The pET plasmids were used for molecular cloning and enzyme expression in *E. coli*.

4.3.2 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (MO, USA) and/or Thermo Fisher Scientific (MA, USA), unless specified elsewhere. For molecular cloning, restriction enzymes and T4 ligase were obtained from New England Biolabs (MA, USA). Phusion Hot Start II DNA polymerase was used for polymerase chain reaction (PCR).

4.3.3 Media and cultivation

For molecular cloning and protein expression, *E. coli* strains were grown in lysogeny broth (LB) containing appropriate antibiotics unless noted otherwise. For *in vivo* characterization of CAT_{Sa} in *E. coli*, M9 hybrid medium [5] with 20 g/L glucose was used. For *C. thermocellum* culture, MTC minimal medium or CTFuD-NY medium [19] was used as specified in the experiments. Optical density (OD) was measured by a spectrophotometer at 600 nm wavelength (Spectronic 200+, Thermo Fisher Scientific, MA, USA).

Table 4-1: A list of plasmids and strains used in this study.

Name	Descriptions	Source
<i>Plasmids</i>		
pNW33N	<i>Bacillus-E. coli</i> shuttle vector, Cm ^R , pBC1 ori for gram positive strains, pBR322 ori for <i>E. coli</i> , source of CAT _{Sa}	Bacillus Genetic Stock Center
pETDuet-1	pBR322 ori, Amp ^R , lacI, T7lac promoter	Novagen
pET_CAT _{Sa}	CAT _{Sa} wild type encoding gene between BamHI, SacI site, pETDuet-1 backbone, 6X His-tag at N-terminus	This study
pET_CAT _{Sa} F97W	F97W site directed variant, pET_CAT _{Sa} backbone	This study
pHS0024	CAT _{Sa} wild type gene under <i>C. thermocellum</i> PgapDH promoter, downstream of Clo1313_2927 for the transcription terminator, tdk operon under cbp promoter substituting with the native cat selection marker, pNW33N plasmid backbone	This study
pHS0024_F97W	CAT _{Sa} F97W site-directed mutated from pHS0024	This study
<i>Strains</i>		
<i>E. coli</i> Top10	Host for molecular cloning, <i>mcrA</i> , $\Delta(mrr-hsdRMS-mcrBC)$, <i>Phi80lacZ(del)M15</i> , $\Delta lacX74$, <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , $\Delta(ara-leu)7697$, <i>galU</i> , <i>galK</i> , <i>rpsL(SmR)</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen
<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> B <i>dcm</i> , <i>ompT</i> , <i>hsdS(rB-mB-)</i> , <i>gal</i>	Invitrogen
M1354	<i>C. thermocellum</i> DSM1313 Δhpt	[18]
HSCT0101	M1354 harboring pHS0024	This study
HSCT0102	M1354 harboring pHS0024_F97W	This study

The plasmids containing mutagenized genes are presented in Table 4-3.

4.3.4 Multiple sequence alignment analysis

Multiple sequence alignment (MSA) analysis was performed using MEGA7 [20]. Protein sequences were aligned by ClustalW [21] and visualized by ESPript 3.0 (<http://esprict.ibcp.fr>) [22]. The key features in protein structures of 3U9F [23], 4CLA [24], and 2XAT [25] were extracted from CAT_SALTI, CAT3_ECOLIX, and CAT4_PSEAE, respectively.

4.3.5 Molecular modeling and docking simulations

4.3.5.1 Three-dimensional (3D) structures

The 3D structure of CAT_{Sa} and alcohols of interest were first generated using Swiss-Model [26] and the ‘Builder’ tools of MOE (Molecular Operating Environment software, version 2019.01), respectively. The 3D structure of the dual substrates-bounded CAT_{Sa} complex (i.e., acetyl-CoA-isobutanol-CAT_{Sa}) was obtained by extracting an isobutanol from the isobutanol-CAT_{Sa} complex and then adding it to the acetyl-CoA-CAT_{Sa} complex. All the structures were prepared by the ‘QuickPrep’ tool of MOE with default parameters and further optimized by energy minimization with the Amber10: EHT force field.

4.3.5.2 Docking simulation

To perform docking simulations, the potential binding pocket was searched using the ‘Site Finder’ tool of MOE. The best-scored site, consistent with the reported catalytic sites [27], was selected for further studies. Docking simulations were performed as previously described [28]. Briefly, acetyl-CoA and each alcohol were docked using the induced fit protocol with the Triangle Matcher placement method and the London ΔG scoring function. After the docking simulations, the best-scored binding pose, showing the crucial interaction between the residue and the substrate at root-mean-square-deviation (RMSD) $< 2 \text{ \AA}$, was selected. As an example, for the acetyl-CoA

docking, the binding pose exhibiting the hydrogen bond between the hydroxyl of Ser-148 and the N⁷¹ of the CoA was chosen [29]. For the alcohol docking, the binding pose showing the hydrogen bond between the N³ of His-189 and the hydroxyl of alcohol was selected [30].

4.3.5.3 *In silico* mutagenesis analysis

In silico mutagenesis analysis of the acetyl-CoA-isobutanol-CAT_{Sa} complex was carried out as previously described [28]. Specifically, the ‘alanine scan’ and ‘residue scan’ tools of MOE were used to identify the potential residue candidates for mutagenesis.

3.3.6 Molecular cloning

4.3.5.4 Plasmid construction

Plasmids were constructed by the standard molecular cloning technique of ligase dependent method and/or Gibson assembly [31] using the primers listed in Table 4-3. The constructed plasmids were introduced into *E. coli* TOP10 by heat shock transformation. Colonies isolated on a selective plate were PCR screened and plasmid purified. The purified plasmids were verified via Sanger sequencing before being transformed into *E. coli* BL21 (DE3). Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis protocol with reduced overlap length [32] or Gibson assembly method [31]. For the *C. thermocellum* engineering, the plasmid pHS005 was constructed first and then modified to pHS0024. pHS0024 has no *hpt* at the downstream of the operon while other sequences of the plasmid are identical to pHS005.

4.3.5.5 Transformation

The conventional chemical transformation and electroporation methods were used for transformation of *E. coli* [33] and *C. thermocellum* [19], respectively. For *C. thermocellum*, the method, however, was slightly modified as described here. First, *C. thermocellum* M1354 (Table

1) was cultured in 50 mL CTFuD-NY medium at 50°C inside an anaerobic chamber (Bactron300, Sheldon manufacturing Inc., OR, USA). The cell culture with OD in a range of 0.8-1.0 was cooled down at room temperature for 20 minutes. Beyond this point, all steps were performed outside the chamber. The cooled cells were harvested at 6,500 x g and 4°C for 20 minutes. The cell pellets were washed twice with ice-chilled Milli-Q water and resuspended in 200 µL of the transformation buffer consisting of 250 mM sucrose and 10% (v/v) glycerol. Several 30 µL aliquots of the electrocompetent cells were immediately stored at -80°C for further use. For electroporation, the electrocompetent cells were thawed on ice and incubated with 500–1,000 ng of methylated plasmids [34] for 10 minutes. Then, the cells were transferred to an ice-chilled 1-mm gap electroporation cuvette (BTX Harvard Apparatus, MA, USA) followed by two consecutive exponential decay pulses with 1.8 kV, 350 Ω, and 25 µF. The pulses usually resulted in a 7.0-8.0 ms time constant. The cells were immediately resuspended in pre-warmed fresh CTFuD-NY and recovered at 50°C under anaerobic condition (90 % N₂, 5% H₂, and 5% CO₂) inside a rubber capped Balch tube. After 0-12 hours of recovery, the cells were mixed with molten CTFuD-NY agar medium supplemented with 15 µg/mL thiamphenicol. Finally, the medium-cell mixture was poured on a petri dish and solidified inside the anaerobic chamber. The plate was incubated at 50°C up to one week until colonies appeared. Transformation efficiency was 2-100 colony forming units per µg plasmid (CFU/µg plasmid).

4.3.6 *In vivo* characterization of CAT_{Sa} and its variants in *E. coli*

For *in vivo* characterization of CAT_{Sa} and its variants in *E. coli*, high-cell density cultures were performed as previously described [35] with an addition of 2 g/L of various alcohols. For *in-situ* extraction of esters, each tube was overlaid with 25% (v/v) hexadecane. To confirm the protein expression of CAT_{Sa} and its variants, 1% (v/v) of stock cells were grown overnight at 37°C and

200 rpm in 15 mL culture tubes containing 5 mL of LB media and antibiotics. Then, 4% (v/v) of the overnight cultures were transferred into 1 mL of LB media containing antibiotics in a 24-well microplate. The cultures were grown at 37°C and 350 rpm using an incubating microplate shaker (Fisher Scientific, PA, USA) until OD reached to 0.4~0.6 and then induced by 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 hours with a Breathe-Easy Sealing Membrane to prevent evaporation and cross contamination (cat# 50-550-304, Research Products International Corp., IL, USA). The protein samples were obtained using the B-PER complete reagent (cat# 89822, Thermo Scientific, MA, USA), according to the manufacturer's instruction and analyzed by SDS-PAGE.

4.3.7 Enzyme characterization

4.3.7.1 His-tag purification

For enzyme expression, an overnight culture was inoculated with a 1:50 ratio in fresh LB medium containing 1 mM IPTG and antibiotics, followed by 18°C overnight incubation (up to 20 hours) in a shaking incubator at 200 rpm. The induced cells were harvested by centrifugation at 4°C, and 4,700 x g for 10 minutes. The cell pellet was then washed once with Millipore water and resuspended in the B-PER complete reagent. After 30 min incubation at room temperature, the mixture was centrifuged at 17,000 x g for 2 minutes. The supernatant was collected and designated as crude extract. For his-tag purification, the crude extract was incubated with HisPur Ni-NTA superflow agarose in a batch as the manufacturer recommends. Then, the resin was washed with at least three volumes of wash buffer, consisting of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.1 mM EDTA. The resin bound proteins were eluted by 300 μ L elution buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 300 mM imidazole, and 0.1 mM EDTA. The eluted sample was then desalted and concentrated via an Amicon filter column with 10 kDa

molecular weight cutoff. Finally, the protein sample was suspended in 200 μ L of 20 mM Tris-HCl buffer (pH 8.0). Protein concentration was measured by the Bradford assay [36] with bovine serum albumin (BSA) as the reference protein.

4.3.7.2 Thermal shift assay

To measure protein melting temperature (T_m), a thermofluor assay was employed with SYPRO Orange [37]. About 10 to 250 μ g of His-tag purified protein was mixed with 5x SYPRO Orange in a 50 μ L final volume in a 96-well qPCR plate. The plate was sealed with PCR caps before running the assay. The StepOne real-time PCR machine (Applied Biosystems, CA, USA) was used to run the assay with the following parameters: ROX reporter, 1°C increment per cycle, one-minute hold at every cycle, and temperature range from 20°C to 98°C. The data was collected, exported, and processed to calculate T_m .

4.3.7.3 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay

Reaction rate for each CAT was determined by a DTNB assay [38] in a 384-well plate. Total reaction volume was 50 μ L with the reaction buffer comprising of 50 mM Tris-HCl (pH 8.0). Concentrations of acetyl-CoA (CoALA Biosciences, TX, USA) and alcohols were varied as specified in each experiment. Final enzyme concentrations of 0.05 μ g/mL and 10 μ g/mL were used for the reactions towards chloramphenicol and alcohols, respectively. Reaction kinetics were collected by measuring absorbance at 412 nm every minute for one hour at 50°C in a microplate reader (Synergy HTX microplate reader, BioTek). The reaction rate was calculated using the extinction coefficient from a standard curve of free coenzyme A (MP Biomedicals, OH, USA) under the same condition. It should be noted that since the maximum operating temperature

recommended for the plate reader is 50°C, the high throughput enzyme assay for CAT at elevated temperature was only performed to determine enzyme kinetics parameters.

4.3.7.4 Calculation of kinetic parameters for reaction rates

The parameters of Michaelis-Menten rate law (eqn. 1) were calculated for each enzyme as follows. First, linear regression was performed on data collected from a microplate reader to identify initial reaction rates, y_i , at different initial substrate concentrations, s_i , where $i = (1, 2, \dots, n)$ is the number of data points collected. Then, these initial reaction rates and associated initial substrate concentrations for all replicates were simultaneously fit to the Michaelis-Menten model (eqn. 1) using robust non-linear regression (eqn. 2) with a soft-L1-loss estimator (eqn. 3) as implemented in the SciPy numerical computing library v1.2.0 [39, 40].

$$v_i = \frac{v_{max} s_i}{K_M + s_i} \quad [\text{eqn. 1}]$$

$$\min_{K_M, v_{max}} \sum_{i=1}^n \rho((v_i(s_i, K_M, v_{max}) - y_i)^2) \quad [\text{eqn. 2}]$$

$$\rho(z) = 2(\sqrt{1 + z}) - 1 \quad [\text{eqn. 3}]$$

The least squares problem determines the parameters K_M and v_{max} by minimizing the difference between the model predicted reaction rates v_i and measured reaction rates y_i (eqn. 2). A smoothing function $\rho(z)$ is used to make the least square problem resistant to outliers (eqn. 3). Due to the unbiased resistance to outliers and the avoidance of errors resulting from conventional linearization methods, robust non-linear regression provides the most precise parameter estimate for the Michaelis-Menten model [41].

4.3.8 Isobutyl acetate production in *C. thermocellum*

4.3.8.1 Cellobiose fermentation

Isobutyl acetate production from cellobiose in *C. thermocellum* strains was performed by

the two-step bioconversion configuration. Cells were first cultured in MTC minimal medium [19] containing 5 g/L cellobiose in a rubber capped Balch tube until OD reached 0.8~1.0. The cells were cooled down at room temperature for 20 minutes and centrifuged at 4,700 x g and 4°C for 20 minutes. After removing the supernatant, cells were resuspended in the same volume of fresh MTC minimal media containing 2 g/L isobutanol in an anaerobic chamber. The cell suspension was then divided into 800 µL in a 2.0 mL screw cap microcentrifuge tube with a 200 µL hexadecane overlay. The cells were incubated at 55°C for 24 hours followed by analysis of gas chromatography coupled with a mass spectrometer (GC/MS) to quantify the amount of isobutyl acetate produced.

4.3.8.2 Cellulose fermentation

For the cellulose fermentation, modified MTC medium (C-MTC medium) was used. 20 g/L of Avicel PH-101 was used as a sole carbon source instead of cellobiose, and 10 g/L of MOPS was added to increase buffer capacity. Initial pH was adjusted to 7.5 by 5M KOH and autoclaved. In an anaerobic chamber, 0.8 mL of overnight cell culture was inoculated in 15.2 mL of C-MTC medium (1:20 inoculation ratio) with 4 mL of overlaid hexadecane. Each tube contained a small magnetic stirrer bar to homogenize cellulose. The rubber capped Balch tube was incubated in a water bath connected with a temperature controller set at 55°C and a magnetic stirring system. Following pH adjustment with 70 µL of 5 M KOH injection, 800 µL of cell culture and 200 µL of hexadecane layer were sampled every 12 hours. Culture pH was maintained within a range of 6.4-7.8 during the fermentation.

Cell growth was monitored by measuring pellet protein. The cell-cellulose pellet from 800 µL sampling volumes was washed twice with Milli-Q water and suspended by 200 µL lysis buffer (0.2 M NaOH, 1% SDS) followed by an hour incubation at room temperature. Then, the solution was neutralized with 50 µL 0.8 M HCl and diluted by 550 µL water. The mixture was centrifuged

at 17,000 x g for 3 minutes. Protein concentration from the supernatant was analyzed by the detergent-compatible Bradford assay (Thermo Scientific, WA, USA). The residual pellet was boiled in a 98°C oven for an hour before quantifying residual cellulose.

Residual cellulose was quantified by the phenol-sulfuric acid method [42] with some modifications. The boiled sample was washed twice with Milli-Q water and suspended in 800 µL water to make equivalent volume to the original. The sample was homogenized by pipetting and vortexing for 10 seconds, and 20 µL of the homogenized sample was transferred to a new 2.0 mL microcentrifuge tube or 96-well plate and dried overnight in a 55°C oven. The dried pellet was suspended in 200 µL of 95% sulfuric acid and incubated for an hour at room temperature. After the pellet was dissolved completely, 20 µL of 5% phenol was added and mixed with the sulfuric acid solution. After 30 min incubation at room temperature, 100 µL of the sample was transferred to a new 96-well plate, and the absorbance at 490 nm was measured. The absorbance was converted to cellulose concentration by the standard curve of Avicel PH-101 treated by the same procedure.

4.3.9 Analytical methods

4.3.9.1 High-performance liquid chromatography (HPLC)

Extracellular metabolites were quantified by using a high-performance liquid chromatography (HPLC) system (Shimadzu Inc., MD, USA). 800 µL of culture samples was centrifuged at 17,000 x g for 3 minutes, then the supernatants were filtered through 0.2 micron filters and run with 10 mM H₂SO₄ mobile phase at 0.6 mL/min on an Aminex HPX-87H (Biorad Inc., CA, USA) column at 50°C. Refractive index detector (RID) and ultra-violet detector (UVD) at 220 nm were used to monitor concentrations of sugars, organic acids, and alcohols.

4.3.9.2 Gas chromatography coupled with mass spectroscopy (GC/MS)

Esters were measured by GC (HP 6890, Agilent, CA, USA) equipped with a MS (HP 5973, Agilent, CA, USA). For the GC system, the Zebron ZB-5 (Phenomenex, CA, USA) capillary column (30 m x 0.25 mm x 0.25 μ m) was used to separate analytes, and helium was used as the carrier with a flow rate of 0.5 mL/min. The oven temperature program was set as follows: 50°C initial temperature, 1°C/min ramp up to 58°C, 25°C/min ramp up to 235°C, 50°C/min ramp up to 300°C, and 2-minutes bake-out at 300°C. 1 μ L of sampled hexadecane layer was injected into the column in the splitless mode with an injector temperature of 280°C.

For the MS system, selected ion mode (SIM) was used to detect and quantify esters with the following parameters: (i) ethyl acetate, m/z 45.00 and 61.00 from 4.2 to 4.6 minute retention time (RT), (ii) isopropyl acetate, m/z 45 and 102 from 4.7 to 5.0 minute RT, (iii) propyl acetate, m/z 59 and 73 from 5.2 to 5.8 minute RT, (iv) ethyl isobutyrate, m/z 73 and 116 from 6.1 to 6.6 minute RT, (v) isobutyl acetate, m/z 61 and 101 from 6.6 to 7.6 minute RT, (vi) butyl acetate, m/z 61 and 116 from 7.7 to 9.2 minute RT, (vii) isobutyl isobutyrate, m/z 89 and 129 from 10.1 to 12.5 minute RT, (viii) benzyl acetate, m/z 108 and 150 from 13.1 to 13.8 minute RT, and (ix) 2-phenethyl acetate, m/z 104 and 121 from 13.8 to 15.5 minute RT. Isoamyl alcohol and isoamyl acetate were used as the internal standard analytes. The esters were identified by RT and quantified by the peak areas and standard curves. Standard curves were determined by using pure esters diluted into hexadecane at concentrations of 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.5 g/L, and 1 g/L.

4.4 Results and Discussion

4.4.1 *In silico* and rapid *in vivo* characterization of a thermostable chloramphenicol acetyltransferase(s) for broad alcohol substrate range

To develop a thermophilic microbial ester production platform, a thermostable AAT is re-

quired. Unfortunately, the AATs known to date are isolated from mesophilic yeasts or plants, and none of them has been reported to be active at a temperature above 50°C. To tackle this problem, we chose CATs to investigate their potential functions as a thermostable AAT because some thermostable CATs have been successfully used as a selection marker in thermophiles [17, 43-46] and others have been shown to perform the acetylation for not only chloramphenicol but various alcohols like AATs [47-50] [7] (Figure 4-1A, Figure 4-5A). As a proof-of-study, we investigated CAT_{sa}, classified as Type A-9, from the plasmid pNW33N for a broad range of alcohol substrates as it has been widely used for genetic engineering in *C. thermocellum* at elevated temperature ($\geq 50^{\circ}\text{C}$) [17, 43, 44].

We first conducted alcohol docking simulations using the homology model. Remarkably, the model predicted binding affinities of short-to-medium chain length alcohols (e.g., ethanol, propanol, isopropanol, butanol, and isobutanol) and aromatic alcohols (e.g., benzyl alcohol and phenethyl alcohol) to the binding pocket. The change in the protein's Gibbs free energy upon the substrate binding was ordered as follows: 2-phenethyl alcohol > benzyl alcohol > isobutanol > butanol > propanol > ethanol > isopropanol (Figure 4-1B). To quickly evaluate the *in silico* docking simulation results experimentally, we next performed *in vivo* characterization of a CAT_{sa}-overexpressing *E. coli* and screened for acetate esters production. Acetyl-CoA was derived from glycolysis while various alcohols were externally supplied to the media. Remarkably, the results exhibited the same trends of specificities of CAT_{sa} towards alcohols as predicted by the *in silico* docking simulation (Figure 4-1B).

The CAT_{sa}-overexpressing *E. coli* produced all the expected acetate esters including ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, benzyl acetate, and 2-phenethyl acetate at titers of 1.1 ± 0.1 , 2.3 ± 0.3 , 0.1 ± 0.0 , 9.8 ± 1.6 , 17.1 ± 6.0 , 152.4 ± 29.5 , and

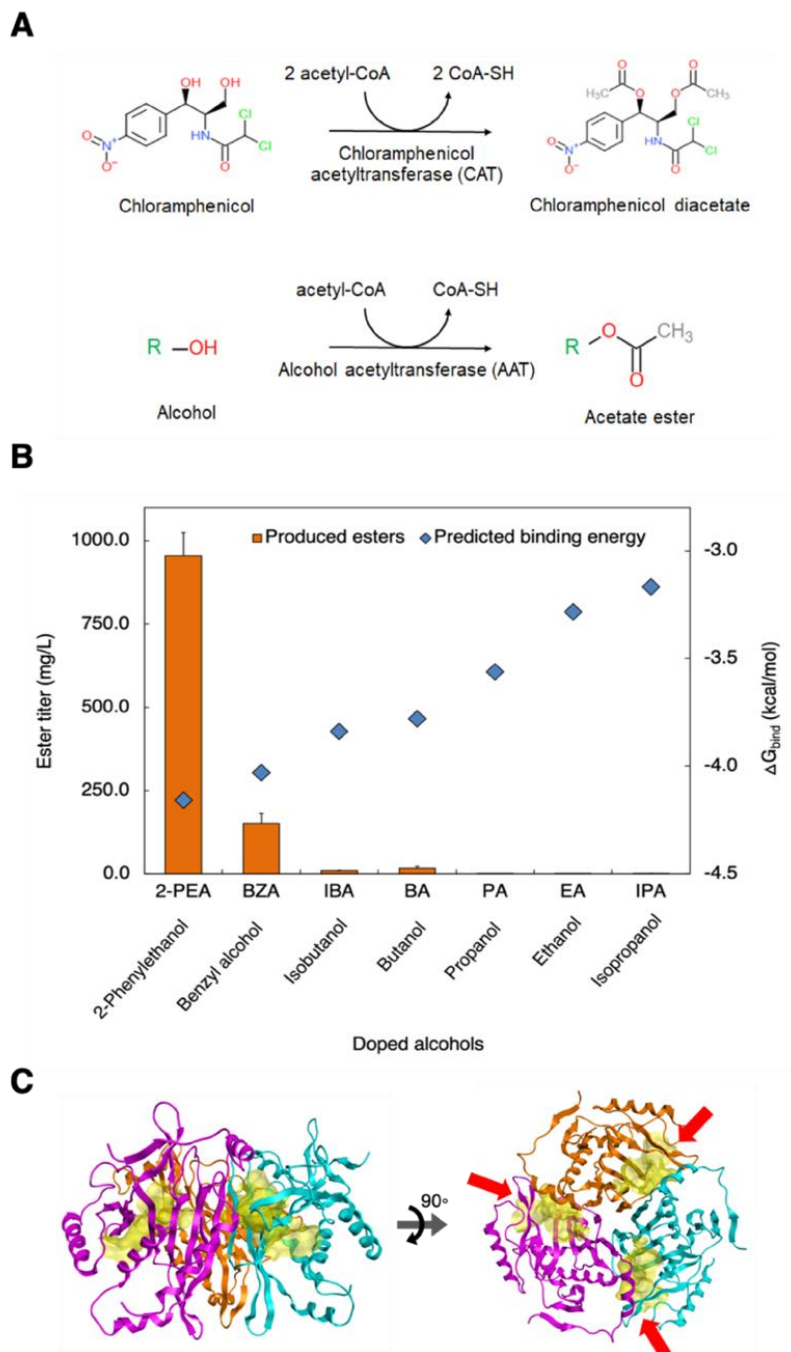


Figure 4-1: Broad substrate specificity of CAT_{Sa}. **(A)** Acetylation of chloramphenicol and alcohol by a chloramphenicol acetyltransferase (CAT) and an alcohol acetyltransferase (AAT), respectively. **(B)** Comparison between the predicted binding free energies for various alcohols bound to the binding pocket of CAT_{Sa} and the titer of esters produced by the CAT_{Sa}-overexpressing *E. coli* with external supply of alcohols. **(C)** Structure of the CAT_{Sa} homology model. The red arrows indicate the binding pockets (in yellow cloud) formulated by the trimeric structure of CAT_{Sa}.

955.3 $\pm$ 69.5 mg/L and specific ester production rates of 0.02 $\pm$ 0.00, 0.05 $\pm$ 0.01, 0.00 $\pm$ 0.00, 0.19 $\pm$ 0.03, 0.34 $\pm$ 0.12, 3.02 $\pm$ 0.57, and 19.27 $\pm$ 1.32 mg/gDCW/h, respectively. We observed that the specific ester production titers and rates are higher for aromatic alcohols than linear, short-chain alcohols likely because the hydrophobic binding pocket of CAT_{Sa} has been evolved towards chloramphenicol [30], an aromatic antibiotic (Figure 4-1C). Specifically, the bulky binding pocket of CAT_{Sa} likely contributes to more interaction with the aromatic substrates than the short, linear-chain alcohols (Figure 4-5B and C).

Overall, thermostable CATs, e.g., CAT_{Sa}, can have broad range of substrate specificities towards linear, short-chain, and aromatic alcohols and hence can be harnessed as AATs for novel ester biosynthesis at elevated temperature.

4.4.2 Discovery of a CAT_{Sa} variant improving conversion of isobutanol and acetyl CoA into isobutyl acetate

Since the *in vivo* activity of CAT_{Sa} is more than 50-fold higher for the aromatic alcohols than isobutanol, we asked whether its activity could be improved for isobutyl acetate biosynthesis. Using the *in-silico* analysis, we started by examining whether any modification of the binding pocket of CAT_{Sa} could improve the activity towards isobutanol. According to the homology model, the binding pocket consists of Tyr-20, Phe-27, Tyr-50, Thr-88, Ile-89, Phe-90, Phe-97, Ser-140, Leu-141, Ser-142, Ile-143, Ile-144, Pro-145, Trp-146, Phe-152, Leu-154, Ile-166, Ile-167, Thr-168, His-189, Asp-193, Gly-194, and Tyr-195, where the His189 and Asp193 are the catalytic sites (Figure 2A). Since chloramphenicol resistance is likely a strong selective pressure throughout evolution, we expected all CATs to exhibit a common binding pocket structure. Unsurprisingly, conserved sequences in the binding pocket were observed by protein sequence alignment of CAT_{Sa} with other CATs of Type A (Figure 4-6A). Especially, Pro-85 and Phe-97 were highly conserved

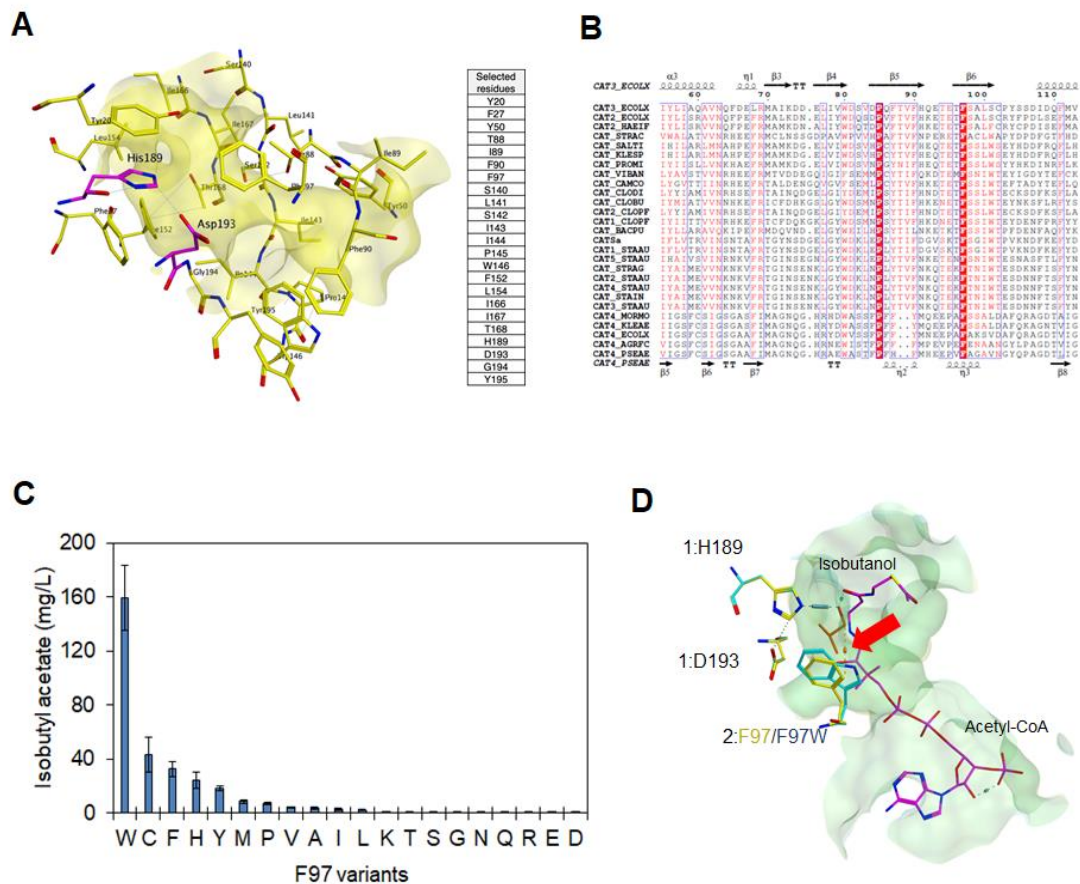


Figure 4-2: Discovery of CAT_{Sa} F97W responsible for enhanced activity towards isobutanol. **(A)** A binding pocket of CAT_{Sa} and associated amino acid residues. The catalytic site residues are in purple. **(B)** Protein sequence alignment of CAT_{Sa} with different CATs. **(C)** Screening of F97 site-saturated mutagenized variants for enhanced isobutyl acetate production in *E. coli*. The letters indicate amino acids substituting F in the wildtype CAT_{Sa}. **(D)** Superposed binding pocket structure of the wildtype and CAT_{Sa} F97W mutant. The red arrow indicates a CH- π interaction between the hydrogen of isobutanol and the indole ring of F97W.

in CATs of not only Type-A but also Type-B (Figure 4-2B and Figure 4-6B).

Based on the binding pocket identified, we performed docking simulation with alanine and residue scans using the acetyl-CoA-isobutanol-CAT_{Sa} complex to identify potential candidates for mutagenesis (Figure 4-7A and B). Remarkably, the top three variant candidates were suggested at the Phe-97 residue (e.g., F97Y, F97W, and F97V). This residue is involved in the formation of a tunnel-like binding pocket [30]. Motivated by the analysis, Phe-97 was chosen for site saturated mutagenesis, and the variants were screened in *E. coli* for isobutyl acetate production by external supply of isobutanol.

Among the variants characterized, the F97W variant exhibited the best performance (Figure 4-2C), with the similar protein expression levels in *E. coli* (Figure 4-8). As compared to the wildtype, the F97W variant enhanced the isobutyl acetate production by 4-fold. Subsequent *in silico* analysis showed that the mutation created a CH- π interaction between the hydrogen of isobutanol and the indole ring of F97W (Figure 4-2D). The model also indicated no change in distance between the isobutanol and active site (His-189) in F97W. Therefore, the CH- π interaction is likely responsible for the improved activity of F97W variant towards isobutyl acetate biosynthesis.

4.4.3 *In vitro* characterization of CAT_{Sa} F97W

Before deploying CAT_{Sa} F97W for isobutyl acetate biosynthesis in the thermophile CBP organism *C. thermocellum*, we checked whether the F97W mutation affected thermal stability of the enzyme. We overexpressed and purified both the wildtype CAT_{Sa} and CAT_{Sa} F97W variant (Figure 4-3A). The SDS-PAGE analysis confirmed the expression and purification of the enzymes by bands with the expected monomer size (25.8 kDa). Thermofluor assay revealed that the F97W variant slightly lowered the wildtype melting point from 72°C to 68.3°C (Figure 4-3B).

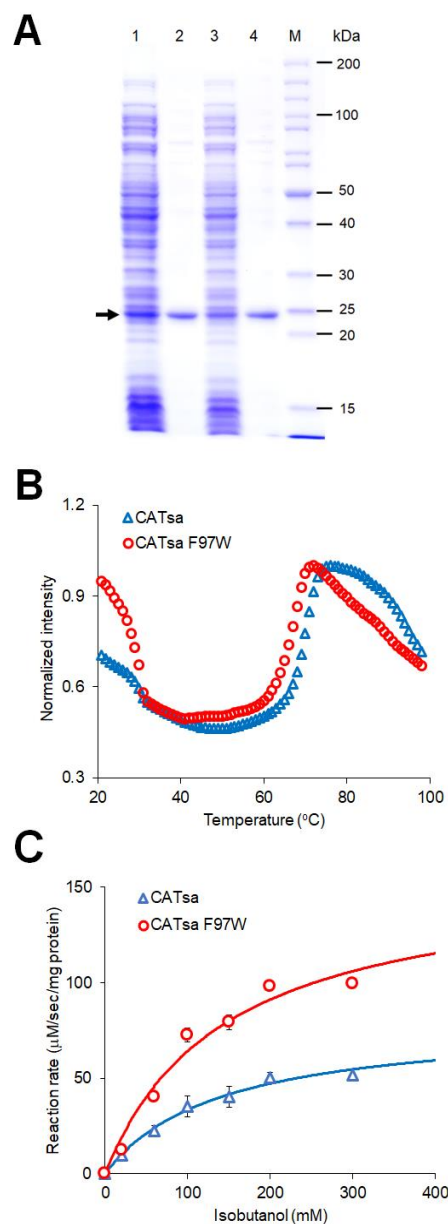


Figure 4-3: *In vitro* characterization of the wildtype CAT_{Sa} and CAT_{Sa} F97W mutant. **(A)** SDS-PAGE of the purified CAT_{Sa} and CAT_{Sa} F97W. The black arrow indicates the expected size of expressed target proteins, including CAT_{Sa} and CAT_{Sa} F97W. Notations: column 1, crude cell extract of IPTG induced *E. coli* BL21(DE3) harboring pET_CAT_{Sa}; column 2, His-tag purified CAT_{Sa}; column 3, crude extract of IPTG induced *E. coli* BL21(DE3) harboring pET_CAT_{Sa} F97W; column 4, His-tag purified CAT_{Sa} F97W; and M, protein ladder. **(B)** Melting curve of CAT_{Sa} and CAT_{Sa} F97W. The intensity was normalized by each maximum value. **(C)** Michaelis-Menten plots of CAT_{Sa} and CAT_{Sa} F97W for various isobutanol concentrations at 50°C. The co-substrate, acetyl-CoA, was supplemented at the saturated concentration of 2 mM. The error bars represent standard deviation of three biological replicates.

Since CAT_{Sa}F97W maintained high melting point, it is possible that CAT_{Sa}F97W still maintains its functionality at high temperature ($\geq 50^{\circ}\text{C}$) but needs to be thoroughly characterized.

Table 4-2 shows the *in vitro* enzymatic activities of both the wildtype CAT_{Sa} and CAT_{Sa}F97W at 50°C . The turnover number (kcat) of CAT_{Sa}F97W was two times higher than that of the wildtype. The increased turnover number of CAT_{Sa}F97W led to 1.9-fold increase in enzymatic efficiency (kcat/ K_M , 4.08 ± 0.62 , 1/M/sec) while the mutation did not result in significant change in K_M . The improved enzymatic efficiency of CAT_{Sa}F97W agrees with the enhanced isobutanol production observed in the *in vivo* characterization using the CAT_{Sa}-overexpressing *E. coli* (Figure 4-2C).

Based on the rigidity of the binding pocket, we originally presumed that mutagenesis on the binding pocket would result in activity loss towards chloramphenicol. Surprisingly, CAT_{Sa}F97W retained the activity towards chloramphenicol (Table 4-2). The F97W mutation decreased kcat but also lowered K_M , resulting in a compensation effect. Turnover number of CAT_{Sa} (kcat, 202.97 ± 3.36 , 1/sec) was similar to the previously reported value by Kobayashi *et al.* [16], but K_M (0.28 ± 0.02 , mM) was about 1.75-fold higher. The difference might attribute to the experimental condition and analysis performed. Kobayashi *et al.* used chloramphenicol in a range of 0.05-0.2 mM for the assay and the Lineweaver-Burk method for analysis, while we used a 0-1.0 mM range with a nonlinear regression analysis method. Interestingly, affinity towards acetyl-CoA was independent of the alcohol co-substrates (Table 4-4), suggesting that the alcohol affinity would be likely the main bottleneck for microbial production of isobutyl acetate.

Taken altogether, the F97W mutation not only resulted in 1.9-fold higher enzymatic efficiency towards isobutanol but also retained thermal stability of CAT_{Sa}. Thus, CAT_{Sa}F97W variant can serve a starting candidate to demonstrate direct biosynthesis of isobutyl acetate at

elevated temperatures by *C. thermocellum*.

4.4.4 Isobutyl acetate production from cellulose at elevated temperature by an engineered *C. thermocellum* overexpressing CAT_{Sa} F97W

We next investigated whether *C. thermocellum* overexpressing CAT_{Sa} F97W could produce isobutyl acetate at elevated temperature. This thermophile was chosen because it has a high cellulolytic activity suitable for CBP, a one-step process configuration for cellulase production, cellulose hydrolysis, and fermentation for direct conversion of lignocellulosic biomass to fuels and chemicals [51]. Furthermore, studies have demonstrated that the wildtype *C. thermocellum* has native metabolism capable of endogenously producing precursor metabolites for ester biosynthesis, such as acetyl-CoA, isobutyryl-CoA, as well as ethanol [52] and higher alcohols (e.g., isobutanol) under high cellulose loading fermentation [53-55] (Figure 4-4A and Figure 4-9A).

We started by generating two isobutyl acetate-producing strains, HSCT0101 and HSCT0102, by introducing the plasmids pHS0024 (harboring the wildtype CAT_{Sa}) and pHS0024_F97W (harboring the mutant CAT_{Sa} F97W) into *C. thermocellum* DSM1313. Colonies were isolated on antibiotics selective plates at 55°C. Successful transformation clearly indicated that CAT_{Sa} F97W conferred the thiamphenicol resistance and hence maintained CAT activity. This result agrees with the *in vitro* enzymatic activity of CAT_{Sa} F97W (Table 4-2).

We next evaluated whether the *C. thermocellum* strains could synthesize isobutyl acetate from cellobiose. Since the endogenous isobutanol production from a typical cellobiose concentration (5 g/L) is low [55], we supplemented the media with 2 g/L isobutanol. Both HSCT0101 and HSCT0102 could produce isobutyl acetate at 55°C as expected. Like the *in vivo* characterization in *E. coli* (Figure 4-2C), HSCT0102 outperformed HSCT0101 with 3.5-fold in-

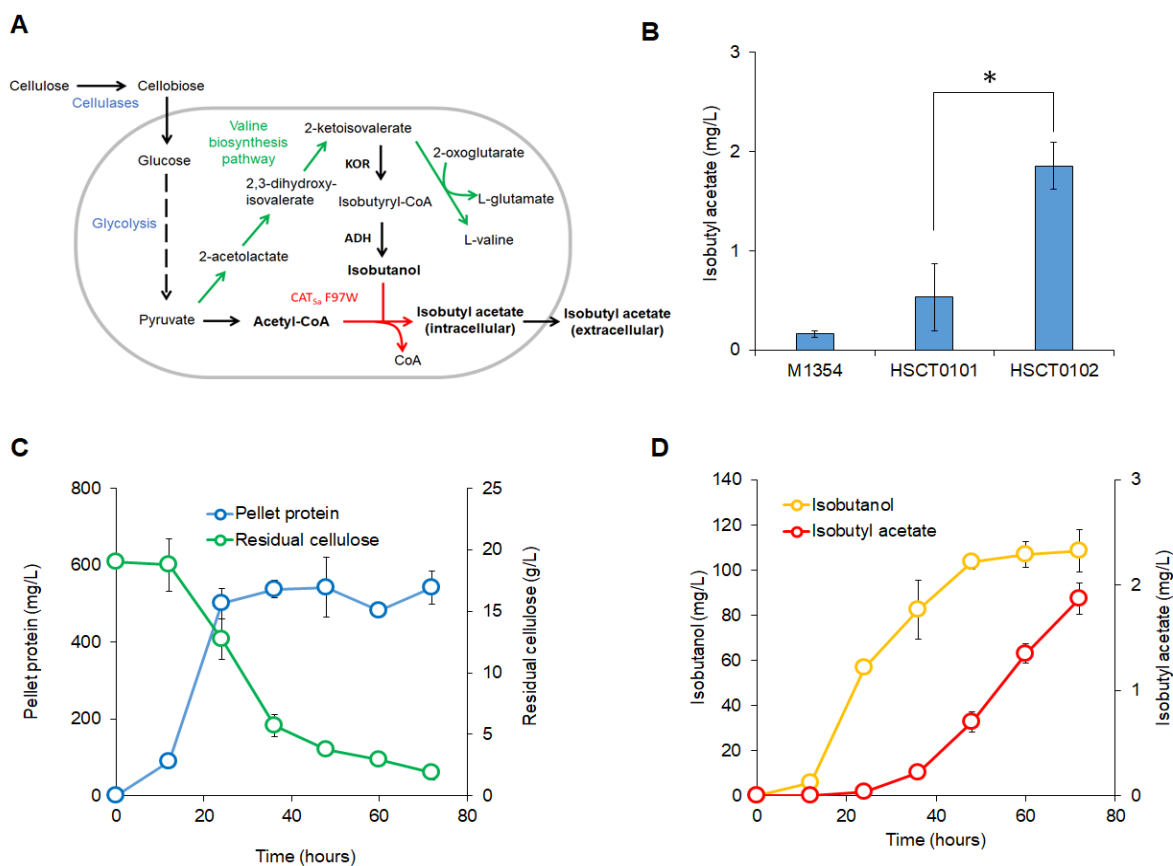


Figure 4-4: Isobutyl acetate production in the engineered *C. thermocellum*. **(A)** A simplified isobutyl acetate production pathway from cellulose in *C. thermocellum*. **(B)** Biosynthesis of isobutyl acetate of the wildtype and engineered *C. thermocellum* strains at 55°C from MTC medium with 5 g/L cellobiose and external supply of 2 g/L isobutanol. Isobutyl acetate was measured after 24 hours from the hexadecane layer of cell cultures. Initial OD of each cell culture was in a range of 0.8–1.0. The error bars represent standard deviation of five biological replicates. Statistical analysis: t-test, “*” p value < 4×10^{-4} , $t = -6.475$, $df = 7$. **(C)** Kinetic profiles of cell growth and residual cellulose of HSCT0102. HSCT0102 was cultured in C-MTC medium with 20 g/L Avicel PH-101. The error bars represent standard deviation of three biological replicates. **(D)** Kinetic profiles of isobutanol and isobutyl acetate production by HSCT0102 in C-MTC medium with 20 g/L Avicel PH-101. The error bars represent standard deviation of three biological replicates. Abbreviation: KOR, 2-ketoisovalerate ferredoxin oxidoreductase; ADH, alcohol dehydrogenase.

crease in isobutyl acetate production (Figure 4-4B). Interestingly, we also observed the parent *C. thermocellum* M1354 produced a trace amount of isobutyl acetate (< 0.1 mg/L) even though this strain does not harbor a CAT (Figure 4-9). This phenomenon was only observed when hexadecane overlay was used during fermentation for ester extraction. One possible explanation is the endogenous activity of esterases in *C. thermocellum* might have been responsible for low isobutyl acetate production while the organic phase overlay helps extract the target ester. It should be noted that the esterase reaction is reversible and more thermodynamically favorable for ester degradation than biosynthesis.

Finally, we tested whether HSCT0102 could endogenously produce isobutyl acetate directly from cellulose at elevated temperature (55°C). After 72 hours, cell mass, containing 550 mg/L of pellet protein, reached 1.04 g/L, and 17 g/L of cellulose were consumed (Figure 4-4C). About 103 mg/L of isobutanol were produced for the first 48 hours, and further increased up to 110 mg/L for additional 24 hours (Figure 4-4D). Besides isobutanol, *C. thermocellum* also produced other fermentative metabolites, including ethanol, formate, acetate, and lactate, as expected (Figure 4-10A and B). For the target isobutyl acetate production, HSCT0102 did not produce isobutyl acetate for the first 24 hours but started accumulating the target product for the next 48 hours. The observed profile of isobutyl acetate production could be attributed to the low substrate affinity of CAT_{Sa} F97W (Table 4-2). The final titer of isobutyl acetate reached 1.9 mg/L. Besides the production of the desirable ester isobutyl acetate, we also observed that HSCT0102 produced other detectable esters such as ethyl acetate, ethyl isobutyrate, and isobutyl isobutyrate (Figure 4-10A, C, and D). Endogenous biosynthesis of these esters could be explained from the complex redox and fermentative metabolism of *C. thermocellum* [54, 56]. *C. thermocellum* can endogenously synthesize the precursor metabolites such as acetyl-CoA and ethanol via the ethanol

Table 4-2: Kinetic parameters of the wildtype CAT_{Sa} and mutant CAT_{Sa} F97W. The reactions were performed at 50°C. The co-substrate, acetyl-CoA, was supplied at the saturated concentration of 2 mM. Melting temperature (T_m) of CAT_{Sa} and CAT_{Sa} F97W are 72.0 ± 0.8, and 68.3 ± 1.2°C, respectively.

	CAT _{Sa}		CAT _{Sa} F97W	
Substrates	Chloramphenicol	Isobutanol	Chloramphenicol	Isobutanol
K _M (mM)	0.28 ± 0.02	138.66 ± 28.92	0.18 ± 0.01	144.77 ± 23.65
k _{cat} (1/sec)	202.97 ± 3.36	0.30 ± 0.03	102.63 ± 2.04	0.59 ± 0.05
k _{cat} /K _M (1/M/sec)	7.37 ± 0.48 x 10 ⁵	2.16 ± 0.45	5.77 ± 0.49 x 10 ⁵	4.08 ± 0.62

biosynthesis pathway and isobutyryl-CoA and isobutanol via the valine biosynthesis (Figure 4-10A). With the availability of four precursor metabolites, *C. thermocellum* could produce ethyl acetate, ethyl isobutyrate, isobutyl acetate, and isobutyl isobutyrate as observed experimentally (Figure 4-10A, C, and D).

Taken altogether, *C. thermocellum* overexpressing CAT_{Sa} successfully produced the target isobutyl acetate from cellulose at elevated temperature (55°C). However, the low titer and conversion rate require optimization to improve isobutyl acetate production in future studies. One of the key metabolic engineering targets is to enhance enzymatic efficiency of CAT_{Sa}. In contrast to *S. cerevisiae*-derived ATF1 that has high specificity towards isobutanol [6] and can be expressed in *E. coli* to achieve a high titer of 17.5 g/L isobutyl acetate and 80% theoretical maximum product yield [7], CAT_{Sa} F97W exhibits a relatively low affinity towards isobutanol. The rationale for utilizing CAT_{Sa} instead of ATF1 is that CAT_{Sa} is thermostable, and this study is the first ever to report its function for ester production at elevated temperatures. Tuning gene expression in *C. thermocellum* is another challenge that needs to be addressed for enhanced ester production.

Since CAT_{Sa} F97W still retains the activity towards chloramphenicol, adaptive evolution strategies such as chemically induced chromosomal evolution (CIChE) can offer a promising strategy to improve the gene expression level [57]. Finally, model-guided optimization at system levels should be implemented for the most effective conversion of cellulose into isobutyl acetate to achieve high production of isobutyl esters and other class of esters [58-60].

4.5 Conclusions

This study demonstrated that a CAT can function and/or be re-purposed as an AAT for novel biosynthesis of designer esters at elevated temperatures. Both *in silico* and *in vivo* characterization

discovered a broad alcohol substrate range of the thermostable chloramphenicol acetyltransferase from *Staphylococcus aureus* (CAT_{Sa}). Discovery of the F97W mutation of CAT_{Sa} by model-guided protein engineering enhanced isobutyl acetate production. This study presented the first report on the consolidated bioprocessing of cellulose into ester(s) by the thermophilic CBP organism *C. thermocellum* harboring an engineered thermostable CAT_{Sa} F97W. Overall, this research helps establish a foundation for engineering non-model organisms for direct conversion of lignocellulosic biomass into designer bioesters.

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4.7 Appendix

Table 4-3: A list of primers used in this study. The bold and underlined letters indicate restriction and site-directed mutation sites, respectively.

Primers	Primer sequence (5' to 3')
<i>Site-saturation mutagenesis of CAT_{sa} F97</i>	
CAT _{sa} _F BamHI	CTCTGGATCCAATGAAC TTTA ATAAAAATTGATTTAG
CAT _{sa} _R SacI	CTCTGAGCTCTTATAAAAAGCCAGTCATTAGGCCTA
F97R_F	TGATGGTGTATCTAAAACAC CGT TCTGGTATTTGGACTC
F97R_R	GTCCAAATACCAGAA ACG TGTTTTAGATACAC
F97K_F	TGATGGTGTATCTAAAACAAA AA TCTGGTATTTGGACTC
F97K_R	GTCCAAATACCAGAT TTTT GTTTTAGATACAC
F97L_F	TGATGGTGTATCTAAAACAC CTG TCTGGTATTTGGACTC
F97L_R	GTCCAAATACCAGAC AG TGTTTTAGATACAC
F97W_F	TGATGGTGTATCTAAAACAT GG TCTGGTATTTGGACTC
F97W_R	GTCCAAATACCAGAC CA TGTTTTAGATACAC
F97I_F	TGATGGTGTATCTAAAACA ATT TCTGGTATTTGGACTC
F97I_R	GTCCAAATACCAGAA ATT TGTTTTAGATACAC
F97G_F	TGATGGTGTATCTAAAACAG GT TCTGGTATTTGGACTC
F97G_R	GTCCAAATACCAGAA AC TGTTTTAGATACAC
CAT _{sa} _F	TGTTTAACTTTAAGAAGGAGATATACCAT
BB_CAT _{sa} _R	TGGATCCTGGCTGTGG
F97A_R	CATTCTTTACAGGAGTCCAAATACCAGAG GG TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97A_F	GG CTCTGGTATTTGGACTCCTGTA
F97N_R	CATTCTTTACAGGAGTCCAAATACCAGAA ATT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97N_F	AA TTCTGGTATTTGGACTCCTGTA
F97D_R	CATTCTTTACAGGAGTCCAAATACCAGAG GT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97D_F	GA CTCTGGTATTTGGACTCCTGTA
F97C_R	CATTCTTTACAGGAGTCCAAATACCAGAA ACA TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97C_F	TG TTCTGGTATTTGGACTCCTGTA
F97Q_R	CATTCTTTACAGGAGTCCAAATACCAGAT TG TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97Q_F	CA ATCTGGTATTTGGACTCCTGTA
F97E_R	CATTCTTTACAGGAGTCCAAATACCAGAC CT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97E_F	GA GTCTGGTATTTGGACTCCTGTA
F97H_R	CATTCTTTACAGGAGTCCAAATACCAGAG GT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97H_F	CA CTCTGGTATTTGGACTCCTGTA
F97M_R	CATTCTTTACAGGAGTCCAAATACCAGAC ATT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97M_F	AT GTCTGGTATTTGGACTCCTGTA
F97P_R	CATTCTTTACAGGAGTCCAAATACCAGAT GG TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97P_F	CA ATCTGGTATTTGGACTCCTGTA
F97S_R	CATTCTTTACAGGAGTCCAAATACCAGAG CT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97S_F	AG CTCTGGTATTTGGACTCCTGTA
F97T_R	CATTCTTTACAGGAGTCCAAATACCAGAC CG TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97T_F	AC GTCTGGTATTTGGACTCCTGTA
F97Y_R	CATTCTTTACAGGAGTCCAAATACCAGAG ACT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97Y_F	GT CTCTGGTATTTGGACTCCTGTA
F97V_R	CATTCTTTACAGGAGTCCAAATACCAGAG TAT TGTTTTAGATACACCATCAAAAATTGTAT
BB_F97V_F	TAC TCTGGTATTTGGACTCCTGTA

Table 4-3: Continued

Primers	Primer sequence (5' to 3')
<i>Clostridium thermocellum</i> engineering	
<i>pHS005</i> construction (<i>C. thermocellum</i> markless gene deletion plasmid)	
pNW33N backbone F	GAGGGGTTTTTGC CAATCCCGTTTGTGAACTAC
pNW33N backbone R	CAGGAAACAGCTATGA CAGGAAACAGCTATGACCATGA
PgapDH F	TCATAGCTGTTTCCTGTAATTACTGTATCTCTCTGGC
PgapDH R	AATTTTATTAAAGTTCATTAATATCGCCTCCTATTGTAA
CAT _{Sa} F	AGGAGGCGATATTAATGAACCTTAATAAAATTGATTTAGACAATTGG
CAT _{Sa} R	ATCATGACGTCGACCTCCTTTATTATAAAAGCCAGTCATTAGGCCTATC
hpt F	AAAGGAGGTCGACGTCATGATAAATCAAATTAAGAAATTTTGG
hpt R	CGGCCGTGTACAATAGCAAAACACTATCTCTCATAC
2927 terminator F	GACAAAGAAGATATGGACTAAAAAATATACAAAGGTTTCTTG
2927 terminator R	GATTATGCGGCCGTGTACAATAGCAAAACACTATCTCTCATACA
MCS2 F	ATTGTACACGGCCGCATAATC
MCS2 R	CAAACGGGATTGGCAAAAACCCCTCAAGACC
bb tdk F	TTTCCCGTTCTCTCTGATTGTGA
bb tdk R	GCAACTATGGATGAACGAAATAGA
tdk F	ATTCGTTTCATCCATAGTTGCAAAAATCCGCTTAAGTCCGCG
tdk R	CAATCAGAGAGAACGGGAAAGTTCCGTATAAAATTAACCGTATG
<i>pHS0024</i> construction (<i>pHS005</i> without <i>hpt</i> gene)	
-hpt F	AAAATATACAAAGGTTTCTTG TG TTTTAATACCGTTATGTTAATATAATG
-hpt R	CACAAGAAACCTTTGTATATTTT TTATAAAAGCCAGTCATTAGGC

Table 4-4: K_M values of CAT_{Sa} and CAT_{Sa} F97W towards acetyl-CoA.

	CAT _{Sa}		CAT _{Sa} F97W	
Co-substrates	Chloramphenicol	Isobutanol	Chloramphenicol	Isobutanol
K_M (mM)	0.08 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	0.08 ± 0.02

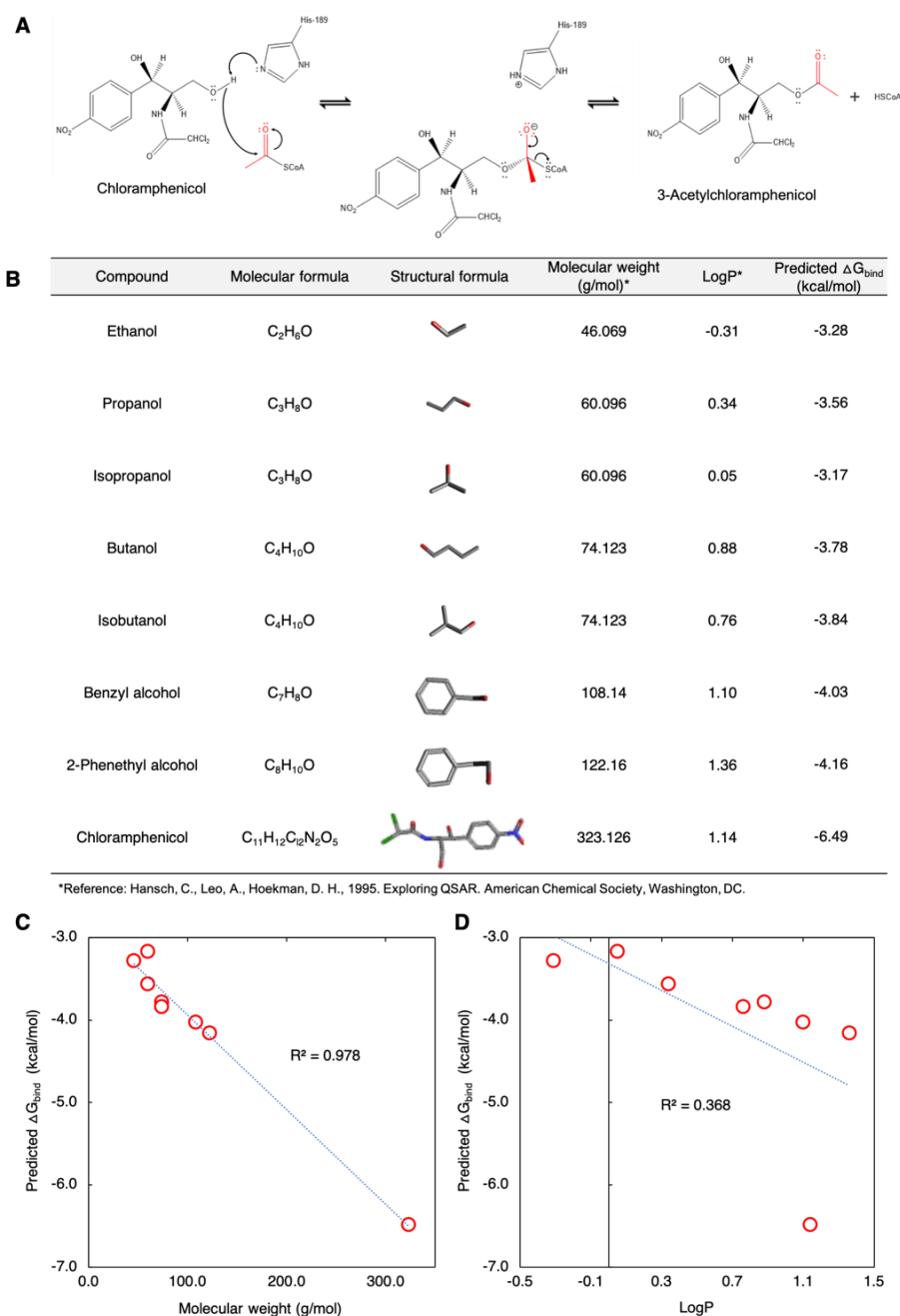


Figure 4-5: (A) Reaction mechanisms of acetylation of chloramphenicol by CAT_{sa}. Briefly, the acetylation of chloramphenicol involves three steps. First, the 3-hydroxyl group of the chloramphenicol is deprotonated by the imidazole ring of histidine of the CAT's active site, generating the activated oxygen. Then, the nucleophilic attack by the oxyanion at the thioester carbonyl carbon of acetyl-CoA generates a tetrahedral intermediate. Finally, loss of the free CoA yields the 3-acetylchloramphenicol. (B) Physical properties of various alcohols used in this study and the predicted ΔG_{bind} from the docking simulation. (C) Correlation between the predicted ΔG_{bind} and the molecular weights of alcohols. (D) Correlation between the predicted ΔG_{bind} and the LogP values of alcohols.

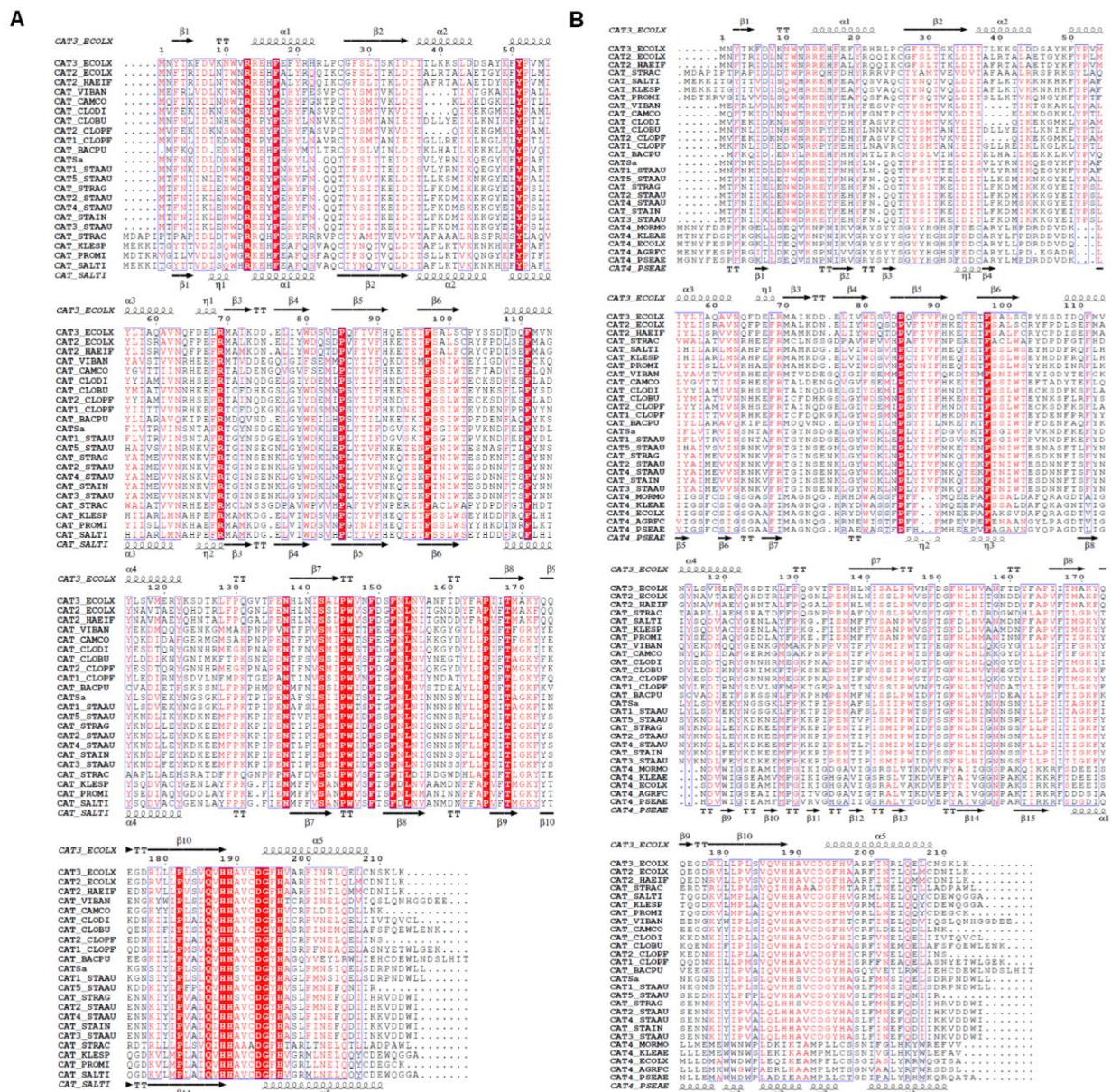


Figure 4-6: Multiple sequence alignment of CATs. (A) Sequence alignment of 22 CATs classified as Type A. (B) Sequence alignment of 27 CATs including Type A and Type B. The highly conserved regions are red highlighted.

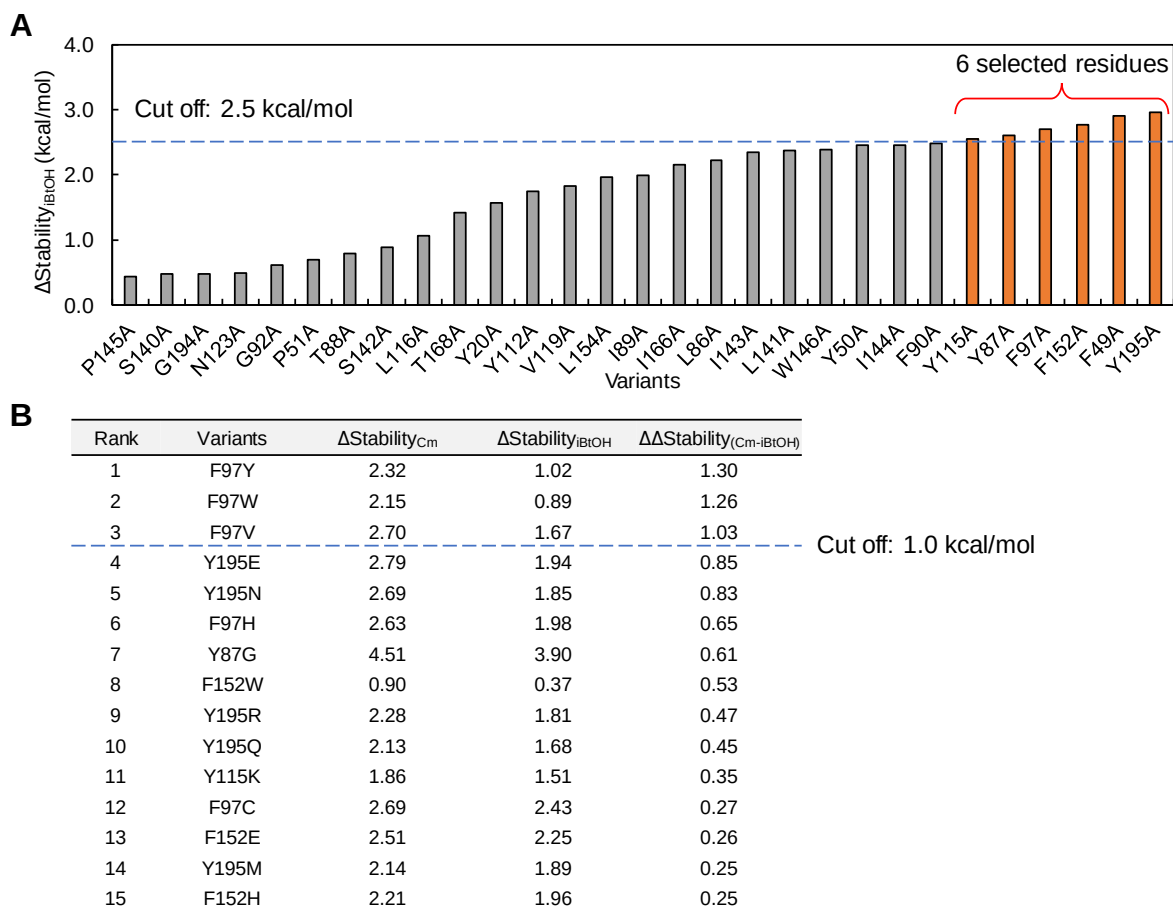


Figure 4-7: (A) Alanine scan of 23 identified residues in the binding pocket of the CAT_{Sa}-isobutanol-acetyl-CoA complex. $\Delta\text{Stability}$ (kcal/mol) represents the relative stability of each variant with respect to its wild type. Positive $\Delta\text{Stability}$ values indicate that the structural stability of the protein-ligands complex is reduced if a specific residue is replaced with alanine, suggesting the significance of that specific residue in the protein-ligands complex. **(B)** Representative residue scan from a selection of six selected residues identified from alanine scan in the binding pocket of the CAT_{Sa}-isobutanol-acetyl-CoA or CAT_{Sa}-chloramphenicol-acetyl-CoA complex. To identify the significant residues in the isobutanol binding exclusively, the $\Delta\Delta\text{Stability}_{(\text{Cm-iBuOH})}$ was calculated by subtracting $\Delta\text{Stability}_{\text{iBuOH}}$ from $\Delta\text{Stability}_{\text{Cm}}$.

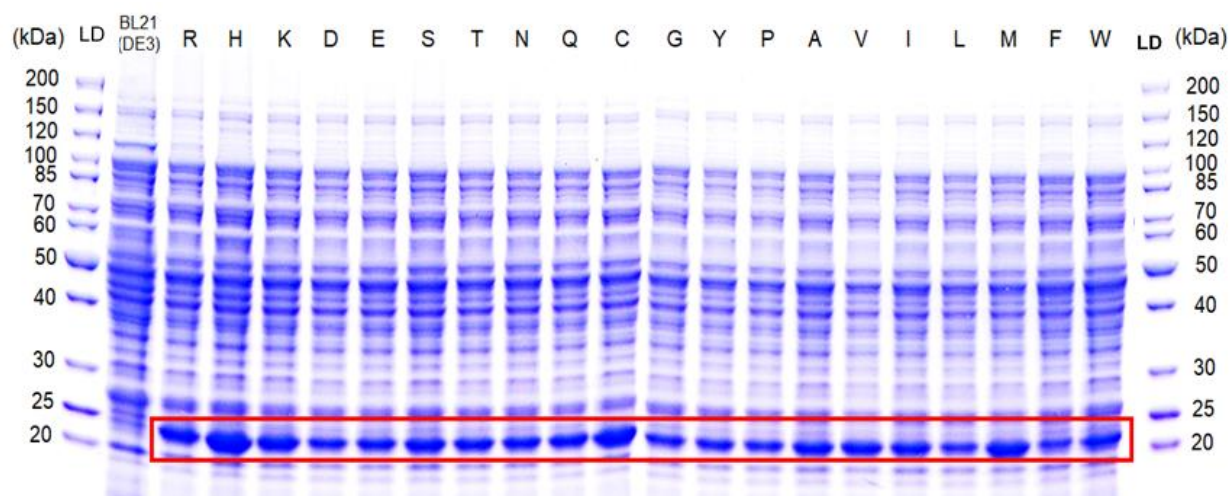


Figure 4-8: SDS-PAGE analysis of *E. coli* crude extracts expressing CAT_{sa} F97 variants. The overexpressed CAT_{sa} F97 variants are shown in the red box. The alphabets annotate the amino acid variants. LD; protein ladder.

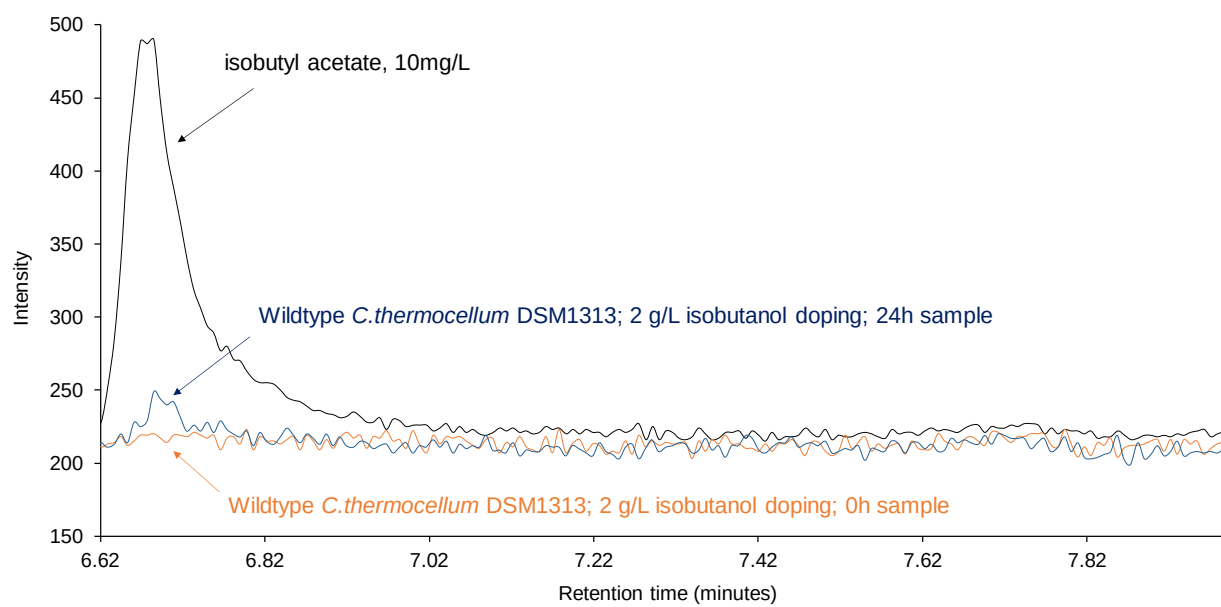


Figure 4-9: An overlaid GC/MS chromatogram showing the trace amount of isobutyl acetate produced by the wildtype *C. thermocellum*.

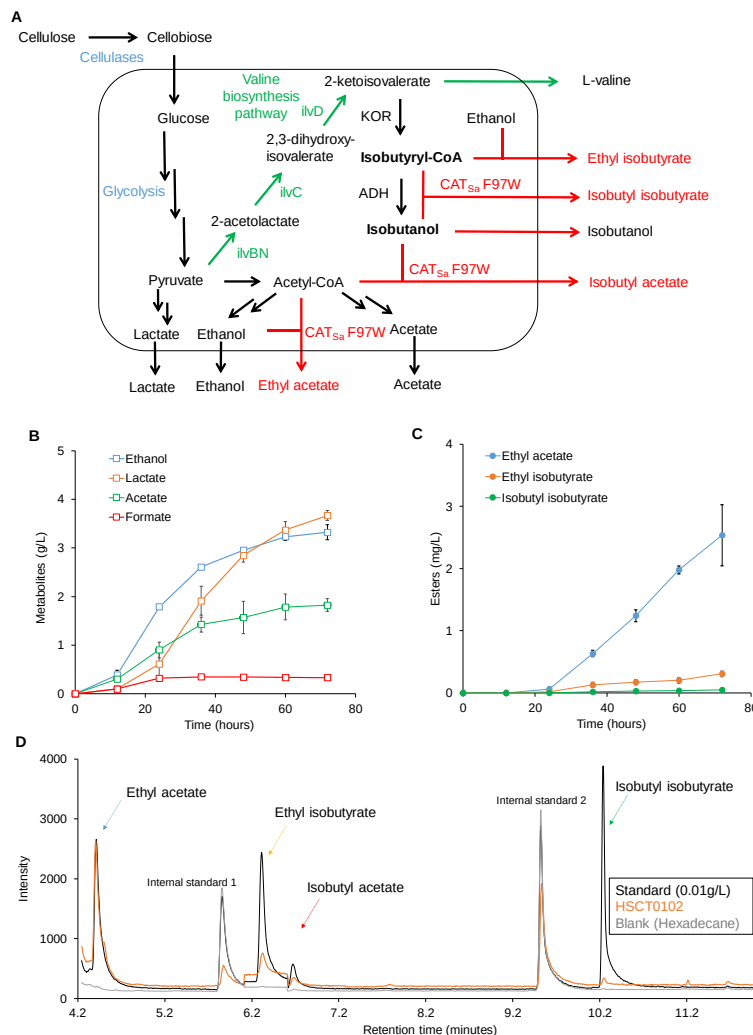


Figure 4-10: Fermentative products from cellulose fermentation of HSCT0102. **(A)** Simplified metabolic pathways of *C. thermocellum* for biosynthesis of native fermentative metabolites and heterologous esters. It should be noted that *C. thermocellum* has two unique routes to endogenously produce isobutanol from 2-ketoisovalerate. The first route, that was experimentally validated [1], uses the 2-ketoisovalerate ferredoxin oxidoreductase (KOR) and alcohol dehydrogenase (ADH) converts 2-ketoisovalerate into isobutyryl-CoA and then isobutanol. The second route, that has not yet been experimentally validated, uses keto-isovalerate decarboxylase (KIVD) and ADH to convert 2-ketoisovalerate to isobutyraldehyde and then isobutanol. Heterologous, thermostable KIVD was engineered in *C. thermocellum* [1]; however, the endogenous acetolactate synthase (*ilvBN*), that catalyzes the first step in valine biosynthesis and might have KIVD activity as observed in *E. coli* [2, 3], has not yet been experimentally confirmed in *C. thermocellum*. **(B)** Kinetic profile of biosynthesis of other esters besides isobutyl acetate, including ethyl acetate, ethyl isobutyrate, and isobutyl butyrate. **(C)** An overlaid GC/MS chromatogram demonstrating ester biosynthesis. **(D)** Kinetic profile of other fermentative metabolites. Abbreviation: IS, internal standard.

Supplementary References

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5 Establishment of a High-Throughput Screening Platform for Rapid Probing Specificities of Alcohol Acyltransferases

5.1 Abstract

Alcohol acyltransferases (AATs) enable microbial biosynthesis of a large space of esters by condensing an alcohol and an acyl-CoA. However, the substrate promiscuity of AATs prevents selectivity microbial biosynthesis of designer esters. Here, we developed a high-throughput microbial screening platform that facilitates rapid identification of efficient AATs for designer ester biosynthesis. First, we established a microplate-based culturing technique with *in situ* fermentation and extraction of esters. We next validated its capability in rapid profiling of the alcohol substrate specificity of 20 variants of CAT_{Sa}, a chloramphenicol acetyltransferase from *Staphylococcus aureus*, for microbial biosynthesis of various acetate esters with various exogenous alcohol supply. Then, by coupling the microplate-based culturing technique with an established colorimetric assay, we developed a high-throughput microbial screening platform for AATs. Finally, we demonstrated that this platform could identify the CAT_{Sa} F97W with enhanced isobutyl acetate (IBA) synthesis as well as reveal three beneficial mutations (P348M, P348A, and P348S) in ATF1_{Sc}, an AAT from *Saccharomyces cerevisiae*, for enhanced butyl acetate production from the engineered variants via computer-guided protein design. In addition to screening of AAT/CAT by direct ester measurement, the platform can also be used to evaluate the effect of AAT/CAT variants expression on cell growth in a high-throughput manner. We anticipate the high-throughput microbial screening platform can be a useful tool to identify novel AATs that have important roles in nature and industrial biocatalysis for designer bioester production.

5.2 Introduction

Esters are an important class of industrial chemicals with broad applications as flavors, fragrances, cosmetics, pharmaceuticals, green solvents, and advanced biofuels [1]. Currently,

esters are mainly produced by chemical synthesis from petroleum-based feedstocks that are neither renewable nor sustainable [2]. Alternatively, microbial conversion has emerged as an alternative route for renewable and sustainable production of esters [3-8]. Critical to the microbial biosynthesis of esters is the requirement of alcohol acyltransferases (AATs, EC 2.3.1.84) that catalyze a thermodynamically-favorable condensation of an alcohol and acyl-CoA in an aqueous environment. Because esters are commonly found in nature such as fruits (e.g., apple [9-11], apricot [12], banana [13], melon [14, 15], papaya [16], and strawberry [13, 17-19]) or yeast fermentation [8, 20], various eukaryotic AATs have been identified and recently exploited for microbial biosynthesis of esters using synthetic biology and metabolic engineering approaches [4, 21-23]. Recent discovery and repurposing of prokaryotic chloramphenicol acetyltransferases (CATs, EC 2.3.1.28) to function as AATs have further expanded the library of ester-producing enzymes [2, 4]. However, due to the substrate promiscuity of these AAT/CAT enzymes, controllable microbial synthesis of designer esters with high selectivity remains a significant challenge [5, 7].

Bioprospecting and protein engineering are promising strategies to find novel AATs with high specificity and activity toward a desired ester [2, 24]. For instance, AAT_{Ac}, an AAT from *Actinidia chinensis*, was engineered to enhance butyl octanoate production and the AAT_{Ac} S99G variant achieved about 4.5-fold improved butyl octanoate production in *Escherichia coli* [25]. Similarly, CAT_{Sa}, a CAT from *Staphylococcus aureus*, was engineered to improve isobutyl acetate (IBA) production and the CAT_{Sa} F97W variant achieved ~3.5-fold improved IBA production in a thermophilic, cellulolytic bacterium *Clostridium thermocellum* via computer-guided rational protein engineering [2]. Further, by combining both bioprospecting and model-guided protein engineering strategies, novel CATs have recently been discovered with improved efficiency, robustness, and

compatibility [24]. Even though engineering AATs/CATs for higher specificity and activities is a promising approach, a large space of novel AATs/CATs is still underexplored.

For screening of a large space of AATs/CATs in a high-throughput manner, *in vitro* assay methods such as i) the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) assay [2, 8, 26] and ii) the α -KGDH (α -ketoglutarate dehydrogenase)-coupled assay [27, 28] can be used. These assays are designed to quantify free CoAs, released from the esterification reaction of AAT/CAT by measuring either the 412 nm absorbance of yellowish TNB (5-thio-2-nitrobenzoic acid) for the DTNB assay or the 340 nm absorbance of NADH (nicotinamide adenine dinucleotide) for the α -KGDH-coupled assay. However, these *in vitro* assays require expensive acyl-CoA reagents and enzyme purification steps.

Alternatively, direct measurement of esters for rapid, high-throughput screening of AAT/CAT specificities and activities *in vivo* can be attractive before determining the catalytic efficiencies in depth for promising enzyme candidates. Here, esters produced by microbes can be extracted with a solvent (e.g., hexane or hexadecane) and then quantified. While the conventional gas chromatography coupled with mass spectrometer (GC/MS) has been used for this approach, it is low-throughput and expensive. For high-throughput screening, the colorimetric assay, based on the hydroxylamine/iron chemistry, can be used. This assay is designed to quantify esters by measuring the absorbance of a colored ferric hydroxamate complex generated via two chemical reactions at 520 nm [29-32].

In this study, we aimed to develop a high-throughput microbial screening platform to identify novel AATs/CATs for biosynthesis of designer esters in a simple, rapid, and efficient manner. We started by establishing a microplate-based culturing technique with *in situ* fermentation and extraction of esters. Then, by coupling the microplate-based culturing technique

with a modified colorimetric assay, we developed a high-throughput microbial screening platform to identify novel AATs. The platform could measure both ester titer and cell growth, which enables rapid profiling of relative specificities and activities of AATs/CATs as well as evaluation of the effect of AATs/CATs expression on microbial health. We next validated the developed high-throughput screening platform by probing the alcohol substrate preference of 20 variants of F97 residue in CAT_{Sa} and identifying ATF1_{Sc} variants for enhanced butyl acetate production from a library of ATF1_{Sc}, generated by computer-guided protein design,. We envision that the developed high-throughput microbial ester synthesis screening platform is a useful tool to identify novel AATs that have important roles in nature and industrial biocatalysis for designer bioester production.

5.2.1 Strains and plasmids

Strains and plasmids used in this study are listed in Table 5-1. Briefly, for molecular cloning, *E. coli* TOP10 was used. For ester production, BL21 (DE3) and EcDL002 [5] were used as a host strain. The pETDuet-1 plasmids containing 20 F97 variants of CAT_{Sa} were used to examine the role of the F97 residue on the alcohol substrate preference in CAT_{Sa} [2]. The pATF1_{Sc} was constructed by subcloning *ATF1* gene in pDL004 [3] into pET29 by Gibson Assembly method [33]. The variants of ATF1_{Sc} were generated by site-directed mutagenesis using the QuickChange method. All the constructed plasmids were introduced into the host strains using chemical transformation. The primers used in this study are listed in Table 5-2. The alphabets annotate the amino acid variants. R; arginine, H; histidine, K; lysine, D; aspartic acid, E; glutamic acid, S; serine, T; threonine, N; asparagine, Q; glutamine, C; cysteine, G; glycine, Y; tyrosine, P; proline, A; alanine, V; valine, I; isoleucine, L; leucine, M; methionine, F; phenylalanine, W; tryptophan.

Table 5-1: A list of plasmids and strains used in this study.

Name	Description	Source
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi80lacZ\Delta M15$ $\Delta lacX74$ <i>recA1</i> <i>araD139</i> $\Delta(ara-leu)7697$ <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
BL21 (DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Invitrogen
EcDL002	TCS083 $\Delta fadE$ (DE3)	[5]
Plasmids		
pCAT _{Sa}	pETDuet-1 carrying CAT _{Sa} ; Amp ^R	[2]
pCAT _{Sa} F97R	pETDuet-1 carrying CAT _{Sa} F97R; Amp ^R	[2]
pCAT _{Sa} F97H	pETDuet-1 carrying CAT _{Sa} F97H; Amp ^R	[2]
pCAT _{Sa} F97K	pETDuet-1 carrying CAT _{Sa} F97K; Amp ^R	[2]
pCAT _{Sa} F97D	pETDuet-1 carrying CAT _{Sa} F97D; Amp ^R	[2]
pCAT _{Sa} F97E	pETDuet-1 carrying CAT _{Sa} F97E; Amp ^R	[2]
pCAT _{Sa} F97S	pETDuet-1 carrying CAT _{Sa} F97S; Amp ^R	[2]
pCAT _{Sa} F97T	pETDuet-1 carrying CAT _{Sa} F97T; Amp ^R	[2]
pCAT _{Sa} F97N	pETDuet-1 carrying CAT _{Sa} F97N; Amp ^R	[2]
pCAT _{Sa} F97Q	pETDuet-1 carrying CAT _{Sa} F97Q; Amp ^R	[2]
pCAT _{Sa} F97C	pETDuet-1 carrying CAT _{Sa} F97C; Amp ^R	[2]
pCAT _{Sa} F97G	pETDuet-1 carrying CAT _{Sa} F97G; Amp ^R	[2]
pCAT _{Sa} F97Y	pETDuet-1 carrying CAT _{Sa} F97Y; Amp ^R	[2]
pCAT _{Sa} F97P	pETDuet-1 carrying CAT _{Sa} F97P; Amp ^R	[2]
pCAT _{Sa} F97A	pETDuet-1 carrying CAT _{Sa} F97A; Amp ^R	[2]
pCAT _{Sa} F97V	pETDuet-1 carrying CAT _{Sa} F97V; Amp ^R	[2]
pCAT _{Sa} F97I	pETDuet-1 carrying CAT _{Sa} F97I; Amp ^R	[2]
pCAT _{Sa} F97L	pETDuet-1 carrying CAT _{Sa} F97L; Amp ^R	[2]
pCAT _{Sa} F97M	pETDuet-1 carrying CAT _{Sa} F97M; Amp ^R	[2]
pCAT _{Sa} F97W	pETDuet-1 carrying CAT _{Sa} F97W; Amp ^R	[2]
pDL004	pETite carrying ATF1 _{Sc} ; Kan ^R	[3]
pATF1 _{Sc}	pET29 carrying ATF1 _{Sc} ; Kan ^R	This study
pATF1 _{Sc} P348W	pET29 carrying ATF1 _{Sc} P348W; Kan ^R	This study
pATF1 _{Sc} P348R	pET29 carrying ATF1 _{Sc} P348R; Kan ^R	This study
pATF1 _{Sc} P348M	pET29 carrying ATF1 _{Sc} P348M; Kan ^R	This study
pATF1 _{Sc} P348H	pET29 carrying ATF1 _{Sc} P348H; Kan ^R	This study
pATF1 _{Sc} P348K	pET29 carrying ATF1 _{Sc} P348K; Kan ^R	This study
pATF1 _{Sc} P348N	pET29 carrying ATF1 _{Sc} P348N; Kan ^R	This study
pATF1 _{Sc} P348I	pET29 carrying ATF1 _{Sc} P348I; Kan ^R	This study
pATF1 _{Sc} P348S	pET29 carrying ATF1 _{Sc} P348S; Kan ^R	This study
pATF1 _{Sc} P348D	pET29 carrying ATF1 _{Sc} P348D; Kan ^R	This study
pATF1 _{Sc} P348C	pET29 carrying ATF1 _{Sc} P348C; Kan ^R	This study
pATF1 _{Sc} P348A	pET29 carrying ATF1 _{Sc} P348A; Kan ^R	This study
pATF1 _{Sc} P348Q	pET29 carrying ATF1 _{Sc} P348Q; Kan ^R	This study

5.2.2 Culture media

For molecular cloning, seed cultures, and protein expression analysis, the Luria-Bertani (LB) medium, comprising of 10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl, was used. For characterization of CAT_{Sa} F97 and ATF1_{Sc} variants, the M9 hybrid medium [5] with 20 g/L glucose was used. 50 µg/mL kanamycin (kan), or 50 µg/mL ampicillin (amp) was used where applicable.

5.2.3 Characterization of CAT_{Sa} F97 variants with external supply of isobutanol

For pre-cultures, 1% (v/v) of stock cells were grown overnight in 5 mL of LB at 37°C and 200 rpm on a 75° angled platform in a New Brunswick Excella E25 (Eppendorf, CT, USA). Next, 5 % (v/v) of pre-cultures were inoculated into 100 µL of the M9 hybrid medium containing 20 g/L of glucose, 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), and 2 g/L of isobutanol in 96-well microplates with or without hexadecane overlays in a 1:1 (v/v) ratio. Then, the microplates were sealed with a plastic adhesive sealing film, SealPlate® (EXCEL Scientific, Inc., CA, USA) to avoid cross contaminations and evaporation the culture medium. Finally, the microplates were incubated at 37°C and 400 rpm for 24 hour (h) in an incubating microplate shaker (Fisher Scientific, PA, USA).

5.2.4 Characterization of CAT_{Sa} F97 variants with external supply of various alcohols

For pre-cultures, single colonies from LB agar plates were inoculated into 100 µL of LB in 96-well microplates using sterile pipette tips. Specifically, the picked single colony by a sterile pipette tip was subsequently mixed with the media in the target well. Then, the pre-cultures were grown overnight at 37°C and 400 rpm in an incubating microplate shaker. For main cultures, 5 % (v/v) of pre-cultures were inoculated into 100 µL of the M9 hybrid media containing 20 g/L of glucose, 0.1 mM of IPTG, and 2 g/L of various alcohols including ethanol, butanol, isobutanol, or

2-phenylethyl alcohol in 96-well microplates with or without hexadecane overlay in a 1:1 (v/v) ratio. Then, the microplates were sealed with a plastic adhesive sealing film and incubated at 37°C and 400 rpm for 24 h in an incubating microplate shaker.

5.2.5 Characterization of ATF1_{Sc} variants with external supply of butanol

The pre-cultures were prepared as described in characterization of CAT_{Sa}F97 variants with external supply of various alcohols. For main cultures, 5 % (v/v) of pre-cultures were inoculated into 120 µL of the M9 hybrid media containing 20 g/L of glucose, 0.1 mM of IPTG, and 2 g/L of butanol in 96-well microplates with hexadecane overlay in a 1:1 (v/v) ratio. Finally, the microplates were sealed with a plastic adhesive sealing film and incubated at 37°C and 400 rpm for 24 h in an incubating microplate shaker.

5.2.6 Molecular modeling and docking simulations

For the computer-guided rational protein engineering of ATF1_{Sc}, first, the 3D structures of ATF1_{Sc}, acetyl-CoA, and butanol were generated as previously described [2]. Then, to perform docking simulations in MOE (Molecular Operating Environment software), the potential binding pocket was identified using the ‘Site Finder’ tool of MOE. After identifying the potential binding pockets, the best hit which includes H191 and D195, the conserved catalytic residues in AATs [34], was chosen. Next, to obtain the acetyl-CoA-butanol-ATF1 complex, the docking simulations were performed as previously described [35]. Briefly, acetyl-CoA and butanol were docked to the binding pocket of ATF1_{Sc}, sequentially. After the docking simulations, the best-scored binding poses were selected.

5.2.7 *In silico* mutagenesis analysis

In silico mutagenesis analysis was carried out as previously described [35]. Specifically,

the ‘residue scan’ tool of MOE was used to identify beneficial mutations with the selected residues. The Δ affinity values (kcal/mol) represent the relative binding affinity of the mutant to the wild type. In short, a more negative value indicates a mutant with better affinity.

5.2.8 Cell growth

The optical density (OD) was measured at 600 nm using a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., VT, USA). The dry cell weight (DCW) of BL21 strains was obtained by multiplication of the optical density of culture broth with a pre-determined conversion factor, 0.385 g/L/OD.

5.2.9 Gas chromatography coupled with mass spectroscopy (GC/MS)

For GC/MS analysis of esters, the microplates were centrifuged at 4,800 x g for 5 min and the hexadecane overlays were used for quantification of esters. The samples were prepared by diluting hexadecane extracts from the cultures with hexadecane containing internal standard (isoamyl alcohol) in a 1:1 (v/v) ratio. Then, 1 μ L of samples were directly injected into a gas chromatograph (GC) HP 6890 equipped with the mass selective detector (MS) HP 5973. For the GC system, helium was used as the carrier gas at a flow rate of 0.5 mL/min and the analytes were separated on a Phenomenex ZB-5 capillary column (30 m x 0.25 mm x 0.25 μ m). The oven temperature was programmed with an initial temperature of 50°C with a 1°C/min ramp to 58°C. Next a 25°C/min ramp was deployed to 235°C and then a 50°C/min ramp was deployed to 300°C. Finally held a temperature of 300°C for 2 minutes to elute any residual non-desired analytes. The injection was performed using the splitless mode with an initial injector temperature of 280°C.

For the MS system, a selected ion monitoring (SIM) mode was deployed to detect analytes. The SIM parameters for detecting esters were as follows: i) for ethyl acetate, ions 45.00, and 61.00

detected from 4.15 to 5.70 min, ii) for isoamyl alcohol (internal standard), ions 45.00, and 88.00 detected from 5.70 to 6.60 min, iii) for isobutyl acetate, ions 61.00, and 101.00 detected from 6.60 to 7.75 min, iv) for butyl acetate, ions 61.00, and 116.00 detected from 7.75 to 13.70 min, and v) for 2-phenethyl acetate, ions 104.00, and 121.00 detected from 13.70 to 13.95 min.

5.2.10 Colorimetric assay for quantification of esters

For colorimetric assay of esters, the previously reported method [32] was slightly modified as described here. Specifically, first, hydroxylamine solutions and stock ferric iron(III) solutions were prepared as previously described [32]. Then, hydroxymates were produced by reacting 60 μ L of hexadecane overlay from the culture with 20 μ L of hydroxylamine stock solution for 10 minute (min). Subsequently, with an addition of 120 μ L of the ferric working solution (1/20-diluted stock ferric iron(III) solutions in ethanol), the reactants were incubated for 5 min, resulting in ferric hydroxamate. Finally, the absorbance of the produced ferric hydroxamate was measured at 520 nm (Ab_{520}) using a BioTek Synergy HT microplate reader. The chemical reactions were incubated in an incubating microplate shaker at room temperature. Hexadecane overlays, hydroxylamine solutions, and stock ferric iron(III) solutions were transferred or aliquoted using a F1-ClipTipTM multichannel pipette (Thermo Fisher Scientific, USA). Esters were quantified using the standard curve.

5.3 Results and Discussion

5.3.1 Establishing a microplate-based culturing technique with *in situ* fermentation and extraction of esters

To develop a high-throughput microbial screening platform (MiSP), we first examined whether the microplate-based culturing technique could be reliably used to monitor cell growth

and continuously extract esters for downstream quantification. We characterized the recombinant BL21 (DE3) strains harboring 20 CAT_{Sa} F97 variants [2] in 96-well plates for production of isobutyl acetate (IBA) with or without solvent (hexadecane) overlay for *in situ* ester extraction (Figure 5-1A). As a basis for comparison, we also performed the high cell density culturing method in shake tubes that was previously employed for AAT screening [2].

The characterization results show that IBA production in microplates followed the same trend of its production observed in high cell density cultures (Figure 5-1B). Strong positive linear correlations ($R^2 \geq 0.98$) in IBA production existed between the microplate-based and high cell density culturing methods (Figure 5-1C and D). The microplate-based culturing method could validate that the CAT_{Sa} F97W variant achieved the highest IBA production among the 20 characterized variants (Table 5-3). While these variants exhibited different activities toward IBA, their cell cultures exhibited similar growth (Figure 5-5)

While IBA production in microplates was almost the same with or without solvent overlays, we found several advantages of using solvent overlays for microbial biosynthesis of esters. First, the solvent overlay enables a more reliable measurement of growth kinetics in microplates by avoiding medium evaporation that caused water condensation (Figure 5-1E) and hence interfered with optical density measurement (Figure 5-5). Second, the solvent overlay simplifies the sample preparation step for quantification of esters and is compatible with a high-throughput workflow, where esters in the solvent layer can be extracted for downstream measurement by either a GC/MS method or a colorimetric assay [3-6, 32]. Third, the solvent overlay helps alleviate the product toxicity during fermentation [36] because esters are known to be inhibitory to microbial health [37].

Overall, the microplate-based culturing method with solvent overlays is reliable and suit-

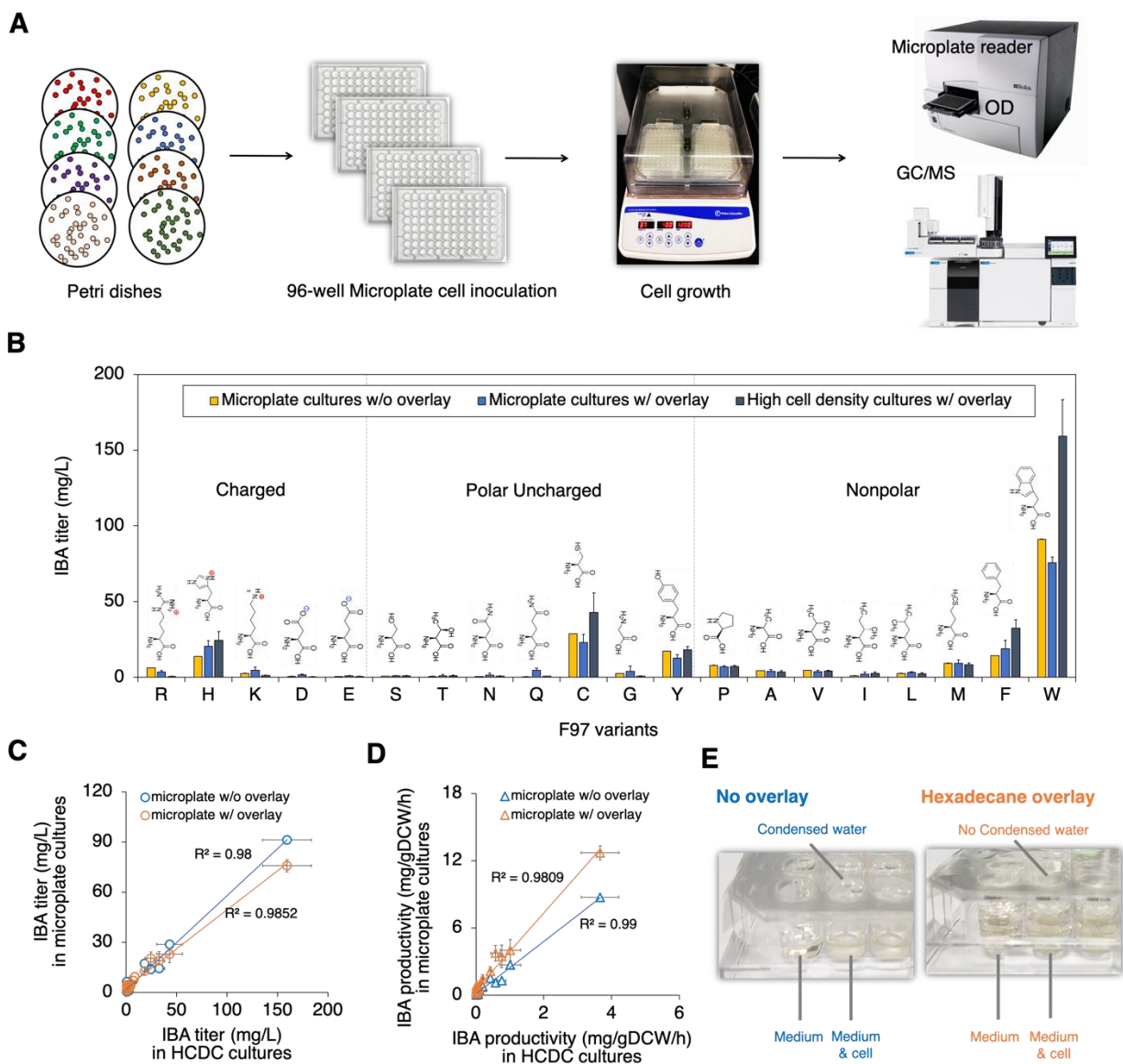


Figure 5-1: A microplate-based culturing method for microbial biosynthesis of esters. **(A)** Workflow of the microplate-based culturing method. **(B)** Comparison of IBA production after 24 hours among three different culturing methods by the recombinant BL21 (DE3) strains carrying CAT_{Sa} F97 variants. **(C-D)** Comparison of **(C)** IBA titer and **(D)** specific productivity measurements among three different culturing methods. The error bars represent standard deviation of four biological replicates ($n=4$). **(E)** Effect of solvent overlay on growth kinetics measurement in the microplate-based culturing method.

able for a rapid, high-throughput *in vivo* screening platform for microbial ester production.

5.3.2 Revealing the influential role of CAT_{Sa} F97 residue by profiling the alcohol

preference of a library of F97 variants with the microplate-based culturing method

Our previous study discovered that the CAT_{Sa} F97W mutant improved its catalytic efficiency towards isobutanol by ~2-fold [2]. We hypothesized that the F97 residue might have an important role in determining the alcohol substrate preference. Using the established microplate-based culturing method, we evaluated whether it could be used for rapid profiling of the alcohol substrate preference of CAT_{Sa} F97 variants. We characterized the recombinant *E. coli* strains carrying 20 CAT_{Sa} F97 variants with exogenous supplementation of alcohols in the media including linear, short-chain alcohols (ethanol, butanol), a branched-chain alcohol (isobutanol), and an aromatic alcohol (2-phenylethyl alcohol) in microplates with hexadecane overlay.

The characterization results showed the mutation in F97 residue changed the ester production profiles (Figure 5-2A, Table 5-4), suggesting that F97 residue plays an important role in determining the alcohol substrate preference of CAT_{Sa}. For IBA production, the F97W variant achieved the highest 6.39-fold improved IBA production as compared to its wildtype, which was consistent with the prior *in vitro* study showing that F97W variant has ~2-fold improved catalytic efficiency towards isobutanol [2]. Among the four target acetate esters investigated, F97W achieved the highest IBA production (91.2 mg/L) while F97C achieved the highest BA production (18.3 mg/L). This result indicates that F97C and F97W variants prefer butanol and isobutanol than the other characterized alcohols, respectively. Similarly, F97H achieved the highest 2-PEA production (182.3 mg/L), showing its alcohol substrate preference shift towards 2-phenethyl alcohol rather than the other alcohols. Remarkably, F97T showed significantly improved selective production of BA (12.4 mg/L), demonstrating the feasibility of production of designer esters using

re-programmed CATs. Figure 5-2B shows that the binding pockets of CATs are formed at the subunit interfaces [38] and the F97 residue located on the opposite subunit of the catalytic residues, H189 and D193. One possible explanation on how only one residue replacement changes the substrate preference of CAT_{Sa} is that the mutation in the F97 residue might dramatically change the size and/or shape of binding pocket by alternating the interactions among subunits of CAT_{Sa}. Taken altogether, the microplate-based culturing method coupled with GC/MS can be employed for rapid profiling of substrate preferences of AATs. The method successfully revealed the important role of the F97 residue in determining the alcohol substrate preference of CAT_{Sa}.

5.3.3 Developing a high-throughput microbial screening platform for ester biosynthesis by integration of the microplate-based culturing method and a colorimetric assay

High-throughput screening of ester biosynthesis has been limited by the use of GC/MS. Since the reactions of esters with hydroxylamine generate hydroxamic acids that form purple complexes with ferric ion, esters can be determined colorimetrically by measuring absorbance at 520 nm (Figure 5-3A) [29-32]. This colorimetric assay has recently been adapted for high-throughput screening of ethyl acetate (EA) production from C5, C6, and C12 carbon sources in *Kluyveromyces marxianus* [32] where cell culture samples were first collected followed by ester extraction with hexane. This protocol is useful but might not be compatible with the microplate-based culturing method in our study because hexane is toxic and hence cannot be used for *in situ* fermentation and extraction, unlike hexadecane. Here, we tested whether the colorimetric assay can be modified and coupled with the microplate-based culturing method to facilitate a high-throughput microbial screening of AATs for ester biosynthesis.

To evaluate whether this colorimetric assay can be modified and coupled with the microplate-based culturing method, we started to test the compatibility of hexadecane with the

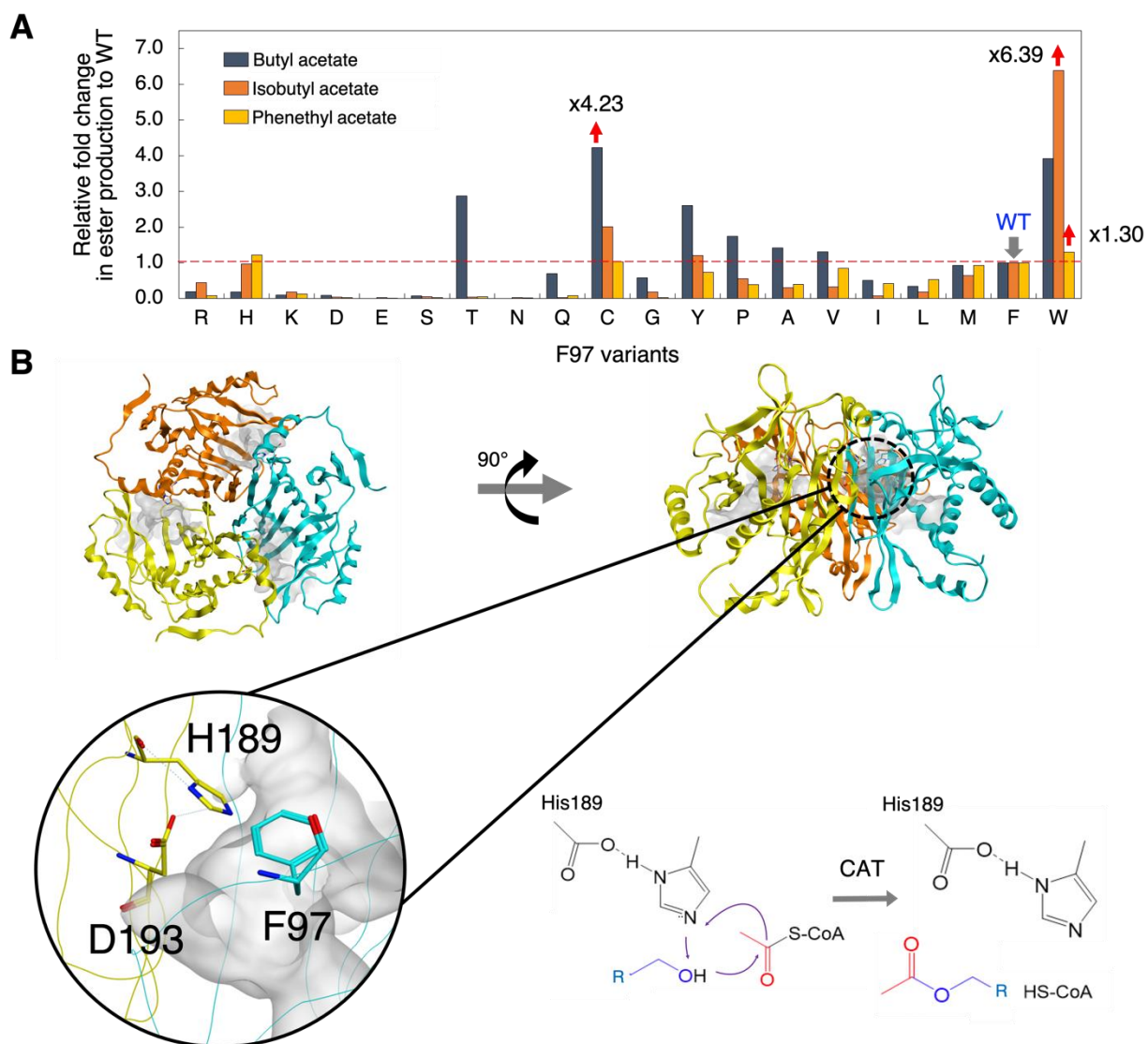


Figure 5-2: Rapid profiling of the alcohol substrate preference of CAT_{Sa} F97 variants. **(A)** Relative fold change in ester production to wild type. **(B)** The 3D structure of homology model of CAT_{Sa} and the reaction mechanism. F97 residue (in green); catalytic residues H189 and D193 (in cyan); binding pockets (in grey clouds).

colorimetric assay by i) quantifying the IBA titer of the hexadecane overlay samples, which have been quantified by GC/MS in the previous study [2], with the colorimetric assay and ii) comparing it with GC/MS results. The results showed that while the IBA titer measured by the colorimetric assay was slightly overestimated compared to that measured by GC/MS (Figure 5-3B), the overall trend of IBA titer was almost identical with a correlation coefficient of 0.972 (Figure 5-3C). We also found that the colorimetric method could overestimate the IBA production due to the inevitable by-product, EA formation, compared to the GC/MS method because the *E. coli* host produces ethanol endogenously, EA can be produced by the unspecific activity of AAT (Figure 5-3D, and Table 5-5).

To avoid this problem, we can use $\Delta Ab_{520}^{adj.}$ values (Ab_{520} of host strain w/ AAT and w/ alcohol doping - Ab_{520} values of host strain w/ AAT and w/o alcohol doping) instead of the ΔAb_{520} values (Ab_{520} values of host strain w/ AAT – Ab_{520} values of host strain w/o AAT) for reliable quantification of target esters. Similarly, we can also use a host strain void of the endogenous pathways causing the biosynthesis of the unwanted alcohol byproduct (e.g., ethanol).

During our protocol development, we observed another interesting phenomenon that the perturbed emulsified layer of an immiscible the hexadecane-ethanol mixture interfered with the measured absorbance and generated irreproducible data. Note that ethanol is originated from the ferric solution used in the colorimetric assay. This problem did not occur in the previous study [32] likely because hexane used for ester extraction is miscible in ethanol. To address this problem, we used a centrifugation step to create the immiscible hexadecane-ethanol mixture with the transparent organic phase and strong purple aqueous phase (Figure 5-6 A and B). We also have validated the reliability of this assay using a IBA standard curve and we could observe an almost perfect linear correlation ($R^2=0.999$) was established between absorbance at 520 nm and IBA concentration wit-

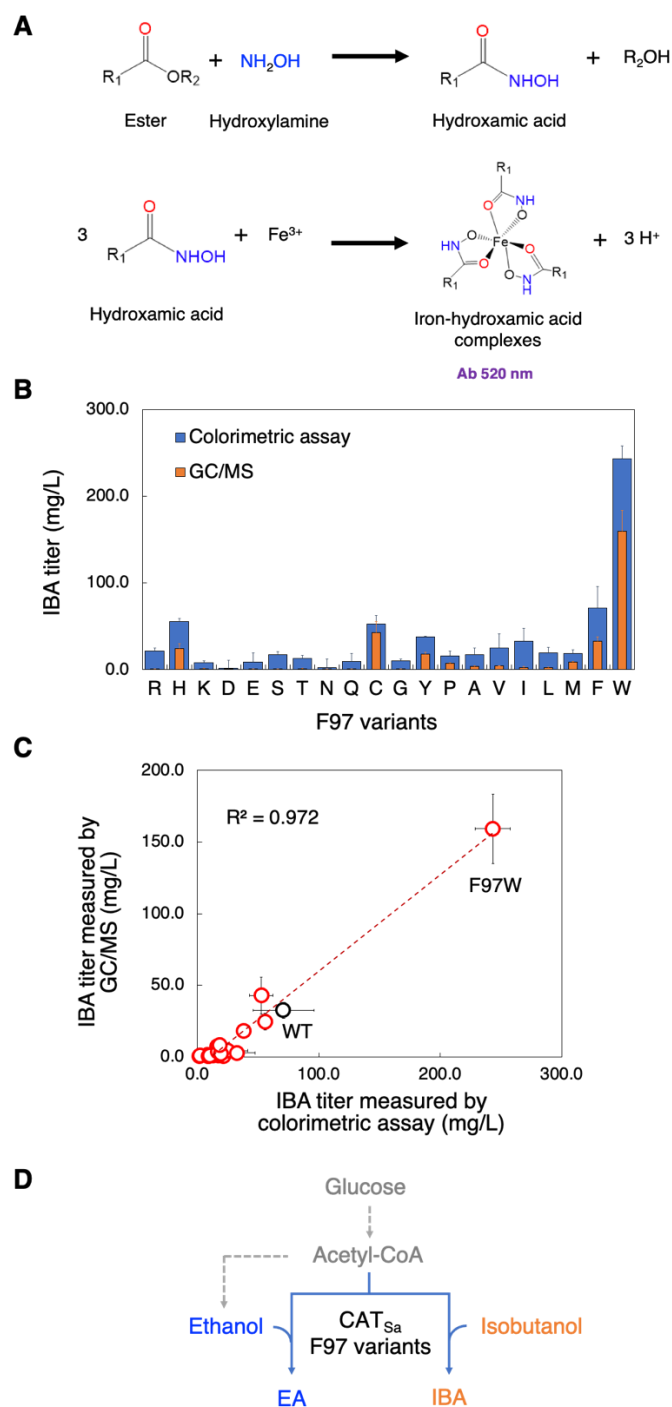


Figure 5-3: (A) Biosynthesis of isobutyl acetate by CAT_{Sa} with isobutanol doping. Dotted lines represent the endogenous ethanol synthesis pathway. Filled line represents the ester synthesis pathway by CAT_{Sa}. (B) Chemical reactions involved in the colorimetric assay. (C) Comparison of IBA titer measured by the colorimetric assay and GC/MS. (D) Correlation between IBA titer measured by GC/MS and the colorimetric assay. The error bars represent standard deviation of three biological replicates ($n=3$).

hin 0-200 mg/L (Figure 5-6C). Taken together, the microplate-based culturing method coupled with a colorimetric assay is suitable for high-throughput microbial screening of AATs/CATs for ester biosynthesis.

5.3.4 Combining the computer-guided protein engineering and high-throughput microbial screening platform to rapid identify ATF1_{Sc} variants for improved BA production

With the established high-throughput microbial screening platform, we applied it to rapidly identify the engineered ATF1_{Sc} mutants for enhanced BA production. We started by generating a library of potential ATF1_{Sc} candidates for improved BA production *in silico* for high-throughput microbial screening. To do this, we first created a 3-D structure of ATF1_{Sc} using the homology model of 15-O-acetyltransferase (PDB:3FP0) best predicted by SWISS-MODEL [39]. We next identified the binding pocket of ATF1_{Sc} for docking simulations of the BA co-substrates, including acetyl-CoA and butanol. Based on the homology model, the binding pocket of ATF1_{Sc} consists of 24 residues including V32, Y36, H191, D195, G196, R197, T316, I347, P348, A349, D350, R352, N370, V371, I374, F376, Y399, I403, L407, K426, L448, S449, N450, V451, F471, and Q473, where the H191 and D195 are the catalytic residues (Figure 5-7A). By performing docking simulations, we generated the acetyl-CoA-butanol-ATF1_{Sc} complex and identified the residues interacting with butanol including V32, Y36, D195, P348, V371, L447, S449, Q473, Q475, and S483 (Figure 5-7B). Finally, we performed the residue scan against these 10 residues to select the top 12 candidates including P348W, P348R, P348M, P348H, P348K, P348N, P348I, P348S, P348D, P348C, P348A, and P348Q for experimental characterization (Figure 5-7C).

To identify the positive ATF1_{Sc} variants for improved BA production, we next characterized the top 12 candidates using TCS083 Δ *fadE* (DE3) [5] as a host strain with or without an exogenous addition of butanol in microplates with hexadecane overlay to obtain Δ Ab₅₂₀^{adj}.

values to avoid the overestimation of the BA titer by endogenous EA production. Then the positive ATF1_{Sc} variants was screened by quantifying the BA titer using the colorimetric assay (Figure 5-8A and B). We also confirmed that there is a very strong linear correlation ($R^2=0.999$) between the absorbance and the concentration of BA standards in the range of 0-200 mg/L (Figure 5-8C). Finally, the colorimetric assay results revealed that the P348M, P348S, or P348A mutation in ATF1_{Sc} improves BA production by 3.34, 2.90, or 2.88-fold, respectively (Figure 5-4A). To confirm this result, we compared the BA titers measured by the colorimetric assay with those by GC/MS. Remarkably, we could observe almost identical BA titers between the two methods (Table 5-6) with a strong linear correlation ($R^2=0.973$) (Figure 5-4B). This result demonstrates the reliability of the developed HTS platform in this study. Interestingly, like the F97 residue in CAT_{Sa} (Figure 5-2B), the P348 residue in ATF1_{Sa} is also located on the opposite side of the catalytic residues including H191 and D195 (Figure 5-4C) and interacts with an alcohol substrate, which might determine the alcohol substrate preference.

Overall, we rationally engineered ATF1_{Sc} for improved BA production through a computer-guided rational protein engineering and rapidly identified the beneficial ATF1_{Sc} variants using the established high-throughput microbial screening platform.

5.4 Conclusions

We developed a high-throughput microbial screening platform to probe specificities of AATs/CATs for designer ester biosynthesis. This platform integrated the microplate culturing method with a modified colorimetric assay previously established, which provides useful information about AAT expression and activity, microbial health, and ester production. For the microplate-based culturing protocol, the use of solvent overlays is critical to minimize medium

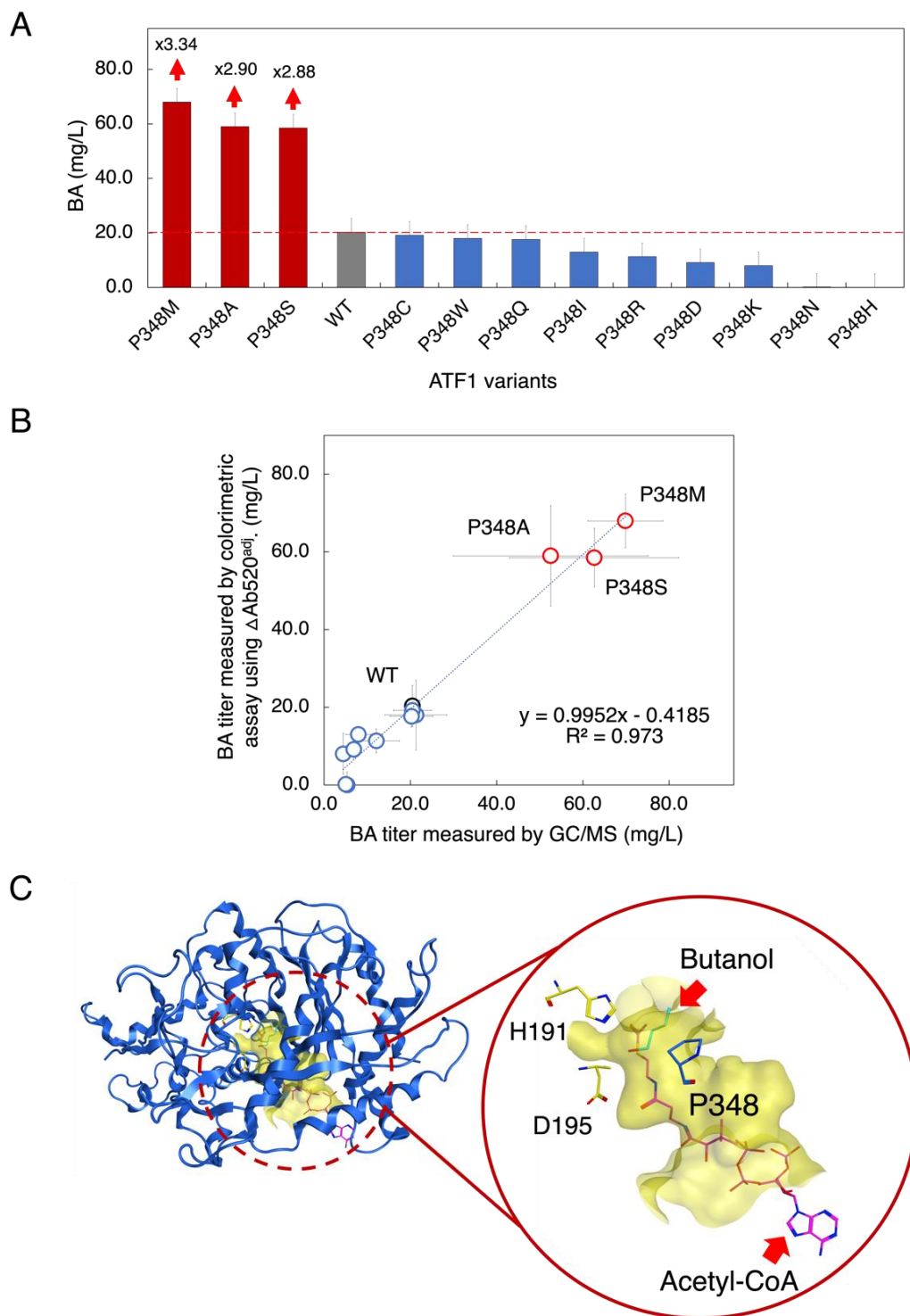


Figure 5-4: Screening of the positive ATF1_{Sc} variants for improved BA production **(A)** BA production of ATF1_{Sc} variants quantified by the colorimetric assay. **(B)** Correlation of BA titer measurement between the colorimetric assay and GC/MS in. **(C)** The location of P348 residue in ATF1_{Sc}. A yellow cloud represents a binding pocket of ATF1_{Sc}. The error bars represent standard deviation of four biological replicates ($n=4$).

evaporation, generate reproducible growth measurement, and eliminate the ester extraction step. For colorimetric assay, the addition of a centrifugation step is crucial to avoid the interference of ethanol-hexadecane immiscible layer that causes irreproducible measurement. The high-throughput microbial screening platform not only confirmed CAT_{Sa} F97W with enhanced isobutyl acetate synthesis but also identified the three ATF1_{Sc} (P348M, P348A, and P348S) variants generated by computer-guided rational protein engineering for enhanced butyl acetate production. Overall, this study presents a high-throughput microbial screening platform for rapid profiling of the alcohol substrate preference of AATs for production of designer esters. We believe that this platform is scalable and compatible with automated microplate handling systems to increase its screening capacity.

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5.6 Appendix

Table 5-2: A list of primers used in this study. The underlined letters indicate site-directed mutation sites of ATF1_{Sc}. F, R, and BB represent forward, reverse, and backbone, respectively.

Primers	Primer sequence (5' to 3')	Description
ATF1 _{Sc} _F	ATAATTTTGTTTAACTTTAAGAAGGAGATATAGATA TGAATGAAATCGATGAGAAAAAT	Primers for constructing the pATF1 _{Sc} by Gibson assembly
ATF1 _{Sc} _R	TTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTG GTGGATAGGGCCTAAAAGGAGAG	
BB_ATF1 _{Sc} _F	ATCCACCACCACCACC	
BB_ATF1 _{Sc} _R	ATCTATATCTCCTTCTTAAAGTTAAACAAAATTATT TCTAG	
P348W_F	ATTTTTATCTGGGCAGATTGCCGCTCACAACTA	Primers for constructing the pATF1 _{Sc} variants by site-directed mutagenesis using the QuickChange method
P348W_R	GGCAATCTGCC <u>CAG</u> ATAAAAAATATCCGTAAGCC	
P348R_F	ATTTTTATCCGTGC <u>AG</u> ATTGCCGCTCACAACTA	
P348R_R	GGCAATCTGC <u>ACG</u> GATAAAAAATATCCGTAAGCC	
P348M_F	ATTTTTATC <u>AT</u> GGCAGATTGCCGCTCACAACTA	
P348M_R	GGCAATCTGC <u>CAT</u> GATAAAAAATATCCGTAAGCC	
P348H_F	ATTTTTATC <u>CAT</u> GCAGATTGCCGCTCACAACTA	
P348H_R	GGCAATCTGC <u>ATG</u> GATAAAAAATATCCGTAAGCC	
P348K_F	ATTTTTATC <u>AAA</u> GCAGATTGCCGCTCACAACTA	
P348K_R	GGCAATCTGC <u>TTT</u> GATAAAAAATATCCGTAAGCC	
P348N_F	ATTTTTATC <u>AAT</u> GCAGATTGCCGCTCACAACTA	
P348N_R	GGCAATCTGC <u>ATT</u> GATAAAAAATATCCGTAAGCC	
P348I_F	ATTTTTATC <u>ATT</u> GCAGATTGCCGCTCACAACTA	
P348I_R	GGCAATCTGC <u>AAT</u> GATAAAAAATATCCGTAAGCC	
P348S_F	ATTTTTATC <u>ACA</u> GCAGATTGCCGCTCACAACTA	
P348S_R	GGCAATCTGC <u>TGT</u> GATAAAAAATATCCGTAAGCC	
P348D_F	ATTTTTATC <u>GAT</u> GCAGATTGCCGCTCACAACTA	
P348D_R	GGCAATCTGC <u>ATC</u> GATAAAAAATATCCGTAAGCC	
P348C_F	ATTTTTATCTGCGCAGATTGCCGCTCACAACTA	
P348C_R	GGCAATCTGC <u>GCA</u> GATAAAAAATATCCGTAAGCC	
P348A_F	ATTTTTATC <u>GCT</u> GCAGATTGCCGCTCACAACTA	
P348A_R	GGCAATCTGC <u>AGC</u> GATAAAAAATATCCGTAAGCC	
P348Q_F	ATTTTTATC <u>CAG</u> GCAGATTGCCGCTCACAACTA	
P348Q_R	GGCAATCTGC <u>CTG</u> GATAAAAAATATCCGTAAGCC	

Table 5-3: Summary of isobutyl acetate (IBA) production in BL21 strains carrying CAT_{sa} F97 variants in various culture methods. WT, wild type.

F97 variants	IBA titer (mg/L)			Specific IBA productivity (mg/gDCW/h)		
	Culture methods					
	Microplates w/o overlay	Microplates w/ overlay	High cell density w/ overlay	Microplates w/o overlay	Microplates w/ overlay	High cell density w/ overlay
R	6.3 ± 1.0	3.5 ± 1.0	0.6 ± 0.1	0.57 ± 0.00	0.59 ± 0.17	0.01 ± 0.00
H	13.7 ± 4.4	20.4 ± 3.8	24.4 ± 5.8	1.13 ± 0.01	3.75 ± 0.69	0.58 ± 0.14
K	2.6 ± 1.0	4.6 ± 2.2	1.3 ± 0.2	0.22 ± 0.01	0.75 ± 0.36	0.03 ± 0.00
D	0.6 ± 0.2	1.6 ± 0.5	0.4 ± 0.1	0.05 ± 0.01	0.26 ± 0.09	0.01 ± 0.00
E	0.4 ± 0.3	1.0 ± 0.2	0.6 ± 0.1	0.03 ± 0.00	0.16 ± 0.04	0.01 ± 0.00
S	0.7 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.06 ± 0.00	0.17 ± 0.05	0.02 ± 0.01
T	0.6 ± 0.2	0.9 ± 1.0	1.0 ± 0.4	0.05 ± 0.02	0.16 ± 0.18	0.02 ± 0.01
N	0.4 ± 0.4	1.5 ± 1.2	0.8 ± 0.1	0.04 ± 0.01	0.26 ± 0.21	0.02 ± 0.00
Q	0.4 ± 0.6	4.6 ± 1.5	0.7 ± 0.1	0.04 ± 0.02	0.83 ± 0.28	0.02 ± 0.00
C	28.5 ± 5.2	23.0 ± 5.4	42.9 ± 12.8	2.70 ± 0.00	4.02 ± 0.94	1.01 ± 0.30
G	2.6 ± 1.7	3.9 ± 3.6	0.9 ± 0.2	0.25 ± 0.01	0.66 ± 0.61	0.02 ± 0.00
Y	17.1 ± 1.2	12.7 ± 2.3	18.2 ± 2.1	1.51 ± 0.01	2.15 ± 0.38	0.42 ± 0.05
P	7.9 ± 0.7	7.0 ± 0.7	7.2 ± 0.7	0.72 ± 0.01	1.06 ± 0.11	0.17 ± 0.02
A	4.3 ± 0.5	3.9 ± 1.2	3.4 ± 1.0	0.41 ± 0.01	0.60 ± 0.18	0.08 ± 0.02
V	4.6 ± 0.4	3.9 ± 0.7	4.3 ± 0.3	0.41 ± 0.01	0.66 ± 0.12	0.10 ± 0.01
I	1.0 ± 0.5	2.2 ± 1.3	2.7 ± 0.9	0.10 ± 0.01	0.38 ± 0.23	0.06 ± 0.02
L	2.7 ± 0.4	3.2 ± 0.5	2.3 ± 0.7	0.27 ± 0.01	0.53 ± 0.09	0.05 ± 0.02
M	9.2 ± 1.8	9.4 ± 2.2	8.4 ± 1.1	0.78 ± 0.01	1.47 ± 0.35	0.20 ± 0.03
F (WT)	14.2 ± 3.0	18.9 ± 5.5	32.6 ± 5.5	1.31 ± 0.01	3.46 ± 1.01	0.76 ± 0.13
W	90.5 ± 41.7	75.7 ± 3.7	159.3 ± 24.1	8.74 ± 0.01	12.72 ± 0.62	3.66 ± 0.55

Table 5-4: The results of rapid profiling the alcohol substrate preference of 20 F97 variants of CAT_{Sa}. WT, wild type; *n.d.*, not detected.

F97 variants	Ester titer (mg/L)			
	Ethyl acetate	Butyl acetate	Isobutyl acetate	2-Phenylethyl acetate
R	<i>n.d.</i>	0.8	6.3	11.8
H	<i>n.d.</i>	0.8	13.8	182.3
K	<i>n.d.</i>	0.4	2.7	19.7
D	<i>n.d.</i>	0.4	0.6	3.8
E	<i>n.d.</i>	<i>n.d.</i>	0.4	1.4
S	<i>n.d.</i>	0.3	0.7	3.5
T	<i>n.d.</i>	12.4	0.6	7.2
N	<i>n.d.</i>	<i>n.d.</i>	0.4	3.3
Q	<i>n.d.</i>	3.0	0.4	12.1
C	<i>n.d.</i>	18.3	28.7	153.9
G	<i>n.d.</i>	2.5	2.6	4.5
Y	<i>n.d.</i>	11.3	17.2	109.9
P	<i>n.d.</i>	7.5	7.9	58.7
A	<i>n.d.</i>	6.1	4.3	59.1
V	<i>n.d.</i>	5.6	4.6	127.0
I	<i>n.d.</i>	2.2	1.1	63.3
L	<i>n.d.</i>	1.5	2.7	79.3
M	<i>n.d.</i>	4.0	9.2	139.0
F (WT)	<i>n.d.</i>	4.3	14.3	149.4
W	0.4	16.9	91.2	194.6

Table 5-5: Comparison of ester titer measured by GC/MS and ΔAb_{520} values. $\Delta Ab_{520} = Ab_{520 \text{ host strain carrying CATSa F97 variants}} - Ab_{520 \text{ host strain}}$. WT, wild type; *n.d.*, not detected. The used hexadecane overlay samples were obtained from high cell density cultures [1].

CAT _{Sa} F97 variants	GC/MS			Colorimetric assay
	Ester titer (mg/L)			ΔAb_{520}
	Ethyl acetate	Isobutyl acetate	Total	
R	<i>n.d.</i>	0.6 ± 0.1	0.6 ± 0.1	0.03 ± 0.00
H	0.0 ± 0.0	24.4 ± 5.8	24.5 ± 5.8	0.07 ± 0.00
K	<i>n.d.</i>	1.3 ± 0.2	1.3 ± 0.2	0.01 ± 0.00
D	<i>n.d.</i>	0.4 ± 0.1	0.4 ± 0.1	0.00 ± 0.01
E	<i>n.d.</i>	0.6 ± 0.1	0.6 ± 0.1	0.01 ± 0.01
S	<i>n.d.</i>	0.9 ± 0.3	0.9 ± 0.3	0.02 ± 0.00
T	<i>n.d.</i>	1.0 ± 0.4	1.0 ± 0.4	0.02 ± 0.00
N	<i>n.d.</i>	0.8 ± 0.1	0.8 ± 0.1	0.00 ± 0.01
Q	<i>n.d.</i>	0.7 ± 0.1	0.7 ± 0.1	0.01 ± 0.01
C	0.1 ± 0.0	42.9 ± 12.8	43.0 ± 12.9	0.06 ± 0.01
G	<i>n.d.</i>	0.9 ± 0.2	0.9 ± 0.2	0.01 ± 0.00
Y	<i>n.d.</i>	18.2 ± 2.1	18.2 ± 2.1	0.05 ± 0.00
P	<i>n.d.</i>	7.2 ± 0.7	7.2 ± 0.7	0.02 ± 0.01
A	<i>n.d.</i>	3.4 ± 1.0	3.4 ± 1.0	0.02 ± 0.01
V	<i>n.d.</i>	4.3 ± 0.3	4.3 ± 0.3	0.03 ± 0.02
I	<i>n.d.</i>	2.7 ± 0.9	2.7 ± 0.9	0.04 ± 0.02
L	<i>n.d.</i>	2.3 ± 0.7	2.3 ± 0.7	0.02 ± 0.01
M	<i>n.d.</i>	8.4 ± 1.1	8.4 ± 1.1	0.02 ± 0.01
F (WT)	0.1 ± 0.0	32.6 ± 5.5	32.6 ± 5.5	0.09 ± 0.03
W	0.2 ± 0.0	159.3 ± 24.1	159.5 ± 24.2	0.29 ± 0.02

Table 5-6: Butyl acetate titer measured by the colorimetric assay and GC/MS. $\Delta Ab_{520}^{adj.} = Ab_{520}^{BtOH} - Ab_{520}^{None}$. Ab_{520}^{BtOH} and Ab_{520}^{None} represent the Ab_{520} values with or without an exogenous addition of 2 g/L butanol, respectively. WT, wild type. Positive hits in bold.

ATF1 _{Sc} variants	Colorimetric assay				GC/MS
	Ab_{520}^{BtOH}	Ab_{520}^{None}	$\Delta Ab_{520}^{adj.}$	Butyl acetate (mg/L) using $\Delta Ab_{520}^{adj.}$	Butyl acetate (mg/L)
WT	0.14 ± 0.01	0.11 ± 0.00	0.03 ± 0.01	20.3 ± 5.3	20.5 ± 1.8
P348W	0.14 ± 0.01	0.11 ± 0.00	0.03 ± 0.01	18.0 ± 9.0	21.3 ± 7.2
P348R	0.12 ± 0.00	0.11 ± 0.00	0.02 ± 0.00	11.3 ± 3.1	12.1 ± 5.4
P348M	0.22 ± 0.00	0.12 ± 0.01	0.10 ± 0.01	68.0 ± 6.9	69.9 ± 8.7
P348H	0.12 ± 0.00	0.12 ± 0.00	0.00 ± 0.01	0.0 ± 3.4	5.3 ± 0.9
P348K	0.12 ± 0.00	0.11 ± 0.01	0.01 ± 0.01	8.0 ± 5.3	4.4 ± 1.0
P348N	0.12 ± 0.00	0.12 ± 0.00	0.00 ± 0.00	0.2 ± 2.0	5.0 ± 1.7
P348I	0.13 ± 0.00	0.11 ± 0.00	0.02 ± 0.00	13.0 ± 1.7	7.9 ± 2.9
P348S	0.20 ± 0.01	0.11 ± 0.00	0.09 ± 0.01	58.5 ± 7.6	62.7 ± 19.6
P348D	0.12 ± 0.00	0.11 ± 0.00	0.01 ± 0.00	9.2 ± 2.4	6.9 ± 1.5
P348C	0.14 ± 0.00	0.11 ± 0.00	0.03 ± 0.00	19.2 ± 1.1	20.5 ± 4.4
P348A	0.20 ± 0.02	0.11 ± 0.00	0.09 ± 0.02	59.0 ± 12.9	52.5 ± 22.7
P348Q	0.13 ± 0.00	0.10 ± 0.00	0.03 ± 0.00	17.7 ± 2.8	20.3 ± 5.0

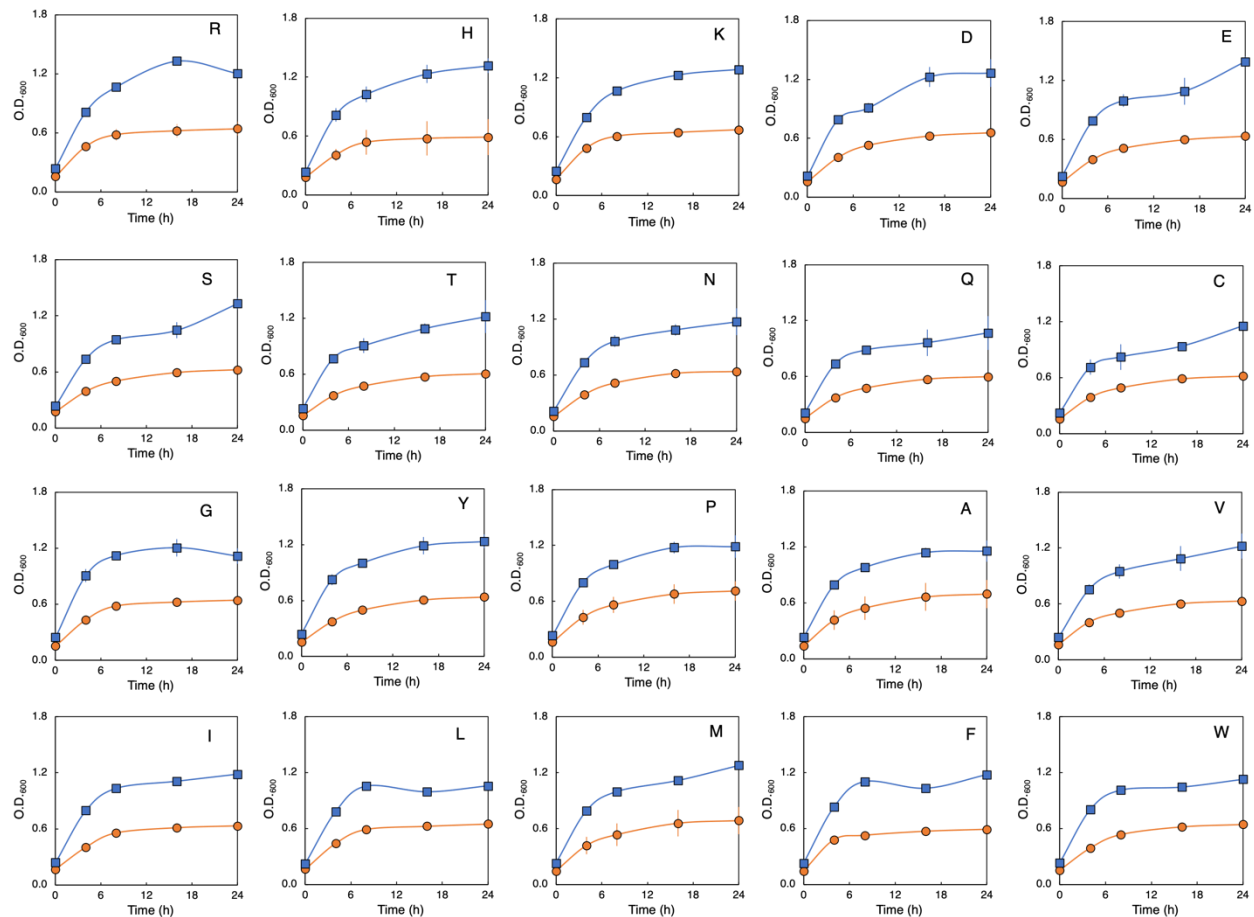


Figure 5-5: Growth curves of BL21 (DE3) strains carrying CAT_{Sa} F97 variants in microplates w/ or w/o hexadecane overlay. Blue square indicates w/o overlay; Orange circle indicates w/ overlay.

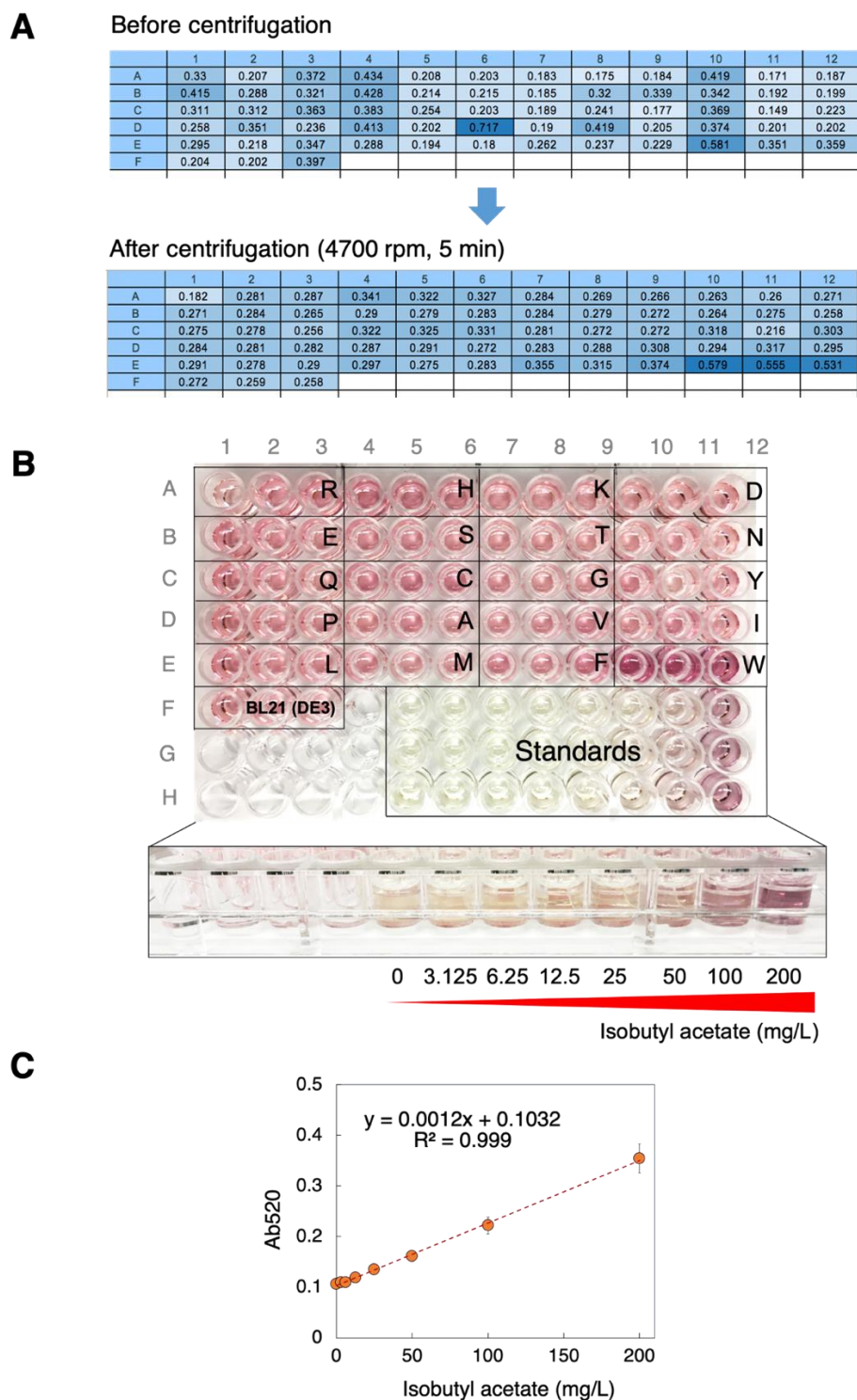


Figure 5-6: (A) Effect of centrifugation on the results of colorimetric assay. (B) The colorimetric assay of hexadecane overlay samples and a series of isobutyl acetate standards with a different concentration. (C) The isobutyl acetate standard curve used for quantification of isobutyl acetate in the colorimetric assay.

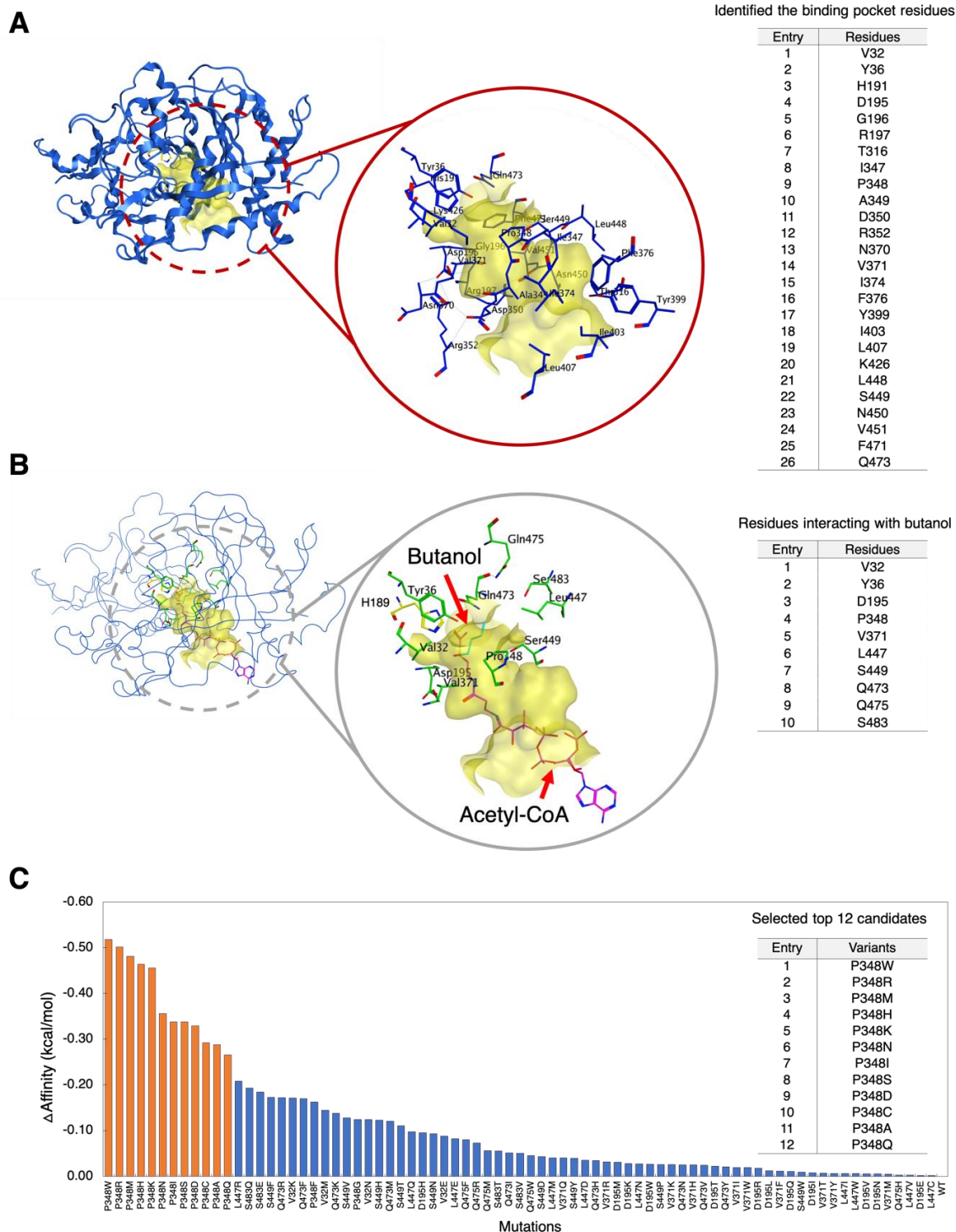


Figure 5-7: (A) The 3D structure of homology model of ATF1_{Sc}. A binding pocket of ATF1_{Sc} (in a yellow cloud); binding pocket residues of ATF1_{Sc} (in blue), **(B)** The 3D structure of acetyl-CoA-butanol-ATF1_{Sc} complex. A binding pocket (in a yellow cloud); residues interacting with butanol in the acetyl-CoA-butanol-ATF1_{Sc} complex (in green). **(C)** Residue scan results of the residues interacting with butanol in the acetyl-CoA-butanol-ATF1_{Sc} complex. The orange bars represent the selected top 12 candidates for further studies.

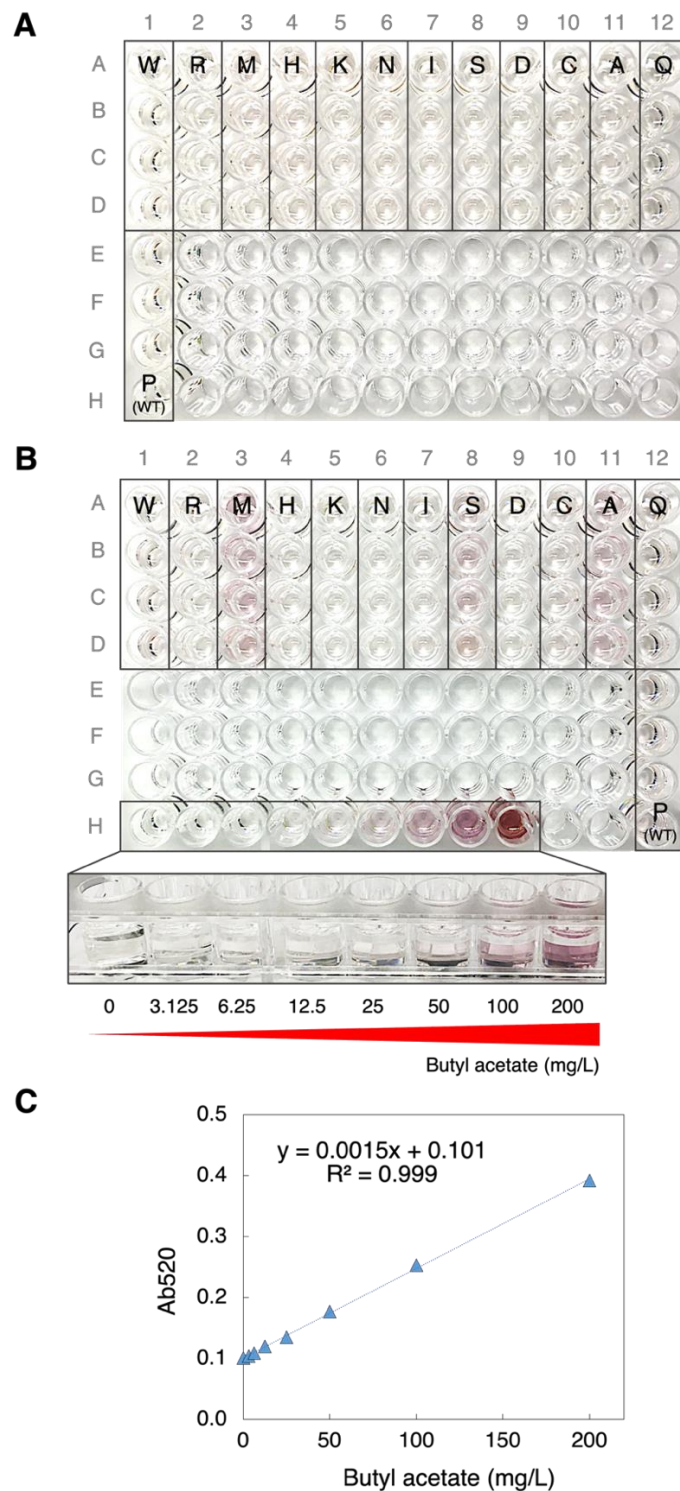


Figure 5-8: The colorimetric assay of hexadecane overlays from the cultures of strains carrying ATF1_{Sc} variants **(A)** without or **(B)** with an exogenous addition of 2 g/L butanol. **(C)** The butyl acetate standard curve used for the quantification of butyl acetate in the colorimetric assay.

Supplementary References

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6 Evaluation of Growth Selection of Butyl Acetate Production Modules with ATF1_{sc} Variants in a Modular (Chassis) Cell

6.1 Abstract

Alcohol acetyltransferases (AATs) are valuable biocatalysts that catalyzing an alcohol and an acyl-CoA into an ester. To date, while microbial ester synthesis platforms has been well established using AATs, its production is still inefficient for industrial-scale production. To achieve efficient ester production, discovery or engineering efficient AATs is essential. However, although several screening methods for rapid discovery of efficient novel AATs or identifying the positive hits of engineered AAT variants have been developed, these screening approaches are not the ideal methods for rapid identification of the best hit from a large library of potential candidates. Thus, we need more efficient and scalable approaches to identify the best hit from a large library.

In this study, we have evaluated and demonstrated the potential of a modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules with a case study of growth selection of a ATF1 variant (ATF1 P348M), engineered for the improved butyl acetate production, in the modular (chassis) cell. Overall, the presented work can help accelerate the discovery of efficient novel AATs or identification of the positive hits of engineered AAT variants from a large library of potential candidates.

6.2 Introduction

Alcohol acetyltransferases (AATs) are valuable biocatalysts that catalyzing an alcohol and an acyl-CoA into an ester (Figure 6-1A) [1, 2]. These enzymes have been studied for microbial production of various esters including short-to-medium chain [3-9], aromatic [10, 11] and terpene esters [12-17] from renewable resources. However, while microbial production of various esters has been well demonstrated, its production is still inefficient for industrial-scale production. Thus,

for a broader spectrum of industrial uses of renewable and sustainable esters, the microbial platforms that can produce a larger library of esters in an efficient and systematic way are required.

To solve this problem, the concept of modular cell design has been proposed [18]. Based on modular cell design, each optimal production strain can be obtained by assembling a modular (chassis) cell with an exchangeable production module(s) in a plug-and-play fashion using a chassis-based modular architecture [19]. Here, a module is defined as an essential and self-contained functional unit, which has standardized interfaces, that enables composition of products by combination [19]. Based on the definition above, an exchangeable production module(s) contains auxiliary regulatory and metabolic pathways, and the modular (chassis) cell interfaces with an exchangeable production module(s) via enzyme synthesis machinery and precursor metabolites [19].

Further, because the modular (chassis) cell has been designed to be auxotrophic by imposing cofactor imbalance and/or insufficient supply of precursor metabolites required for cell growth [18], the production module(s) can be designed to balance redox, and/or supply energy/precursor metabolites that required to rescue the growth defect of the modular (chassis) cell to enable growth-coupled product formation in a plug-and-play fashion [20]. Based on this design principle, because the modular (chassis) cell contains core metabolic phenotypes shared among production modules, the desirable phenotype of each production strain is determined by the production module. Thus, with this approach, the best production strains or modules can be screened or selected with growth [18]. For example, the modular (chassis) cell with the stronger production module achieves the faster cell growth, substrate consumption, and product formation [21]. However, to achieve efficient ester production, although the modular (chassis) cell offers a

frame for rapid development of efficient ester production strains, it still requires efficient ester production modules.

To construct efficient ester production modules, because AATs have a critical role in biosynthesis of esters, discovery or engineering a large library of efficient AATs is essential [22]. To date, for rapid discovery of efficient novel AATs or identifying the positive hits of engineered AAT variants, several screening methods including i) a thin-layer chromatography (TLC) assay [23], ii) a hydroxylamine/ferric iron assay [24-27], iii) a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay [28-30], and iv) a coupled-enzyme assay with α -ketoglutarate dehydrogenase (α -KGDH) [31, 32] have been developed. However, because with these screening approaches, each individual candidate must be evaluated for the desired property with multiple steps, they were not the ideal methods for rapid identification of the best hit from a large library of potential candidates.

Compared with the screening methods, selection methods offer more efficient and scalable approaches to identify the best hit from a large library by directly eliminating undesired candidates with a certain selective pressure to the library [33]. Specifically, growth selection platform provides a facile and universal selection platform by employing growth as an easily measurable readout which allows for substantially higher throughput ($>10^6$ samples per round of selection) as compared to the 96-well plate-based screening methods [34-36]. Fortunately, because the modular (chassis) cell offers a selection pressure through growth coupling between a chassis and a production module, the modular (chassis) cell can be used as a growth selection platform for identification of superior enzymes or production modules [21]. However, to date, while growth selection of other enzymes such as pyruvate decarboxylase (PDC) in an ethanol production module has been demonstrated in the modular (chassis) cell [21], its studies on AATs or ester production modules has been limited majorly due to a lack of AAT variants.

In our previous work (Chapter 5), because we could obtain ATF1_{Sc} variants (AAT from *Saccharomyces cerevisiae*) such as ATF1_{Sc} P348M that showed improved butyl acetate (BA) production in *E. coli*, here, we evaluated the potential of the modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules using the ATF1_{Sc} variants. Specifically, to evaluate the potential of the modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules, we first constructed the modular cells carrying butyl acetate production modules with ATF1_{Sc} wildtype or ATF1_{Sc} P348M variant. Then by comparing the growth of the modular (chassis) cell (TCS095 (DE3)) with its derivatives including the modular (chassis) cell carrying butyl acetate production modules with ATF1_{Sc} wildtype (EcJWBA) or ATF1_{Sc} P348M variant (EcJWBA_{mut}), the potential of the modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules has been evaluated. Overall, this work can help facilitate rapid discovery of efficient novel AATs or identifying the positive hits of engineered AAT variants in the modular (chassis) cell.

6.3 Materials and Methods

6.3.1 Strains and plasmids

Strains and plasmids used in this study are listed in Table 6-1. For molecular cloning, *E. coli* TOP10 was used. For validation of a butanol production module and growth selection of AAT variants, TCS095 [21] were used as a host strain. All the constructed plasmids were introduced into host strains using chemical transformation. The primers used in this study are listed in Table 6-2.

Table 6-1: A list of plasmids and strains used in this study.

Name	Description	Source
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX</i> 74 <i>recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
MG1655 (DE3)	F ⁻ λ ⁻ (DE3)	[37]
TCS095 (DE3)	MG1655 Δ <i>ldhA</i> ::Δ <i>frdA</i> ::Δ <i>sfcA</i> ::Δ <i>maeB</i> ::Δ <i>zwf</i> ::Δ <i>ndh</i> ::Δ <i>pta</i> :: Δ <i>poxB</i> ::Δ <i>adhE</i> (DE3)	[21]
EcDL112	TCS095 (DE3)/ pCT24	[21]
EcJWBtOH	TCS095 (DE3)/ pBtOH	This study
EcJWBA	TCS095 (DE3)/ pBtOH pATF1	This study
EcJWBA _{mut}	TCS095 (DE3)/ pBtOH pATF1 P348M	This study
EcJWATF1	TCS095 (DE3)/ pATF1	This study
EcJWATF1 _{mut}	TCS095 (DE3)/ pATF1 P348M	This study
Plasmids		
pCT24	pETite P _{T7lac} :: <i>pdcc::adhB</i> ; Kan ^R	[38]
pBtOH	pACYCD P _{T7lac} :: <i>atoB::hbd::crt</i> -P _{T7lac} :: <i>ter::adhE2</i> ; Cm ^R	This study
pATF1	pET29 P _{T7lac} :: <i>ATF1</i> _{Sc} ; Kan ^R	Chapter 5
pATF1 P348M	pET29 P _{T7lac} :: <i>ATF1</i> _{Sc} P348M; Kan ^R	Chapter 5

Table 6-2: A list of primers used in this study. F, R, and BB represent forward, reverse, and backbone, respectively.

Primers	Primer sequence (5' to 3')
adhE2_F	GTAGAAAGGTTTCGACAGGATTTAAAAAGAGGAGAAAATGAAAGTGACCA ATCAAAAAGAG
adhE2_R	CGCAGCAGCGGTTTCTTTACCAGACTCGAGTTAGAACTCTTTATGTAAA TGTCTTTGAG
BB_BtOH_F	TAACTCGAGTCTGGTAAAGAAACC
BB_BtOH_R	TTTCTCCTCTTTTTAAATCCTGTGCGAACCTTTCTAC

6.3.2 Culture media

For molecular cloning, and seed cultures, LB (Lysogeny Broth) medium was used. For validation of butanol production module, TBD₅₀, TB (Terrific Broth) medium containing 50 g/L glucose was used (without supplementation with glycerol). For growth selection of ATF1 variants, M9D₁₀, M9 medium containing 10 g/L glucose was used. 30 µg/mL chloramphenicol (Cm), and/or 50 µg/mL kanamycin (Kan) was added to the media for selection where applicable.

6.3.3 Validation of a butanol production module

For seed cultures, 1% (v/v) of stock cells were grown overnight in 5 mL of LB medium with appropriate antibiotics on a 75° angled platform in a New Brunswick Excella E25 at 37 °C and 200 rpm. Next, 1% (v/v) of seed cultures inoculated in 500 mL baffled flasks containing 50 ml of TBD₅₀ medium with appropriate antibiotics. The cells were aerobically grown in shaking incubators at 37°C, 200 rpm and induced at an O.D.₆₀₀ of 0.6~0.8 with 0.1 mM of IPTG at 28°C. After 2 hours of induction, 10 ml of the cultures were allocated into 25 mL anaerobic culture tubes and each tube was overlaid with 1ml hexadecane (20% (v/v)) for *in situ* ester recovery. Finally, the anaerobic culture tubes were sealed with a rubber stopper inside the anaerobic chamber and grown for another 24 hours on a 75° angled platform in shaking incubators at 28°C, 200 rpm. All anaerobic culture tubes were prepared inside the anaerobic chamber contains a mixture of gases (90% N₂, 5% H₂, and 5% CO₂). The experiments were performed with biological triplicates.

6.3.4 Growth selection of an efficient butyl acetate production module with ATF1_{Sc} variants

For seed cultures, the single colonies from LB agar plates were inoculated into 5 mL of LB medium with appropriate antibiotics on a 75° angled platform in a New Brunswick Excella E25 at

37 °C and 200 rpm. Next, 1% (v/v) of seed cultures were transferred into 5 mL of M9D₁₀ medium with appropriate antibiotics to adapt the cell to a defined environment. Then, the cells were aerobically grown overnight in shaking incubators at 37°C, 200 rpm. Next, 1% (v/v) of the cultures were inoculated in 5 mL of M9D₁₀ medium with appropriate antibiotics and the cells were grown for 4 hours at 37 °C and 200 rpm. Then the cells were induced with 0.1 mM of IPTG at 28°C for 2 hours. Lastly, the induced cells were transferred into 25 mL anaerobic culture tubes containing 10 mL of M9D₁₀ with an initial O.D.₆₀₀ of ~0.05 and the tubes were sealed with a rubber stopper inside the anaerobic chamber. Finally, the anaerobic culture tubes were characterized on a 75° angled platform in a New Brunswick Excella E25 at 28 °C and 200 rpm. All anaerobic culture tubes were prepared inside the anaerobic chamber contains a mixture of gases (90% N₂, 5% H₂, and 5% CO₂). The experiments were performed with biological replicates.

6.3.5 Cell growth

The optical density (OD) was measured at 600 nm using a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., VT, USA).

6.3.6 Statistics

Statistical analysis was performed with Microsoft Excel using the two-tailed unpaired Student's *t* test.

6.3.7 High performance liquid chromatography (HPLC)

Metabolites were quantified by using the Shimadzu HPLC system (Shimadzu Inc., MD, USA) equipped with the Aminex HPX-87H cation exchange column (BioRad Inc., CA, USA) heated at 50°C. A mobile phase of 10 mN H₂SO₄ was used at a flow rate of 0.6 mL/min. Detection was made with the reflective index detector (RID).

6.4 Results and Discussion

6.4.1 Construction and validation of a butanol production module

To evaluate the potential of the modular (chassis) cell for growth selection of ester production modules, we aimed to compare the growth of modular cells carrying butyl acetate production modules with different AATs including ATF1_{sc} wildtype and its P348M variant which achieved ~3-fold improved butyl acetate production as compared to its wildtype (Chapter 5). Accordingly, we began to construct a butanol production module because butyl acetate can be synthesized by condensing butanol and acetyl-CoA by an ATF1_{sc} (Figure 6-1B). Note that acetyl-CoA is endogenously produced by the cell.

To construct a butanol production module, we first amplified the two DNA fragments including i) butyl-CoA synthesis pathway from pDL2 [38] using a set of primers BB_BtOH_F and BB_BtOH_R and ii) *adhE2* from genomic DNA of *Clostridium acetobutylicum* using a set of primers adhE2_F and adhE2_R. Then, by assembling these two DNA fragments with Gibson Assembly method [39], a butanol production module, pBtOH, has been constructed. After constructing the butanol production module, we next introduced it into the modular (chassis) cell, TCS095 (DE3), resulting in the butanol production strain, EcJWBtOH (Figure 6-1C and Table 6-1). Finally, to validate the butanol production module, we characterized EcJWBtOH.

The characterization results showed that EcJWBtOH produced 2.03 ± 0.01 g/L of butanol from 7.35 ± 0.10 g/L of glucose after 24 h of culture, showing that the constructed butanol production module is functional in the modular (chassis) cell (Figure 6-1D). After validating a butanol production module, we next constructed butyl acetate production strains by introducing pATF1 or pATF1 P348M into EcJWBtOH, resulting EcJWBA and EcJWBAmut, respectively (Figure 6-1C and Table 6-1). Finally, to evaluate the potential of the modular (chassis) cell as a

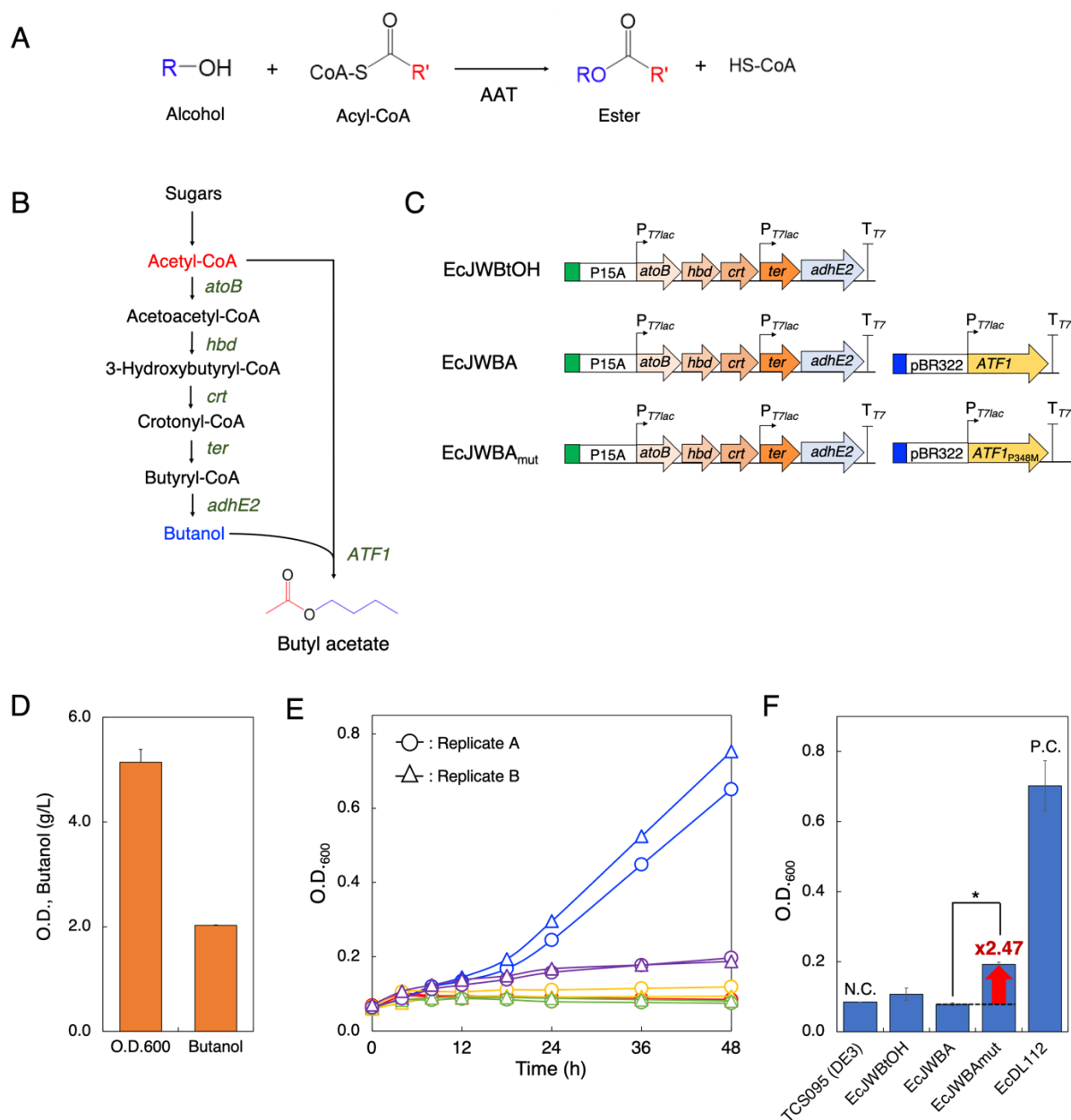


Figure 6-1: (A) Esterification reaction by an alcohol acyltransferase (AAT). (B) Biosynthetic pathway of butyl acetate from glucose. (C) Validation of a butanol production module in EcJWBtOH. (D) Schematic of strains carrying butyl acetate production modules with ATF1_{Sc} wildtype (EcJWBA) or ATF1_{Sc} P348M variant (EcJWBA_{mut}). (E) Growth selection of an efficient ATF1_{Sc} variant. TCS095 (DE3) in red; EcDL112 in blue; EcJWBA in green; EcJWBA_{mut} in purple. TCS095 (DE3) was used as a negative control. EcDL112 carrying an ethanol production module was used as a positive control. (F) Comparison of O.D.₆₀₀ values between the modular (chassis) cell (TCS095 (DE3)) and its derivatives after 48 h of cultures in the anaerobic culture tubes. N.C. and P.C. represent a negative control and a positive control, respectively. **p* < 0.002 (Student's t-test).

growth selection platform of efficient AATs or ester production modules, EcJWBtOH, EcJWBA, and EcJWBAmut were characterized together with the modular (chassis) cell without a production module, TCS095 (DE3), as a negative control [21], and the modular (chassis) cell carrying an ethanol production module, EcDL112, as a positive control [21].

6.4.2 Growth selection of an efficient butyl acetate production module with ATF1_{Sc} variants in a modular (chassis) cell

The characterization results showed that while EcJWBtOH and EcJWBA grew almost as similarly as TCS095 (DE3) (negative control), EcJWBAmut achieved ~2.5-fold improved growth as compared to its counterpart, EcJWBA (Figure 6-1E and F). Specifically, after 48 h of characterization, the O.D.₆₀₀ value of EcJWBAmut was 0.193 ± 0.005 while that of EcJWBA was 0.078 ± 0.004 (Figure 6-1F and Table 6-3). Note that EcDL112 (positive control) achieved the best growth among the characterized five strains as we expected (Figure 6-1E and F). This result shows the potential of the modular (chassis) cell as a growth selection platform for discovery of efficient novel AATs or identification of the positive hits of engineered AAT variants with growth as an easily measurable readout. It also suggests that the modular (chassis) cell can be used for growth-coupled ester production for improved ester production via adaptive laboratory evolution.

One possible explanation for this phenotype (growth) difference between EcJWBA and EcJWBAmut is because ATF1_{Sc} P348M of a butyl acetate production module in EcJWBAmut has higher activity compared to that of its wildtype in EcJWBA, ATF1_{Sc} P348M in EcJWBAmut provides the stronger driving force to pull carbon fluxes to butyl acetate by enabling ~2.5-fold improved growth of the modular (chassis) cell (Figure 6-1F). Note that the only carbon outlet in butyl acetate production is butanol formation [40] and based on the conventional “push-and-pull” metabolic engineering strategy [41], the carbon fluxes can be pulled into butyl acetate by ATF1_{Sc}

Table 6-3: Summary of growth selection of an efficient butyl acetate production module with ATF1_{sc} variants in a modular (chassis) cell. A and B indicate biological replicates.

Time (h)	Strains									
	TCS095 (DE3)		EcJWBtOH		EcJWBA		EcJWBA _{mut}		EcDL112	
	A	B	A	B	A	B	A	B	A	B
0	0.069	0.063	0.066	0.060	0.062	0.067	0.064	0.071	0.063	0.063
4	0.100	0.093	0.105	0.076	0.083	0.083	0.088	0.107	0.088	0.088
8	0.095	0.092	0.107	0.089	0.083	0.089	0.113	0.124	0.120	0.122
12	0.095	0.090	0.106	0.093	0.087	0.090	0.124	0.138	0.135	0.145
18	0.092	0.093	0.111	0.095	0.084	0.090	0.139	0.149	0.168	0.194
24	0.090	0.089	0.111	0.092	0.079	0.088	0.158	0.168	0.245	0.296
36	0.087	0.087	0.115	0.093	0.077	0.085	0.178	0.178	0.448	0.525
48	0.084	0.085	0.119	0.094	0.075	0.081	0.197	0.188	0.651	0.753

variants (Figure 6-1B). However, while this work shows the potential of the modular (chassis) cell as a growth selection platform for efficient AATs or ester production modules, further work is necessary i) to elucidate the underlying principle in growth selection of efficient AATs or ester production modules and ii) to demonstrate improved ester production via growth-coupling with a large library.

6.5 Conclusions

As a proof-of-concept, here, we have evaluated the potential of the modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules with a case study of growth selection of butyl acetate production modules with ATF1_{Sc} variants (ATF1_{Sc} wildtype and its P348M) in the modular (chassis) cell. Interestingly, we could observe improved growth in the modular (chassis) cell carrying a better butyl acetate production module. This result suggests the potential for growth selection of efficient AATs or ester production modules as well as growth-coupled ester production in the modular (chassis) cell. Overall, this work can help accelerate the discovery of efficient novel AATs or identification of the positive hits of engineered AAT variants from a large library of potential candidates by allowing the use of simple phenotype (growth) as an easy readout. As this platform matures, it is expected to enable rapid development of efficient production strains for production of various designer esters via growth coupling between the modular (chassis) cell and an efficient ester production module with selected AATs.

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7 Final Conclusions

Despite significant advances in genome-scale modeling and metabolic engineering, the current strain development is still limited by the vast biochemical space in nature. The use of modular (chassis) cells can enable us to develop optimal production strains for various products with minimal experimental effort. However, for the use of modular (chassis) cells in production of various biochemicals such as a library of esters, there are three major challenges including i) a vast biochemical space; ii) a compatibility between a chassis cell and production modules; and iii) low specificity of pathway enzymes such as alcohol acyltransferases (AATs) towards target substrate(s). To address these challenges, first, a framework for rapid design, construction, and validation of synthetic metabolic pathways have been developed with a case study of production of C4-derived esters. Second, the compatibility between a chassis cell and production modules have been improved by a combinatorial protein solubilization strategies. Third, an *in silico* rational protein design protocol for altering the alcohol substrate specificity of AATs has been developed. Fourth, a high-throughput screening (HTS) platform has been established to accelerate the identification of beneficial mutations in AATs for improved production of designer esters. Finally, the potential of a modular (chassis) cell as a growth selection platform of efficient AATs or ester production modules has been evaluated using butyl acetate production modules with AAT variants. Furthermore, to expand a library of esters that can be produced from renewable resources, modular design principles have been used to establish a lactate ester platform.

Overall, this work contributes to validating the modular cell theory by offering a framework for rapid design, construction, and validation of synthetic metabolic pathways in a modular (chassis) cell with a case study of production of various esters.

8 Recommendations

Although this work tackled the challenges that we can encounter in construction of production modules with knowledge-based metabolic engineering strategies, this process can be accelerated by using computational tools. For example, at the design stages, basic information on each biological part comprised of a production module such as genetic parts (e.g., promoter strength, ribosome binding site strength, and so on) and enzymes (e.g., kinetics, cost, metabolic burdens, *in vivo* solubility, *in vivo* concentrations, and so on) can be predicted to aid better decision-making for design of novel and efficient production modules. Then, at the build stages, based on the information obtained from the predictions, each biological part can be assembled into a production module. One suggestion, here, is developing and the use of a scoring matrix would help to incorporate the data from multi-layered model predictions to bridge the gap between the predictions and experimental implementation. Biosensors can also be used for improving product formation via dynamic flux control. For example, for the synthesis of an ester, because two different precursors (an alcohol and an acyl-CoA) are required at a 1:1 ratio, the carbon fluxes and/or cellular resources should be allocated equally between these two molecules. Thus, to address this problem, transcription factor-based biosensors can be used to regulate gene expression of the biosynthesis pathways of an alcohol and an acyl-CoA.

One of the biggest challenges in growth selection of efficient ester production modules and/or growth-coupled ester production in a modular (chassis) cell is the fastest growing strain is not necessarily the best ester producer. Specifically, because ester production modules are composed of three different submodules including i) an alcohol synthesis, ii) an acyl-CoA synthesis, and iii) an AATase, it is possible that while these submodules are coupled to growth,

their combination to an ester pathway is not. Therefore, with growth, we cannot differentiate the best ester producer from the best precursor producers such as an alcohol, an acyl-CoA, or a combination thereof. In other words, although we could select the winner with ester production modules, for example, with ethyl butyrate production modules, growth selection could identify the best ethyl butyrate producer, but also could select for the best ethanol or butyl-CoA producer. Thus, to examine the potential of a modular (chassis) cell in growth selection of ester production modules and/or growth-coupled ester production, we need to use the same alcohol and acyl-CoA production modules by varying the AATase. However, to date, this study could not be conducted because variants of AATs were not available.

Here, to obtain variants of AATs, a rational protein engineering protocol and a high-throughput ester synthesis screening platform have been developed. Then, by using a combination of these two techniques, variants of ATF1_{sc} for improved butyl acetate production were identified. Although this work showed the potential of a modular (chassis) cell in growth coupling of ester production modules using butyl acetate production modules with ATF1 variants, further work is required i) to elucidate the underlying principle in growth selection of efficient AATs or ester production modules and ii) to demonstrate growth-coupled ester production with a large library. The framework presented in this work enables the design and construction of a library of ester production modules with AAT variants, hence contributing to the validation of modular design principles with a case study of production of various esters.

As this technology matures, natural esters can be produced using microbes from renewable resources in a sustainable and systematic way. For example, 2-phenethyl acetate has rose smell. Thus, if the market demand for natural rose smell increasing beyond what nature can offer, microbes can be used to produce 2-phenethyl acetate, by simply assembling the submodules

including i) an alcohol synthesis (e.g., 2-phenylethyl alcohol), ii) an acyl-CoA synthesis (e.g., endogenous acetyl-CoA), and iii) an AATase that specialized for condensing these two precursors (e.g., ATF1_{sc}).

While this is an example of the systematic modular ester synthesis at the molecular level, this platform can be easily moved to the cellular level to eliminate multiple cloning and transformation steps as well as to harness the specialty of each strain by taking advantage of the benefits of modular design principles. For example, a library of alcohol and ester producing strains can rapidly constructed by introducing alcohol production modules and ester synthesis modules into the strains A and B, respectively. Then, by using a “mix-and-match” approach at the cellular level, a large array of esters can be produced without further strain development while harnessing the specialty of each strain. For example, alcohol producing strain can utilize carbon source A (e.g., glucose), whereas ester producing strain can utilize carbon source B. (e.g., xylose). However, to establish a robust modular co-culture system, further studies on engineering of stable consortium(a) are required.

In summary, continued efforts in these areas would enable to establish a rapid, efficient, and systematic strain development platform for production of a large space of molecules (e.g., a large array of esters) while providing knowledge on engineering of efficient production modules as well as modular (chassis) cells.

VITA

Jong-Won Lee was born to In-Kyun Lee and Sun-Jin Jung on January 11, 1986 in Busan, South Korea. Jong-Won has an older brother, Jong-Moon Lee. Jong-Won graduated from Busan Dong-Sung High School in 2004. He attended Ajou University, earning a Bachelor of Engineering degree in Biological Engineering in 2008. He then performed compulsory military service as a Second and First Lieutenant in the Republic of Korea Army through the ROTC program for over 2 years. He continued studies at Seoul National University while being supported by Brain Korea 21 scholarships and earned a Master of Science degree in Bioengineering in 2012. During this time, he worked under the supervision of Dr. Jin-Ho Seo to study the production of 3-hydroxypropionic acid from xylose in recombinant *Escherichia coli*. He then moved on to Il-Dong Pharmaceutical Co., Ltd.'s Central Research Institute where he worked as a Research Scientist performing microbial production of biopharmaceuticals. Three years later he accepted a fellowship through the Bredesen Center for Interdisciplinary Research and Education at the University of Tennessee, Knoxville (UTK) in 2015 where he earned his PhD in Energy Science and Engineering. During this time, he worked in the lab of Dr. Cong T. Trinh in the Department of Chemical and Biomolecular Engineering at the UTK studying rapid design, construction, and validation of synthetic metabolic pathways in a modular *Escherichia coli* (chassis) cell. Jong-Won has a passion of contributing to the rapid development of industrial strains for accelerating the commercialization of biochemicals(fuels) from renewable resources. He plans to continue his career in the field of metabolic engineering to dedicate himself to the efforts for replacing current environmentally harmful chemical reactions with greener biological processes to build a sustainable future for the next generations.