

Design and Construction of Designer Bioester Libraries for Validation of the Modular Cell Theory

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

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December 2016

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Dedicated to my parents

Mitch and Sherry

and my grandparents

Barbra, Betty, Jay and Sam

Acknowledgements

First, I would like to thank and praise my savior, Jesus Christ. Without him, none of this would be possible.

Second, I would like to immensely thank my advisor, Dr. Cong T. Trinh, for all of the guidance and insight that has been instrumental to my research. The stimulating discussion and teachings have allowed me to pursue some of my crazy ideas and develop interesting techniques. I would not be writing this otherwise.

I would like to thank my committee members, Gladys Alexandre, Eric Boder, Paul Dalhaimer and my collaborators, Fu-Min Menn and Adam Guss for their discussions and support for my PhD. I am immensely grateful for Amy Brewer, Rita Gray, Amber Tipton and Jennifer Wolfenbarger for their general guidance and help with ordering my research materials. I am additionally very grateful for the Department of Chemical and Biomolecular Engineering at the University of Tennessee and the BioEnergy Science Center at Oak Ridge National Lab for admitting me to the program and their continual support for my research. I am immensely grateful for Laura Jarboe for sparking my initial interest in pursuing my graduate degree and passion for research at Iowa State University. I would additionally like to thank both Elsevier and Wiley publishing groups for publishing my works in this dissertation.

I would specifically like to thank Tyler Bennett, Brian Fane, R. Adam Thompson, and Michael Wierzbicki for lending their ear at any time of the day, sometimes at 3 am, discussing all ideas, and for their great friendship. The countless conversations, ideas, and

guidance throughout my graduate research would not be completed without them. I would like to thank my closest friends from home and Iowa State University for their friendship and their overwhelming support and interest in my research, in particular Emily Davenport, Greg Goin, Cody Hopkins, KC Keim, Ryan Kincade, Chuck Light, Brett Mech, Cody Moore, Jake O'Brien, Jarred O'Brien, and Mark Spero. I would additionally like to thank my closest friends I have developed at the University of Tennessee for their friendship, support, ideas and discussions, in particular, Rob Atkinson, Alex Meyers, Beth Conerty. I would also like to thank my lab mates for their friendship, support, discussions and for smelling my bacterial cultures, validating I wasn't crazy, in particular Lorenzo Briganti, D.J. Conner, Sergio Garcia, Julie Hipp, Drew Kirkpatrick, Jong-Won Lee, Katie Lutes, Brian Mendoza, Dr. Paulo Avilo Neto, Dr. Narayan Niraula, Dr. Seunghyun Ryu, Kevin Spellman, Caleb Walker, Brandon Wilbanks, and Akshitha Yarrabothoula. I would also like to further thank Brandon Wilbanks for all of the help and discussions with various aspects of my research and for making sacrifices to take time points in the middle of the night. I could not have done it all without him.

Finally, I would like to thank my family members including grandparents, aunts, uncles, cousins, siblings, and parents for all of their unfathomable encouragement love, kindness, and support. I would have never accomplished this work without them. I am grateful for my sisters Shannon and Allyson for all of their laughs and phone calls always checking in with me. I am also grateful for my parents Mitch and Sherry who without their instilled drive, fight, and determination, I would not be here today. I love you all.

Abstract

Renewable and sustainable fuels and chemicals are required for mankind to reduce their dependence on petroleum. Metabolic engineering and synthetic biology have provided avenues for production of renewable fuels and chemicals by using waste feedstocks derived from biomass, municipal, and off gases, such as carbon dioxide and methane, using microbial cell factories. However, development of optimal microbial cell factories has been a challenge due to the vast combinations of pathways, genetic parts, and hosts to produce a targeted product. The purpose of this work is for validation of the modular cell theory via rational pathway design and testing for development of optimal microbial cell factories. This dissertation is divided up into four different parts.

Part I focuses on engineering and production of butyrate ester libraries for use as fuels, flavors, fragrances and solvents, specifically ethyl butyrate, isopropyl butyrate, and isobutyl butyrate using a modular chassis cell derived from the modular cell theory. Part II focuses on the synthesis of designer esters from waste organic acids, the carboxylates, as well as characterizing the enzyme responsible for condensing ester precursor molecules for novel activity using the modular chassis cell. Part III focuses on the expansion of part II by modulating an ester precursor molecule for the production of novel esters that can be used as next generation biofuels. Part IV focuses on further validating the modular cell theory by using growth-based selection for ethanol production by varying open reading frames and genetic parts.

The work presented will validate and further provide insights to modular cell theory and ester biosynthesis from fermentable sugars and organic acids.

Table of Contents

1	Introduction	1
1.1	References	8
2	Engineering Modular Ester Fermentative Pathways in <i>Escherichia coli</i>.....	15
2.1	Abstract	16
2.2	Introduction	17
2.2.1	Materials and Methods.....	21
2.2.2	Strain construction	21
2.2.3	Plasmid/pathway construction	21
2.2.3.1	Construction of the butyryl-CoA submodule	26
2.2.3.2	Construction of the butyryl-CoA plus AAT submodule	27
2.2.3.3	Construction of the ethanol production submodule.....	27
2.2.3.4	Construction of the isopropanol production submodule.....	28
2.2.3.5	Construction of the isobutanol production submodule.....	28
2.2.4	Medium and cell culturing techniques.....	29
2.2.4.1	Culture media	29
2.2.4.2	Strain characterization for ester production.....	29
2.2.5	Analytical methods	31
2.2.5.1	High performance liquid chromatography (HPLC).....	31
2.2.5.2	Gas chromatography coupled with mass spectroscopy (GC/MS).....	31
2.3	Results	32
2.3.1	Establishing the butyrate ester fermentative pathways in <i>E. coli</i>	32
2.3.1.1	Investigating targeted butyrate ester production by alcohol addition..	33
2.3.1.2	Demonstrating extracellular secretion of butyrate esters	35
2.3.1.3	Engineering <i>E. coli</i> base strain for endogenous butyrate ester production from glucose	37
2.3.2	Optimizing endogenous production of butyrate esters	40
2.3.2.1	Endogenous butyrate ester production by the modular strain	40
2.3.2.2	Enhancing ester production by in-situ fermentation and extraction....	42

2.3.3	Demonstrating obligate ester fermentative pathways in the modular strain	43
2.4	Discussion	46
2.5	References	50
3	Expanding the Modular Ester Fermentative Pathways for Combinatorial Biosynthesis of Esters from Volatile Organic Acids	59
3.1	Abstract	60
3.2	Introduction	62
3.3	Materials and Methods	64
3.3.1	Strains and plasmids	64
3.3.1.1	Strains	64
3.3.1.2	Plasmids.....	65
3.3.1.3	Media and cell culturing conditions	71
3.3.2	Analytical methods	72
3.3.2.1	High performance liquid chromatography (HPLC).....	72
3.3.2.2	Gas chromatography coupled with mass spectroscopy (GC/MS).	73
3.3.3	Bioinformatics.....	74
3.4	Results	75
3.4.1	Establishing the acid-to-ester fermentative pathways in <i>E. coli</i>	75
3.4.2	Exploring combinatorial biosynthesis of esters	78
3.4.2.1	Ester production from co-fermentation of acetic acid and glucose.	78
3.4.2.2	Ester production from co-fermentation of glucose and propionic acid.	81
3.4.2.3	Ester production from co-fermentation of glucose and butyric acid. ..	86
3.4.2.4	Ester production from co-fermentation of glucose and pentanoic acid.	87
3.4.2.5	Ester production from co-fermentation of glucose and hexanoic acid.	89
3.4.3	Ester production from co-fermentation of glucose and mixed VOAs	90
3.4.3.1	Use of single cultures for upgrading mixed VOAs to target esters.	90
3.4.3.2	Use of mixed cultures for upgrading mixed VOAs to target esters.....	94
3.5	Discussion	95
3.6	References	99

4	Microbial Synthesis of a Branched-Chain Ester Platform from Organic Waste Carboxylates.....	106
4.1	Abstract	107
4.2	Introduction	109
4.3	Materials and Methods	110
4.3.1	Plasmids and strains.....	110
4.3.2	Media and cell culturing conditions.....	112
4.3.3	Analytical methods	113
4.3.4	Calculation of Octane Normalized Mass Energy Density (ONMED).....	115
4.4	Results and Discussion.....	115
4.4.1	Design of microbial biosynthesis of branched-chain ester platform	115
4.4.2	Expanding combinatorial biosynthesis of ester platforms	118
4.4.2.1	Microbial biosynthesis of an acetate ester platform.	118
4.4.2.2	Microbial biosynthesis of a propionate ester platform.	120
4.4.2.3	Microbial biosynthesis of a butyrate ester platform.	122
4.4.2.4	Microbial biosynthesis of a pentanoate ester platform.....	123
4.4.2.5	Microbial biosynthesis of a hexanoate ester platform.	123
4.5	Conclusion.....	124
4.6	References	130
5	Enabling Directed Metabolic Pathway Evolution via Growth Selection of Modular Cell.....	132
5.1	Abstract	133
5.2	Introduction	134
5.3	Materials and Methods	136
5.3.1	Strain construction	136
5.3.2	Plasmid/pathway construction	139
5.3.2.1	Construction of the PDC modules.	139
5.3.2.2	Construction of the ethanol production modules.....	140
5.3.3	Medium and cell culturing techniques.....	140
5.3.3.1	Culture media.	140

5.3.3.2	Strain characterization.	141
5.3.3.3	Evolution characterization.	141
5.3.3.4	Random mutagenesis.	142
5.3.4	2.4. Analytical methods	144
5.3.4.1	High performance liquid chromatography (HPLC).....	144
5.4	Results	144
5.4.1	Establishing production modules.....	144
5.4.1.1	Modular cell cannot function without an exchangeable production module.	144
5.4.1.2	Engineering PDC modules for growth-based selection.....	145
5.4.2	Demonstrating pathway selection by modular cell	147
5.4.3	Enabling directed evolution by modular cell	150
5.4.3.1	Directed evolution of EcDL108-EcDL112.....	150
5.4.3.2	Characterization of evolved EcDL108-EcDL112.	152
5.4.3.3	Random mutagenesis for selection of an optimal PDC.	155
5.4.4	Demonstrating modulation of genetic parts to enable degree of coupling between modular cell and production module	158
5.5	Discussion	161
5.6	References	165
6	Conclusion	168
Vita.		169

List of Tables

Table 2-1: A list of strains and plasmids.....	22
Table 2-2: A list of primers for plasmid and strain construction and validation	23
Table 2-3: Production of butyrate esters by EcDL201-6 in high-cell density fermentation experiments after 24 hr.	39
Table 3-1: A list of plasmids and strains.....	67
Table 3-2: A table of primers used.....	68
Table 3-3: Ester titers of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single VOAs after 24h.....	82
Table 3-4: Specific ester productivities of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h.....	83
Table 3-5: Ester yields on glucose of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h.	84
Table 3-6: Fermentation data of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h.	85
Table 3-7: Ester titers of EcDL102, EcDL104, EcDL105, and mixed cultures of EcDL102, EcDL104, and EcDL105 for co-fermentation of glucose and 5 g/L mixed organic acids after 24h.	94
Table 4-1: A list of plasmids and strains.....	111
Table 4-2: A list of primers used	112
Table 4-3: Ester titers of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single carboxylates after 24h.	126
Table 4-4: Ester yields on glucose of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h.....	127
Table 4-5: Specific ester productivities of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h.....	128
Table 4-6: Fermentation data of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h.	129
Table 5-1: A list of strains and plasmids used in this study.....	137

Table 5-2: A list of primers for plasmid construction and validation.....	138
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List of Figures

Figure 2-1: A simplified metabolic network for producing esters.....	19
Figure 2-2: <i>In-vivo</i> butyrate ester production via exogenous short chain alcohol addition.	34
Figure 2-3: Extracellular secretion of butyrate esters and organic solvent effects on cell growth.	36
Figure 2-4: Endogenous production of ethyl butyrate, isopropyl butyrate, and butyl butyrate.	38
Figure 2-5: Residual alcohol formation from endogenous butyrate production.....	40
Figure 2-6: Demonstration of modular cell growth coupling.	44
Figure 2-7: Fermentation kinetics of endogenous ethyl butyrate production by the coupled modular strain EcDL204 in the <i>in-situ</i> high-glucose fermentation and extraction.	45
Figure 3-1: Acid to ester pathways, carboxylate acids and their potential esters, and acid-to-ester design.	66
Figure 3-2: Phylogenetic analysis and structural and catalytic motifs of AATs.	77
Figure 3-3: Percent of esters that are secreted into hexadecane.	79
Figure 3-4: Ester production from exogenous addition of C ₂ -C ₆ acids.	80
Figure 3-5: AAT specificity from alcohols and acyl-CoAs.....	91
Figure 3-6: Mixed acid ester production.....	93
Figure 4-1: Branched chain ester synthesis.	116
Figure 4-2: Ester production of EcDL207, 208, and 209 after 24 h from co-fermentation of glucose and organic acids.	119
Figure 4-3: Physical properties of carboxylates, alcohols, and esters.	121
Figure 5-1: Homoethanol pathway for modular cell growth selection.	146
Figure 5-2: Fermentation kinetics for EcDL107-EcDL112.....	148
Figure 5-3: Directed evolution data for EcDL108-EcDL112 for 150 generations.....	151

Figure 5-4: Fermentation kinetics, including cell growth, glucose consumption, ethanol production, and specific growth rate with specific ethanol production rate for EcDL109- EcDL112 at generation 75, compared to initial fermentation kinetics.	153
Figure 5-5: Growth kinetics of the evolved plasmid in the unevolved chassis host compared to the initial growth kinetics for EcDL108, EcDL110, EcDL111.	154
Figure 5-6: Growth kinetics of the evolved host chassis with unevolved pCT15 and pDL017-pDL021.....	155
Figure 5-7: Initial kinetic growth rates of random mutagenesis study before splitting anaerobic growth into triplicates.....	156
Figure 5-8: Growth kinetics for pDL020 and randomly mutated pDL020 in the host chassis cell.	157
Figure 5-9: Maximum growth rate and ethanol production rates of random mutagenesis plasmids.	158
Figure 5-10: Growth kinetics of TCS083, EcDL107 EcDL116.	159
Figure 5-11: Analysis of degrees of coupling of the modular chassis cell with ethanol production modules including pCT24, pAY1, and pAY3.	160

1 Introduction

Non-renewable based feedstocks such as petroleum, coal, and natural gas are the basis for most consumer products and are non-sustainable and environmentally harmful (Hill et al., 2006). In 2015, the United States consumed over 97.5 quadrillion British thermal units of energy with less than 10% being supplied from renewable resources (EIA, 2016). Renewable resources need to be developed to reduce our dependence on non-renewable feedstocks. Therefore, utilization of energy from the sun, e.g. biomass, helps reduce our dependence on nonrenewable feedstocks and provides a renewable and sustainable alternative route for making biologically derived products.

Several generations of biomass feedstocks have been explored over the years for production of industrial platform chemicals and have been seen as both renewable and sustainable. First generation of biomass feedstocks applied agricultural crops, such as corn, to make renewable chemicals, specifically ethanol, however sparked a blistering fuel-vs-food debate (Naik et al., 2010). Second generation biomass applied greener technologies, i.e. thermochemical and biochemical processing, towards lignocellulosic biomass for renewable chemical production, but the necessity of year-round crop for sustainability and biomass recalcitrance becomes direct issues (Naik et al., 2010). Third generation biomass utilized genetically modified crops for reducing recalcitrance and providing more fermentable sugars for microbes as well as algae for product formation (Yang et al., 2015). Lastly, fourth generation of biomass used energy storage crops and organic wastes from various sources, such as organic fruit peels, paper waste, and organic life waste (Yang et

al., 2015). These generations of biomass feedstocks provide flexibility for novel biotransformation to achieve a more sustainable chemical economy independent from petroleum. However, engineering biocatalysts (or microorganisms) is essential for conversion of biomass substrates into desired biochemicals.

Laboratory workhorse, *Escherichia coli*, has been engineered to produce a plethora of chemicals from biomass based fermentable sugars, such as alcohols (Atsumi et al., 2008; Ingram et al., 1987; Trinh et al., 2011), biodiesels (Wierzbicki et al., 2016; Zhang et al., 2012), pharmaceuticals (Martin et al., 2003), and plastics (Jung et al., 2010). However, prior to this work, *E. coli* had not been engineered to produce short chain esters, such as butyrate esters, directly from glucose or other carbon sources. Esters represent a broad class of industrial chemicals that can be used in various industries including flavor, fragrances, solvents, lubricants, and fuels. The top 10 companies in flavor and fragrance industry, alone, generated over \$24 billion dollars in sales in 2014 according to www.leffingwell.com which include ester production.

Esters are traditionally produced via Fisher esterification by catalyzing an acid and alcohol with high heat and additional strong acid (Riemenschneider and Bolt, 2000). Conversely, esters can be produced via natural extraction from their food sources, post separation, (e.g. apples (Mattheis et al., 1991), banana (Tressl and Drawert, 1973), grapes (Stern et al., 1967), melon (Wyllie and Leach, 1990), oranges (Schultz et al., 1967), peach (Sevenants and Jennings, 1966), pear (Rizzolo et al., 2005), pineapple (Connell, 1964) and strawberry (Larsen et al., 1992)), or microbially synthesized using yeast (Suomalainen and Lehtonen, 1979; Verstrepen et al., 2003). However, natural extraction reignites the food-vs-fuel debate by redirecting biomass and nutrition away from human consumption. Thus,

using microbial biocatalysts and renewable based feedstocks would be advantageous to generate esters.

Esters can be biologically produced in microbes using three different methods: i) condensation of an acyl-CoA and an alcohol via an alcohol ac(et)yltransferase (AAT), ii) dehydration of a hemiacetal with conversion of NAD(P)^+ to NAD(P)H via an alcohol dehydrogenase (ADH), and iii) conversion of a ketone with conversion of NAD(P)H to NAD(P)^+ via a Baeyer-Villager monooxygenase (BVMO) (Park et al., 2009). The lack of a cofactor or oxygen utilization might lend distinct advantage towards AATs, which are redox neutral, allowing for cofactors to be directed towards product precursors, such as alcohols. Thus, engineering alcohols and acyl-CoAs, to be condensed via AATs, generates scientific questions to produce specific or libraries of targeted products, including; i) where are alcohols and/or acyl-CoAs naturally produced? ii) how can we engineer production of alcohols and/or acyl-CoAs? and iii) what AAT is best for engineered alcohols and acyl-CoAs to generate a targeted ester?

Microbial engineering for alcohol formation is well studied and reviewed (Atsumi and Liao, 2008; Chen et al., 2013a; Clomburg and Gonzalez, 2010; Lamsen and Atsumi, 2012; Li et al., 2010; Wang et al., 2012; Yan and Liao, 2009)). Some of these alcohols, e.g. ethanol and butanol, can be derived from CoA pathways (Jones and Woods, 1986) which can be truncated to solely produce the precursor CoA moiety for ester synthesis. However, this route of CoA generation is limited and extensive mining and construction of CoA pathways from glucose would be time consuming and expensive. Thus, finding alternative routes for generating CoA moieties becomes advantageous for both chemical production and *in vivo* enzyme screening for ester production.

Acyl-CoA transferases, (ACTs) are a general class of enzymes (E.C. 2.8.3.X) that can convert acyl acids and a CoA, primarily acetyl-CoA, to their respective acyl-CoA counterpart and a CoA based acid, e.g. acetate. This enzyme lends a hand to CoA generation for ester biosynthesis. *Clostridium propionicum* encompasses one of these enzymes, propionyl-CoA transferase, PCT, that exhibits some promiscuous activity for substrates toward CoA formation (Schweiger and Buckel, 1984). PCT is promising for the production of generating novel CoA molecules from acyl acids. If AATs are capable of utilizing unique acyl-CoAs, the next question is where can acyl acids be found in nature?

Anaerobic digesters are a mixed consortia of microorganisms that are capable for biomass degradation to generate acyl acids (Agler et al., 2011; Holtzapple, 2015; Holtzapple et al., 1999; Thanakoses et al., 2003). Anaerobic digesters produce C₂-C₆ linear, saturated, carboxylic acids, also known as the carboxylates. These carboxylates are often inhibitory towards the cell and have little intrinsic value. However, this platform can be biologically upgraded to produce a plethora of chemicals, including alcohols as well as potential esters (Holtzapple et al., 1999; Levy et al., 1981; Steinbusch et al., 2008), from all generations of biomass. Nevertheless, ester formation might be limited by the AATs that are used for condensation, and thus far alcohol and acyl-CoAs testing has been limited to a small library (Aharoni et al., 2000; Balbontin et al., 2010; Beekwilder et al., 2004; Crowhurst et al., 2008; Cumplido-Laso et al., 2012; Defilippi et al., 2005; Dunemann et al., 2011; El-Sharkawy et al., 2005; Galaz et al., 2013; Gonzalez-Aguero et al., 2009; Gonzalez et al., 2009; Gunther et al., 2011; Li et al., 2007; Li et al., 2006; Lucchetta et al., 2007; Morales-Quintana et al., 2011; Morales-Quintana et al., 2015; Morales-Quintana et al., 2012; Morales-Quintana et al., 2013; Olías et al., 2002; Saerens et al., 2006; Souleyre

et al., 2005; Verstrepen et al., 2003; Zhang et al., 2010). Therefore, further understanding of AATs would be advantageous for directed product formation from carboxylate producing CoAs and alcohols. Utilizing ACT enzymes, coupled with engineered alcohol formation, leads toward advantageous screening of AAT activity and increasing the number of esters that can be produced from engineered metabolic pathways. However, engineering optimal microbial cell factories to produce esters at high titer, rates, and yields is necessary.

Computational tools have been developed for understanding and modeling cellular metabolism (Antoniewicz, 2015; Edwards et al., 2001; Fell and Small, 1986; Orth et al., 2010; Papin et al., 2004; Schilling et al., 2000; Schuster et al., 2000; Trinh et al., 2009; Varma and Palsson, 1994; Wiechert, 2001) and for rationally designing microbial strains (Burgard et al., 2003; Flowers et al., 2013; Hadicke and Klamt, 2010; Hadicke and Klamt, 2011; Trinh et al., 2008). Additionally, synthetic biology is continually developing genetic parts, e.g. promoters, ribosome binding sites, terminators (Blazeck et al., 2011; Cambray et al., 2013; Chen et al., 2013b; Cox et al., 2007; Mutalik et al., 2013; Mutalik et al., 2012; Na et al., 2010; Redden and Alper, 2015; Rhodius and Mutalik, 2010; Salis et al., 2009; Seo et al., 2013) that can be used for modulating product pathways. Generating combinatorial libraries of product pathways has been heavily investigated using modulation of similar enzymes and transcriptional parts (Du and Ryan, 2015; Freestone and Zhao, 2016; Jin et al., 2016; Jones et al., 2015; Li et al., 2013; Schaerli and Isalan, 2013; Smanski et al., 2014). Thus generating and screening combinatorial libraries of microbial cell factories that use strain design aspects and synthetic biology is not only daunting but can become prohibitive.

The modular cell theory (MODCELL) was designed as a rational approach to optimize a chassis cell for engineered product formation (Trinh et al., 2015). MODCELL was designed to generate auxotrophic modular cells that contain essential metabolic pathways and couple growth to an auxiliary pathway, i.e. desired production pathway, by balancing cellular redox and precursor metabolites. If an auxiliary pathway is not available, the cell does not sustain growth. Modular cells are designed for minimal strain iterative steps for combinatorial synthesis of biochemicals and are built using plug-and-play production modules for generating optimal microbial cell factories. Modular cells can also be a powerful selection platform; that is the most efficient production module couples to the modular cell which grows the fastest. Therefore, this selection platform can easily be applied to combinatorial libraries, for producing esters and alcohols, resulting in the most optimal product pathway being selected with minimal rounds of culture transfer.

This work is my effort to validate the modular cell theory by applying modular pathway design principles to a modular chassis cell, derived from the modular cell theory, for production of esters and ethanol. Validation of the modular cell theory consists of four main chapters. Chapter 2 describes the design and construction of a butyrate ester platform with enhanced butyrate ester formation and demonstrating initial product growth coupling using the modular chassis cell. Chapter 3 describes the design and construction of a carboxylate to ester platform as well as screening AATs for *in vivo* activity and specificity using the modular cell. Chapter 4 describes expanding the carboxylate to ester platform for the synthesis of novel branched chain esters as potential drop-in biofuels. Chapter 5 describes the selection of an optimal pyruvate decarboxylase for ethanol production from

a small library and a combinatorial library as well as selection of an optimal ethanol pathway by varying genetic parts. I envision this work to be the basis for refining and streamlining the theory, design, and construction of optimal microbial cell factories for the production of biochemicals.

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2 Engineering Modular Ester Fermentative Pathways in *Escherichia coli*

Summary: This chapter is based on the published paper: Layton, Donovan S., and Cong T. Trinh. "Engineering modular ester fermentative pathways in *Escherichia coli*." *Metabolic Engineering* 26 (2014): 77-88.

2.1 Abstract

Sensation profiles are observed all around us and are made up of many different molecules, such as esters. These profiles can be mimicked in everyday items for their uses in foods, beverages, cosmetics, perfumes, solvents, and biofuels. Here, we developed a systematic ‘natural’ way to derive these products via fermentative biosynthesis. Each ester fermentative pathway was designed as an exchangeable ester production module for generating two precursors— alcohols and acyl-CoAs that were condensed by an alcohol acyltransferase to produce a combinatorial library of unique esters. As a proof-of-principle, we coupled these ester modules with an engineered, modular, *E. coli* chassis in a plug-and-play fashion to create microbial cell factories for enhanced anaerobic production of a butyrate ester library. We demonstrated tight coupling between the modular chassis and ester modules for enhanced product biosynthesis, an engineered phenotype useful for directed metabolic pathway evolution. Compared to the wildtype, the engineered cell factories yielded up to 48-fold increase in butyrate ester production from glucose.

2.2 Introduction

Chemicals and transportation fuels are mainly derived from petroleum-based feedstocks, which are not renewable or sustainable. Their increasing demand has adversely affected market prices, the environment, and national energy security (Hill et al., 2006). To tackle this problem, recent research has focused on exploiting microbial conversion routes to produce these chemicals and fuels from renewable and sustainable biomass feedstocks (Lynd et al., 2008; Somerville et al., 2010; Stephanopoulos, 2008). Some of the challenges are to overcome biomass recalcitrance, and engineer novel microbial biocatalysts that can efficiently convert lignocellulosic biomass into target biochemicals and biofuels and replace those synthesized from petroleum-based feedstocks in a competitive manner (Himmel et al., 2007; Stephanopoulos, 2007).

Esters are valuable chemicals that have broad applications in foods, beverages, cosmetics, perfumes, solvents, and biofuels. Most esters R_1COOR_2 are currently produced by the Fisher esterification of an organic acid R_1COOH and an alcohol R_2OH derived from the petroleum-based feedstocks under hazardous conditions using corrosive acid/base and high temperature (Riemenschneider and Bolt, 2000). The diversity of R_1 and R_2 moieties consisting of linear, branched, even, odd, saturated, unsaturated, and/or aromatic carbon structures can cover a large combinatorial space of esters with unique properties. Since esters are commonly found in living species, such as plants and microbes, there is great potential to produce these esters via microbial conversion routes from renewable and sustainable feedstocks.

In living cells, esters can be esterified from an ac(et)yl-CoA (or aryl-CoA) and an alcohol via an alcohol acyltransferase (AAT). Alcohols such as ethanol (Ohta et al., 1991;

Trinh et al., 2008), propanol (Jain and Yan, 2011; Jun Choi et al., 2012; Shen and Liao, 2008; Srirangan et al., 2013) , isopropanol (Hanai et al., 2007; Lee et al., 2012), butanol (Bond-Watts et al., 2011; Inui et al., 2008; Lee et al., 2008; Nielsen et al., 2009), and isobutanol (Atsumi et al., 2008; Trinh et al., 2011) can be synthesized from either fermentative or Ehrlich pathways, and ac(et)yl-CoAs from the *de novo* fatty acid biosynthesis (Handke et al., 2011; Magnuson et al., 1993) or reverse β -oxidation cycle (Dellomonaco et al., 2011) (Figure 2-1).

With the diversity of alcohol and ac(et)yl-CoA synthesis, a combinatorial library of esters can be synthesized, and many of these esters can be found in nature such as fruits-bananas (Beekwilder et al., 2004; Harada et al., 1985), melons (El-Sharkawy et al., 2005; Lucchetta et al., 2007; Shalit et al., 2001; Yahyaoui et al., 2002), strawberries (Aharoni et al., 2000; Olías et al., 2002; Perez et al., 1993; Perez et al., 1996), and plant waxes (Kunst and Samuels, 2003; Kunst and Samuels, 2009; Samuels et al., 2008) or brewery yeast (Suomalainen and Lehtonen, 1979). For instance, esterification of isobutanol and butyryl-CoA yields isobutyl butyrate, which has a fruity aroma and can be used as food supplement or solvent for lacquer and nitrocellulose. Some of these “natural” esters can also be synthesized by recombinant hosts such as *E. coli*, *L. lactis*, and *C. acetobutylicum* expressing different AATs (Aharoni et al., 2000; Beekwilder et al., 2004; El-Sharkawy et al., 2005; Harada et al., 1985; Hernandez et al., 2007; Lucchetta et al., 2007; Olías et al., 2002; Park et al., 2009; Perez et al., 1993; Perez et al., 1996; Rodriguez et al., 2014; Shalit et al., 2001; Vadali et al., 2004; Yahyaoui et al., 2002). Recently, recombinant *S. cerevisiae* and *E. coli* were also engineered to produce fatty acid methyl (Menendez-Bravo et al., 2014; Nawabi et al., 2011), ethyl, (Kalscheuer et al., 2006; Runguphan and Keasling, 2014; Shi et al.,

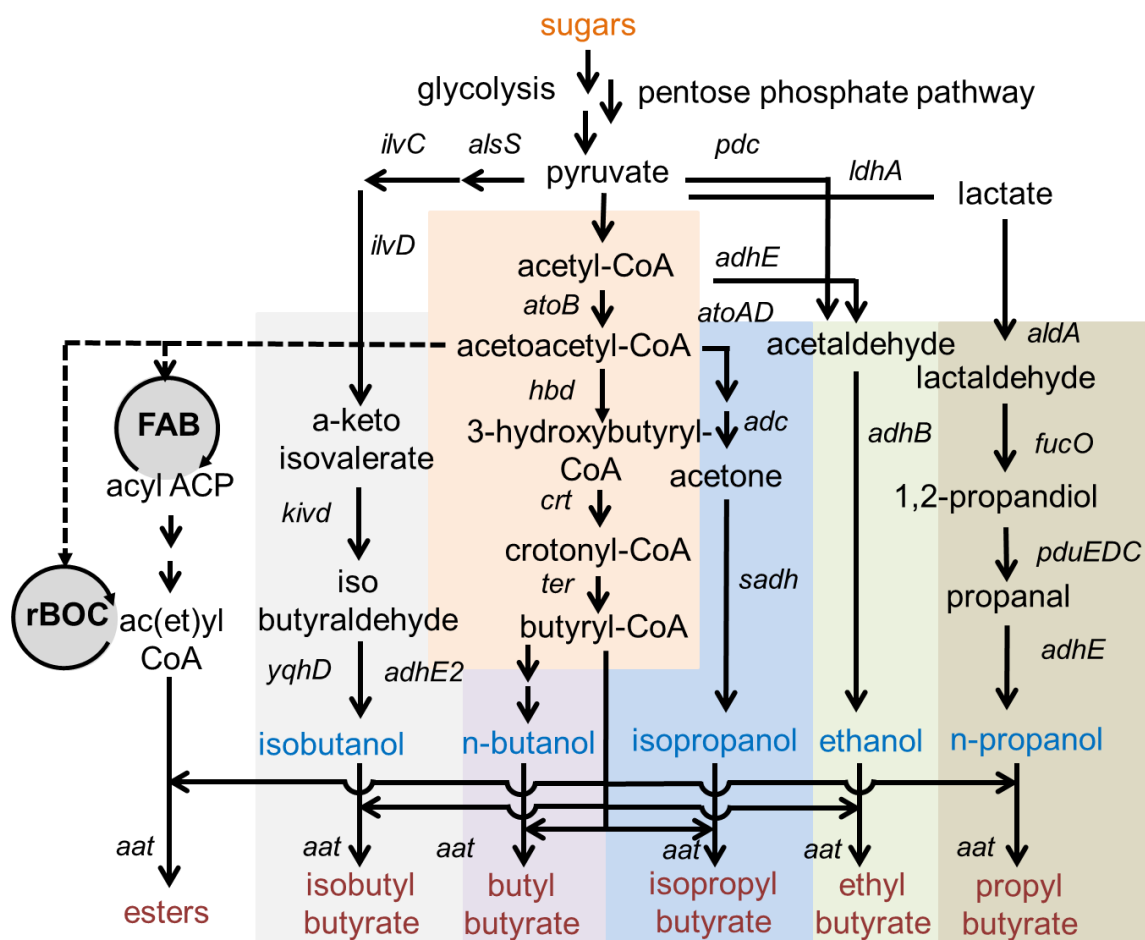


Figure 2-1: A simplified metabolic network for producing esters.

The simplified network demonstrates the production of esters via esterification of alcohols (e.g., ethanol, propanol, isopropanol, butanol, and isobutanol) and acyl-CoAs from biomass-derived fermentable sugars by microbial conversion routes. Acyl-CoAs can be derived from the fermentative pathways, the fatty acid biosynthesis pathway (FAB), or the reverse β -oxidation cycle (rBOC).

2014; Steen et al., 2010; Yu et al., 2011) and short-chain esters as biodiesels (Guo et al., 2014), which expands the ester solution space beyond what has been previously mentioned.

In this study, we designed and constructed modular ester producing pathways that tightly couple with a modular *E. coli* chassis designed for efficient combinatorial biosynthesis of novel esters under anaerobic conditions. Anaerobic fermentation is the most efficient and economical route to produce biochemicals and biofuels such as esters due to the following reasons: 1) the reducing equivalent NADH generated from sugar degradation can be effectively recycled by modular ester fermentative pathways to enhance ester production and 2) scale-up fermentation processes are much simpler and less expensive since the supply and precise control of air are not required and contamination issues can be minimized (Blanch, 2012; Huang and Zhang, 2011; Trinh, 2012; Trinh et al., 2011). We designed each ester fermentative pathway as an exchangeable ester production module for producing two precursors, alcohols and acyl-CoAs, which were then condensed by an alcohol acyltransferase to produce a combinatorial library of unique esters. As a proof-of-principle, we coupled each of the fermentative ester modules with the modular chassis in a plug-and-play fashion to create modular microbial cell factories for enhanced production of a butyrate ester library. We demonstrated these cell factories could secrete esters extracellularly and enhance ester production via *in-situ* fermentation and extraction. By performing kinetic characterization of the optimized ethyl butyrate production strain, we demonstrated tight coupling between the modular chassis and the ester production modules for enhanced butyrate ester production, an engineered novel phenotype useful for the directed metabolic pathway evolution.

2.2.1 Materials and Methods

2.2.2 Strain construction

Table 2-1 lists strains used in this study. *E. coli* TOP10 was primarily used for molecular cloning. *E. coli* MG1655 and its derivatives were used for ester production studies. Mutants with chromosomal gene deletion were constructed by using the P1 transduction (Trinh et al., 2006). To construct mutants for expressing T7 promoter, the prophage λ DE3 was used to insert a T7 polymerase gene into the specific site of the mutant chromosome by using a commercial kit (cat#69734-3, Novagen Inc.) Mutants with gene deletion or/and addition were confirmed by PCR amplification with the primers listed in Table 2-2.

2.2.3 Plasmid/pathway construction

Modular metabolic pathway engineering strategy (Tseng and Prather, 2012; Xu et al., 2013; Xu et al., 2012) was applied to design ester fermentative pathways as exchangeable ester production modules that are derived from multiple sub-modules. In general, each ester production module consists of an acyl-CoA production submodule, an AAT production submodule, and an alcohol production submodule. Each submodule is designed as one operon that contains at least one gene under a consecutive T7 promoter. The acyl-CoA and AAT production submodules are organized in a pETite* vector backbone using the ampicillin selection marker. The alcohol production submodule is organized in a different pETite* vector using the kanamycin selection marker. This modular metabolic pathway engineering enables quick replacement of ribosome binding sites, promoter strengths, and origin of replications for manipulating plasmid copy number to control and optimize metabolic fluxes through the ester fermentative pathways.

Table 2-1: A list of strains and plasmids

Plasmids/Strains	Genotypes	Sources
Plasmids		
pCP20	<i>flp, bla, cat, cl857</i> λts	Yale collection
pCOLA	kan ⁺	Novagen
pETite C-His	pBR322 <i>ori</i> ; kan ⁺	Lucigen
pETite*	kan ^R	this study
pCT13	pCOLA P _{T7} ::RBS:: <i>alsS</i> ::RBS:: <i>ilvC</i> ::RBS:: <i>ilvD</i> –P _{T7} ::RBS:: <i>kivd</i> ::RBS:: <i>adhE</i> ::T _{T7} ; kan ⁺	Trinh 2011
pCT24	pETite* P _{T7} ::RBS:: <i>pdC</i> ::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pCT79	pETite* P _{T7} ::RBS:: <i>atoD</i> ::RBS:: <i>atoA</i> ::RBS:: <i>thl</i> ::RBS:: <i>adc</i> ::RBS:: <i>sadh</i> ::T _{T7} ; kan ⁺	this study
pAY7	pETite* P _{T7} :: <i>atoD</i> :: <i>atoA</i> ; kan ⁺	this study
pDL1	pETite* SAAT; kan ⁺	this study
pDL2	pETite* P _{T7} ::RBS:: <i>atoB</i> ::RBS:: <i>hbd</i> ::RBS:: <i>crt</i> ::RBS:: <i>ter</i> ::T _{T7} ; kan ⁺	this study
pDL3	pETite* P _{T7} ::RBS:: <i>atoB</i> ::RBS:: <i>hbd</i> ::RBS:: <i>crt</i> ::RBS:: <i>ter</i> ::RBS:: <i>T_{T7}::saat</i> ::T _{T7} ; amp ⁺	this study
Strains		
<i>T. denticola</i>	wildtype	ATCC 35405
<i>C. acetobutylicum</i>	wildtype	ATCC 824
TOP10	F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>) 7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
JW5020-1	BW25113 Δ <i>fadE</i> ::kan ⁺	CGSC#11134
MG1655	F ⁺ λ [–]	ATCC 700926
TCS083	MG1655, Δ <i>zwf</i> ::Δ <i>ndh</i> ::Δ <i>sfcA</i> ::Δ <i>maeB</i> ::Δ <i>ldhA</i> ::Δ <i>frdA</i> ::Δ <i>poxB</i> ::Δ <i>pta</i> ::kan [–] (cured)	Trinh 2008
EcDL001	MG1655 (IDE3) Δ <i>fadE</i> ::kan [–] (cured)	this study
EcDL002	TCS083 (IDE3) Δ <i>fadE</i> ::kan [–] (cured)	this study
EcDL101	EcDL001 carrying pDL3; amp ⁺	this study
EcDL201	EcDL001 carrying pDL3 and pCT24; kan ⁺ and amp ⁺	this study
EcDL202	EcDL001 carrying pDL3 and pCT13; kan ⁺ and amp ⁺	this study
EcDL203	EcDL001 carrying pDL3 and pCT79; kan ⁺ and amp ⁺	this study
EcDL204	EcDL002 carrying pDL3 and pCT24; kan ⁺ and amp ⁺	this study
EcDL205	EcDL002 carrying pDL3 and pCT13; kan ⁺ and amp ⁺	this study
EcDL206	EcDL002 carrying pDL3 and pCT79; kan ⁺ and amp ⁺	this study

Table 2-2: A list of primers for plasmid and strain construction and validation

Primer name	Sequences
Primers used to build the butyryl-CoA submodule (pDL2)	
DL_0001	5'-CATCATCACCACCATCACTAA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
DL_0003	5'- TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATA CAT ATGAAAAATTGTGTCATCGT-3'
DL_0004	5'-TTAATTCAACCGTTCAATCAC-3'
DL_0005	5'- CGGTCAGGGAATTGCGATGGTGATTGAACGGTTGAA TTAAA AAGAGGAGAAAATGAAAAAGGTATGTGTTATAG-3'
DL_0006	5'-TTATTTTGAATAATCGTAGAAAC-3'
DL_0007	5'- AAGAAAATCAGGAAAAGGTTTCTACGATTATTCAAA ATAAA AAGAGGAGAAAATGGA ACTAAACAATGTCATC-3'
DL_0008	5'-CTATCTATTTTTGAAGCCTTC-3'
DL_0009	5'- CATAGAGAAAAGAAAAATTGAAGGCTTCAAAAATAG ATAG AAAGAGGAGAAAAT GATTGTAAAACCAATGGTTAG-3'
DL_0010	5'- GCCGCTCTATTAGTGATGGTGGTGATGATGTAAATC CTGTC GAACCTT T-3'
Primers used to build the ethanol submodule (pCT24)	
P006_f	5'- GAAGGAGATATACATATGAGTTATACTGTCGGTACCT ATTT AGCGGAG-3'
P006_r	5'- GTGATGGTGGTGATGATGCTCGAGTTAGGATCCCTAG AGGA GCTTGTTAACAGG-3'
P007_f	5'- AAAAAACTCGAGTTAGGATCCTTAGAAAGCGCTCAG GAAGAG -3'
P007_r	5'- AAAAAAGAATTCATGAGATCTAAGGAGATATAATGG CTTCT TCAACTTTTTAT-3'

Table 2-2 continued

Primer name	Sequences
Primers used to build the isopropanol submodule (pCT79)	
AY.22f	5'-AAAAAACATATGAAAACAAAATTGATGACATTA-3'
AY.22r	5'-AAAAAAAGATCTTTATTTGCTCTCCTGTGAAAC-3'
AY.23f	5'- AAAAAAGGATCCAAGGAGATATAATGGATGCGAAAC AAC GTATT-3'
AY.23r	5'-AAAAAACTCGAGTCATAAATCACCCCGTTGCGT-3'
NN35	5'-ACTTACTATTACTACATCTCTcatTATATCTCCTTtcaTA AATCACCCCGTTGCGTATT3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
NN36	5'- AATACGCAACGGGGTGATTTATGAAAGGAGATATAAT GAGAGAT GT AGTAATAGT AAGT-3'
NN37	5'- TTTAATTACTTCATCCTTTAACATTATATCTCCTTTTAG TCTCTTTC AA CTACGAGAG C-3'
NN38	5'- GCTCTCGTAGTTGAAAGAGACTAAAAGGAGATATAAT GTTAAAG GAT GAAGTAATT AAA-3'
NN39	5'- ACCTAGCATTGCAAAACCTTTCATTATATCTCCTTTTA CTTA AGATAATCATATATAAC-3'
NN40	5'- GTTATATATGATTATCTTAAGTAAAAGGAGATATAAT GAAA GGTTTTGCAATGCTAGGT-3'
MW.sadh.r	5'- GTGGCGGCCGCTCTATTAGTGATGGTGGTGATGATGT TATA ATATAACTACTGCTTTAAT-3'
Primers used to build the AAT submodule (pDL1)	
DL_0001	5'-CATCATCACCACCATCACTAA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
pETiteDL_0011	5'- GAAATAATTTTGTTTAACTATAAGAAGGAGATATACA TATG GAGAAAATTGAGGTCAG-3'
pETiteDL_0012	5'- GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTA AATT AAGGTCTTTGGAG-3'

Table 2-2 continued

Primer name	Sequences
Primers used to build the butyryl-CoA plus AAT submodule (pDL3)	
DL_0003	5'- TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATA CATA TGAAAAATTGTGTCATCGT-3'
DL_0014	5'-ATATCAAGCTTGAATTCGTTACCCGG-3'
pETiteDL_0015	5'- GGAGGAACTATATCCGGGTAACGAATTCAAGCTTGAT ATTA ATACGACTCACTATAGGG-3'
pETiteDL_0016	5'-GTCCAGTTACGCTGGAGTCTGAGGCTC-3'
DL_0013	5'-GAGCCTCAGACTCCAGCGTA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
Primers used for knockout verification	
P043_KO_ldhA_f	5'-TTTCTGGCGGATTTTATCG-3'
P043_KO_ldhA_r	5'-CGTCAACGGCACAAGAATAA-3'
P051_KO_frdA_f	5'-AGTTGATGCAACCGGAGAAC-3'
P051_KO_frdA_r	5'-ACGGCGAGACAAATTTTACG-3'
maeA_fwd_KO	5'-CAGCGTAGTAAATAACCCAACC-3'
maeA_rev_KO	5'-GACAGCTTAACGGCTTTGTAG-3'
P044_KO_zwf_f	5'-CGATGAACGGTCGAAGTTTT-3'
P044_KO_zwf_r	5'-TGCCATAGCAGCAATACTCG-3'
P049_KO_ndh_f	5'-GCAGACGCACAAATTCAAGA-3'
P049_KO_ndh_r	5'-ACGGGAACACCTCCTTCTTT-3'
maeB_fwd_KO	5'-GATGATAATGGCGAATGGAC-3'
maeB_rev_KO	5'-CGTTCTTTATCCATGAGTCG-3'
P045_KO_pta_f	5'-TCACTGGTGGTATCGGTGAA-3'
P045_KO_pta_r	5'-GAATGCGAAATGAGTGTGGA -3'
P046_KO_poxB_f	5'-ATGGATATCGTCGGGTTTGA-3'
P046_KO_poxB_r	5'-AAGCAATAACGTTCCGGTTG-3'
fadE_fwd_KO	5'-CGCATTATTCGGCCTACGGTTC-3'
fadE_rev_KO	5'-CCAGACTCCGTTTGTAAATGCAACAC-3'
DE3_fwd	5'-ATGAACACGATTAACATCGC-3'
DE3_rev	5'-TTACGCGAACGCGAAGTC-3'

Genes encoding the ester fermentative pathways are organized in plasmids. Table 2-1 shows a list of plasmids used and generated in this study, and Table 2-2 presents a list of primers used for the plasmid construction and validation. All plasmids were constructed using the pETite* backbone that was modified from pETite to be compatible with the BglBrick Gene Assembly method (Anderson et al., 2010). The XhoI restriction site in the kanamycin gene of pETite* and EcoRI restriction site downstream of the T7 promoter was removed via site-directed mutagenesis (cat#210519-5, Agilent Inc.) by using the primers P004_f/P004_r and P013_f/P013_r, respectively. A new BglII restriction site that is 50 nucleotides upstream of the T7 promoter was created by using the primers P012_f/P012_r.

2.2.3.1 Construction of the butyryl-CoA submodule

The design of the butyryl-CoA production submodule is pDL2, pETite* P_{T7}::RBS::*atoB*::RBS::*hbd*::RBS::*crt*::RBS::*ter*::T_{T7}, where P_{T7} and T_{T7} are T7 promoter and T7 terminator, respectively. This submodule converts acetyl-CoA to butyryl-CoA that is based on the fermentative butanol pathway of *Clostridium acetobutylicum* and was previously demonstrated to be functional in *E. coli* (Inui et al., 2008). The plasmid pDL2 was assembled by the Gibson Gene Assembly method (Gibson et al., 2009) using 5 DNA fragments: the backbone fragment amplified from pETite* using the primers DL_0001/DL_0002, the gene *atoB* amplified from the genomic DNA of *E. coli* MG1655 using the primers DL_0003/DL_0004, the genes *hbd* and *crt* amplified from *C. acetobutylicum* using the primers DL_0005/DL_0006 and DL_0007/DL_0008, respectively, and the gene *ter* amplified from *Treponema denticola* using the primers DL_0009/DL_0010.

2.2.3.2 Construction of the butyryl-CoA plus AAT submodule

The SAAT gene was used to construct the butyryl-CoA plus AAT submodule–pDL3 (pETite* P_{T7}::RBS::*atoB*::RBS::*hbd*::RBS::*crt*::RBS::*ter*::RBS::T_{T7}::*saat*::T_{T7}). SAAT was derived from the strawberry alcohol acyltransferase and provided by Dr. Jules Beekwilder (Plant Research International) as a kind gift. This gene was amplified from the plasmid pRSET-SAAT (Aharoni et al., 2000) by using the primers DL_0011/DL_0012 and inserted into pETite* to create pDL1. The assembly of the butyryl-CoA plus SAAT submodule to form pDL3 was performed by using the Gibson Gene Assembly method with 3 DNA pieces: the butyryl-CoA operon amplified from pDL2 using the primers DL_0003/DL_0014, the AAT operon amplified from pDL1 using the primers DL_0015/DL_0016, and the backbone pETite* amplified using the primers DL_0013/DL_0002.

2.2.3.3 Construction of the ethanol production submodule

The ethanol production submodule (pCT24, pETite* P_{T7}::RBS::*pdh*::RBS::*adhB*::T_{T7}) was designed to convert pyruvate to ethanol. The plasmid pCT24 was assembled by the BglBrick Gene Assembly method using 3 DNA pieces: the gene *pdh* amplified from pLOI297 using the primers P006_f/P006_r and digested with NdeI/BamHI, the gene *adhB* amplified from pLOI297 using the primers P007_f/P007_r and digested with BglII/XhoI, and the vector backbone pETite* doubly digested with NdeI/XhoI.

2.2.3.4 Construction of the isopropanol production submodule

The isopropanol production submodule— pCT79 (pETite* P_{T7}::RBS::*atoD*::RBS::*atoA*::RBS:: *thiL*::RBS::*adc*::RBS::*sadH*::T_{T7}) was designed to convert acetyl-CoA to isopropanol based on the previous study (Hanai et al., 2007). We first constructed pAY7 (pETite* P_{T7}::RBS::*atoD*::RBS::*atoA*::T_{T7}) by amplifying the genes *atoD* and *atoA* from the genomic DNA of *E. coli* MG1655 with the primers AY.22f/AY.22r and AY.23f/AY.23r, respectively and ligating them into the pETite* by the BglBrick Gene Assembly method. We then constructed pCT79 by the Gibson Gene Assembly method with 4 DNA pieces: the backbone fragment amplified from pETite* with the primers NN35/DL_0002, the genes *thiL*, *adc* and *sadH* amplified from the genomic DNA of *C. acetobutylicum* using the primers NN36/NN37, NN38/NN39, and NN40/MW.sadH.r, respectively.

2.2.3.5 Construction of the isobutanol production submodule.

The isobutanol production submodule— pCT13 pCOLA P_{T7}::RBS::*alsS*::RBS::*ilvC*::RBS::*ilvD*—P_{T7}::RBS::*kivd*:: RBS::*adhE*::T_{T7}) was previously constructed (Trinh et al., 2011).

All plasmid constructs were checked by enzyme digestion, PCR amplification, and sequencing before characterization.

2.2.4 Medium and cell culturing techniques

2.2.4.1 Culture media

For molecular cloning, Luria-Bertani (LB) was used with supplementation of antibiotic where applicable. Antibiotics at working concentrations of 50 µg/mL kanamycin (kan), 100 µg/mL ampicillin (amp) were used to maintain the selection of single plasmids. For dual plasmids, two antibiotics were used for selection and concentrations of each antibiotic were used at half of their working concentrations for single plasmid selection.

For ester production experiments, the M9 hybrid medium (pH~7) was used, consisting of 100 mL/L of 10X M9 salts, 1 mL/L of 1 M MgSO₄, 100 µL/L of 1M CaCl₂, 1 mL/L of stock thiamine HCl solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, and appropriate antibiotics. Unless specified, 20 g/L glucose was used in the M9 hybrid medium. The stock 10X M9 salt solution contained 67.8 g/L Na₂HPO₄, 30 g/L KH₂PO₄, 5 g/L NaCl, and 10 g/L NH₄Cl. For fermentation experiments using glucose greater than 20 g/L, the M9 hybrid medium was also supplemented with 50 mM MOPS for buffering capacity.

2.2.4.2 Strain characterization for ester production

For high-cell density fermentation experiments, cells were grown overnight in culture tubes containing the M9 hybrid medium, and subcultured the next morning until the exponential phase (OD_{600nm}~2.0) was reached. Next cells were transferred in a fresh M9 hybrid medium with an initial OD_{600nm}~0.05, grown to OD_{600nm}~5.0, and then induced with IPTG at a working concentration of 0.5 mM for 30 minutes to activate production

modules. To set consistent characterization conditions among strains, cells were then spun down, resuspended in a fresh M9 hybrid medium containing 0.5 mM IPTG, and transferred into 15 mL polypropylene centrifuge tubes with a working volume of 10 mL. For exogenous alcohol addition experiments, the stock alcohols were added to reach a concentration of ~2 g/L. The tubes were wrapped in PTFE tape to seal tube threading and capped to ensure complete anaerobic conditions. The residue oxygen in the medium and head space should be exhausted less than 1 hr. For endogenous ester production experiments, characterization was carried out in the same fashion as the exogenous alcohol additions for ethyl butyrate, isopropyl butyrate, and isobutyl butyrate production. For isopropyl butyrate production, ~10 g/L of acetate was also added into the medium due to the necessary conversion of acetate to produce isopropanol.

Cells were grown on a 75° angled platform in a New Brunswick Excella E25 at 37°C and 175 rpm. After 24 hr, the culture tubes were submerged in an ice water bath for at least 1 hr to condense any volatiles. Whole-cells and cell supernatants were stored at -20°C for subsequent metabolite analysis.

For the *in-situ* high-glucose fermentation and extraction experiments, cells were first grown overnight in culture tubes with the M9 hybrid medium, subcultured into capped flasks the next morning to an $OD_{600nm} \sim 0.05$, and grown to $OD_{600nm} \sim 1.0$. Cells were then subcultured a second time into anaerobic bottles with fresh medium with dodecane overlay (a 1:10 volume ratio of dodecane to medium), an initial $OD_{600nm} \sim 0.1$, and a final working volume of 120 mL. Cells were grown to $OD_{600nm} \sim 2.0$ and induced with IPTG at a working concentration of 0.2 mM. Sampling and pH (~7) adjustment with 5M KOH were performed every 24 hr. The anaerobic bottles were set up in a way to reduce the CO₂

pressure build up by rerouting the pressure to an external tube with dodecane to create a closed, anaerobic, system to produce ethyl butyrate. All characterization experiments were performed at least in biological triplicates.

2.2.5 Analytical methods

2.2.5.1 High performance liquid chromatography (HPLC)

Metabolites from cell supernatants were quantified by using a Shimadzu HPLC system equipped with RID and UV-Vis detectors, and an Aminex HPX-87H cation exchange column (BioRad Inc.) A sample was first filtered through a 0.2- μ m filter unit, then loaded into the column operated at 50°C, and eluted with the 10 mN H₂SO₄ mobile phase running at a flow rate of 0.6 mL/min (Trinh et al., 2011).

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

For the aqueous phase analysis, 500 μ L of samples (cells plus supernatant) were transferred to a 2 mL polypropylene microcentrifuge tube with a screw cap containing 100-200 mg of glass beads (0.25-0.30 mm in diameter), 60 μ L of 6N HCl, and 500 μ L of hexane solution containing ~3 mg/L amyl acetate as an internal standard. The cells were lysed by bead bashing for 5 minutes using a Biospec Mini BeadBeater 16 and then centrifuged at 13,300xg for 1 minute. The extractants of the organic layer were diluted 10 fold in hexane with internal standard and used for the GC/MS analysis. For the organic phase analysis conducted in the *in-situ* fermentation and extraction experiments, samples in the dodecane layer were used directly for the GC/MS analysis.

All esters were analyzed by using the HP6890 GC/MS system equipped with a 30m $\times$ 0.25mm i.d., 0.25 μ m film thickness column plus an attached 10 m guard column (Zebron ZB-5, Phenomenex Inc.) and a HP 5973 mass selective detector. A selected ion mode (SIM) method was deployed to analyze 1 μ L of samples. The GC method was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C then a 25°C/min ramp was deployed to 180°C. The final ramp was then issued to a final temperature of 300°C at a rate of 50°C/min. The injection was performed using a splitless mode with an initial MS source temperature of 160°C. The carrier gas used was helium flowing at a rate of 0.5 mL/min. The detection was delayed 6.5 minutes due to solvent delay and then esters were detected using the following SIM parameters: ions 56.10 and 73.10 detected from 6.50-7.20 minutes for isobutyl acetate, ions 71.1, 88.10, and 116.0 detected from 7.20-7.85 minutes for ethyl butyrate, ions 56.10 and 73.00 detected from 7.85-8.50 minutes for butyl acetate, ions 71.00 and 89.10 detected from 8.50-10.15 minutes for isopropyl butyrate and propyl butyrate, ions 70.10 and 101.00 detected from 10.15-10.70 minutes for amyl acetate, ions 71.10 and 114.10 detected from 10.70-11.25 minutes for isobutyl butyrate, and ions 71.10, 89.10, and 116.00 detected from 11.25- t_{final} minutes for butyl butyrate.

2.3 Results

2.3.1 Establishing the butyrate ester fermentative pathways in *E. coli*

Butyrate esters can be produced directly from sugars by esterification of butyryl-CoA and alcohols via alcohol acyl transferases (AATs). High production of butyrate esters depends on not only the high fluxes of butyryl-CoA- and alcohol-producing pathways, but also enzyme specificity and activity of AATs. To establish the butyrate ester fermentative

pathway in *E. coli*, first we tested the function of the butyryl-CoA plus SAAT production submodule under anaerobic conditions. SAAT was chosen because it had broad substrate specificity (Aharoni et al., 2000).

2.3.1.1 Investigating targeted butyrate ester production by alcohol addition

We first constructed the *E. coli* base strain, EcDL001 derived from *E. coli* MG1655, containing the disrupted acyl-CoA dehydrogenase FadE which blocks the degradation of acyl-CoA pool required for the ester biosynthesis (Clark, 1981). We then introduced the Butyryl-CoA plus SAAT production module, pDL3, into EcDL001 to yield EcDL101. We characterized EcDL101 in high-cell density fermentation experiments to test the function of pDL3 and its alcohol specificity. This was done by externally adding various short-chain alcohols including ethanol, propanol, isopropanol, butanol, and isobutanol at working concentrations of 2 g/L. The results show that EcDL101 successfully produced the targeted butyrate esters with exogenous addition of alcohols under anaerobic conditions (Figure 2-2). EcDL101 produced ethyl butyrate with a titer of 6.00 ± 0.13 mg/L and a specific ester production rate of 80.02 ± 3.26 $\mu\text{g/gDCW/hr}$ after 24 hr by exogenous ethanol addition to the medium (Figure 2-2B). Similarly, addition of propanol yielded 5.21 ± 0.10 mg/L and 69.16 ± 1.65 $\mu\text{g/gDCW/hr}$ of propyl butyrate; addition of isopropanol produced 4.31 ± 0.28 mg/L and 57.13 ± 2.97 $\mu\text{g/gDCW/hr}$ of isopropyl butyrate; addition of isobutanol achieved 2.08 ± 0.07 mg/L and 27.00 ± 1.02 $\mu\text{g/gDCW/hr}$ of isobutyl butyrate; and addition of butanol produced 2.37 ± 0.04 mg/L and 30.55 ± 1.24 $\mu\text{g/gDCW/hr}$ (Figure 2-2B).

Of note, EcDL101 also produced ethyl butyrate as byproduct in some alcohol

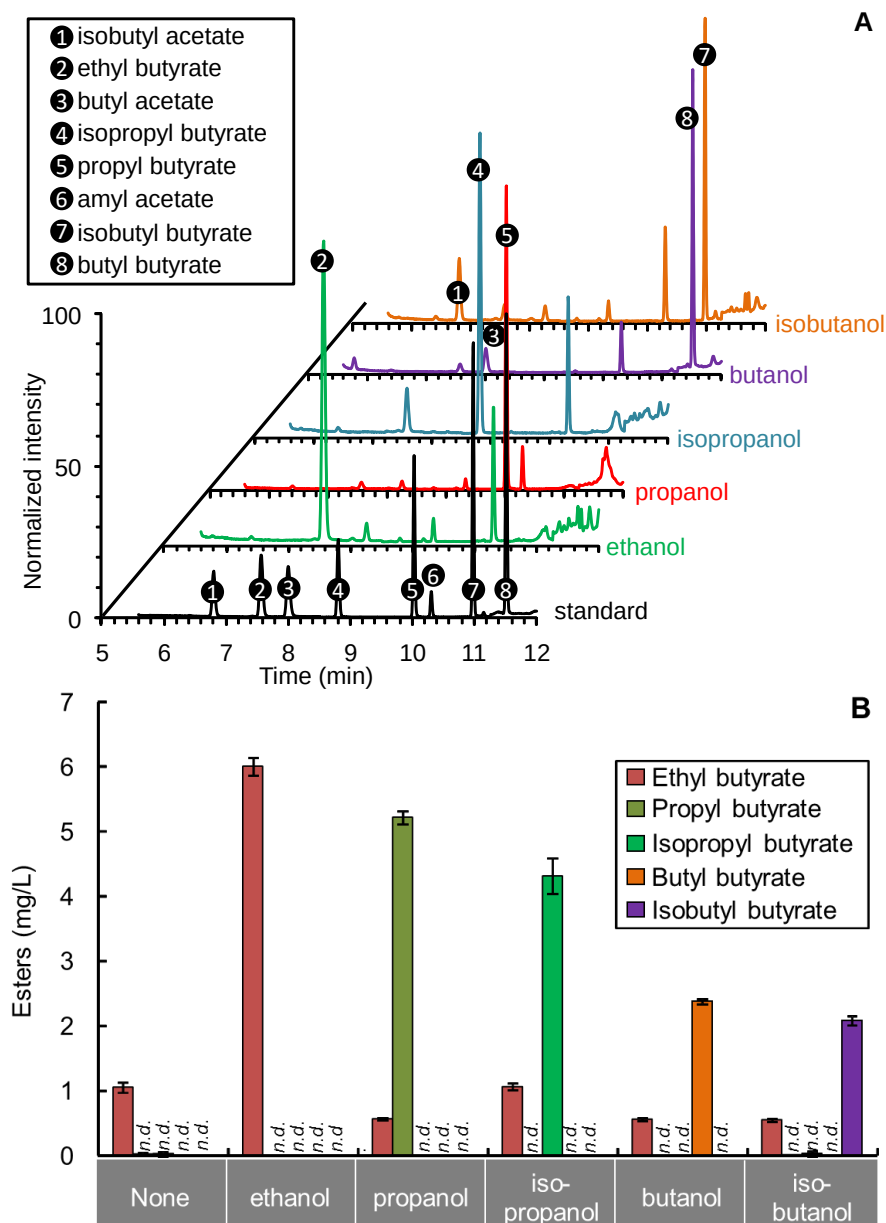


Figure 2-2: *In-vivo* butyrate ester production via exogenous short chain alcohol addition. Short chain alcohols added were ethanol, propanol, isopropanol, butanol, and isobutanol at a working concentration of 2 g/L. **A.** GC/MS chromatograms of butyrate esters produced by EcDL101. **B.** *In-vivo* butyrate ester production by EcDL101. *n.d.*: not detected.

addition experiments but at lower concentrations and rates than the controlled experiments. This was likely due to the availability of endogenous acetyl-CoA and the function of native aldehyde/alcohol dehydrogenases such as *adhE* for converting acetyl-CoA to ethanol. Taken together, EcDL101 achieved the highest ethyl butyrate production and exhibited different specificity towards different added alcohols with the following order: ethanol > propanol > isopropanol > butanol > isobutanol.

2.3.1.2 Demonstrating extracellular secretion of butyrate esters

We hypothesized that *E. coli* produced and secreted butyrate esters extracellularly due to the aroma produced. To test this hypothesis, we analyzed the butyrate ester production by using two different extraction methods with hexane as an extraction solvent. The first method extracted esters from the whole-cell culture which were mechanically lysed. The second method extracted esters by using only the cell supernatant. The results show that at least 76% of butyrate esters were secreted extracellularly by EcDL101 (Figure 2-3A).

It is interesting to observe that 99% of ethyl butyrate was excreted extracellularly while only 76%~89% was excreted for the longer, bulkier esters such as isobutyl butyrate. This difference could be due to the limitations placed on molecular diffusion through the cell membrane or due to active pumping mechanisms. This discovery leads to future investigation of the limits placed on the ester secretion mechanisms found in *E. coli* and perhaps allow for engineering of targeted pumps for further excretion for the enhancement of ester production.

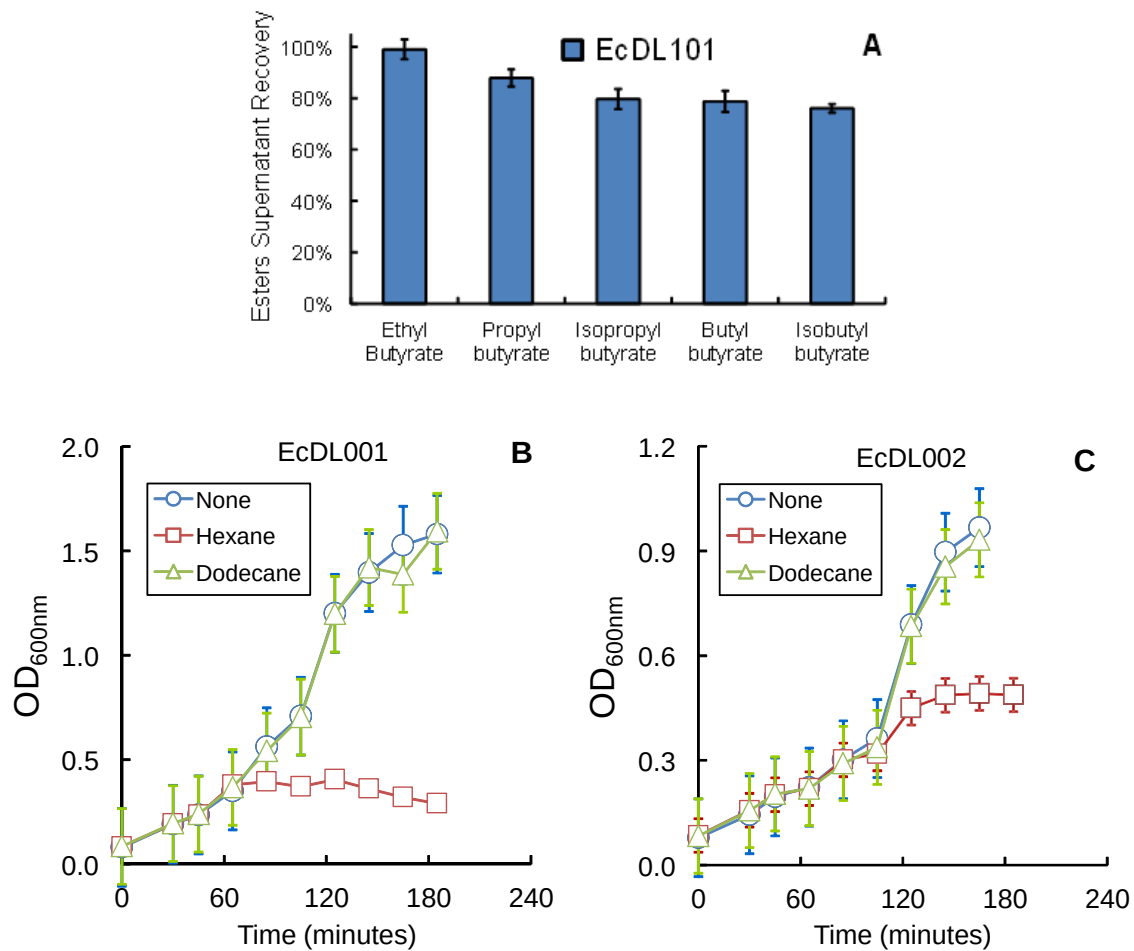


Figure 2-3: Extracellular secretion of butyrate esters and organic solvent effects on cell growth. **A.** Demonstration of extracellular secretion of butyrate esters by EcDL101. **B.** Effect of overlaying organic solvents, hexane and dodecane, on cell growth of EcDL001 and EcDL002 (**C**).

2.3.1.3 Engineering *E. coli* base strain for endogenous butyrate ester production from glucose

The issue with exogenous alcohol addition for ester production is the inhibitory effect of alcohols on cellular function and product formation, and the cost of alcohols themselves. Thus, it is advantageous to endogenously produce the alcohols, along with butyryl-CoA, to produce butyrate esters directly from sugars. We constructed the butyrate ester fermentative pathways by simply combining the butyryl CoA plus SAAT production submodule (pDL3) with any alcohol submodules. In this study, we focused on characterizing the ethyl butyrate production module (pDL3/pCT24), isopropyl butyrate module (pDL3/pCT77), and isobutyl butyrate module (pDL3/pCT13). The base strain carrying these modules are named as EcDL201, EcDL202, and EcDL203 and are designed to produce ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively, from glucose under anaerobic conditions.

We characterized EcDL201, EcDL202, and EcDL203 in high-cell density fermentation experiments. The results show all characterized base strains successfully produced the targeted butyrate esters directly from glucose under anaerobic conditions (Figure 2-4, Table 2-3). After 24 hr, EcDL201 produced ethyl butyrate at a titer of 3.21 ± 0.14 mg/L and a specific ester production rate of 39.17 ± 2.00 μ g/gDCW/hr; EcDL202 produced isopropyl butyrate at 0.18 ± 0.02 mg/L and 2.64 ± 0.26 μ g/gDCW/hr; and EcDL203 produced isobutyl butyrate at 0.26 ± 0.00 mg/L and 3.71 ± 0.06 μ g/gDCW/hr. Besides the targeted butyrate esters, EcDL202 and EcDL203 also produced high level of ethyl butyrate as a byproduct. Taken all together, ethyl butyrate was produced with the highest specificity

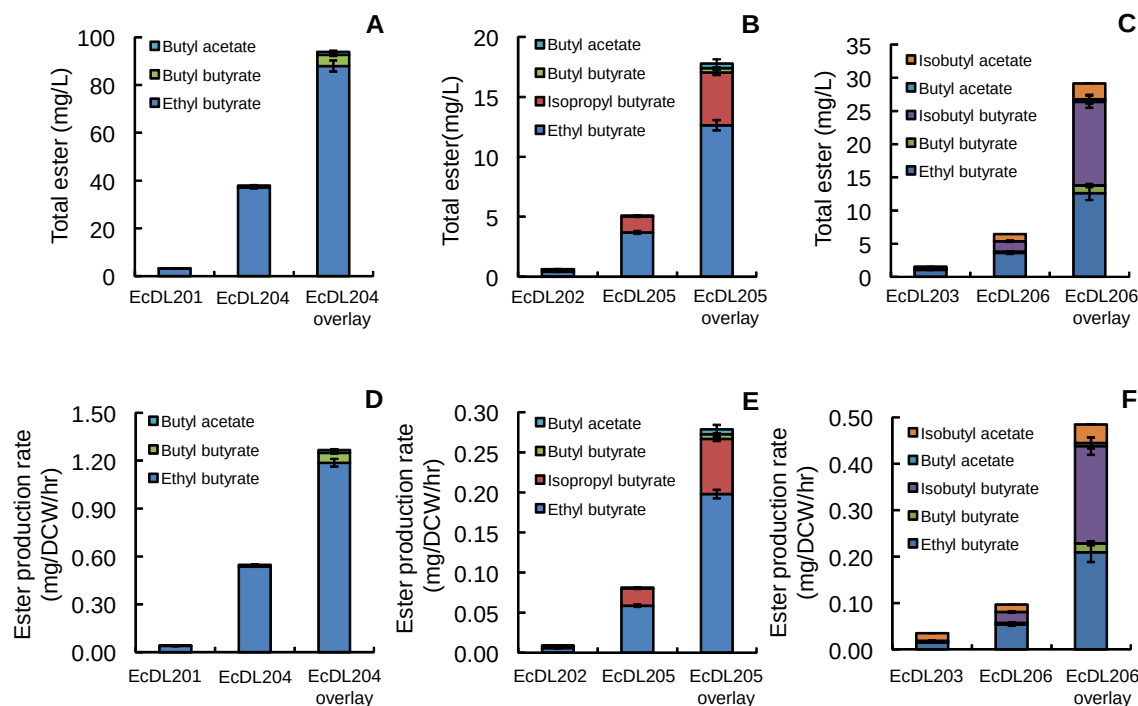


Figure 2-4: Endogenous production of ethyl butyrate, isopropyl butyrate, and butyl butyrate. Endogenous production was directly from glucose by the base strains (EcDL201, EcDL202, and EcDL203) and modular strains (EcDL204, EcDL205 and EcDL206) after 24 hr. Panel A-C: ester titers produced, Panels D-F: specific ester production rates. Dodecane overlay was used for solvent extraction during *in-situ* fermentation and extraction.

(99%) and rate by EcDL201 but the trend appeared to be reciprocal for production of isopropyl butyrate (29%) by EcDL202 and isobutyl butyrate (19%) by EcDL203. This could be due to flux imbalance for synthesizing the ester precursors, butyryl CoA and alcohols, as well as the specificity of SAAT. Residue alcohols were produced in a range of 1.63-5.97 g/L in total for all characterized strains (Fig 2-5).

Table 2-3: Production of butyrate esters by EcDL201-6 in high-cell density fermentation experiments after 24 hr. The highlighted cells of the table correspond to the expected butyrate esters produced by the engineered strains. Values shown in parenthesis are product specificity. *n.d.*: not detected by GC/MS.

Strains	Ethyl butyrate	Isopropyl butyrate	Isobutyl butyrate	Butyl butyrate	Isobutyl acetate	Butyl acetate	Total	Total ester yield
	mg/L (%)						mg/g	
EcDL201	3.21±0.14 (99%)	<i>n.d.</i>	<i>n.d.</i>	0.02±0.00 (1%)	<i>n.d.</i>	<i>n.d.</i>	3.24±0.14	0.16±0.01
EcDL202	0.41±0.01 (66%)	0.18±0.02 (30%)	<i>n.d.</i>	0.03±0.00 (4%)	<i>n.d.</i>	<i>n.d.</i>	0.62±0.01	0.1±0.01
EcDL203	1.07±0.01 (69%)	<i>n.d.</i>	0.26±0.00 (2%)	0.03±0.00 (17%)	0.19±0.01 (12%)	<i>n.d.</i>	1.37±0.01	0.16±0
EcDL204	37.16±0.43 (98%)	<i>n.d.</i>	<i>n.d.</i>	0.75±0.16 (2%)	<i>n.d.</i>	<i>n.d.</i>	37.91±0.41	2.69±0.05
EcDL205	3.68±0.12 (72%)	1.34±0.05 (26%)	<i>n.d.</i>	0.07±0.00 (2%)	<i>n.d.</i>	<i>n.d.</i>	5.09±0.17	0.36±0.01
EcDL206	3.61±0.16 (56%)	<i>n.d.</i>	1.54±0.09 (24%)	0.23±0.02 (3%)	1.08±0.02 (17%)	<i>n.d.</i>	5.38±0.28	1.04±0.02
EcDL204 (overlay)	87.93±2.26 (94%)	<i>n.d.</i>	<i>n.d.</i>	4.62±0.40 (5%)	<i>n.d.</i>	1.29±0.42 (1%)	93.85±3.05	15.3±0.43
EcDL205 (overlay)	12.63±0.42 (71%)	4.40±0.22 (25%)	<i>n.d.</i>	0.37±0.04 (2%)	<i>n.d.</i>	0.38±0.34 (2%)	17.79±0.71	1.8±0.07
EcDL206 (overlay)	12.64±1.07 (43%)	<i>n.d.</i>	12.60±0.93 (43%)	1.14±0.24 (4%)	2.39±0.25 (8%)	0.40±0.69 (2%)	28.77±2.48	2.87±0.23

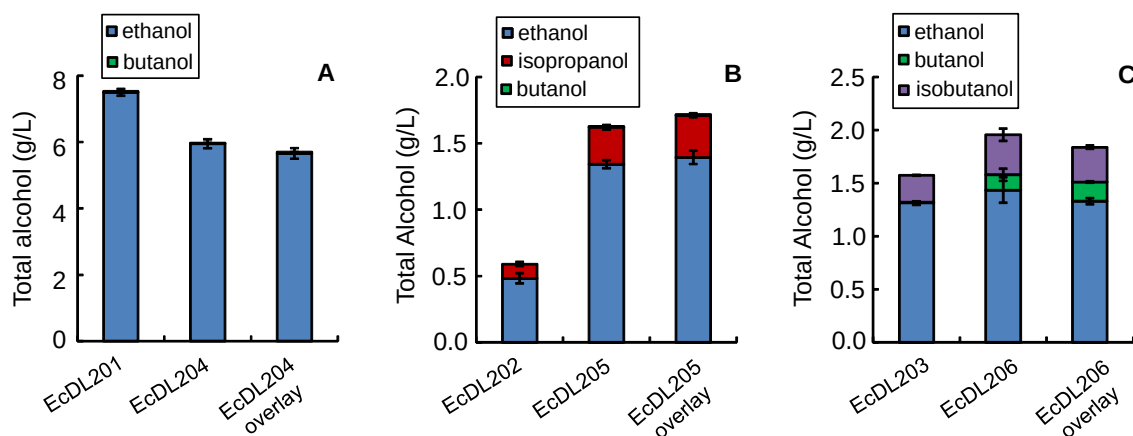


Figure 2-5: Residual alcohol formation from endogenous butyrate production. **(A)** EcDL201 and EcDL204, **(B)** EcDL202 and EcDL205, and **(C)** EcDL203 and EcDL206. Production of butyrate esters by EcDL201, EcDL202, EcDL203, EcDL204, EcDL205, and EcDL206 in high-cell density fermentation experiments after 24 hr. The highlighted cells of the table correspond to the targeted butyrate esters produced by the engineered strains. Values shown in parenthesis are product specificity. *n.d.*: not detected by GC/MS.

2.3.2 Optimizing endogenous production of butyrate esters

2.3.2.1 Endogenous butyrate ester production by the modular strain

Low level of ester production in the base strain is expected because it has multiple competitive fermentative pathways. To enhance butyrate ester production, we next designed and constructed the modular chassis EcDL002 (having 9 gene knockouts and 1 gene knockin, see Table 2-1). This chassis had the disrupted FadE and was derived from TCS083 that was previously designed by elementary mode analysis (EMA), constructed, and validated for enhanced ethanol production under anaerobic conditions (Trinh et al., 2008).

In brief, TCS083 was designed to block all major fermentative pathways, oxidative pentose phosphate pathway, and inefficient electron transport system (see (Trinh et al., 2006; Trinh et al., 2008) for details). Based on EMA, the modular chassis could tightly

couple growth with enhanced production of not only ethanol but also other alcohols (butanol and isobutanol) (Trinh, 2012) and their associated esters under anaerobic conditions (Liu, 2014) (this novel phenotype experimentally validated and presented later in the text).

We constructed EcDL204, EcDL205, and EcDL206 by transforming the ethyl butyrate, isopropyl butyrate, and isobutyl butyrate production modules into EcDL002, respectively. We then characterized these coupled modular strains in high-cell density fermentation experiments for enhanced butyrate ester production directly from glucose. The result shows that the coupled modular strains successfully produced the targeted butyrate esters endogenously from glucose under anaerobic conditions and outperformed the base strains characterized above (Figure 2-4, Table 2-3).

EcDL204 produced ethyl butyrate at a titer of 37.16 ± 0.43 mg/L (98% specificity) and a specific ester production rate of 536.62 ± 1.74 μ g/gDCW/hr after 24 hr, yielding ~14-fold higher ethyl butyrate production than the base strain EcDL201. Likewise, EcDL205 produced isopropyl butyrate at 1.34 ± 0.05 mg/L (26% specificity) and 21.37 ± 0.58 μ g/gDCW/hr, reaching ~8 fold higher isopropyl butyrate production than the base strain EcDL202. EcDL206 produced isobutyl butyrate at 1.54 ± 0.09 mg/L (29% specificity) and 23.15 ± 1.44 μ g/gDCW/hr, achieving ~6 fold higher isobutyl butyrate production than the base strain EcDL203. Among the engineered modular strains, EcDL204 produced ethyl butyrate with the highest titer, rate, and specificity. Like EcDL202-3, EcDL205-6 produced a large fraction of ethyl butyrate as byproduct with the same reason as previously described.

2.3.2.2 Enhancing ester production by *in-situ* fermentation and extraction

Since butyrate esters have low solubility in the aqueous phase and are secreted extracellularly, they can be isolated from the fermentation broth via solvent extraction. This simultaneous *in-situ* fermentation and extraction strategy can potentially increase ester production, minimize product cytotoxicity on cell growth, and reduce downstream separation costs. Therefore, we first examined some common candidate solvents– hexane and dodecane to determine whether they can be used for the *in-situ* fermentation and extraction. We characterized the growth of the base strain (EcDL001) and modular strain (EcDL002) on these two extraction solvents. The result shows that hexane was toxic to cell growth while dodecane was not (Figure 2-3B, 3C). Therefore, we decided to use dodecane for *in-situ* fermentation and extraction to enhance ester production in subsequent studies.

We characterized the coupled modular strains EcDL204, EcDL205, and EcDL206 in high cell density experiments with *in-situ* extraction for enhanced production of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively. The results show that all characterized strains significantly improved targeted ester production with *in-situ* extraction. After 24 hr, EcDL204 increased the ethyl butyrate production about 2.2 fold from 37.16 ± 0.43 mg/L (or $536.62 \mu\text{g/gDCW/hr}$) to 87.93 ± 2.26 mg/L ($1187.35 \pm 24.32 \mu\text{g/gDCW/hr}$). Likewise, EcDL205 improved the isopropyl butyrate production about 3.2 fold from 1.34 ± 0.05 mg/L (or $21.37 \pm 0.58 \mu\text{g/gDCW/hr}$) to 4.40 ± 0.22 mg/L (or $68.96 \pm 2.91 \mu\text{g/gDCW/hr}$). EcDL206 increased the isobutyl butyrate production about 9.0 fold from 1.54 ± 0.09 mg/L (or $23.15 \pm 1.44 \mu\text{g/gDCW/hr}$) to 12.60 ± 0.93 mg/L (or $209.10 \pm 18.58 \mu\text{g/gDCW/hr}$). The yields of ethyl butyrate, isopropyl butyrate, and isobutyl

butyrate on glucose were 15.3 ± 0.43 , 1.8 ± 0.07 , and 2.87 ± 0.23 mg ester/g glucose for EcDL204, 205, and 206, respectively (Table 2-3).

Overall, deploying the coupled modular strains together with *in-situ* fermentation and extraction resulted in ~27-fold increase in ethyl butyrate production, ~24 fold in isopropyl butyrate production, and ~48 fold in isobutyl butyrate production over the base strain.

2.3.3 Demonstrating obligate ester fermentative pathways in the modular strain

We performed the anaerobic growth kinetics of the coupled modular strains to demonstrate that modular ester producing pathways as obligate fermentative pathways. We chose EcDL204 as a model candidate because it can produce ethyl butyrate with high specificity, titer, and rate. First, we performed the ester-module dependent growth experiments to

demonstrate tight coupling between the ester module and modular chassis under anaerobic conditions. We characterized EcDL001, EcDL002, and EcDL204 in the anaerobic rubber capped tubes sparged with nitrogen as shown in Figure 2-6. The results show that EcDL001, a positive control, could grow without carrying any ester production modules as expected because its genotype is quite similar to the wildtype *E. coli* MG1655. On the contrary, the modular chassis EcDL002, a negative control, could not grow anaerobically because it did not carry any ester production module. However, the coupled modular strain EcDL204 (EcDL002 pDL3/pCT24) could recover growth anaerobically by carrying the

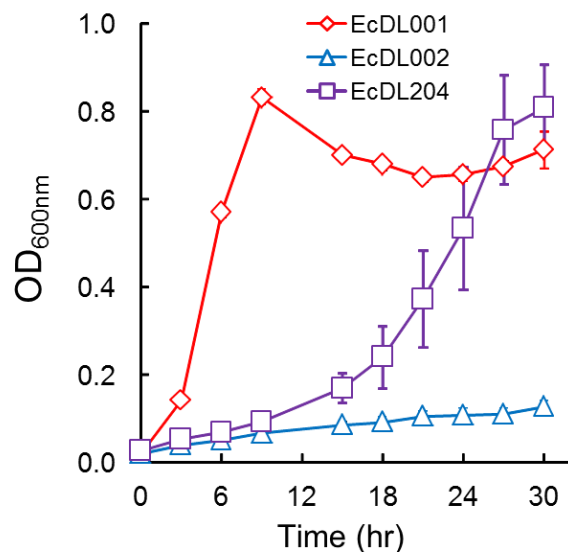


Figure 2-6: Demonstration of modular cell growth coupling. Growth kinetics study demonstrating the tight coupling between the modular chassis and ethyl butyrate production module as an obligate ester fermentative pathway. Positive control strain: EcDL001, negative control strain: EcDL002, and test strain: EcDL204.

ethyl butyrate production module. The result clearly demonstrates the tight coupling between the modular chassis and ester production modules. This engineered phenotype is very useful for the directed metabolic pathway evolution to enhanced ester production because ester-overproducing mutants can be isolated based on a simple and effective growth selection (Fong et al., 2005; Jantama et al., 2008; Shen et al., 2011; Trinh and Srienc, 2009; Unrean and Srienc, 2011).

Next, we investigated the fermentation kinetics of EcDL204 by characterizing it in an *in-situ* high-glucose fermentation and extraction experiments with low cell inoculation. The results show that EcDL204 could grow and couple with the ethyl butyrate production module under anaerobic conditions. The coupled modular strain produced ethyl butyrate during both the (0-24 hr) growth and (24-96 hr) stationary phases (Figure 2-7A). After 96

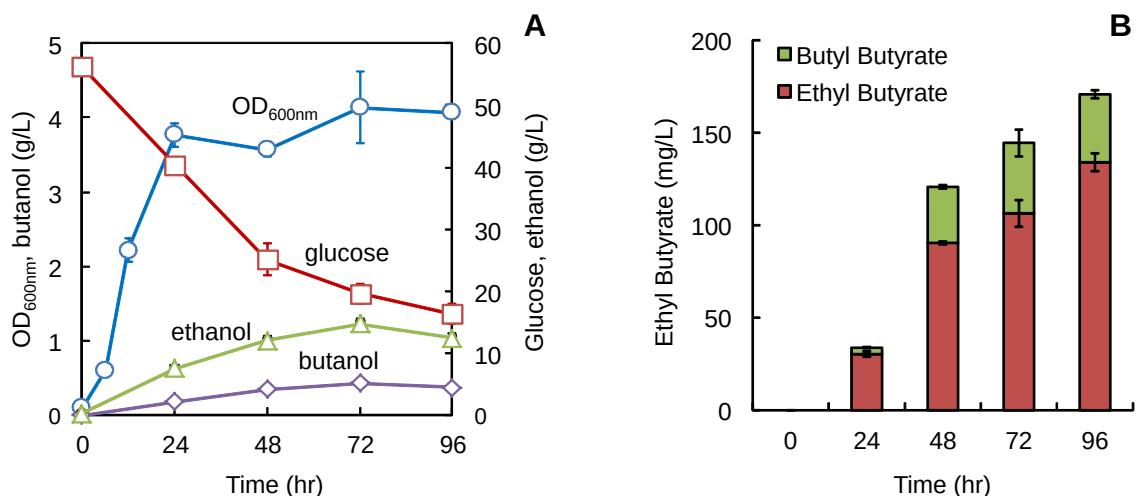


Figure 2-7: Fermentation kinetics of endogenous ethyl butyrate production by the coupled modular strain EcDL204 in the *in-situ* high-glucose fermentation and extraction. Panel **A** shows the metabolic profiles of cell growth (OD_{600nm}), glucose consumption, and alcohol production. Panel **B**. shows kinetic ethyl butyrate production.

hr, EcDL204 produced ethyl butyrate up to 134.00 ± 4.83 mg/L (78% specificity), about 1.52-fold increase from the previous 24 hr experiment (Figure 2-7B), and 36.83 ± 2.13 mg/L of butyl butyrate. The yield of total ester production was 4.36 ± 0.35 mg/g glucose. It is of interest to note that residue alcohols, 12.39 g/L of ethanol and 0.37 g/L butanol, were also observed at the end of fermentation. Production levels of residue alcohols, suggest 1) low turnover of the alcohol acyltransferase, 2) high ethanol flux, and 3) a relatively high production (22% of total produced esters) of butyl butyrate as a byproduct when compared to the 99% product specificity observed in the high-cell density fermentation experiments. This result provides insight to the efficiency and future optimization of the butyrate ester modules.

2.4 Discussion

Esters represent a diverse class of unique molecules with a broad range of applications that can be synthesized combinatorically by condensation of acyl-CoA and alcohol moieties. By using the modular pathway design, we presented a systematic approach to construct and characterize ester production with the focus on exploring the modular butyrate ester fermentative pathways. The base strain EcDL101 could overproduce a variety of butyrate esters such as ethyl butyrate, propyl butyrate, isopropyl butyrate, butyl butyrate, and isobutyl butyrate with exogenous addition of short-chain alcohols, where each one of these bioesters can be used as an additive for flavor and fragrances. This strain can potentially produce a variety of butyrate esters with exogenous supply of other alcohols, which are currently being explored in our laboratory. Of note, EcDL101 (or EcDL002/pDL3) can be deployed as a platform to screen other novel AATs besides SAAT to make unique butyrate esters. Likewise, this modular design strategy can be exploited to screen for other acyl-CoA plus AAT submodules to produce other esters. This whole-cell screening method is expected to be more sensitive, quicker, and cheaper than the traditional screening method because extracellularly secreted esters can be collected in an organic layer, and this method does not require AATs to be expressed, purified, and characterized with exogenous addition of both acyl-CoAs and alcohols.

Energetic concerns are of high priority when developing modular ester fermentative pathways to ensure cofactor balancing. By utilizing anaerobic conditions, we can use the butyrate ester production module as an effective electron sink and remove the competitive effect of an oxygen electron sink to direct reducing equivalents toward enhanced ester production. Based on elementary mode analysis, there exist efficient pathways that could

support anaerobic growth and butyrate ester production during growth and no-growth phases. During the no-growth phase, the maximum theoretical yields of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate are 0.67, 0.47, and 0.67 Cmol ester/Cmol glucose (or 0.43, 0.29, and 0.40 g ester/g glucose), respectively (Liu, 2014). In this study, we successfully demonstrated the design, construction, and characterization of modular butyrate ester pathways as obligate fermentative pathways for combinatorial biosynthesis of butyrate esters directly from fermentable sugars under anaerobic conditions.

Compared to the base strain, the coupled modular strains EcDL204-6 achieved 27, 24, and 48-fold increase in production of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively, from glucose via fermentation and extraction with dodecane overlay. Kinetic characterization of EcDL204 achieved a titer of 134 mg/L in the high glucose fermentation and extraction, the highest titer reported to date. Despite achieving significant increase in butyrate ester production by the engineered strains, the product yields were still low. It is expected that the target ester production will significantly be improved with medium optimization and characterization in controlled bioreactors with either *in-situ* solvent extraction or *in-situ* gas stripping. In addition, it is important to further enhance the butyrate ester production by balancing and enhancing fluxes through the butyrate ester production modules by manipulating promoters (Cox et al., 2007), ribosome binding sites (Anderson et al., 2010; Salis et al., 2009), and plasmid copy numbers (Kittleson et al., 2011) and harnessing efficient AATs.

As shown in Figure 2-1, esters constitute a large space of unique molecules seen in nature such as fruits and plants. Our research is fundamentally different from a recent work by Rodriguez *et al.* (Rodriguez et al., 2014). Here, we laid out the foundation to engineer

modular ester fermentative pathways as exchangeable ester production modules. We investigated the biosynthesis of butyrate esters (such as ethyl butyrate, isopropyl butyrate, and isobutyl butyrate) directly from glucose under anaerobic conditions while Rodriguez *et al.* focused on the biosynthesis of acetate esters. It is interesting to observe that ATF1 from *S. cerevisiae* had high specificity towards acetate esters such as isobutyl acetate in Rodriguez *et al.*'s report but not others such as butyrate esters of this study. In addition, we engineered our modular chassis that coupled with ester production modules for anaerobic production of targeted esters. However, strains employed in Rodriguez *et al.*'s work were derived from JCL260 and likely cannot support anaerobic production of reduced metabolites such as butanol, isobutanol, and derived esters directly from sugars under anaerobic conditions due to redox imbalance leading to growth inhibition (Trinh, 2012; Trinh et al., 2011).

Since coupled modular strains in this study could produce esters under anaerobic conditions and secrete them for easy extraction, they have great advantages for scale-up fermentation processes. Furthermore, esters produced via microbial conversion routes are considered as “natural” with valuable and broad applications as flavor and fragrance products. The other advantage to produce esters in a recombinant host, such as *E. coli*, is the flexibility to achieve high product selectivity by manipulating orthogonal acyl-CoA, alcohol pathways, and AATs. As demonstrated in this study, we introduced the heterologous butyryl-CoA, ethanol, isopropanol, and isobutanol pathways and SAAT for making butyrate esters with relatively high selectivity with some unavoidable byproducts due to endogenous production of acetyl-CoA and ethanol in *E. coli* and broad substrate

specificity of SAAT. We believe this issue can be addressed by screening for novel, highly-specific AATs that are abundant in nature.

Indeed, the diversity of alcohol ac(et)yltransferases span over every higher order eukaryote that produces a flavor and fragrance, and are capable of condensing a plethora of alcohols and CoA moieties to form esters. We see this in every fruit we taste, and every flower we smell. Using this enzyme class enables us to engineer alcohol and CoA pathways to produce vibrant, desirable molecules. Nature has already perfected these enzymes, but the issue at hand lies in the screening process for higher specificity, faster turnover. Unfortunately, screening every enzyme found in nature for desired product formation is nearly impossible, but, utilizing techniques such as random mutagenesis, high throughput screening, and molecular dynamic simulations, will allow further understanding and development of specificity across this diverse enzyme class. By coupling plug-and-play designer alcohol and CoA modules with the designed modular chassis, demonstration of a combinatorial product library points toward a library of esters limited only by the user's imagination.

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3 Expanding the Modular Ester Fermentative Pathways for Combinatorial Biosynthesis of Esters from Volatile Organic Acids

Summary: This chapter is based on the published paper: Layton, Donovan S., and Cong T. Trinh. "Expanding the modular ester fermentative pathways for combinatorial biosynthesis of esters from volatile organic acids." *Biotechnology and bioengineering* 113 (2016): 1764-1776.

3.1 Abstract

Volatile organic acids are byproducts of fermentative metabolism, e.g., anaerobic digestion of lignocellulosic biomass or organic wastes, which are often times undesired inhibiting cell growth and reducing directed formation of the desired products. Here, we devised a general framework for upgrading these volatile organic acids to high-value esters that can be used as flavors, fragrances, solvents, and biofuels. This framework employs the acid-to-ester modules, consisting of the AAT (alcohol acyltransferase) plus ACT (acyl CoA transferase) submodule and the alcohol submodule, for co-fermentation of sugars and organic acids to acyl CoAs and alcohols to form a combinatorial library of esters. By assembling these modules with the engineered *Escherichia coli* modular chassis cell, we developed microbial manufacturing platforms to perform the following functions: i) rapid *in vivo* screening of novel AATs for their catalytic activities, ii) expanding combinatorial biosynthesis of unique fermentative esters, and iii) upgrading volatile organic acids to esters using single or mixed cell cultures. To demonstrate this framework, we screened for a set of 5 unique and divergent AATs from multiple species, and were able to determine their novel activities as well as produce a library of 12 out of the 13 expected esters from co-fermentation of sugars and (C2-C4) volatile organic acids. We envision the developed framework to be valuable for *in vivo* characterization of a repertoire of not-well-

characterized natural AATs, for expanding the combinatorial biosynthesis of fermentative esters, and for upgrading volatile organic acids to high-value esters.

3.2 Introduction

Harnessing renewable or waste feedstocks (e.g., switchgrass, corn stover, agricultural residue or municipal solid waste) can provide an alternative route for sustainable production of chemicals via fermentation while benefiting the environment and reducing our dependence on the petroleum source that is neither renewable nor sustainable (Bokinsky et al., 2011; Dale, 2015; Silva et al., 2013; Thanakoses et al., 2003). The carboxylate platform is the emerging technology that can directly convert these renewable or waste feedstocks into the carboxylates, dominantly comprised of short-chain (C2-C6) volatile organic acids (VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids) (Agler et al., 2011; Chang et al., 2010; Holtzapple, 2015). This technology employs robust consortia of microorganisms that are found in cattle rumens, anaerobic digesters, termite hindguts or swamps, and are very efficient for fermentation of renewable or waste feedstocks with minimum requirement of feedstock pre-processing, such as sterilization and biomass pretreatment. The carboxylates, after fermentation, can be upgraded to hydrocarbon fuels or chemicals via traditional chemical conversion (Agler et al., 2011; Holtzapple, 2015; Napora-Wijata et al., 2014). However, some current challenges to the carboxylate platform are to alleviate the toxicity of acids inhibiting microbes and carboxylate production, and to minimize the competitive conversion of sugars and/or carboxylates to unwanted byproducts such as methane, hydrogen, and/or carbon dioxide.

Different from the carboxylates, esters can be directly used with broader applications such as flavors, fragrances, solvents, and biofuels. By using alcohol acyl transferases (AATs), cells can synthesize esters by condensing acyl CoAs and alcohols from biomass-derived fermentable sugars and/or organic acids. Recent studies have

successfully engineered recombinant *E. coli* strains to produce ester platforms including acetate esters (Rodriguez et al., 2014; Tai et al., 2015; Tashiro et al., 2015) and butyrate esters (Layton and Trinh, 2014) from fermentable sugars. Anaerobic ester production can be captured by *in situ* fermentation and extraction, which can minimize product toxicity. In comparison to the carboxylates (Günther et al., 2011), esters constitute a larger space of novel and unique molecules (Layton and Trinh, 2014). Even though there is a great potential to upgrade the carboxylate platform to ester platform via fermentation, the approach has not yet been demonstrated.

The key to expand the ester platform from the carboxylate platform is to have novel AATs with desirable catalytic activities. However, the functions of these AATs are poorly understood even though AATs are abundant in nature, as found in fruits and plants. The main limitation is the characterization of these enzymes, which depends on the use of expensive, unstable substrates such as acyl CoAs and low throughput GC/MS screening method. Currently, most of the AATs are characterized with a small library of substrates, primarily acetyl CoA and alcohols (Beekwilder et al., 2004; El-Sharkawy et al., 2005) to produce acetate esters; however, the activities of these AATs towards the biosynthesis of (>C₂) acylates are poorly understood (Balbontín et al., 2010; Beekwilder et al., 2004; El-Sharkawy et al., 2005; Verstrepen et al., 2003). Therefore, the development of new analytical methods to characterize these abundant AATs with their novel potential functions together with assistance of bioinformatics (e.g., protein structure, molecular dynamic simulation (Galaz et al., 2013; Morales-Quintana et al., 2011; Morales-Quintana et al., 2012)) can provide fundamental understanding of functional roles of these AATs in

their natural fruits, plants, and flowers as well as facilitate the upgrading of the carboxylate to ester platforms for broad industrial applications.

In this study, we developed a general framework to upgrade the carboxylates to esters by co-fermentation of sugars and (C2-C6) VOAs that are dominant in the carboxylate platform. In this framework, we applied modular pathway engineering to design the acid-to-ester modules, consisting of the AAT (alcohol acyltransferase) plus ACT (acyl CoA transferase) submodule and the alcohol submodule, that generate acyl CoAs and alcohols from sugars and VOAs to form a combinatorial library of esters. By assembling these modules with the engineered *Escherichia coli* modular chassis cell, we developed novel microbial manufacturing platforms and applied them for i) rapid *in vivo* screening of novel AATs for their catalytic activities, ii) expanding the combinatorial biosynthesis of 12 unique fermentative esters, including acetate esters (e.g., ethyl acetate, acyl acetate) as well as acylate esters (e.g., ethyl acylate, acyl acylate), and iii) demonstrating the upgrading of carboxylates to higher-value esters by using single and/or mixed cell cultures.

3.3 Materials and Methods

3.3.1 Strains and plasmids

3.3.1.1 Strains

Table 3-1 shows a list of strains and plasmids used in this study. Both *Clostridium propionicum* and *Saccharomyces cerevisiae* were obtained from the ATCC strain collection, and were used for genomic DNA extraction. *E. coli* TOP10 strain was used for molecular cloning. For ester production, we deployed the engineered *E. coli* modular

chassis cell EcDL002 as the ester production host (Layton and Trinh, 2014; Trinh, 2015). By transforming the modules pDL009-pDL013 into EcDL002 via electroporation (Sambrook, 2001), we created the ester production strains EcDL102-106, respectively.

3.3.1.2 Plasmids.

In this study, the ester fermentative pathways were designed as exchangeable ester production modules that are assembled from multiple sub-modules (Layton and Trinh, 2014). The acid-to-ester production module consisted of a propionyl-CoA transferase (PCT, belonging to the general class of ACT) plus AAT submodule and the ethanol production submodule (Figure 3-1). These submodules were organized in plasmids and transcribed by the T7 promoter. Table 3-1 shows a list of plasmids used and generated in this study, and Table 3-2 presents a list of primers used for the plasmid construction and validation.

All plasmids were derived from the pETite* backbone (Layton and Trinh, 2014) and constructed by using the Gibson gene assembly method (Gibson et al., 2009).

Ethanol submodule. The ethanol production submodule, pCT24, was designed to convert pyruvate to ethanol and was previously constructed (Layton and Trinh, 2014).

AAT submodules. Five AAT genes, including ATF1 and ATF2 of *S. cerevisiae* (Verstrepen et al., 2003), SAAT of *Fragaria ananassa* (Aharoni et al., 2000), VAAT of *F. vesca* (Beekwilder et al., 2004), and AeAAT of *Actinidia eriantha* (Günther et al., 2011), were harnessed to construct the AAT submodules that condense acyl CoAs and alcohols to produce esters.

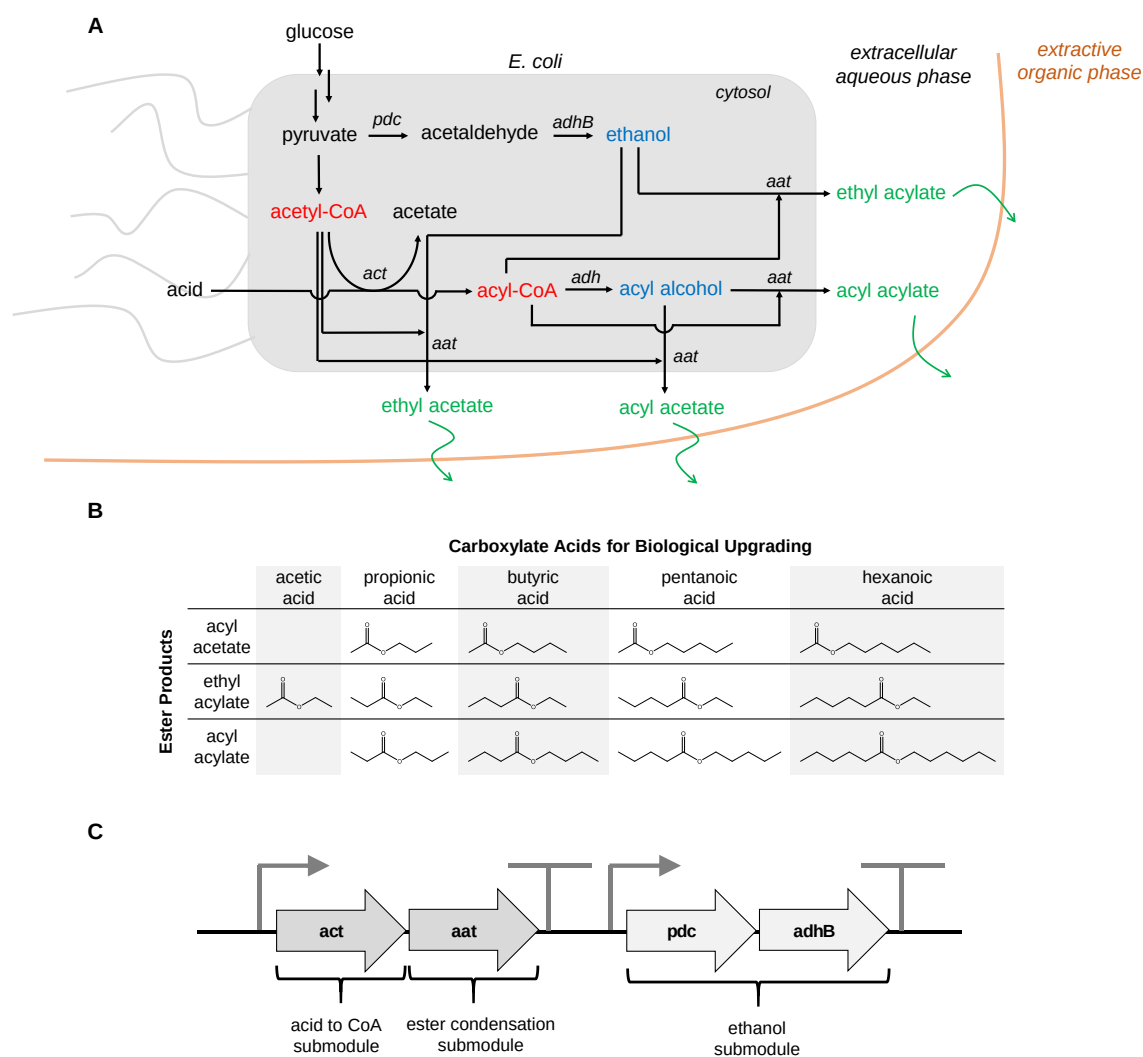


Figure 3-1: Acid to ester pathways, carboxylate acids and their potential esters, and acid-to-ester design. **(A)** Acid-to-ester pathways for combinatorial biosynthesis of esters from co-fermentation of sugars and fatty acids via *in situ* fermentation and extraction. **(B)** Potential esters produced from the carboxylate platform. **(C)** Design of the acid-to-ester production module.

Table 3-1: A list of plasmids and strains

Plasmids/Strains	Genotypes	Sources
<i>Plasmids</i>		
pCP20	<i>flp, bla, cat, cl857</i> λ <i>ts</i>	Yale collection
pCOLA	kan ⁺	Novagen
pETite C-His	pBR322 <i>ori</i> ; kan ⁺	Lucigen
pETite*	kan ^R	(Layton and Trinh, 2014)
pCT24	pETite* P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	(Layton and Trinh, 2014)
pDL04	pETite* <i>atf1</i> ; kan ⁺	this study
pDL05	pETite* <i>atf2</i> ; kan ⁺	this study
pDL01	pETite* SAAT; kan ⁺	(Layton and Trinh, 2014)
pDL06	pETite* VAAT; kan ⁺	this study
pDL08	pETite* AeAT9; kan ⁺	this study
pDL09	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS:: <i>atf1</i> ::T _{T7} ::P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pDL10	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS:: <i>atf2</i> ::T _{T7} ::P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pDL11	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::SAAT::T _{T7} ::P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pDL12	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::VAAT::T _{T7} ::P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pDL13	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::AeAT9::T _{T7} ::P _{T7} ::RBS:: <i>pd</i> c::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
<i>Strains</i>		
<i>C. propionicum</i>	wildtype	ATCC 25522
BY4741	MAT a, <i>ura3d0, his3-d200, leu2-d0, met15-d0</i>	ATCC 201388
TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara leu</i>) 7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
EcDL002	TCS083 (λ DE3) Δ <i>fadE</i> ::kan ⁻ (cured)	(Layton and Trinh, 2014)
EcDL102	EcDL002 carrying pDL09; kan ⁺	this study
EcDL103	EcDL002 carrying pDL10; kan ⁺	this study
EcDL104	EcDL002 carrying pDL11; kan ⁺	this study
EcDL105	EcDL002 carrying pDL12; kan ⁺	this study
EcDL106	EcDL002 carrying pDL13; kan ⁺	this study

Table 3-2: A table of primers used

Primer name	Sequences
<i>Primers used to build the AAT submodule (pDL004-006)</i>	
DL_0001	5'-CATCATCACCACCATCACTAA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
DL_0011	5'-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3'
DL_0012	5'-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATT AAGGTCTTTGGAG-3'
DL_0017	5'-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3'
DL_0018	5'-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGCGGATAA CATACGTAGACCG-3'
DL_0019	5'-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG AATGAAATCGATGAGAA-3'
DL_0020	5'-GCCGCTCTATTAGTGATGGTGGTGATGATGCTAAGGGCCTA AAAGGAGAG-3'
DL_0021	5'-TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGGAAGATATAGAAGGATA-3'
DL_0022	5'-GCCGCTCTATTAGTGATGGTGGTGATGATGTTAAAGCGACG CAAATTCGC-3'
<i>Primers used to build the pct_AAT+ethanol module (pDL009-014)</i>	
DL_0023	5'-AAATAATTTTGTTTAACTATAAGAAGGAGATATACATATGA GAAAGGTTCCCATTATTAC-3'
DL_0024	5'-TCAGGACTTCATTTCTTCAG-3'
DL_0014	5'-ATATCAAGCTTGAATTCGTTACCCGG-3'
DL_0015	5'-GGAGGAAGTATATCCGGGTAACGAATTCAAGCTTGATATTA ATACGACTCACTATAGGG-3'
DL_0016	5'-GTCCAGTTACGCTGGAGTCTGAGGCTC-3'
DL_0013	5'-GAGCCTCAGACTCCAGCGTA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
DL_0025	5'-CTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGAAT GAAATCGATGAGAAAAATC-3'
DL_0026	5'-GGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAAGATATAGAAGGATAC-3'
DL_0027	5'-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAGAAAATTGAGGTCAG-3'
DL_0028	5'- TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3'
DL_0029	5'-GGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATA TGGCAAGCTCTGTGCGTCTG-3'

Table 3-2 continued*gBlock sequences for AeAT9*

	5'-
AeAT9_1	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACAT ATGGCAAGCTCTGTGCGTCTGGTTAAAAAACCAGTCCTGG TTGCCCCGGTTGATCCGACCCCGAGCACCGTTCTGTCTCTG AGCTCCCTGGACTCTCAGCTGTTTCTGCGTTTCCCAATCGA ATACCTGCTGGTTTATGCTTCTCCGCATGGCGTCGACCGTG CGGTTACCGCGGCACGTGTGAAAGCAGCACTGGCCCGTTTC CCTGGTTCCGTACTACCCGCTGGCGGGTCGCGTCAAGACC CGTCCGGATTCCACGGGCTGGACGTGGTGTGCCAGGCTC AGGGCGCAGGCCTGCTGGAAGCAGTATCCGACTACACTG CAAGCGACTTCCAGCGTGCGCCGCGTTCCGTAACCGAGTG GCGTAAACTGCTGCTGGTGGAAGTGTTCAAAGTCGTACCG CCGCTGGTTGTCCAGCTGACCTGGCTGAGCGACGGCTGCG TTGCGCTGGGCGTGGGTTTCAGCCACTGCGTGATCGACGG CATTGGTTCCTCTGAATTCCTGAACCTGTTTCGCGGAAGTG GCAACCGGTTCGTGCCCCGCTGAGCGAATTTCAACCGAAGC CGGTTTGGGATCGCCACCTGCTGAACAGCGCAGGCCGTAC TAACCTGGGCACCCACCCAGAATTCGGTCGCGTACCGGAC CTGAGCGGCTTTGTAAACGCGTTTTACCCAGGAGCGTCTGT CCCCTACTAGCATCACTTTTGACAAAAC-3'
	5'-
AeAT9_2	AGGAGCGTCTGTCCCCTACTAGCATCACTTTTGACAAAAC TTGGCTGAAAGAACTGAAGAACATCGCAATGAGCACCAG CCAGCCGGGTGAATTCCCGTATACTTCCTTCGAAGTACTG TCCGGTCATATTTGGCGCTCCTGGGCCCCGCTCTCTGAACCT GCCAGCGAAACAGGTGCTGAAACTGCTGTTTTCTATCAAC ATTCGCAATCGCGTGAAACCGTCCCTGCCAGCAGGTTACT ACGGCAATGCTTTCGTGCTGGGCTGCGCGCAAACCAGCGT GAAGGATCTGACCGAAAAAGGCCTGGGTATTGCGCCGA TCTGGTGCGTGGTGCAAAAGAACGTGTTGGCGACGAATAC GCACGTGAAGTGGTTGAATCCGTGAGCTGGCCGCGTCGCG CTAGCCCGGATTCTGTAGGTGTTCTGATCATCTCCCAATG GTCTCGCCTGGGTCTGGACCGTGTTGACTTCGGTCTGGGC CGTCCGGTTCAGGTAGGTCCGATCTGCTGTGACCGTTACT GCCTGTTTCTGCCGGTTCGCGATCGTACTGAATCTGTAAA AGTTATGGTGGCGGTGCCGACCAGCGCAGTCGATCGCTAC GAATACTTTATCCGTAGCCCGTACAGCCATCATCACCACC ATCACTAATAGAGCGGCCGCCACC-3'

The SAAT submodule, pDL001, that contained the SAAT gene, was previously constructed (Layton and Trinh, 2014). To create the ATF1 submodule, pDL004, the ATF1 gene was amplified from the genomic DNA of *S. cerevisiae* by using the primers DL_0019/DL_0020, and inserted into the pETite* backbone amplified by using the primers DL_0001/DL_0002. To create the ATF2 submodule, pDL005, the ATF2 gene was amplified from the genomic DNA of *S. cerevisiae* by using the primers DL_0021/DL_0022, and inserted into the pETite* backbone. To create the VAAT submodule, pDL006, the VAAT gene was amplified from the plasmid pRSET-VAAT (Beekwilder et al., 2004) by using DL_0017/DL_0018, and inserted into the pETite* backbone. To create the AeAT9 submodule, pDL007, the AeAT9 gene was assembled from two gBlocks (e.g., AeAT9_1 and AeAT9_2) synthesized via IDTDNA (Coralville, Iowa, USA), and inserted into the pETite* backbone.

Acid-to-ester production modules. Each acid-to-ester production module (including pDL009, pDL010, pDL011, pDL012, or pDL013) was created by assembling 4 DNA fragments including i) the PCT gene amplified from the genomic DNA of *C. propionicum* using the primers DL_0023/DL0024, ii) the ATF1 gene (amplified from the plasmid pDL004 using primers DL_0025/DL_0014), the ATF2 gene (pDL005, DL_0026/DL_0014), the SAAT gene (pDL001, DL_0027/DL_0014), the VAAT gene (pDL006, DL_0028/DL_0014), or the AeAT9 gene (pDL007, DL_0029/DL_00140), iii) the ethanol submodule amplified from pCT24 using the primers DL_0015/DL_0016, and iv) the pETite* backbone amplified using the primers DL_0013/DL_0002.

3.3.1.3 Media and cell culturing conditions

Culture media. For molecular cloning, the Luria-Bertani (LB) complex medium, that contained 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl, and 50 μ g/mL kanamycin (if applicable), was used. For the acid-to-ester production experiments, the M9 hybrid medium (pH~7) was used, consisting of 100 mL/L of 10X M9 salts, 1 mL/L of 1 M MgSO₄, 100 μ L/L of 1M CaCl₂, 1 mL/L of stock thiamine solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, 20 g/L glucose and 50 μ g/mL kanamycin. The stock 10x M9 salt solution contained 67.8 g/L Na₂HPO₄, 30 g/L KH₂PO₄, 5 g/L NaCl, and 10 g/L NH₄Cl. In addition, about 2 g/L organic acid each was supplemented to the M9 hybrid medium. The VOAs used for this study included acetic, propionic, butyric, valeric, and hexanoic acids that are dominantly found (>95%) in the carboxylate platforms (Holtzapfel, 2015). For the acid-to-ester production experiments using mixed VOAs, we used the working concentration of 5 g/L with the composition mimicking those found in the countercurrent fermentation of corn stover by pig manure, e.g., 40% (w/v) acetic acid, 18% propionic acid, 20% butyric acid, 11% pentanoic acid, and 11% hexanoic acid (Thanakoses et al., 2003).

Cell culturing conditions. *In situ*, high-cell density fermentation and extraction experiments were performed for ester production. Specifically, cells were grown overnight in 15 mL culture tubes containing the M9 hybrid medium, and subcultured the next morning until the exponential phase (OD_{600nm} ~2.0, 1 OD ~0.5 g DCW/L) was reached. Next, cells were transferred in a fresh M9 hybrid medium with an initial OD_{600nm} ~0.05, grown to OD_{600nm} ~5.0, and then induced with IPTG at a working concentration of 0.5 mM at 37 °C for 30 minutes to activate the ester production modules. To set consistent

characterization conditions among strains (e.g., fresh and consistent supply of glucose and other nutrients), cells were then spun down, resuspended in a fresh M9 hybrid medium containing ~2 g/L organic acid and 0.5 mM IPTG, and transferred into 15 mL glass centrifuge tubes with a working volume of 10 mL. Each tube was overlaid with 1 mL hexadecane for *in situ* fermentation and extraction. The tubes were wrapped in PTFE tape to seal tube threading and capped to ensure complete anaerobic conditions. The residue oxygen in the medium and head space should be exhausted in less than 1 h.

Cells were grown on a 75° angled platform in a New Brunswick Excella E25 at 37°C and 175 rpm. Whole-cells and cell supernatants were collected and stored at -20°C for subsequent metabolite analysis while hexadecane overlay was stored at room temperature for ester analysis. All experiments were performed with at least three biological replicates.

3.3.2 Analytical methods

3.3.2.1 High performance liquid chromatography (HPLC).

Metabolites (sugars, organic acids, and alcohols) from culture supernatants were quantified by using the Shimadzu HPLC system equipped with the RID and UV-Vis detectors (Shimadzu Inc., Columbia, MD, USA), and the Aminex HPX-87H cation exchange column (BioRad Inc., Hercules, CA, USA). Samples were first filtered through 0.2-μm filter units, loaded into the column operated at 50°C, and eluted with the 10 mM H₂SO₄ mobile phase running at a flow rate of 0.8 mL/min.

3.3.2.2 Gas chromatography coupled with mass spectroscopy (GC/MS).

To identify and quantify esters being produced, GC/MS samples were processed from both the aqueous and organic phases from the *in situ*, high-cell density fermentation and extraction experiments. For the aqueous phase, 500 μ L of whole-cells and supernatants were transferred to a 2 mL polypropylene microcentrifuge tube with a screw cap containing 100-200 mg of glass beads (0.25-0.30 mm in diameter), 60 μ L of 6N HCl, and 500 μ L of hexadecane solution containing 5.8 mg/L amyl acetate as an internal standard. The cells were lysed by bead bashing for 4 minutes using a Biospec Mini BeadBeater 16 and then centrifuged at 13,300xg for 1 minute. The GC/MS samples were collected from the organic layer and directly used for GC/MS runs.

For the organic phase from the *in situ*, high-cell density fermentation and extraction experiments, the extractants were diluted with hexadecane containing internal standard in a 1:1 (v/v) ratio, and then directly used for GC/MS runs.

All esters were analyzed by using the HP6890 GC/MS system equipped with a 30m $\times$ 0.25mm i.d., 0.25 μ m film thickness column plus an attached 10 m guard column (Zebron ZB-5, Phenomenex Inc.) and a HP 5973 mass selective detector. A selected ion mode (SIM) method was deployed to analyze 1 μ L of samples. The GC method was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C then a 25°C/min ramp was deployed to 235°C. The final ramp was then issued to a final temperature of 300°C at a rate of 50°C/min to elute any residual non desired analytes. The injection was performed using a splitless mode with an initial MS source temperature of 200°C. The carrier gas used was helium flowing at a rate of 0.5 mL/min. The detection of the desired products was accomplished using the following SIM parameters: i) ions 45.00, 61.00, 70.00, and

85.00 detected from 0-5.40 minutes for ethyl acetate; ii) ions 57.00, 74.00, and 102.00 detected from 5.40-7.20 minutes for ethyl propionate and propyl acetate where propyl acetate and ethyl propionate were separated further using their parent ions for quantification if necessary; iii) ions 71.10, 88.10, and 116.00 detected from 7.20-7.71 minutes for ethyl butyrate; iv) ions 57.00, 75.00, and 87.00 detected from 7.71-7.98 minutes for propyl propionate; v) ions 56.00, 61.00, 73.00 from 7.98-9.40 minutes for butyl acetate; vi) ions 61.00, 70.00, and 87.00 from 9.40-9.90 minutes for isoamyl acetate; vii) ions 85.00, 88.00, and 101.00 from 9.90-10.20 minutes for ethyl pentanoate; viii) ions 70.10 and 101.00 from 10.20-11.25 minutes for amyl acetate; ix) ions 71.10, 89.10, and 101.00 for butyl butyrate from 11.25-11.53 minutes; x) ions 60.00, 88.00, and 99.00 from 11.53-11.66 for ethyl hexanoate; xi) ions 56.00, 61.00, and 84.00 from 11.66-13.00 minutes for hexyl acetate; and xii) ions 70.0, 85.00, and 103.00 from 13.00- t_{final} minutes for pentyl pentanoate.

For our GC/MS analysis, the lower limit for quantifying ester production is within the range of 10 $\mu\text{g/mL}$ with good signal to noise ratio ($>3:1$).

3.3.3 Bioinformatics

For sequence alignment and phylogenetic analysis, each protein sequence was retrieved from NCBI, and was inputted into MEGA6 (Tamura et al., 2013) and aligned via MUSCLE (Edgar, 2004). The phylogenetic tree was generated using the neighbor-joining algorithm with a 1000 bootstrap value. Template proteins used for the analysis include: ATF1 (AJU14295.1, gene bank ID), ATF2 (AJR99982.1), SAAT (AAG13130.1), VAAT (AAN07090.1), and AeAT9 (HO772635)

3.4 Results

3.4.1 Establishing the acid-to-ester fermentative pathways in *E. coli*

VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids, are the dominant byproducts of fermentation, for instance, the carboxylate platform (Holtzapple, 2015). To upgrade these acids into high-value esters, we developed a general framework to engineer microbial cell factories that co-utilize fermentable sugars (e.g., glucose) and fatty acids (e.g., VOAs) for combinatorial biosynthesis of target esters (Figure 3-1). Sugars are primarily used as the carbon and energy source for cell growth as well as production of enzymes and the precursor acetyl CoA for ester biosynthesis. We also engineered the heterologous ethanol pathway from *Zymomonas mobilis* for conversion of sugars into the additional precursor ethanol to expand the biosynthesis of the fermentative ester library. Under anaerobic conditions, fatty acids, that have higher degrees of reduction than sugars, are primarily converted to the precursors, acyl CoAs and acyl alcohols, for the ester biosynthesis. This general framework is tailored to generate a fermentative ester library including acetate esters (e.g., ethyl acetate, acyl acetate) and acylate esters (e.g., ethyl acylate, acyl acylate) via co-utilization of sugars and fatty acids. The esters produced are collected in the organic overlay during the *in situ* fermentation and extraction.

We designed the acid-to-ester pathways as production modules consisting of the ethanol submodule and the PCT plus AAT submodule. The ethanol module, containing PDC and AdhB of *Z. mobilis*, was previously engineered and functional (Layton and Trinh, 2014). The broad substrate specificity of the bi-functional aldehyde/alcohol dehydrogenase AdhB was purposely used in the design to reduce acyl CoAs to acyl alcohols. It should be

noted that the endogenous bi-functional alcohol/aldehyde dehydrogenases, such as AdhE, can also reduce acyl CoAs to their respective acyl alcohols. To build the PCT plus AAT submodule, we chose the enzyme PCT that is known to exhibit broad substrate specificity towards various VOAs for producing acyl CoAs (Schweiger and Buckel, 1984). Due to the limited information about the catalytic and specific function of AATs towards production of our target esters, we first mined a library of potential AAT sequences from literatures, NCBI, and Uniprot database (Figure 3-2) (Aharoni et al., 2000; Balbontín et al., 2010; Beekwilder et al., 2004; Cumplido-Laso et al., 2012; El-Sharkawy et al., 2005; Feng et al., 2014; González-Agüero et al., 2009; Harada et al., 1985; Li et al., 2006; Lucchetta et al., 2007; Olías et al., 2002; Park et al., 2009; Perez et al., 1993; Perez et al., 1996; Shalit et al., 2001; Souleyre et al., 2005; Yahyaoui et al., 2002). From this library, we chose 5 AATs that were both similar in sequence and/or organism but divergent in substrate preference to test production of our target esters. These AATs belong to the BAHD acyltransferase superfamily (St-Pierre and Luca, 2000) and possess the conserved BAHD acyltransferase motifs, e.g., H-X-X-X-D for catalytic functions and D-F-G-W-G for structural integrity (Galaz et al., 2013; Günther et al., 2011; Hansson et al., 2002; Ma et al., 2005). In this study, we chose the ATF1 and ATF2 of *S. cerevisiae* because they are the most divergent AATs that have only 37% sequence identity between each other, and encompass the capability to convert C2-C6 alcohols to their respective acetate counterpart (Verstrepen et al., 2003). Both the SAAT and VAAT enzymes, derived from the same strawberry genus but different species, were chosen because they have relatively high protein sequence similarity (88% identity) and exhibit broad substrate specificity (Beekwilder et al., 2004). Finally, AeAT9

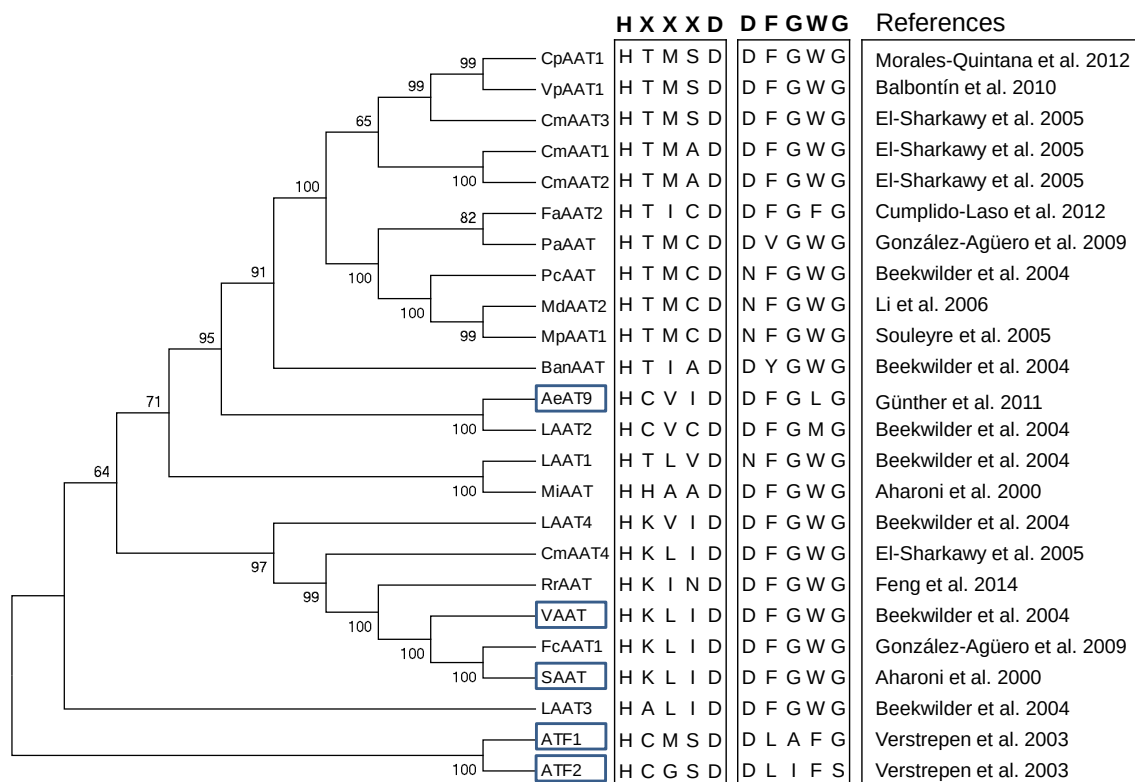


Figure 3-2: Phylogenetic analysis and structural and catalytic motifs of AATs. The conserved motifs are H-X-X-X-D for catalytic functions and D-F-G-W-G for structural integrity

has divergent sequence identity from each of the other sequences used, and has also been shown to have broad substrate specificity (Günther et al., 2011; Morales-Quintana et al., 2012).

We constructed a total of 5 acid-to-ester production modules, pDL009, pDL010, pDL011, pDL012, and pDL013, each of which contains the PCT plus AAT submodule and the ethanol submodule. All of these modules have the identical structural genes except AATs. Specifically, pDL009, pDL010, pDL011, pDL012, and pDL013, carry the ATF1, ATF2, SAAT, VAAT, and AeAT9 genes, respectively. By transforming these modules

into the previously engineered modular chassis cell EcDL002, we created 5 microbial cell factories, EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106, to evaluate the acid to ester conversion as well as specificity of AATs.

3.4.2 Exploring combinatorial biosynthesis of esters

We characterized the strains EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 by the *in situ*, high-cell density fermentation and extraction method with co-fermentation of sugars and individual VOAs, including acetic, propionic, butyric, pentanoic, and hexanoic acids, that are dominant fermentative products of the carboxylate platform. This characterization method is designed for rapid *in vivo* screening of the acid-to-ester production modules for combinatorial biosynthesis of target esters and evaluation of the specificity of the AATs under the same physiological conditions. Throughout this section, we reported only the production of esters that are in the hexadecane overlay used for the *in situ* fermentation and extraction. For a reference to calculate the total ester production, one could estimate 80% of the esters were secreted extracellularly based on our previous study (Layton and Trinh, 2014), and >80% of these extracellular esters were extracted in the hexadecane overlay (Figure 3-3).

3.4.2.1 Ester production from co-fermentation of acetic acid and glucose.

Strain characterization shows that most of the engineered strains produced ethyl acetate as the sole fermentative ester with different efficiency (Figure 3-4A). Among the strains, EcDL105 produced ethyl acetate with the highest level of 19.64 ± 3.20 mg/L after

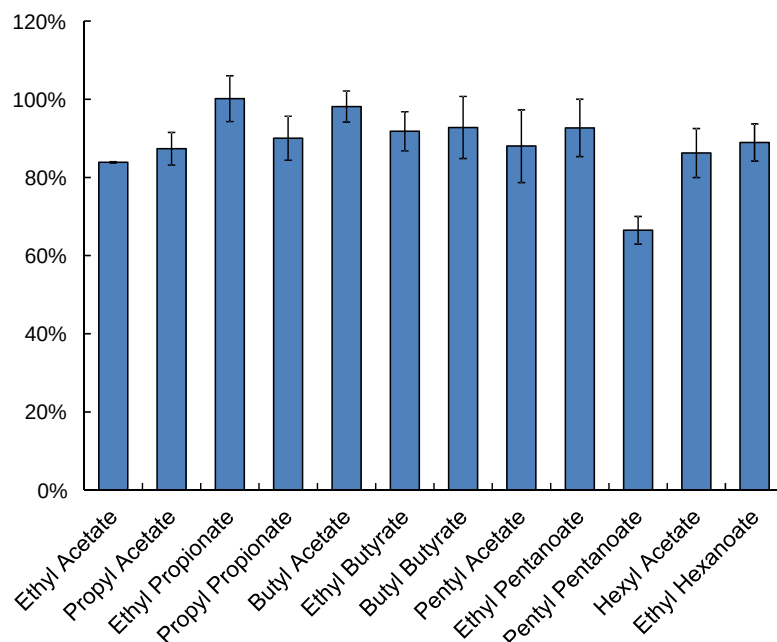


Figure 3-3: Percent of esters that are secreted into hexadecane. Percent of esters that were extracted into hexadecane overlay from the aqueous phase after 24 h. Extraction percentage is defined as the ratio of the final amount of ester in the overlay ($t = 24$ h) to the initial amount added to the medium ($t = 0$ h) multiplied by 100%. To estimate the extraction percentages, culture medium containing 22 mg/L of each ester only (without cell culture) was overlaid with hexadecane and incubated for 24 h at 37°C in sealed 15 mL tubes, which mimicked the condition of high-cell density fermentation and extraction experiments.

24 h while EcDL102, EcDL103, EcDL104, and EcDL106 generated 5.88 ± 0.58 mg/L, 3.01 ± 0.56 mg/L, 10.58 ± 3.61 mg/L, and 1.19 ± 0.19 mg/L ethyl acetate, respectively.

From these results, we arranged the AAT preference of ethanol and acetyl-CoA to produce ethyl acetate in the decreasing order as follows: VAAT (highest), SAAT, ATF1, ATF2, and AeAT9 (lowest). It should be noted that since we observed insignificant change in growth (OD_{5-7}) in our experiments, either ester titers (mg/L) or ester specific productivities ($\mu\text{g/g DCW/h}$) can be used to compare the AAT activities from the strains

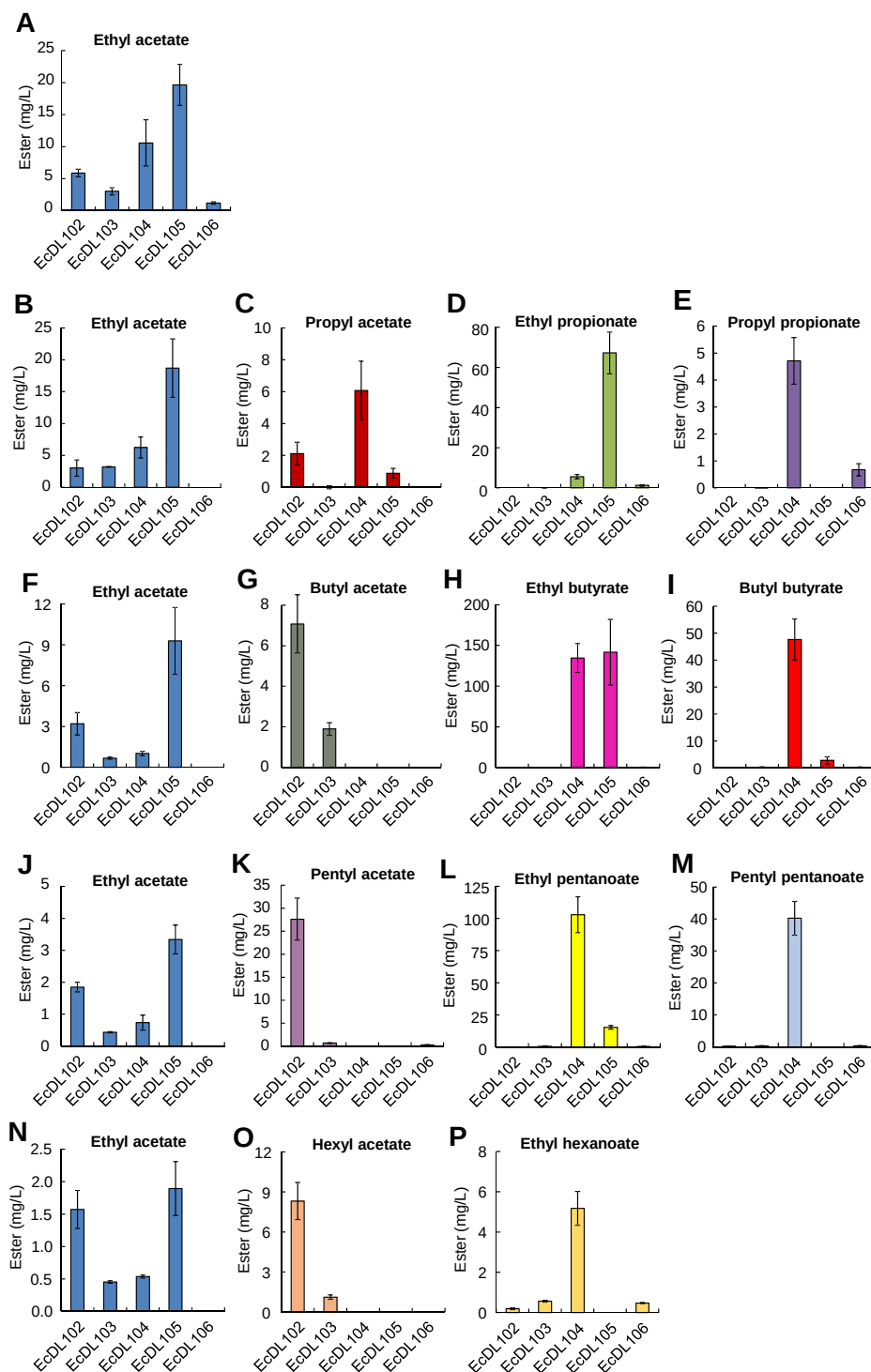


Figure 3-4: Ester production from exogenous addition of C₂-C₆ acids. (A) acetic acid, (B-E) propionic acid, (F-I) butyric acid, (J-M) pentanoic acid, and (N-P) hexanoic acid after 24 h. Each panel represents an ester that is produced by 5 microbial cell factories, EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 that express ATF1, AFT2, SAAT, VAAT, and AeAT9, respectively.

characterized. Besides the presented figure, these values are also reported in Tables 3-3 and 3-4. Table 3-5 and Table 3-6 report ester yields on glucose and remaining fermentation data, respectively.

3.4.2.2 Ester production from co-fermentation of glucose and propionic acid.

Our acid-to-ester module design can potentially generate 2 acetate esters (e.g., ethyl acetate and propionyl acetate) and 2 propionate esters (e.g., ethyl propionate and propyl propionate) from co-fermentation of glucose and propionic acid. Strain characterization confirms production of these esters (Figure 3-4B-E). However, none of the engineered strains alone was capable of producing all of the target esters likely because these strains expressed AATs with different specificities. Among the strains, EcDL105 produced the highest amount of total esters (86.81 ± 15.30 mg/L) whereas the total ester production were 5.12 ± 2.00 mg/L for EcDL102, 1.15 ± 0.29 mg/L for EcDL103, 22.62 ± 5.42 mg/L for EcDL104, and 1.87 ± 0.61 mg/L for EcDL106 (Figure 3-4B-E, Table 3-3). Consistent with the exogenous addition of acetic acid, most of the strains produced ethyl acetate, and EcDL105 produced the highest level of 18.69 ± 4.58 mg/L (Figure 3-4B). For propyl acetate, EcDL104 produced the highest amount of 6.07 ± 1.87 mg/L while EcDL102, EcDL103, and EcDL105 generated 2.11 ± 0.71 mg/L, 0.49 ± 0.09 mg/L, and 0.88 ± 0.30 mg/L, respectively. EcDL106 did not produce propyl acetate (Figure 3-4C).

The results show distinct substrate specificity among AATs for production of propionate esters. For ethyl propionate, EcDL105 produced the highest amount of 67.24 ± 10.41 mg/L, about 10.1 times higher than EcDL104 (5.60 ± 1.06 mg/L), 56.0 times higher

Table 3-3: Ester titers of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single VOAs after 24h. The acyl acetate, ethyl acylate, and acyl acylate columns correspond to the acids added. For instance, with the exogenous addition of pentanoic acid, the acyl acetate, ethyl acylate, and acyl acylate columns represent pentyl acetate, ethyl pentanoate, and pentyl pentanoate, respectively. Abbreviations: n.a: not applicable; n.d.: not detected.

	Ester titer (mg/L)					
	AATs	Ethyl acetate	Acyl acetate	Ethyl acylate	Acyl acylate	Total
Acetic acid doping	EcDL102	5.88 ± 0.58	n.a.	n.a.	n.a.	5.88 ± 0.58
	EcDL103	3.01 ± 0.56	n.a.	n.a.	n.a.	3.01 ± 0.56
	EcDL104	10.58 ± 3.61	n.a.	n.a.	n.a.	10.58 ± 3.61
	EcDL105	19.64 ± 3.20	n.a.	n.a.	n.a.	19.64 ± 3.20
	EcDL106	1.19 ± 0.19	n.a.	n.a.	n.a.	1.19 ± 0.19
Propionic acid doping	EcDL102	3.01 ± 1.28	2.11 ± 0.71	n.d.	n.d.	5.12 ± 2.00
	EcDL103	3.20 ± 0.05	0.49 ± 0.09	0.17 ± 0.14	n.d.	3.21 ± 0.29
	EcDL104	6.24 ± 1.65	6.07 ± 1.84	5.60 ± 1.06	4.72 ± 0.87	22.62 ± 5.42
	EcDL105	18.69 ± 4.58	0.88 ± 0.30	67.24 ± 10.41	n.d.	86.81 ± 15.30
	EcDL106	n.d.	n.d.	1.20 ± 0.38	0.68 ± 0.23	1.87 ± 0.61
Butyric acid doping	EcDL102	3.20 ± 0.82	7.08 ± 1.44	0.21 ± 0.01	0.19 ± 0.03	10.69 ± 2.30
	EcDL103	0.68 ± 0.07	1.90 ± 0.31	0.43 ± 0.05	0.28 ± 0.03	3.29 ± 0.46
	EcDL104	1.03 ± 0.17	n.d.	134.43 ± 17.66	47.63 ± 7.63	183.09 ± 25.46
	EcDL105	9.29 ± 2.45	n.d.	141.6 ± 40.2	2.76 ± 1.35	153.65 ± 44.00
	EcDL106	n.d.	n.d.	0.44 ± 0.40	0.17 ± 0.15	0.61 ± 0.55
Pentanoic acid doping	EcDL102	1.85 ± 0.15	27.64 ± 4.57	0.18 ± 0.01	0.18 ± 0.03	29.85 ± 4.77
	EcDL103	0.44 ± 0.02	0.70 ± 0.09	0.62 ± 0.03	0.29 ± 0.12	2.04 ± 0.25
	EcDL104	0.74 ± 0.24	n.d.	102.86 ± 13.92	40.25 ± 5.28	143.85 ± 19.44
	EcDL105	3.34 ± 0.45	n.d.	15.42 ± 1.38	n.d.	18.76 ± 1.83
	EcDL106	n.d.	0.30 ± 0.05	0.49 ± 0.05	0.33 ± 0.04	1.12 ± 0.14
Hexanoic acid doping	EcDL102	1.57 ± 0.29	8.32 ± 1.39	0.19 ± 0.05	n.d.	10.08 ± 1.73
	EcDL103	0.45 ± 0.02	1.12 ± 0.17	0.56 ± 0.04	n.d.	2.14 ± 0.23
	EcDL104	0.54 ± 0.02	n.d.	5.17 ± 0.84	n.d.	5.71 ± 0.86
	EcDL105	1.89 ± 0.42	n.d.	n.d.	n.d.	1.89 ± 0.42
	EcDL106	n.d.	n.d.	0.47 ± 0.03	n.d.	0.47 ± 0.03

Table 3-4: Specific ester productivities of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h.

		Specific ester production rates (µg/gDCW/h)				
		AATs	Ethyl acetate	Acyl acetate	Ethyl acylate	Acyl acylate
Acetic acid doping	EcDL102	84.88 ± 9.20	n.a.	n.a.	n.a.	
	EcDL103	23.58 ± 7.45	n.a.	n.a.	n.a.	
	EcDL104	148.83 ± 48.39	n.a.	n.a.	n.a.	
	EcDL105	325.18 ± 54.22	n.a.	n.a.	n.a.	
	EcDL106	17.94 ± 2.94	n.a.	n.a.	n.a.	
Propionic acid doping	EcDL102	41.91 ± 17.50	29.36 ± 9.6	n.d.	n.d.	
	EcDL103	0.68 ± 0.84	0.67 ± 1.40	0.24 ± 1.94	n.d.	
	EcDL104	86.36 ± 20.6	84.06 ± 23.17	77.65 ± 12.96	65.42 ± 10.44	
	EcDL105	277.24 ± 119.68	13.18 ± 6.81	986.8 ± 351.11	n.d.	
	EcDL106	n.d.	n.d.	17.43 ± 5.43	9.86 ± 3.19	
Butyric acid doping	EcDL102	45.01 ± 11.22	99.49 ± 19.76	2.97 ± 0.13	2.73 ± 0.41	
	EcDL103	9.52 ± 0.97	26.6 ± 4.40	6.04 ± 0.68	3.96 ± 0.42	
	EcDL104	14.95 ± 1.85	n.d.	1957.87 ± 247.4	692.81 ± 100.37	
	EcDL105	147.82 ± 38.75	n.d.	2253.04 ± 636.74	43.95 ± 21.49	
	EcDL106	n.d.	n.d.	6.77 ± 6.22	2.62 ± 2.30	
Pentanoic acid doping	EcDL102	47.43 ± 36.39	306.95 ± 158.18	4.82 ± 3.90	3.01 ± 0.92	
	EcDL103	6.24 ± 0.21	9.96 ± 1.15	8.90 ± 0.41	4.08 ± 1.71	
	EcDL104	10.97 ± 3.49	n.d.	1533.47 ± 223.21	600.15 ± 85.89	
	EcDL105	54.76 ± 7.74	n.d.	252.48 ± 19.76	n.d.	
	EcDL106	n.d.	4.48 ± 0.62	7.22 ± 0.64	4.88 ± 0.59	
Hexanoic acid doping	EcDL102	22.92 ± 4.14	121.42 ± 20.09	2.74 ± 0.66	n.d.	
	EcDL103	6.94 ± 0.32	17.07 ± 2.47	8.63 ± 0.66	n.d.	
	EcDL104	8.65 ± 0.51	n.d.	83.32 ± 14.57	n.d.	
	EcDL105	29.52 ± 6.7	n.d.	n.d.	n.d.	
	EcDL106	n.d.	n.d.	6.78 ± 0.36	n.d.	

Table 3-5: Ester yields on glucose of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h.

		Ester yields (mg/g)			
	AATs	Ethyl Acetate	Acyl Acetate	Ethyl Acylate	Acyl Acylate
Acetic acid doping	EcDL102	0.56 ± 0.05	n.a.	n.a.	n.a.
	EcDL103	0.10 ± 0.03	n.a.	n.a.	n.a.
	EcDL104	0.56 ± 0.19	n.a.	n.a.	n.a.
	EcDL105	1.30 ± 0.20	n.a.	n.a.	n.a.
	EcDL106	0.07 ± 0.01	n.a.	n.a.	n.a.
Propionic acid doping	EcDL102	0.27 ± 0.12	0.19 ± 0.06	n.d.	n.d.
	EcDL103	0.03 ± 0.00	0.03 ± 0.01	0.01 ± 0.01	n.d.
	EcDL104	0.33 ± 0.09	0.32 ± 0.10	0.29 ± 0.06	0.25 ± 0.05
	EcDL105	1.18 ± 0.40	0.06 ± 0.02	4.22 ± 1.10	n.d.
	EcDL106	n.d.	n.d.	0.06 ± 0.02	0.04 ± 0.01
Butyric acid doping	EcDL102	0.30 ± 0.08	0.67 ± 0.15	0.02 ± 0.00	0.02 ± 0.00
	EcDL103	0.04 ± 0.00	0.11 ± 0.02	0.03 ± 0.00	0.02 ± 0.00
	EcDL104	0.05 ± 0.01	n.d.	6.98 ± 0.92	2.47 ± 0.40
	EcDL105	0.57 ± 0.14	n.d.	8.68 ± 2.34	0.17 ± 0.08
	EcDL106	n.d.	n.d.	0.03 ± 0.02	0.01 ± 0.01
Pentanoic acid doping	EcDL102	0.70 ± 0.11	10.45 ± 2.48	0.07 ± 0.01	0.07 ± 0.02
	EcDL103	0.06 ± 0.00	0.11 ± 0.01	0.09 ± 0.01	0.05 ± 0.02
	EcDL104	0.03 ± 0.01	n.d.	4.96 ± 0.21	1.95 ± 0.12
	EcDL105	0.45 ± 0.11	n.d.	2.19 ± 0.50	n.d.
	EcDL106	n.d.	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Hexanoic acid doping	EcDL102	0.18 ± 0.03	0.95 ± 0.21	0.02 ± 0.00	n.d.
	EcDL103	0.04 ± 0.00	0.10 ± 0.02	0.05 ± 0.01	n.d.
	EcDL104	0.03 ± 0.00	n.d.	0.29 ± 0.05	n.d.
	EcDL105	0.17 ± 0.04	n.d.	n.d.	n.d.
	EcDL106	n.d.	n.d.	0.03 ± 0.00	n.d.

Table 3-6: Fermentation data of EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 for co-fermentation of glucose and single organic acids after 24h. The subscripts i and f are referred to the initial time (t = 0 h) and final time (t = 24 h) of fermentation, respectively.

	Strain	OD _i	OD _f	Glucose _i (g/L)	Acid _i (g/L)	ΔGlucose (g/L)	ΔAcid (g/L)	Ethanol (g/L)	Acyl Alcohol (g/L)
Acetic acid doping	EcDL102	5.06	6.86 ± 0.12	19.01	2.65	10.56±0.21	2.16 ± 0.05	3.68 ± 0.08	n.a.
	EcDL103	5.08	8.20 ± 0.14	19.01	2.65	17.94±0.95	1.72 ± 0.17	6.28 ± 0.68	n.a.
	EcDL104	4.96	7.22 ± 0.21	19.01	2.65	19.01±0	1.92 ± 0.03	6.57 ± 0.06	n.a.
	EcDL105	5.08	5.31 ± 0.09	19.01	2.65	15.13±0.09	1.74 ± 0.01	5.39 ± 0.03	n.a.
	EcDL106	5.04	6.37 ± 0.06	19.01	2.65	17.92±0.44	2.03 ± 0.03	6.64 ± 0.28	n.a.
Propionic acid doping	EcDL102	5.06	7.26 ± 0.20	19.12	3.06	11.24±0.78	2.24 ± 0.02	3.61 ± 0.28	0.45 ± 0.06
	EcDL103	5.08	7.51 ± 0.20	19.12	3.06	16.94±0.27	1.99 ± 0.12	5.26 ± 0.11	0.42 ± 0.03
	EcDL104	4.96	7.41 ± 0.32	19.12	3.06	19.12±0	1.51 ± 0.04	6.11 ± 0.06	0.97 ± 0.03
	EcDL105	5.08	7.42 ± 0.35	19.12	3.06	14.93±0.04	2.66 ± 0.12	4.15 ± 0.15	0.15 ± 0.01
	EcDL106	5.04	6.74 ± 0.13	19.12	3.06	18.56±0.58	2.21 ± 0.09	6.57 ± 0.18	0.35 ± 0.05
Butyric acid doping	EcDL102	5.06	7.17 ± 0.12	19.25	1.87	10.65±0.28	1.70 ± 0.05	3.91 ± 0.07	0.26 ± 0.03
	EcDL103	5.08	7.19 ± 0.16	19.25	1.87	16.74±0.1	1.54 ± 0.03	6.28 ± 0.54	0.25 ± 0.02
	EcDL104	4.96	6.85 ± 0.59	19.25	1.87	19.25±0	1.48 ± 0.02	6.56 ± 0.17	0.47 ± 0.02
	EcDL105	5.08	5.79 ± 0.10	19.25	1.87	16.46±0.35	1.58 ± 0.03	5.62 ± 0.35	0.06 ± 0.00
	EcDL106	5.04	6.07 ± 0.07	19.25	1.87	17.46±0.25	1.64 ± 0.04	6.71 ± 0.20	0.27 ± 0.01
Pentanoic acid doping	EcDL102	5.06	6.98 ± 0.11	19.15	2.05	3.26±1.02	1.70 ± 0.05	3.41 ± 0.02	0.16 ± 0.02
	EcDL103	5.08	6.92 ± 0.14	19.15	2.05	6.77±0.47	2.14 ± 0.04*	5.04 ± 0.52	0.07 ± 0.01
	EcDL104	4.96	6.59 ± 0.26	19.15	2.05	19.15±0	1.80 ± 0.01	6.41 ± 0.15	0.19 ± 0.01
	EcDL105	5.08	5.42 ± 0.22	19.15	2.05	7.3±0.62	2.08 ± 0.03*	5.05 ± 0.14	0.00 ± 0.00
	EcDL106	5.04	6.53 ± 0.25	19.15	2.05	19.15±0	1.58 ± 0.04	6.65 ± 0.19	0.18 ± 0.03
Hexanoic acid doping	EcDL102	5.06	6.73 ± 0.10	19.18	2.15	8.88±0.57	1.78 ± 0.05	3.09 ± 0.07	0.00 ± 0.00
	EcDL103	5.08	6.18 ± 0.06	19.18	2.15	11.39±0.75	2.03 ± 0.15	4.57 ± 0.26	0.00 ± 0.00
	EcDL104	4.96	5.74 ± 0.40	19.18	2.15	18.06±0.97	2.03 ± 0.03	6.36 ± 0.46	0.00 ± 0.00
	EcDL105	5.08	5.97 ± 0.22	19.18	2.15	11.42±0.07	2.20 ± 0.09*	3.83 ± 0.08	0.00 ± 0.00
	EcDL106	5.04	6.93 ± 0.41	19.18	2.15	16.14±1.37	1.85 ± 0.01	6.43 ± 0.29	0.00 ± 0.00

than EcDL106 (1.20 ± 0.38 mg/L), and 395.5 times higher than EcDL103 (0.17 ± 0.14 mg/L) (Figure 3-4D). EcDL102 did not produce any ethyl propionate. Like the propyl acetate production, EcDL104 generated propyl propionate at the highest level of 4.72 ± 0.87 mg/L while EcDL106 produced much less, 0.68 ± 0.23 mg/L. EcDL102 and EcDL105 did not produce any significant amount of propyl propionate (Figure 3-4E). Taken altogether, the results exhibit some notable trends of AAT specificity from co-fermentation of glucose and propionic acid. The VAAT of EcDL105 has substrate specificity for ethanol and acetyl CoA/propionyl CoA to produce ethyl acetate and ethyl propionate while the SAAT of EcDL104 has substrate specificity for propanol and acetyl CoA/propionyl CoA to produce propyl acetate and propyl propionate. In contrast, ATF1 shows substrate preference for ethanol/propanol and acetyl CoA to produce ethyl acetate and propyl acetate.

3.4.2.3 Ester production from co-fermentation of glucose and butyric acid.

Strain characterization confirms the production of two acetate esters (e.g., ethyl acetate, butyl acetate) and two butyrate esters (e.g., ethyl butyrate and butyl butyrate) from co-fermentation of glucose and butyric acid (Figure 3-4F-I). Both EcDL104 and EcDL105 produced the highest amount of total esters, e.g., 183.09 ± 25.46 mg/L and 153.65 ± 44.00 mg/L, respectively while the total ester production were 10.69 ± 2.30 mg/L for EcDL102, 3.29 ± 0.46 mg/L for EcDL103, and 0.61 ± 0.55 mg/L for EcDL106 (Figure 3-4F-I, Table 3-3). Like the exogenous addition of propionic acid, none of the engineered strains alone was capable of producing all of these target esters. For the ethyl acetate production, the same trend of AAT specificity was observed, where EcDL105 produced the highest amount of 9.29 ± 2.45 mg/L among the strains characterized (Figure 3-4F). For butyl acetate, only

EcDL102 and EcDL103 produced at the levels of 7.08 ± 1.44 mg/L and 1.90 ± 0.31 mg/L, respectively (Figure 3-4G). Clearly, ATF1 of EcDL102 has higher activity than ATF2 of EcDL103. In comparison with SAAT, VAAT, and AeAT9, both ATF1 and ATF2 have substrate preference for longer chain alcohol and acetyl CoA to produce acyl acetate.

In addition, the results show very distinct AAT specificities for butyrate ester production. Only EcDL104 and EcDL105 produced ethyl butyrate at relatively high levels of 134.43 ± 17.66 mg/L and 141.60 ± 40.20 mg/L, respectively (Figure 3-4H). In contrast, all of the strains produced butyl butyrate (Figure 3-4I). EcDL104 made butyl butyrate at the highest level of 47.63 ± 7.63 mg/L while EcDL102, EcDL103, EcDL105, and EcDL106 produced much less, 0.19 ± 0.03 mg/L, 0.28 ± 0.03 mg/L, 2.76 ± 1.35 mg/L, and 0.17 ± 0.15 mg/L, respectively.

Taken altogether, we can draw some notable trends of substrate preference among AATs from co-fermentation of glucose and butyric acid. The VAAT of EcDL105 again has substrate specificity for ethanol and acyl CoA/butyryl CoA to produce ethyl acetate and ethyl butyrate while the SAAT of EcDL104 has substrate preferences for ethanol/butanol and butyryl CoA to produce ethyl butyrate and butyl butyrate. Differently, both ATF1 and ATF2 have distinct substrate preference for butanol and acetyl CoA to produce butyl acetate.

3.4.2.4 Ester production from co-fermentation of glucose and pentanoic acid.

Strain characterization shows that all four of the expected esters, including two acetate esters (e.g., ethyl acetate, pentyl acetate) and two pentanoate esters (e.g., ethyl pentanoate, pentyl pentanoate), were produced (Figure 3J-M). EcDL104 produced the

highest amount of total esters (143.85 ± 19.44 mg/L) while the total production were 29.85 ± 4.77 mg/L for EcDL102, 2.04 ± 0.25 mg/L for EcDL103, 18.76 ± 1.83 mg/L for EcDL105, and 1.12 ± 0.14 mg/L for EcDL106 (Figure 3J-M, Table 3-3). Even though the production of ethyl acetate was low, we observed the same trend of AAT specificity for ethyl acetate among the engineered strains (Figure 3-4J). Like the exogenous addition of propionic and butyric acids, EcDL102 produced pentyl acetate at the highest level of 27.64 ± 4.57 mg/L while EcDL103 and EcDL106 produced this ester at significantly lower levels of 0.70 ± 0.09 mg/L and 0.30 ± 0.05 mg/L (Figure 3-4K).

Likewise, EcDL104 and EcDL105 are the main producers of acylate esters. EcDL104 produced ethyl pentanoate at the highest level of 102.86 ± 13.92 mg/L, about 6.67 higher than EcDL105 (15.42 ± 1.38 mg/L) (Figure 3-4L). It should be noted that this trend has switched from exogenous addition of butyric to pentanoic acids. EcDL102, EcDL103, and EcDL106 produced ethyl pentanoate at much lower levels of 0.18 ± 0.01 mg/L, 0.62 ± 0.03 mg/L, 0.49 ± 0.05 mg/L, respectively. Besides high production of ethyl pentanoate, EcDL104 also made pentyl pentanoate at the highest level of 40.25 ± 5.28 mg/L (Figure 3-4M).

Taken altogether, the results clearly show that the VAAT of EcDL105 has substrate preference for ethanol and acetyl CoA/pentyl CoA to produce ethyl acetate and ethyl pentanoate for co-fermentation of glucose and pentanoic acid while the SAAT of EcDL104 has distinct substrate preference for ethanol/pentanol and pentyl CoA to produce ethyl pentanoate and pentyl pentanoate. Different from both the VAAT and SAAT, the ATF1 of EcDL102 has high preference for pentanol and acetyl CoA to produce pentyl acetate.

3.4.2.5 Ester production from co-fermentation of glucose and hexanoic acid.

Unlike exogenous addition of propionic, butyric, and pentanoic acids, only three out of 4 expected esters, including ethyl acetate, hexyl acetate, and ethyl hexanoate, were produced by co-fermentation of glucose and hexanoic acid (Figure 3-4N-P). In comparison to other exogenous addition of acids, the total ester production was relatively low, 10.08 ± 1.73 mg/L for EcDL102 (highest), 2.14 ± 0.23 mg/L for EcDL103, 5.71 ± 0.86 mg/L for EcDL104, 1.89 ± 0.42 mg/L for EcDL105, and 0.47 ± 0.03 mg/L for EcDL106 (Figure 3-4N-P, Table 3-3). Production of ethyl acetate exhibited the same trend as exogenous addition of other VOAs (Figure 3-4N). EcDL102 was the main producer of hexyl acetate, generating 8.32 ± 1.39 mg/L, about 7.43 times higher than EcDL103 (Figure 3-4O).

Like exogenous addition of longer chain VOAs, EcDL104 was the main producer of ethyl hexanoate, 5.17 ± 0.84 mg/L while EcDL102, EcDL103, and EcDL106 produced this ester at significantly lower levels of 0.19 ± 0.05 mg/L, 0.56 ± 0.04 mg/L, and 0.47 ± 0.03 mg/L (Figure 3-4P). However, we did not observe any significant production of ethyl hexanoate by EcDL105.

In summary, it is possible to produce 12 out of the 13 possible esters with exogenous addition of C2-C6 VOAs using the designed acid-to-ester production modules. The engineered strains EcDL102, EcDL103, EcDL104, EcDL105, and EcDL106 that carry these modules with different AAT specificities, generated 10, 11, 9, 6, and 9 unique esters, respectively. The ATF1 of EcDL102, SAAT of EcDL104, and VAAT of EcDL105 were the most active among the five AATs tested for ester production, and exhibited distinct substrate specificity. The ATF1 preferred acetyl CoA and acyl alcohols to produce acyl

acyl acetate with the highest activity towards pentanol (Figure 3-5A).

Both the SAAT and VAAT prefer acyl CoAs and ethanol to produce ethyl acylate (Figure 3-5B, 5C). However, VAAT is more specific to C2-C4 acyl CoAs while SAAT is more specific to C4-C6 acyl CoAs. Among the AATs characterized, the SAAT is the only one that has dominant substrate preference towards acyl CoAs and acyl alcohols to produce acyl acylate with the highest activities towards butyl butyrate and pentyl pentanoate (Figure 3-5D). In addition, it was observed that the production of esters was reduced for the fermentation of longer chain acids (e.g., hexanoic acid). This reduction might be caused by toxicity of the exogenous acids, converted intermediates, and/or substrate availability. Further studies beyond our proof of concept would need to be conducted to understand the causes.

3.4.3 Ester production from co-fermentation of glucose and mixed VOAs

3.4.3.1 Use of single cultures for upgrading mixed VOAs to target esters.

Anaerobic digestion, such as the carboxylate platform, is very robust for converting lignocellulosic biomass or biomass waste into C2-C6 VOAs that dominantly contain acetic, propionic, butyric, pentanoic, and hexanoic acids (Holtzapfel, 2015). Since the strains EcDL102, DL104, and DL105 have the most active AATs for production of ethyl acetate, acyl (>C2) acetate, ethyl acylate, and acyl (C>2) acylate, we investigated these acid-to-ester production platforms for their capabilities to upgrade mixed VOAs to high-value esters. Strain characterization shows that EcDL105 produced the highest amount of total esters (138.76 ± 35.42 mg/L), about 1.25 times slightly higher than EcDL104 (110.66

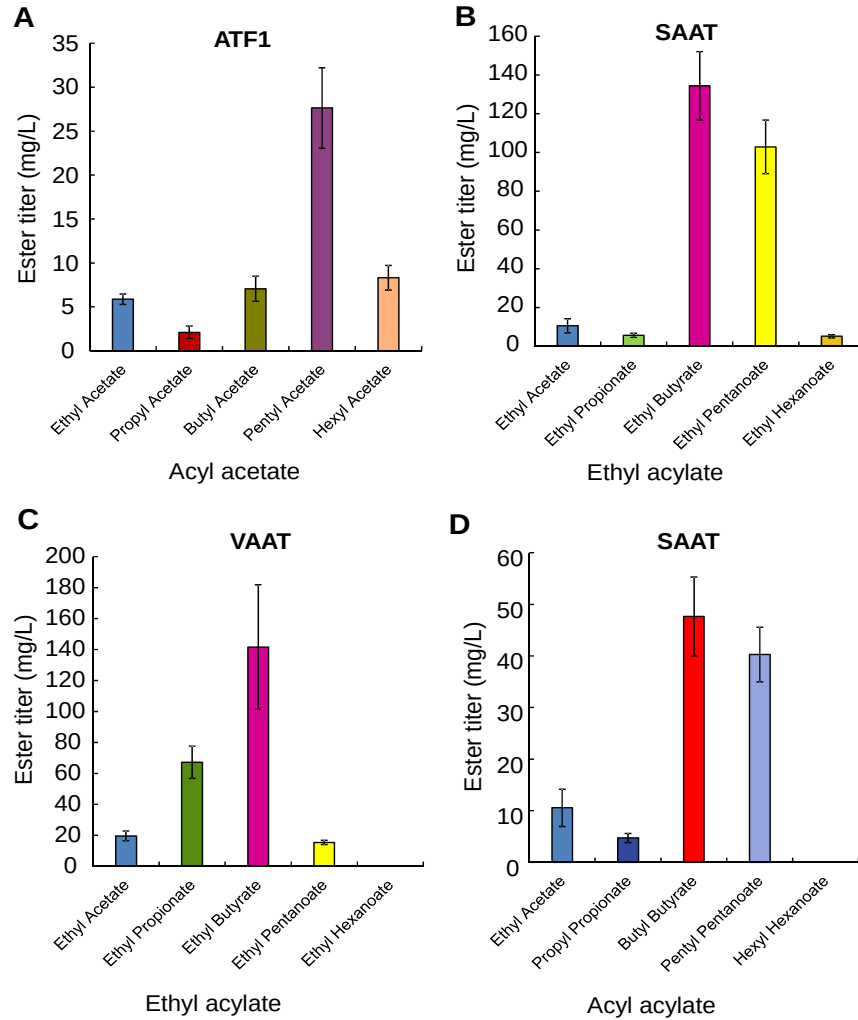


Figure 3-5: AAT specificity from alcohols and acyl-CoAs. Specificity of ester production by ATF1 of EcDL102, SAAT of EcDL104, and VAAT of EcDL05 from co-fermentation of glucose and single VOAs after 24 h. **(A)** Acyl acetate production by ATF1. **(B)** Ethyl acylate production by SAAT. **(C)** Ethyl acylate production by VAAT. **(D)** Acyl acylate production by SAAT.

± 14.10 mg/L) and 4.68 times higher than EcDL102 (29.67 ± 6.13 mg/L) after 24h (Figure 3-6, Table 3-7). EcDL102 produced 7 out of 10 esters that were produced from exogenous addition of individual VOAs. Consistently, acyl acetates were the most dominant with high production levels of ethyl acetate (10.45 ± 2.58 mg/L) and pentyl acetate (9.46 ± 1.65 mg/L). Different from EcDL102, EcDL104 primarily produced ethyl acylate with the highest production of ethyl butyrate (56.80 ± 8.42 mg/L) followed by ethyl pentanoate (40.86 ± 3.85 mg/L) (Figures 3-6A, 3-6C, Table 3-7). EcDL104 also produced acyl acylate, e.g., butyl butyrate at the highest level of 2.32 ± 0.14 mg/L among significantly lower levels of propyl propionate and pentyl pentanoate. Likewise, EcDL105 produced a total of 8 esters. Interestingly, two additional esters, propyl propionate and ethyl hexanoate, that were not observed from single addition of VOAs, were also produced in small quantities, possibly due to the less inhibition of acids and availability of ester precursors. Similar to EcDL104, EcDL105 primarily produced ethyl butyrate (101.77 ± 25.16 mg/L), followed by ethyl propionate (15.88 ± 4.93 mg/L) and ethyl pentanoate (7.10 ± 1.17 mg/L) (Figures 3-6A, 3-6D, Table 3-7). Consistent with single exogenous addition, EcDL105 produced more ethyl acetate than EcDL102 and EcDL104.

Overall, the total ester production levels and the distribution of ester products from the co-fermentation of glucose and mixed VOAs are very consistent with the substrate specificities of ATF1 (of EcDL102), SAAT (of EcDL104), and VAAT (of EcDL05) that were identified from exogenous addition of individual VOAs.



Figure 3-6: Mixed acid ester production. (A) Ester production and **(B-E)** fraction of ester profiles of EcDL102, EcDL104, EcDL105, and mixed cultures of EcDL102, EcDL104, and EcDL105 for co-fermentation of glucose and 5 g/L total mixed organic acids after 24h.

Table 3-7: Ester titers of EcDL102, EcDL104, EcDL105, and mixed cultures of EcDL102, EcDL104, and EcDL105 for co-fermentation of glucose and 5 g/L mixed organic acids after 24h.

Esters	Ester titer (mg/L)			
	EcDL102	EcDL104	EcDL105	Mixed cultures
Ethyl Acetate	10.45 ± 2.58	3.69 ± 0.67	11.44 ± 3.55	6.52 ± 2.67
Propyl Acetate	2.98 ± 0.50	0.73 ± 0.21	0.47 ± 0.14	2.02 ± 1.20
Ethyl Propionate	n.d.	1.89 ± 0.32	15.88 ± 4.93	2.65 ± 0.21
Propyl Propionate	n.d.	0.23 ± 0.32	0.61 ± 0.13	0.36 ± 0.04
Butyl Acetate	4.45 ± 0.68	n.d.	n.d.	2.96 ± 1.40
Ethyl Butyrate	n.d.	56.8 ± 8.42	101.77 ± 25.16	44.34 ± 5.02
Butyl Butyrate	0.10 ± 0.01	2.32 ± 0.14	1.03 ± 0.25	1.31 ± 0.27
Pentyl Acetate	9.46 ± 1.65	n.d.	n.d.	3.14 ± 1.11
Ethyl Pentanoate	n.d.	40.86 ± 3.85	7.10 ± 1.17	17.49 ± 1.24
Pentyl Pentanoate	n.d.	0.80 ± 0.03	n.d.	0.34 ± 0.05
Hexyl Acetate	2.02 ± 0.63	n.d.	n.d.	1.03 ± 0.18
Ethyl Hexanoate	0.12 ± 0.04	3.35 ± 0.15	0.43 ± 0.02	1.50 ± 0.05
Total	29.67 ± 6.13	110.66 ± 14.10	138.76 ± 35.42	83.64 ± 13.43

3.4.3.2 Use of mixed cultures for upgrading mixed VOAs to target esters.

To produce the entire spectrum of target esters from co-fermentation of glucose and mixed VOAs, we used the mixed cultures of EcDL102, EcDL104, and EcDL105 in equal amount for the *in situ* fermentation and extraction. Strain characterization, indeed, confirms the production of 12 out of 13 expected esters except hexyl hexanoate, which is consistent with the exogenous addition of individual VOAs (Figures 3-6A, 3-6E, Table 3-7). The total ester production of the mixed cultures was 83.64 ± 13.43 mg/L, lower than the single cultures of EcDL104 and EcDL105 but higher than the single culture of EcDL102.

Among the esters, ethyl butyrate was produced at the highest amount (44.34 ± 5.02 mg/L) followed by ethyl pentanoate (17.49 ± 1.24 mg/L). The results are expected because

both SAAT (of EcDL104) and VAAT (of EcDL105) are specific to ethyl acylate production and have higher activities than ATF1 (of EcDL105) that is specific to acyl acetate production.

3.5 Discussion

In this study, we established a general framework for upgrading VOAs to high-value esters that have broad industrial applications such as fragrances, flavors, solvents, and biofuels. By assembling the acid-to-ester modules with the established *E. coli* modular chassis cell, we developed microbial manufacturing platforms to perform the following functions: i) rapid *in vivo* screening of novel AATs for their catalytic activities, ii) expanding the combinatorial biosynthesis of unique fermentative esters, and iii) upgrading carboxylates to higher-value esters using single or mixed cell cultures.

Among the five AATs screened, ATF1 of *S. cerevisiae*, SAAT of *F. ananassa*, and VAAT of *F. vesca* were the most active towards the carboxylate library, and exhibited activities for 10, 9, and 9 out of 13 unique fermentative esters, respectively. ATF1, SAAT, and VAAT had substrate preference for biosynthesis of acyl (C4-C6) acetate, ethyl (C2-C4) acylate, and ethyl (C4-C6) acylate, respectively. Different from ATF1 and VAAT, SAAT also exhibited high substrate preference for biosynthesis of acyl acylates (e.g., propyl propionate, butyl butyrate, and pentyl pentanoate). Based on the total ester production from exogenous addition of single or mixed VOAs, both VAAT and SAAT had higher activities than ATF1. Examination of the highly conserved catalytic (H-X-X-X-D) and structural (D-F-G-W-G) motifs (Figure 3-2) offered some hypothesis of substrate specificity for the characterized AATs. However, there are more factors influencing

substrate specificity than simply the catalytic and structural motifs; for instance, SAAT and VAAT have both identical catalytic and structural motifs but different substrate specificity. Using molecular dynamics simulations should help elucidate modes of action for substrate specificity, and guide protein engineering efforts of the characterized, and other, AATs for *in vivo* engineering of ester formation (Galaz et al., 2013; Morales-Quintana et al., 2012; Morales-Quintana et al., 2013).

The conventional method to characterize AAT activities is time-consuming and expensive because this method i) requires protein expression, purification, and characterization, ii) uses expensive substrates (e.g., acyl CoAs) that sometimes are not available and hence limit the space of substrates tested, and iii) relies on the low throughput GC/MS method to quantify esters produced. In contrast, our *in vivo* screening method developed can address some of these limitations. By using cheap substrates such as mixed VOAs, the *in vivo* method can screen for AAT activities for combinatorial biosynthesis of more than 10 unique esters simultaneously, and access their relative substrate preferences as determined for ATF1 of EcDL102, SAAT of EcDL104, and VAAT of EcDL105 in our studies. In addition, the *in situ* high cell density fermentation and extraction eliminates many steps of protein expression, purification, enzyme assay, and ester extraction for GC/MS while evaluating the functional *in vivo* activities of AATs in heterologous hosts. In our study, the *in vivo* method not only confirmed the known activities of ATF1, SAAT, and VAAT for the biosynthesis of acetate esters but also discovered their new catalytic functions towards the biosynthesis of ethyl acylate, acyl acylate, and perhaps more. The knowledge of activities of AATs will be useful for fundamental understanding of their

functional roles in natural environments such as fruits and flowers as well as for elucidation of protein structures and catalytic functions for rational novel protein engineering.

The design of the acid-to-ester modules is quite flexible to enable expansion of the combinatorial biosynthesis of esters. The acid-to-ester module of this study can potentially generate a set of 13 unique esters by using a mixture of short, linear (C2-C6) VOAs that are dominantly found in the anaerobic digestion of lignocellulosic biomass or biomass wastes. By replacing the ethanol submodule with other branched, short-chain alcohol modules (e.g., isopropanol, isobutanol, isopentanol, and isopentenol), we can potentially generate a library of 24 additional unique esters. By manipulating AATs with controllable specificities as well as employing single and/or mixed cell cultures for fermentation, it is possible to tailor the acid-to-ester modules to produce desirable esters as high-purity chemicals or mixed, designer bioesters.

VOAs investigated in this study are the dominant fermentative products of the carboxylate platform (e.g., anaerobic digestion of lignocellulosic biomass or biomass wastes) and have great potential for downstream upgrading to fuels and high-value chemicals via chemical conversion (Agler et al., 2011; Holtzapple, 2015). Our study shows the alternative microbial conversion route that can be deployed for directly upgrading these VOAs to higher-value esters. Since VOAs are toxic to cells, upgrading these VOAs to esters for convenient extraction helps alleviate the acid toxicity while producing higher-value chemicals. This approach can be potentially realized by deploying engineered, compatible acid-to-ester strains to the microbial communities of the anaerobic digester. Even though the proof-of-concept for upgrading VOAs to esters is demonstrated, the yields, titers, and productivities for the acid-to-ester conversion are low (Tables 3-3-7)

likely due to VOA toxicity, non-optimized cultivation conditions, non-optimized acid-to-ester production modules, and/or appropriate choice of the production hosts. In our study, relatively high amount of ethanol secreted to the medium (see Table 3-6) implies that carbon fluxes through ACT and/or AAT submodules might have been limiting potentially due to low catalytic efficiency and poor protein expression/folding of ACT and AATs (Zhu et al., 2015). Addressing these limitations is necessary for enhanced acid-to-ester conversion in the future study.

In summary, we envision that our developed framework is valuable and powerful for *in vivo* characterization of a repertoire of not-well-characterized natural AATs, for expanding the combinatorial biosynthesis of fermentative esters, and for upgrading volatile organic acids to higher-value esters.

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4 Microbial Synthesis of a Branched- Chain Ester Platform from Organic Waste Carboxylates

Summary: This chapter is based on the published paper: Layton, Donovan S., and Cong T. Trinh. "Microbial synthesis of a branched-chain ester platform from organic waste carboxylates." *Metabolic Engineering Communications* 3 (2016): 245-251.

4.1 Abstract

Processing of lignocellulosic biomass or organic wastes produces a plethora of chemicals such as short, linear carboxylic acids that make up the carboxylate platform from anaerobic digestion. While this carboxylate platform has low-value and is inhibitory to microbes during fermentation, it can be biologically upgraded to high-value products. In this study, we expanded our general framework for biological upgrading of carboxylates to branched-chain ester platform by using three highly active alcohol acyltransferases (AATs) for alcohol and acyl CoA condensation and modulating the alcohol moiety from ethanol to isobutanol in a modular chassis cell. With this framework, we demonstrated the production of an ester platform comprised of 16 out of all 18 potential esters, including acetate, propionate, butanoate, pentanoate, and hexanoate esters, from the 5 linear, saturated C₂-C₆ carboxylic acids. Among these esters, 5 new branched-chain esters, including isobutyl acetate, isobutyl propionate, isobutyl butyrate, isobutyl pentanoate, and isobutyl hexanoate were synthesized *in vivo*. During 24 h *in situ* fermentation and extraction, one of the engineered strains, EcDL208 harnessing the SAAT of *Fragaria ananassa* produced ~63 mg/L of a mixture of butyl and isobutyl butyrates from glucose and butyrate co-fermentation and ~127 mg/L of a mixture of isobutyl and pentyl pentanoates from glucose and pentanoate co-fermentation, with high specificity. These

butyrate and pentanoate esters are potential drop-in liquid fuels. This study provides better understanding of functional roles of AATs for microbial biosynthesis of branched-chain esters and expands the potential use of these esters as drop-in biofuels beyond their conventional flavor, fragrance, and solvent applications.

4.2 Introduction

The natural, efficient consolidated bioprocessing of lignocellulosic biomass or organic wastes is anaerobic digestion (Agler et al., 2011; Jonsson and Martin, 2016). In this process, a consortium of mixed microbes (e.g. anaerobic digesters) can degrade organic wastes directly into the carboxylate platform (e.g., linear and saturated C₂-C₆ organic acids) without the stipulation of any pretreatment (Batstone and Virdis, 2014; Thanakoses et al., 2003). While these carboxylates have low values and are inhibitory to microbes, they can be biologically upgraded to a large space of high-value chemicals such as esters that are widely used in flavor, fragrance, and solvent industries. Certain carboxylate-derived esters have high hydrophobicity for easy separation from fermentation and encompass high combustion properties that can be used as biodiesels or jet fuels (Chuck and Donnelly, 2014; Contino et al., 2011; Kallio et al., 2014).

Biologically upgrading of the carboxylate to ester platforms has recently been demonstrated (Layton and Trinh, 2016). This conversion was achieved by a modular cell (Trinh et al., 2015) tightly integrated with an engineered acid-to-ester production module – a modular heterologous pathway comprised of an alcohol production submodule, an acid to acyl CoA synthesis submodule, and alcohol and an acyl CoA condensation submodule. The flexible design of these modules served several purposes: i) expanding the biosynthesis of the ester platform in a plug-and-play fashion using a pure culture or a consortium of mixed cultures and ii) screening alcohol acyl transferases (AATs) for their novel *in vivo* activities. Understanding the catalysis of the AAT condensation reaction is critical for efficient ester biosynthesis but is currently limited. Some recent studies have aimed at understanding AAT specificities using various techniques, from whole-cell *in vivo* approaches using the

carboxylate platform (Layton and Trinh, 2016) or additions from the 2-keto acid synthesis pathway (Rodriguez et al., 2014) to *in vitro* enzymatic assays (Lin et al., 2016) and *in silico* protein modeling (Morales-Quintana et al., 2011; Morales-Quintana et al., 2015; Morales-Quintana et al., 2012; Morales-Quintana et al., 2013). To date, the biological upgrading of the carboxylate to ester platforms has only been demonstrated using the ethanol production module, and understanding of whether the targeted AATs have activity towards other alcohols has not yet been investigated.

In this study, we biologically upgraded the carboxylate to branched-chain ester platforms by modulating the alcohol submodule from ethanol to isobutanol. Using the engineered *Escherichia coli* modular cell, we explored the functional roles of three AATs of the acid-to-ester module for the potential synthesis of 18 unique esters from the 5 linear, saturated C₂-C₆ carboxylic acids commonly found in the carboxylate platform. Microbial biosynthesis of the ester platform with longer- and branched-chain alcohols beyond ethanol modulate the ester flavor and fragrance properties as well as improves the energy density of these esters that can potentially be used as pure or blended biodiesels and jet fuels.

4.3 Materials and Methods

4.3.1 Plasmids and strains

The list of plasmids and strains used in this study is presented in Table 4-1. The fermentative branched-chain ester pathway was designed as an exchangeable production module comprised of an alcohol submodule and an acyl-CoA transferase (ACT) plus AAT submodule (Layton and Trinh, 2016). Each submodule carried genes organized in operons

of a plasmid under T7 promoters. The isobutanol submodule pCT13 was previously constructed (Trinh et al., 2011). Each ACT plus AAT submodule (e.g., pDL014, pDL015, or pDL016) was created by assembling 3 DNA fragments including i) the propionyl-CoA transferase (PCT, belonging to the general class of ACT) gene amplified from the genomic DNA of *Clostridium propionicum* using the primers DL_0023/DL0024, ii) the ATF1 gene (amplified from the plasmid pDL004 using primers DL_0025/DL_0020), the SAAT gene (pDL001, DL_0012/DL_0027), or the VAAT gene (pDL006, DL_0018/DL_0028), and iii) the pETite* backbone amplified using the primers DL_0001/DL_0002.

The engineered *E. coli* modular chassis cell EcDL002 was deployed as the ester production host (Layton and Trinh, 2014). By transforming the submodules pCT13 and

Table 4-1: A list of plasmids and strains

Plasmids/Strains	Genotypes	Sources
<i>Plasmids</i>		
pETite*	kan ^R	(Layton and Trinh, 2014)
pDL001	pETite* SAAT; kan+	(Layton and Trinh, 2016)
pDL004	pETite* <i>atf1</i> ; kan+	(Layton and Trinh, 2016)
pDL006	pETite* VAAT; kan+	(Layton and Trinh, 2016)
pCT13	pCOLA-P _{T7} ::RBS:: <i>alsS</i> ::RBS:: <i>ilvC</i> ::RBS:: <i>ilvD</i> -P _{T7} ::RBS:: <i>kivd</i> ::RBS:: <i>adhE</i> ::T _{T7} ; kan ^R	Trinh et al 2011
pDL014	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::ATF1::T _{T7} ; amp ^R	this study
pDL015	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::SAAT::T _{T7} ; amp ^R	this study
pDL016	pETite* P _{T7} ::RBS:: <i>pct</i> ::RBS::VAAT::T _{T7} ; amp ^R	this study
<i>Strains</i>		
<i>C. propionicum</i>	Wildtype	ATCC 25522
EcDL002	TCS083 (λDE3) Δ <i>fadE</i> ::kan ⁻ (cured)	(Layton and Trinh, 2014)
EcDL207	EcDL002 pCT13 + pDL014; kan ^R amp ^R	this study
EcDL208	EcDL002 pCT13 + pDL015; kan ^R amp ^R	this study
EcDL209	EcDL002 pCT13 + pDL016; kan ^R amp ^R	this study

Table 4-2: A list of primers used

Primers	Sequences
DL_0001	5'-CATCATCACCACCATCACTAA-3'
DL_0002	5'-ATGTATATCTCCTTCTTATAGTTAAAC-3'
DL_0012	5'-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATT AAGGTCTTTGGAG-3'
DL_0018	5'-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGCCGATA ACATACGTAGACCG-3'
DL_0020	5'-GCCGCTCTATTAGTGATGGTGGTGATGATGCTAAGGGCCTA AAAGGAGAG-3'
DL_0023	5'-AAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG AGAAAGGTTCCTTATTAC-3'
DL_0024	5'-TCAGGACTTCATTTCTTCAG-3'
DL_0025	5'-CTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGAA TGAAATCGATGAGAAAAATC-3'
DL_0027	5'-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAGAAAATTGAGGTCAG-3'
DL_0028	5'-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACAT ATGGAGAAAATTGAGGTCAG-3'

pDL014-pDL016 into EcDL002 via electroporation (Sambrook, 2001), we created the ester production strains EcDL207-209, respectively.

4.3.2 Media and cell culturing conditions

The medium (pH~7) used for the acid-to-ester production experiments contained 100 mL/L of 10X M9 salts, 1 mL/L of 1 M MgSO₄, 100 µL/L of 1M CaCl₂, 1 mL/L of stock thiamine solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, 2 g/L organic acid (e.g., acetic, propionic, butyric, pentanoic, or hexanoic acid), 20 g/L glucose, 25 µg/mL kanamycin, and 50 µg/mL ampicillin. The stock 10x M9 salt solution contained 67.8 g/L Na₂HPO₄, 30 g/L KH₂PO₄, 5 g/L NaCl, and 10 g/L NH₄Cl. The organic acids used for the acid-to-ester production experiments are the dominant chemicals present in the carboxylate platform (Holtzapple, 2015).

Ester production was carried out via *in situ*, high-cell density fermentation and extraction using the hexadecane organic overlay as previously described (Layton and Trinh, 2016). Briefly, the fermentation was conducted in a 75° angled platform in a New Brunswick Excella E25 at 37°C and 175 rpm for 24 h under anaerobic conditions. Whole-cells and cell supernatants were collected and stored at -20°C for subsequent metabolite analysis while hexadecane overlay was stored at room temperature for ester analysis. All experiments were performed with at least three biological replicates.

4.3.3 Analytical methods

Sugars, organic acids, and alcohols from culture supernatants were analyzed by the high pressure liquid chromatography (HPLC) technique. Produced esters were captured by hexadecane organic overlay and were quantified by gas chromatography coupled with mass spectroscopy (GC/MS) technique. The sample preparation for HPLC and GC/MS, data analyses, and instruments were described in detail previously (Layton and Trinh, 2016) while the running methods were slightly modified to capture the new branched chain esters.

All esters were analyzed by using the HP6890 GC/MS system equipped with a 30m × 0.25mm i.d., 0.25µm film thickness column plus an attached 10 m guard column (Zebron ZB-5, Phenomenex Inc.) and a HP 5973 mass selective detector. A selected ion mode (SIM) method was deployed to analyze 1 µL of samples. The GC method was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C then a 25°C/min ramp was deployed to 235°C. The final ramp was then issued to a final temperature of 300°C at a rate of 50°C/min to elute any residual non desired analytes. The injection was performed

using a splitless mode with an initial MS source temperature of 200°C. The carrier gas used was helium flowing at a rate of 0.5 mL/min.

The detection of the desired products was accomplished using the following SIM parameters: ions 45.00, 61.00, 70.00, and 85.00 detected from 0-4.70 minutes for ethyl acetate; ions 59.00, 61.00, and 87.00 detected from 4.70-5.50 minutes for isopropyl acetate; ions 57.00, 74.00, and 102.00 detected from 5.50-6.15 minutes for ethyl propionate and propyl acetate where propyl acetate and were separated further using their parent ions for quantification if necessary; ions 56.00, 73.00, 101.00 detected from 6.60-7.20 minutes for isobutyl acetate; ions 71.10, 88.10, and 116.00 detected from 7.20-7.71 minutes for ethyl butyrate; ions 57.00, 75.00, and 87.00 detected from 7.71-7.98 minutes for propyl propionate; ions 56.00, 61.00, 73.00 from 7.98-8.50 minutes for butyl acetate; ions 71.00, 89.10, and 130.00 from 8.50-9.20 minutes; for isopropyl butyrate; ions 56.00, 57.00, 75.00, and 87.00 from 9.20-9.53 minutes for isobutyl propionate detection; ions 61.00, 70.00, and 87.00 from 9.53-9.90 minutes for isoamyl acetate; ions 85.00, 88.00, and 101.00 from 9.90-10.20 minutes for ethyl pentanoate; ions 70.10 and 101.00 from 10.20-10.80 minutes for amyl acetate; ions 71.00, 89.00, and 101.00 from 10.80-11.25 for isobutyl butyrate; ions 71.10, 89.10, and 101.00 for butyl butyrate from 11.25-11.53 minutes; x) ions 60.00, 88.00, and 99.00 from 11.53-11.66 for ethyl hexanoate; ions 56.00, 61.00, and 84.00 from 11.66-11.87 minutes for hexyl acetate; ions 60.00, 99.00, 117.00 from 11.87 to 12.08 for isopropyl hexanoate, ions 57.00, 85.00, and 103.00 from 12.08-12.89 minutes for isobutyl pentanoate; ions 71.00, 99.00, and 117.00 from 12.89-13.20 for isobutyl hexanoate; and ions 70.0, 85.00, and 103.00 from 13.20- t_{final} minutes for pentyl pentanoate.

For isobutyl pentanoate, because commercial standards were not available, the concentration was estimated from the parent ion extraction and used for comparison against pentyl pentanoate for quantification. For our GC/MS analysis, the lower limit for quantifying ester production is within the range of 10 µg/mL with good signal to noise ratio (>3:1).

4.3.4 Calculation of Octane Normalized Mass Energy Density (ONMED)

The standard heat of combustion of each chemical (kJ/kg) was estimated based on average bond energies (Zumdahl, 2000). The ONMED of a chemical is defined as the ratio of its standard heat of combustion to that of octane.

4.4 Results and Discussion

4.4.1 Design of microbial biosynthesis of branched-chain ester platform

The general framework for biological upgrading of the carboxylate to branched-chain ester platforms utilizes the acid-to-ester pathway (Figure 4-1A). This framework was built upon our previously established foundation (Layton and Trinh, 2016) by modulating the alcohol submodule from ethanol to isobutanol. In brief, the designed acid-to-ester pathway contained the isobutanol submodule for conversion of pyruvate to isobutanol and the PCT plus AAT submodule that converts carboxylates to acyl CoAs and condenses them with alcohols to produce esters (Figure 4-1B).

As the isobutanol submodule contains the overexpression of an *E. coli* alcohol/aldehyde dehydrogenase (AdhE), it can reduce acyl CoAs from carboxylates to

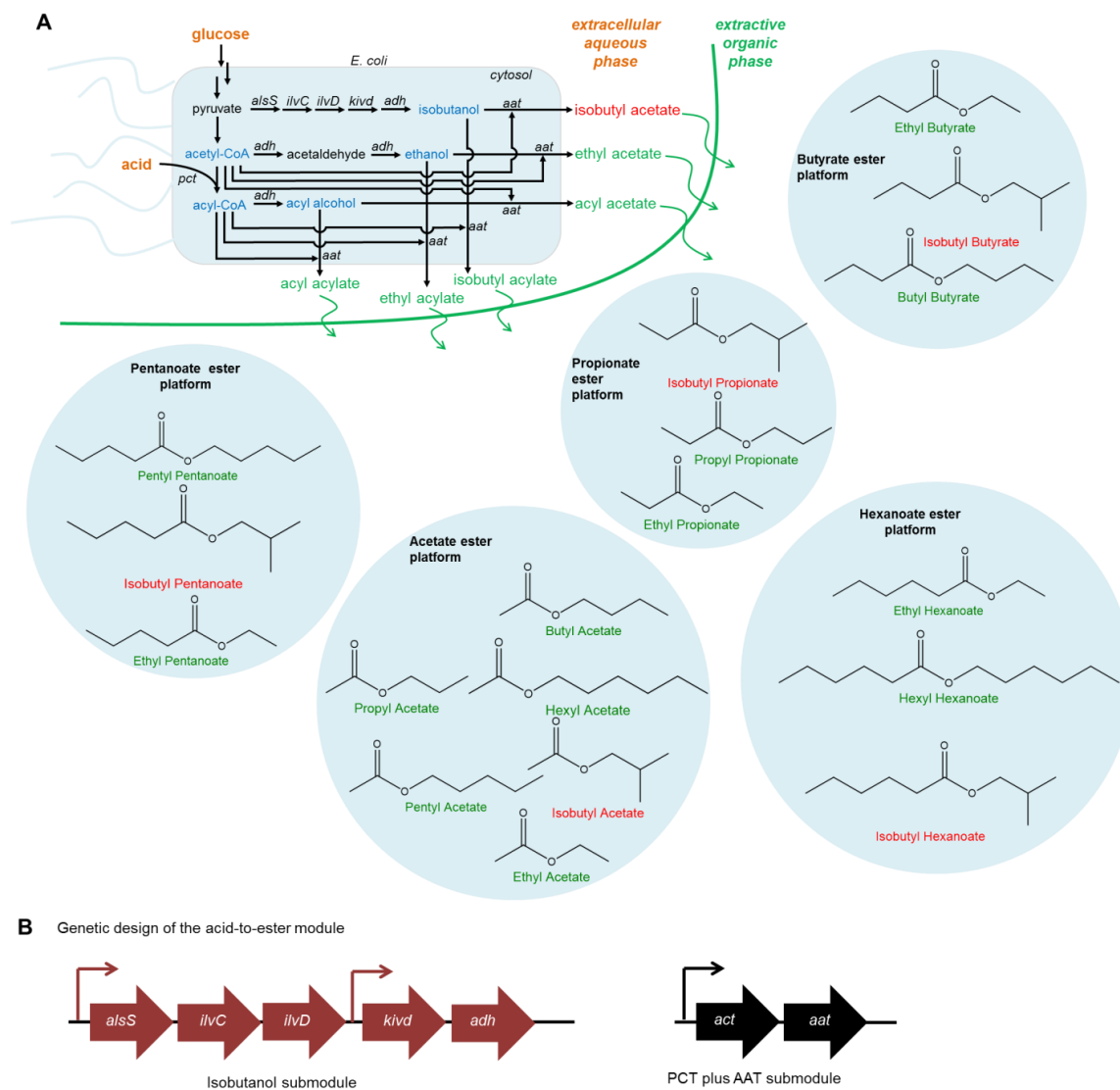


Figure 4-1: Branched chain ester synthesis. **(A)** Microbial biosynthesis of branched-chain ester platform from the carboxylates. **(B)** Genetic design of the acid-to-ester module.

alcohols that can be used for ester biosynthesis by the PCT plus AAT submodule. In our design, we used the PCT of *C. propionicum* because it exhibits broad substrate specificity towards C₂-C₆ carboxylates to produce their respective CoA counterparts (Layton and Trinh, 2016; Schweiger and Buckel, 1984). We also used three most active AATs, including ATF1 of *Saccharomyces cerevisiae*, SAAT of *Fragaria ananassa*, and VAAT of *F. vesca*, that encompass various substrate preferences to test for the branched-chain ester biosynthesis (Layton and Trinh, 2016). Specifically, ATF1 exhibits substrate preference towards longer-chain acetate esters, while SAAT and VAAT have substrate preferences towards C₄-C₆ ethyl acylates and C₂-C₄ ethyl acylates, respectively. In addition, SAAT has specificity towards acyl acylates.

By modulating the alcohol submodule from ethanol to isobutanol, the designed framework can expand the ester production library from 13 to 18 potential esters by co-fermentation of glucose and five linear, saturated C₂-C₆ carboxylates (Figure 4-1A). The five new branched-chain esters that can be synthesized microbially from the carboxylate platform include isobutyl acetate, propionate, butyrate, pentanoate, and hexanoate (Figure 4-1B). Ester synthesis depends on the availability of precursor metabolites, acyl CoAs and alcohols, and the broad substrate activities of AATs. In this study, we characterized three strains harnessing the acid-to-ester pathways with various AATs while other heterologous genes and their constructs were identical. These engineered strains are EcDL207, 208, and 209 and carry ATF1, SAAT and VAAT, respectively.

4.4.2 Expanding combinatorial biosynthesis of ester platforms

4.4.2.1 *Microbial biosynthesis of an acetate ester platform.*

A total of 5 targeted acetate esters including acetyl, propyl, butyl, pentyl, and hexyl acetates could be potentially synthesized from 5 carboxylates. Our results show that i) a mixture of ethyl and isobutyl acetates could be produced from co-fermentation of glucose and acetate (Figure 4-2A); ii) a mixture of ethyl, propyl, and isobutyl acetates from co-fermentation of glucose and propionate (Figure 4-2B); iii) a mixture of ethyl, butyl, and isobutyl acetates from co-fermentation of glucose and butyrate (Figure 4-2C); iv) a mixture of ethyl, isobutyl, and pentyl acetates from co-fermentation of glucose and pentanoate (Figure 4-2D); and v) a mixture of ethyl, isobutyl, and hexyl acetates from co-fermentation of glucose and hexanoate (Figure 4-2E).

Due to the various AAT specificities and precursor availability, it is anticipated that each characterized strain might not be able to produce all expected acetate esters for each co-fermentation of glucose and a targeted carboxylate as previously observed (Layton and Trinh, 2016).

Among the characterized strains and acetate esters, EcDL207 produced pentyl acetate at the highest level of 66.30 ± 13.44 mg/L after 24 h from the co-fermentation of glucose and pentanoate (Figure 4-2D). EcDL207 also synthesized 31.17 ± 0.32 mg/L hexyl acetate (Figure 4-2E), 20.26 ± 2.82 mg/L isobutyl acetate (Figure 4-2A) and 10.22 ± 0.85 mg/L butyl acetate (Figure 4-2C) at much higher titers than EcDL208 and EcDL209.

The observed phenotypes of EcDL207 were consistent with our previous study (Layton and Trinh, 2016) where ATF1 exhibited high specificity towards acetate esters. If

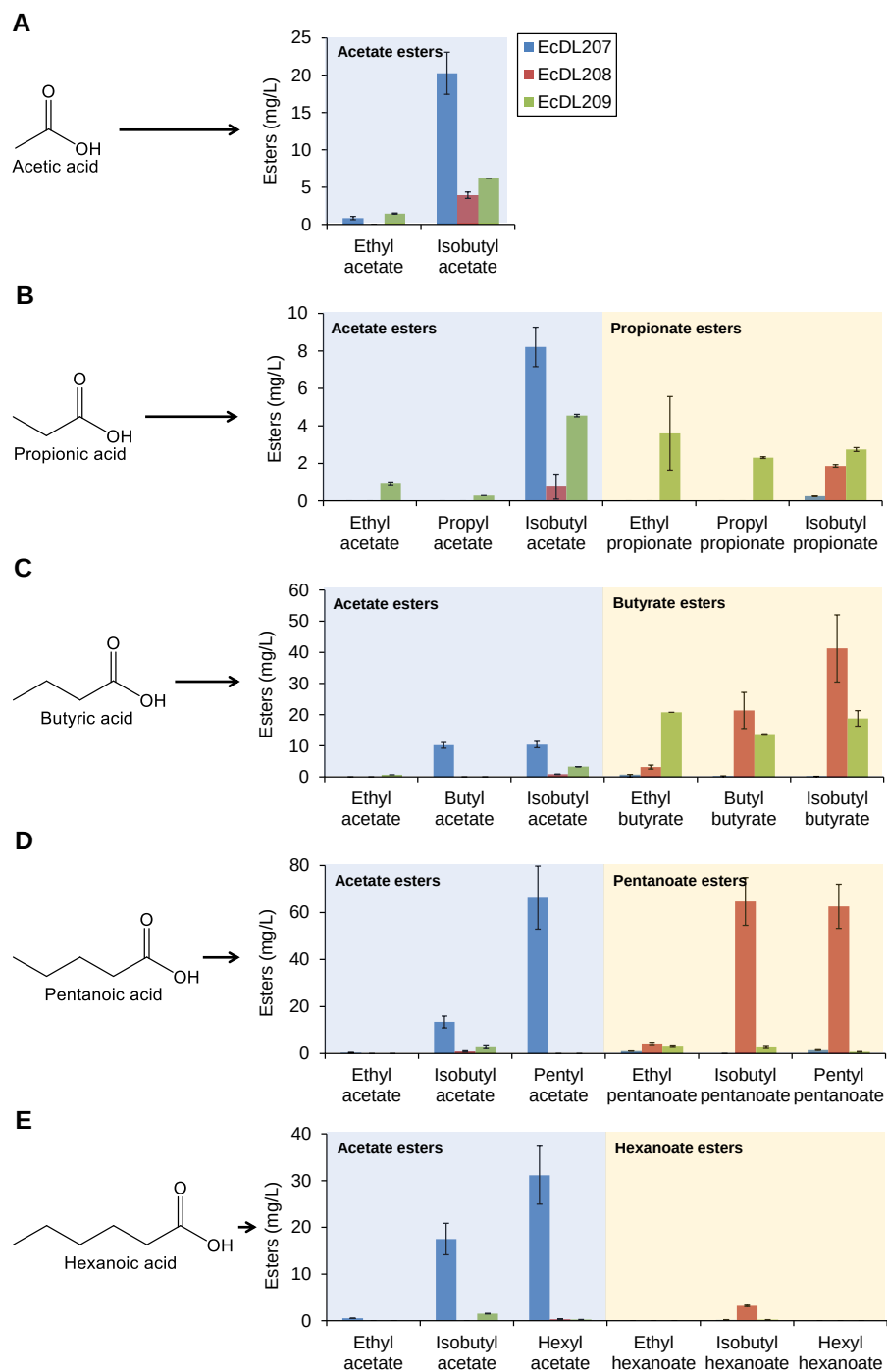


Figure 4-2: Ester production of EcDL207, 208, and 209 after 24 h from co-fermentation of glucose and organic acids. Panel (A) depicts acetic acid, (B) propionic acid, (C) butyric acid, (D) pentanoic acid, and (E) hexanoic acid.

the application were tailored for production of an acetate ester platform, ATF1 would be a strong candidate to use for the acid-to-ester pathway.

Both carboxylate and acetate ester platforms have distinct physical properties. The fruity smell of esters makes them unique presenting broad applications in flavor, fragrance, and solvent industries. While acetate and most of the carboxylates are very soluble in water, causing toxicity to microbes during fermentation, the acetate esters have significant reduction in water solubility (Figure 4-3A) and can be easily extracted during fermentation as implemented in our study. In addition, biological upgrading of acetate to acetate esters resulted in the improved ONMED values making them suitable for biofuel applications (Figure 4-3B). For instance, isobutyl acetate (0.639) has a higher ONMED value than ethanol (0.615) and acetic acid (0.312), and has been tested as a biofuel blend (Olson et al., 2003). Lower solubility of isobutyl acetate in water (~7 g/L) in comparison to acetic acid (complete solubility) and isobutanol (~88 g/L) is very advantageous for *in situ* fermentation and extraction.

4.4.2.2 Microbial biosynthesis of a propionate ester platform.

Co-fermentation of glucose and propionate could generate a propionate ester platform comprised of ethyl, propyl, and isobutyl propionates (Figure 4-2B). Among the characterized strains, only EcDL209 could produce all three propionate esters while EcDL207 and EcDL208 could synthesize only isobutyl propionate. Isobutyl propionate was produced at the highest titer of 3.60 ± 1.96 (mg/L) by EcDL209 among propionate esters and characterized strains.

It is interesting to notice that EcDL209 harnessing VAAT produced little ethyl

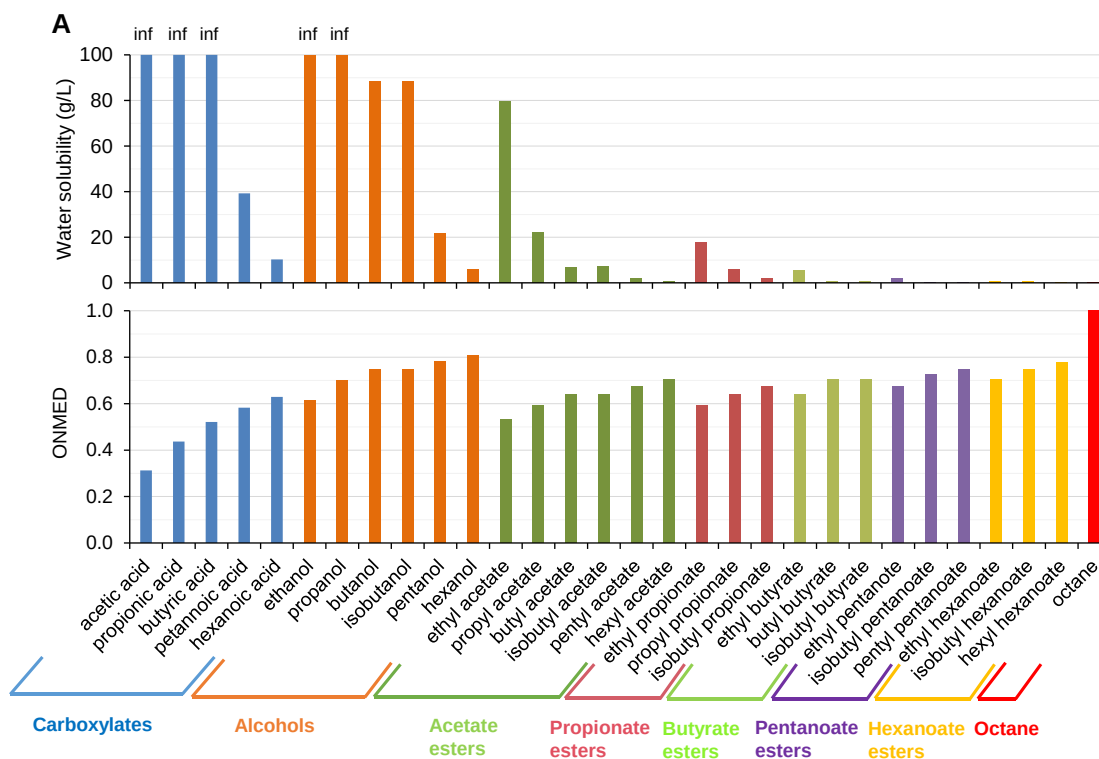


Figure 4-3: Physical properties of carboxylates, alcohols, and esters. Panel (A) depicts water solubility (g/L). inf: complete solubility. Panel (B) depicts octane normalized mass energy density (ONMED).

propionate. In our previous study, however, we observed that the VAAT exhibited a relatively high activity towards ethyl propionate production with a titer up to 67.24 ± 10.41 mg/L when the ethanol module was used instead of the isobutanol module (Layton and Trinh, 2016). Altogether, these results suggest that the insufficient generation of ethanol in EcDL207, 208 and 209 might have resulted in low ethyl propionate production. Among acylate esters, the production of propionate esters was the lowest (Figure 4-2).

Propyl acetate, propyl propionate, and isobutyl propionate, each have unique intrinsic physical properties including ONMED values 0.594, 0.639 and 0.675, respectively as well as fruity odors and relatively low water solubility (Figure 4-3). The

ONMED values of these propionate esters are all slightly lower than propanol (0.697) but higher than ethanol (0.615), which allow them to be blended in biofuels for increasing their octane.

4.4.2.3 Microbial biosynthesis of a butyrate ester platform.

From co-fermentation of glucose and butyrate, all characterized strains EcDL207-209 produced the targeted butyrate ester platform, consisting of ethyl, butyl, and isobutyl butyrates. EcDL208 produced 21.34 ± 13.76 mg/L butyl butyrate and 41.25 ± 18.76 mg/L isobutyl butyrate at the highest levels among the characterized strains while EcDL209 produced ethyl butyrate at the highest titer of 20.76 ± 0.00 (mg/L). Consistent with the previous study (Layton and Trinh, 2016), SAAT of EcDL208 shows substrate preferences towards C₄-C₆ acyl CoAs and short-chain alcohols while VAAT of EcDL209 exhibits substrate preferences toward C₂-C₄ acyl CoAs and ethanol. As expected, ATF1 of EcDL207 produced some amount of butyl and isobutyl acetates because it has substrate preferences towards acetyl CoA and short-chain alcohols.

Butyric acid is completely soluble in water and is very toxic to microbes (Figure 4-3). However, biological upgrading of butyrate can generate a butyrate ester platform with unique properties. The most distinct feature is the odor difference between rancid butyrate and its derived pleasant butyrate esters. Both butyl and isobutyl butyrate have very low water solubility (<0.7 g/L), which is advantageous for simultaneous fermentation, separation, and extraction process development (Figure 4-3). These butyrate esters also have higher ONMED value than ethanol and propanol, which make them suitable for

biofuel application. For instance, butyl butyrate has been recently tested as a potential jet fuel alternative (Chuck and Donnelly, 2014).

4.4.2.4 *Microbial biosynthesis of a pentanoate ester platform.*

Biological upgrading of pentanoate could expand the pentanoate ester platform to include ethyl, pentyl, and isobutyl pentanoates. Among the characterized strains, EcDL208 could produce all three targeted pentanoate esters at the highest titers, including 3.90 ± 0.50 mg/L ethyl pentanoate, 64.71 ± 10.19 mg/L isobutyl pentanoate, and 62.63 ± 0.75 mg/L pentyl pentanoate. The high production of the targeted pentanoate ester platform conferred the substrate preference of SAAT used in EcDL208 (Layton and Trinh, 2016).

Like butyric acid, pentanoic acid exhibits a rancid odor. However, biologically-upgraded pentanoate esters have pleasant smells and tastes, and hence are known for their wide use in flavor and fragrance industries. Unlike pentanoic acid, its derived pentanoate esters are mostly insoluble and are advantageous for *in situ* fermentation and extraction. Since ethyl, isobutyl, and pentyl pentanoates have ONMED values of 0.675, 0.727, and 0.747, respectively, which are close to the isobutanol ONMED (0.749), these esters can be potentially used as drop-in biofuels beyond their conventional flavor, fragrance, and solvent applications. For instance, ethyl pentanoate has undergone road trials and demonstrated stable performance when blended (10%) with gasoline (Lange et al., 2010).

4.4.2.5 *Microbial biosynthesis of a hexanoate ester platform.*

Co-fermentation of hexanoate and glucose could potentially yield a hexanoate ester platform including ethyl, isobutyl, and hexyl hexanoates. The characterized strains,

however, could only synthesize isobutyl hexanoate, neither ethyl hexanoate nor hexyl hexanoate. EcDL208 produced isobutyl hexanoate with the highest titer of 3.21 ± 0.15 mg/L. SAAT of EcDL208 was also first shown to have the substrate specificity for isobutanol and hexanoyl CoA to produce isobutyl hexanoate. Different from the previous study where the ethanol submodule was used instead of the isobutanol submodule (Layton and Trinh, 2016), both SAAT and VAAT exhibited activities towards ethyl hexanoate production despite low titers. These results suggest that inefficient supply of precursor metabolites in EcDL208 and EcDL209 might have limited ethyl hexanoate biosynthesis investigated in this study. Currently, we have not been able to synthesize hexyl hexanoate likely due to low activity of the characterized AATs towards this ester.

Not only does isobutyl hexanoate have one of the highest ONMED values at 0.747 (comparable with pentyl pentanoate) among the biologically upgraded esters, but it also exhibits little to no solubility in aqueous solutions (Figure 4-3). Interestingly, ethyl octanoate that also has the same ONMED as isobutyl hexanoate has recently been tested and demonstrated for its use in A-1 jet fuel (Chuck and Donnelly, 2014). The physical property of isobutyl hexanoate makes it a potential candidate for jet fuel application.

4.5 Conclusion

Biological upgrading low-value carboxylates, derived from lignocellulosic biomass or organic wastes, to high-value ester platforms has significant potential. In this study, we expanded our general, flexible framework for this biological upgrading. By deploying the ester production strains harnessing the acid-to-ester modules with various AATs, we demonstrated the microbial biosynthesis of 16 out of the total 18 potential esters including

5 new branched-chained esters – isobutyl acetate, isobutyl propionate, isobutyl butyrate, isobutyl pentanoate, and isobutyl hexanoate. Not only did we confirm the substrate preferences of ATF1 (EcDL207) towards long-chain acetate esters, SAAT (EcDL208) towards acyl acylates, and VAAT (EcDL209) towards ethyl C₂-C₄ acylates, but also demonstrated their activities towards branched-chain esters. Since our study aimed to expand the ester platform, there is much room to enhance ester production that is currently low in future studies (Tables 4-3-4-6). Many promising strategies can be employed to improve the ester platform production such as pathway optimization (e.g., modulating promoter, ribosome binding site, gene orthologs to balance and optimize pathway fluxes) and process conditions (e.g. temperature, medium, pH, substrate feeding, *in situ* extraction and fermentation).

Table 4-3: Ester titers of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single carboxylates after 24h. The acyl acetate, ethyl acylate, isobutyl acylate, and acyl acylate columns correspond to the acids added. For instance, with the exogenous addition of pentanoic acid, the acyl acetate, ethyl acylate, isobutyl acylate, and acyl acylate columns represent pentyl acetate, ethyl pentanoate, isobutyl pentanoate, and pentyl pentanoate, respectively. Abbreviations: n.a: not applicable; n.d.: not detected.

		Ester titer (mg/L)						
	Strains	Ethyl Acetate	Isobutyl Acetate	Acyl Acetate	Ethyl Acylate	Isobutyl acylate	Acyl Acylate	Total
Acetic acid doping	EcDL207	0.86 ± 0.22	20.26 ± 2.82	n.a.	n.a.	n.a.	n.a.	21.11 ± 3.04
	EcDL208	n.d.	3.92 ± 0.44	n.a.	n.a.	n.a.	n.a.	3.92 ± 0.44
	EcDL209	1.46 ± 0.07	6.17 ± 0.01	n.a.	n.a.	n.a.	n.a.	7.64 ± 0.08
Propionic acid doping	EcDL207	n.d.	8.22 ± 1.06	n.d.	n.d.	0.25 ± 0.01	n.d.	8.47 ± 1.06
	EcDL208	n.d.	0.76 ± 0.67	n.d.	n.d.	1.86 ± 0.07	n.d.	2.62 ± 0.73
	EcDL209	0.91 ± 0.1	4.55 ± 0.07	0.28 ± 0	3.6 ± 1.96	2.74 ± 0.10	2.3 ± 0.04	14.38 ± 2.28
Butyric acid doping	EcDL207	n.d.	10.38 ± 1.05	10.22 ± 0.85	0.77 ± 0.03	0.18 ± 0.02	0.25 ± 0.10	21.81 ± 2.05
	EcDL208	n.d.	0.88 ± 0.09	n.d.	3.17 ± 0.66	41.25 ± 10.8	21.34 ± 5.81	66.64 ± 17.36
	EcDL209	0.7 ± 0.01	3.32 ± 0.02	n.d.	20.76 ± 0.00	18.76 ± 2.51	13.76 ± 0.07	57.3 ± 2.61
Pentanoic acid doping	EcDL207	0.36 ± 0.18	13.44 ± 2.53	66.3 ± 13.45	1.06 ± 0.03	n.d.	1.48 ± 0.14	82.65 ± 16.34
	EcDL208	n.d.	0.93 ± 0.26	n.d.	3.9 ± 0.50	64.71 ± 10.19	62.63 ± 9.45	132.17 ± 20.39
	EcDL209	n.d.	2.72 ± 0.59	n.d.	2.95 ± 0.31	2.61 ± 0.44	0.75 ± 0.1	9.02 ± 1.43
Hexanoic acid doping	EcDL207	0.53 ± 0.02	17.49 ± 3.37	31.17 ± 6.21	n.d.	0.17 ± 0.05	n.d.	49.36 ± 9.66
	EcDL208	n.d.	n.d.	0.32 ± 0.06	n.d.	3.21 ± 0.15	n.d.	3.53 ± 0.21
	EcDL209	n.d.	1.53 ± 0.07	0.27 ± 0.01	n.d.	0.23 ± 0.01	n.d.	2.03 ± 0.09

Table 4-4: Ester yields on glucose of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h. Ester yields are calculated based on glucose consumption.

		Ester yields (mg/g)					
	Strains	Ethyl Acetate	Isobutyl Acetate	Acyl Acetate	Ethyl Acylate	Isobutyl acylate	Acyl Acylate
Acetic acid doping	EcDL207	0.09 ± 0.02	2.21 ± 0.29	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcDL208	0 ± 0	0.33 ± 0.04	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcDL209	0.08 ± 0.07	0.33 ± 0.28	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Propionic acid doping	EcDL207	<i>n.d.</i>	0.9 ± 0.12	<i>n.d.</i>	<i>n.d.</i>	0.03 ± 0	<i>n.d.</i>
	EcDL208	<i>n.d.</i>	0.07 ± 0.06	<i>n.d.</i>	<i>n.d.</i>	0.17 ± 0.01	<i>n.d.</i>
	EcDL209	0.07 ± 0.01	0.35 ± 0.01	0.02 ± 0	0.24 ± 0.12	0.21 ± 0	0.12 ± 0.1
Butyric acid doping	EcDL207	<i>n.d.</i>	1.29 ± 0.04	1.27 ± 0.08	0.1 ± 0.01	0.02 ± 0	0.03 ± 0.02
	EcDL208	<i>n.d.</i>	0.08 ± 0	<i>n.d.</i>	0.3 ± 0.04	3.86 ± 0.79	2 ± 0.42
	EcDL209	0.04 ± 0.03	0.28 ± 0.04	0.06 ± 0.11	1.8 ± 0.29	1.52 ± 0.27	1.25 ± 0.26
Pentanoic acid doping	EcDL207	0.03 ± 0.04	1.73 ± 0.52	8.54 ± 2.74	0.13 ± 0.01	<i>n.d.</i>	0.19 ± 0.04
	EcDL208	<i>n.d.</i>	0.08 ± 0.04	<i>n.d.</i>	0.32 ± 0.13	5.35 ± 2.31	5.17 ± 2.2
	EcDL209	<i>n.d.</i>	0.26 ± 0.08	<i>n.d.</i>	0.27 ± 0.05	0.24 ± 0.06	0.07 ± 0
Hexanoic acid doping	EcDL207	0.07 ± 0	2.25 ± 0.45	4.01 ± 0.81	<i>n.d.</i>	0.02 ± 0.01	<i>n.d.</i>
	EcDL208	<i>n.d.</i>	<i>n.d.</i>	0.03 ± 0.01	<i>n.d.</i>	0.29 ± 0.03	<i>n.d.</i>
	EcDL209	<i>n.d.</i>	0.11 ± 0.1	0.02 ± 0.01	<i>n.d.</i>	0.02 ± 0	<i>n.d.</i>

Table 4-5: Specific ester productivities of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h.

		Specific ester production rates (µg/gDCW/h)					
	Strains	Ethyl Acetate	Isobutyl Acetate	Acyl Acetate	Ethyl Acylate	Isobutyl acylate	Acyl Acylate
Acetic acid doping	EcDL207	12.15 ± 2.92	286.72 ± 36.12	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcDL208	<i>n.d.</i>	62.68 ± 6.14	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	EcDL209	16.17 ± 14.02	68.29 ± 59.15	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Propionic acid doping	EcDL207	<i>n.d.</i>	121.08 ± 15.64	<i>n.d.</i>	<i>n.d.</i>	3.72 ± 0.13	<i>n.d.</i>
	EcDL208	<i>n.d.</i>	11.98 ± 10.56	<i>n.d.</i>	<i>n.d.</i>	29.38 ± 1.52	<i>n.d.</i>
	EcDL209	15.47 ± 1.77	77.3 ± 1.6	4.79 ± 0.05	52.36 ± 28.55	46.49 ± 1.73	26.05 ± 22.57
Butyric acid doping	EcDL207	<i>n.d.</i>	149.22 ± 15.5	146.78 ± 12.34	11.07 ± 0.48	2.64 ± 0.24	3.67 ± 1.52
	EcDL208	<i>n.d.</i>	13.68 ± 1.34	<i>n.d.</i>	49.35 ± 10.07	642.9 ± 166.53	332.52 ± 89.48
	EcDL209	7.73 ± 6.69	57.59 ± 4.3	13.04 ± 22.59	367.57 ± 40.27	311.37 ± 41.8	255.56 ± 47.15
Pentanoic acid doping	EcDL207	3.5 ± 3.51	194.06 ± 38.28	957.31 ± 200.92	15.34 ± 0.6	<i>n.d.</i>	21.39 ± 2.21
	EcDL208	<i>n.d.</i>	14.29 ± 3.82	<i>n.d.</i>	59.84 ± 7.11	992.32 ± 145.81	960.54 ± 134.84
	EcDL209	<i>n.d.</i>	44.93 ± 10.03	<i>n.d.</i>	48.75 ± 5.61	43.24 ± 7.62	12.38 ± 1.69
Hexanoic acid doping	EcDL207	7.51 ± 0.39	249.22 ± 50.98	444.12 ± 93.22	<i>n.d.</i>	2.46 ± 0.74	<i>n.d.</i>
	EcDL208	<i>n.d.</i>	<i>n.d.</i>	5.16 ± 0.98	<i>n.d.</i>	51.08 ± 2.28	<i>n.d.</i>
	EcDL209	<i>n.d.</i>	16.65 ± 14.43	3.44 ± 1.77	<i>n.d.</i>	3.63 ± 0.24	<i>n.d.</i>

Table 4-6: Fermentation data of EcDL207, EcDL208, and EcDL209 for co-fermentation of glucose and single organic acids after 24h. The subscripts i and f are referred to the initial time (t = 0 h) and final time (t = 24 h) of fermentation, respectively.

	Strains	OD _i	OD _f	pH _i	pH _f	glucose _i (g/L)	glucose _f (g/L)	acid _i (g/L)	acid _f (g/L)	ethanol (g/L)	isobutanol (g/L)	acyl alcohol (g/L)
Acetic acid doping	EcDL207	5.12	7.02±0.16	7	6	20.42±0.05	11.45±0.54	2.26±0	1.99±0.1	1.99±0.1	0.55±0.04	0
	EcDL208	5.2	5.54±0.14	7	5.84	20.42±0.05	8.8±0.02	2.26±0	2.06±0	2.06±0	1.34±0.01	0
	EcDL209	5	5.42±0.09	7	5.72	20.42±0.05	6.67±2.31	2.26±0	1.85±0.63	1.85±0.63	0.56±0.2	0
Propionic acid doping	EcDL207	5.12	6.55±0.04	7	6.05	20.04±0.04	10.89±0.64	2.28±0	2.37±0.15	0.7±0.04	0.54±0.06	N.D.
	EcDL208	5.2	5.7±0.17	7	5.83	20.04±0.04	8.98±0.07	2.28±0	2.38±0.02	0.68±0.01	1.28±0.03	N.D.
	EcDL209	5	5.13±0.1	7	5.7	20.04±0.04	7.09±0.48	2.28±0	2.15±0.15	1.43±0.11	0.57±0.02	0.3±0.02
Butyric acid doping	EcDL207	5.12	6.85±0.09	7	6	19.48±1.11	11.4±1.09	2.24±0.23	1.42±0.13	3.67±0.33	0.9±0.08	N.D.
	EcDL208	5.2	5.83±0.06	7	5.88	19.48±1.11	8.88±0.24	2.24±0.23	1.33±0.03	3.39±0.09	1.45±0.03	N.D.
	EcDL209	5	5.37±0.05	7	5.68	19.48±1.11	7±0.88	2.24±0.23	1.33±0.03	3.5±0.64	0.46±0.12	N.D.
Pentanoic acid doping	EcDL207	5.12	6.81±0.29	7	5.98	19.96±0.13	12.01±1.06	2.3±0.01	1.53±0.14	1.06±0.09	0.74±0.05	N.D.
	EcDL208	5.2	6.01±0.12	7	5.87	19.96±0.13	9.56±0.1	2.3±0.01	1.33±0.01	1.09±0.02	1.36±0.02	N.D.
	EcDL209	5	5.41±0.13	7	5.7	19.96±0.13	9.04±1.83	2.3±0.01	1.93±0.41	1.49±0.21	0.48±0.07	N.D.
Hexanoic acid doping	EcDL207	5.12	6.97±0.22	7	5.98	20.08±0.17	12.3±0.12	2.1±0	2.11±0.01	1.32±0.01	0.58±0.02	N.D.
	EcDL208	5.2	5.61±0.05	7	5.9	20.08±0.17	9.06±1.14	2.1±0	1.69±0.21	1.17±0.14	0.96±0.13	N.D.
	EcDL209	5	5.47±0.16	7	5.8	20.08±0.17	10.74±0.02	2.1±0	2.11±0.01	1.77±0.15	0.59±0.07	N.D.

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5 Enabling Directed Metabolic Pathway Evolution via Growth Selection of Modular Cell

5.1 Abstract

To produce a target chemical at high yields, titers, and productivities, various combinations of available genetic parts for expression system can result in a large number of microbial cell factories generated for characterization. This engineering approach will become laborious and expensive when developing optimal strains for production of a large space of biochemicals due to extensive screening. Modular cell design offers a promising solution for rapid generation of optimal microbial cell factories using plug-and-play production modules. Here, we validate the modular cell theory by demonstrating: i) tight coupling between the modular chassis cell and a production module, an heterologous ethanol pathway as a testbed, ii) selection of an optimal pyruvate decarboxylase (PDC) of the ethanol production module based on growth selection, iii) discovery of new function of a hypothetical PDC protein, iv) improvement of growth rate and ethanol production rate by evolving the modular chassis cell and PDCs, iv) selection of the most optimal PDC from a library of 10^8 randomly mutagenized PDC, and vi) selection of an optimal ethanol pathway using a library of promoters with variable transcriptional strength. We envision that the modular cell design is a platform technology for rapid development of optimal microbial cell factories for combinatorial synthesis of biochemicals.

5.2 Introduction

Engineering efficient microbial biocatalysts to produce targeted chemicals at high titers, rates, and yields has been the primary focus of metabolic engineering. This has been traditionally achieved by genetic knockouts, knockins, and overexpression systems (Stephanopoulos, 1999). Several strategies have been used for characterizing cellular metabolism such as flux balance analysis (FBA) (Edwards et al., 2001; Fell and Small, 1986; Orth et al., 2010) metabolic flux analysis (MFA) (Antoniewicz, 2015; Varma and Palsson, 1994; Wiechert, 2001) and metabolic pathway analysis (MPA) (Papin et al., 2004; Schilling et al., 2000; Schuster et al., 2000; Trinh et al., 2009). Many methods have built upon the foundational FBA, MFA, and MPA to rationally engineer novel strains for enhanced product formation such as OptKnock (Burgard et al., 2003), MMF (Trinh et al., 2008), cMCS (Hädicke and Klamt, 2011), SMET (Flowers et al., 2013), EMRA (Lee et al., 2014). Strain optimization techniques use a metabolic or genomic network but do not incorporate the intrinsic variability that can be seen in over-expression or under-expression system parts, such as promoters, ribosome binding sites, terminators, and origins of replication.

Synthetic biology has added an additional layer to expression systems by developing a vast number of genetic parts for product pathways. Several tools have been developed for understanding and expanding the possible space of genetic parts including RBS modulation (Na et al., 2010; Salis et al., 2009; Seo et al., 2013), promoter elements (Cox et al., 2007; Mutalik et al., 2013; Rhodius et al., 2012), and terminator elements (Cambray et al., 2013; Chen et al., 2013; Redden and Alper, 2015). Combinatorial libraries

have been generated using several of these parts (Du and Ryan, 2015; Freestone and Zhao, 2016; Jones et al., 2015; Li et al., 2013; Lütke-Eversloh and Stephanopoulos, 2008; Schaerli and Isalan, 2013; Smanski et al., 2014). There have been significant efforts for generating and gathering genetic parts by the International Genetic Engineering Machinery (iGEM) foundation, which houses thousands of synthetic parts for researchers to use (www.igem.org). However, characterizing and screening all combinations of genetic parts for an optimal pathway would be nearly impossible without rational strain theory.

The modular cell theory has been recently developed to elucidate rationally designed efficient modular cells that couple cellular growth and product biosynthesis (Trinh et al., 2015). This theory utilizes MPA-based frameworks to design a modular chassis cell that is auxotrophic, containing essential metabolic pathways for sufficient biomass formation if an effective production module is present for balancing redox, energy, and intracellular metabolites. Modular cells are designed to minimize strain optimization iterations for producing combinatorial libraries of chemicals using exchangeable plug-and-play production modules and to provide a growth-based selection platform i.e. the tighter the coupling between the modular chassis cell and exchangeable production module, the faster the growth rate. Recently, the modular chassis cell has been demonstrated for enhanced product biosynthesis from plug-and-play production modules (Layton and Trinh, 2014; Layton and Trinh, 2016a; Layton and Trinh, 2016b; Wierzbicki et al., 2016). However, demonstrating the selection platform that the modular cell encompasses has yet to be fully explored.

In this study, we applied modular cell theory to demonstrate tight coupling between cell growth and product formation using the modular chassis cell as an efficient selection platform. We demonstrated the requirement of an effective redox sink for coupling growth and product formation and selected for an optimal PDC, with varying degrees of coupling, from a library of six PDCs derived from eukaryotic and prokaryotic organisms. Using a directed evolution approach, we evolved five of our six PDCs for enhanced growth rates and ethanol production rates. We rapidly screened for a library of 10^8 PDCs derived from random mutagenesis and were able to select for an optimal PDC. Lastly, we demonstrated additional selection capability of the modular cell by using a library of ethanol modules with various transcriptional strengths.

5.3 Materials and Methods

5.3.1 Strain construction

Table 5-1 lists strains used in this study. TCS083 (Trinh et al., 2011) and its derivatives were used for growth coupling studies and *E. coli* TOP10 was used for molecular cloning. TCS095 was constructed using P1 transduction for chromosomal gene deletion from TCS083 (Trinh et al., 2006). The prophage λ DE3 was used to insert a T7 polymerase gene into the specific site of TCS083 or TCS095 by using a commercial kit for strains expressing a T7 promoter (cat#69734-3, Novagen Inc.) All mutants and plasmids were PCR confirmed with the primers used listed in Table 5-2.

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

Plasmids/Strains	Genotype	Source
<i>Plasmids</i>		
pETite*	kan ^R	(Layton and Trinh, 2014)
pCT15	pETite* <i>cdc</i> ; kan ⁺	this study
pDL017	pETite* PDC1 _{Sc} ; kan ⁺	this study
pDL018	pETite* PDC5 _{Sc} ; kan ⁺	this study
pDL019	pETite* PDC6 _{Sc} ; kan ⁺	this study
pDL020	pETite* PDC _{ppa} ; kan ⁺	this study
pDL021	pETite* PDC _{Yli} ; kan ⁺	this study
pCT24	pETite* P _{T7} ::RBS:: <i>cdc</i> ::RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	(Layton and Trinh, 2014)
pAY1	pETite* P _{BBa_J23100} ::RBS:: <i>cdc</i> :: RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
pAY3	pETite* P _{BBa_J23108} ::RBS:: <i>cdc</i> :: RBS:: <i>adhB</i> ::T _{T7} ; kan ⁺	this study
<i>Strains</i>		
<i>S. cerevisiae</i>	MAT a, <i>ura3d0</i> , <i>his3-d200</i> , <i>leu2-d0</i> , <i>met15-d0</i>	ATCC 201388
<i>P. pastoris</i>	Wildtype	ATCC 28485
<i>Y. lipolytica</i>	MATA <i>ura3-302 leu2-270 xpr2-322 axp2-ΔNU49 XPR2::SUC2</i>	ATCC MYA-2613
TOP10	F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG</i>	Invitrogen
TCS083	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>	(Trinh et al., 2008)
TCS083 (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> (λDE3)	this study
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> (λDE3)	this study
EcDL107	TCS083 (DE3) carrying pCT15; kan ⁺	this study
EcDL108	TCS083 (DE3) carrying pDL017; kan ⁺	this study
EcDL109	TCS083 (DE3) carrying pDL018; kan ⁺	this study
EcDL110	TCS083 (DE3) carrying pDL019; kan ⁺	this study
EcDL111	TCS083 (DE3) carrying pDL020; kan ⁺	this study
EcDL112	TCS083 (DE3) carrying pDL021; kan ⁺	this study
EcDL113	TCS095 (DE3) carrying pCT24; kan ⁺	this study
EcDL114	TCS095 (DE3) carrying pAY1; kan ⁺	this study
EcDL115	TCS095 (DE3) carrying pAY3; kan ⁺	this study
EcDL116	TCS095 (DE3) carrying pCT15; kan ⁺	this study

Table 1-2: A list of primers for plasmid construction and validation

Primer name	Sequences
DL_0001	CATCATCACCACCATCACTAA
DL_0002	ATGTATATCTCCTTCTTATAGTTAAAC
DL_0036	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGTCTGAAATTACTTTGGG
DL_0037	CCGCTCTATTAGTGATGGTGGTGATGATGTTATTGCTTAGCG TTGGTAGC
DL_0038	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGTCTGAAATAACCTTAGG
DL_0039	GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTATTGTT TAGCGTTAGTAGC
DL_0040	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGTCTGAAATTACTCTTGG
DL_0041	GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTATTGTT TGGCATTGTAGC
DL_0042	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGGCTGAAATAACACTAGG
DL_0043	GCCGCTCTATTAGTGATGGTGGTGATGATGTTAAGCTGCGTT GGTCTTGG
DL_0044	AATAATTTTGTTTAACTATAAGAAGGAGATATACATATGAG CGACTCCGAACCCCAAATG
DL_0045	GCCGCTCTATTAGTGATGGTGGTGATGATGCTAAACGTTGG TCTTGGCAG
P006_f:	GAAGGAGATATACATATGAGTTATACTGTCCGTACCTATTT AGCGGAG
P006_r:	GTGATGGTGGTGATGATGCTCGAGTTAGGATCCCTAGAGGA GCTTGTTAACAGG
AY.6r	AAAAAAAGATCTGACGAATTCTCTAGATATCGCTCAATACT G
AY.7f	AAAAAAAGATCTTTGACGGCTAGCTCAGTCCTAGGTACAGT GCTAGCAAGGAGATATACATATGAGTTATACTGTC
AY.10f	AAAAAAAGATCTCTGACAGCTAGCTCAGTCCTAGGTATAAT GCTAGCAAGGAGATATACATATGAGTTATACTGTC
P043_KO_ldhA_f	TTTCTGGCGGATTTTTATCG
P043_KO_ldhA_r	CGTCAACGGCACAAGAATAA
P051_KO_frdA_f	AGTTGATGCAACCGGAGAAC
P051_KO_frdA_r	ACGGCGAGACAAATTTTACG
maeA_fwd_KO	CAGCGTAGTAAATAACCCAACC
maeA_rev_KO	GACAGCTTAACGGCTTTGTAG
P044_KO_zwf_f	CGATGAACGGTTCGAAGTTTT
P044_KO_zwf_r	TGCCATAGCAGCAATACTCG
P049_KO_ndh_f	GCAGACGCACAAATTCAAGA
P049_KO_ndh_r	ACGGGAACACCTCCTTCTTT
maeB_fwd_KO	GATGATAATGGCGAATGGAC
maeB_rev_KO	CGTTCTTTATCCATGAGTCG
P045_KO_pta_f	TCACTGGTGGTATCGGTGAA
P045_KO_pta_r	GAATGCGAAATGAGTGTGGA

Table 5.2: continued

Primer name	Sequences
P046_KO_poxB_f	ATGGATATCGTCGGGTTTGA
P046_KO_poxB_r	AAGCAATAACGTTCCGGTTG
P047_KO_adhE_F	AGAAAGCGTCAGGCAGTGTT
P047_KO_adhE_R	AAAGCGATGCTGAAAGGTGT
DE3_fwd	ATGAACACGATTAAACATCGC
DE3_rev	TTACGCGAACGCGAAGTC

5.3.2 Plasmid/pathway construction

5.3.2.1 Construction of the PDC modules.

The *S. cerevisiae*, *P. pastoris*, and *Y. lipolytica* PDC modules were constructed using the Gibson assembly method (Gibson, 2009) using the pETite* as a vector backbone (Layton and Trinh, 2014). Table 2 shows a list of primers used for this study. The backbone piece was amplified from pETite* using primers DL_0001/DL_0002. The module containing PDC1_{Sc} was amplified from *Saccharomyces cerevisiae* (*S. cerevisiae*) cDNA using primers DL_0036/ DL_0037 and then inserted into the pETite* backbone, to generate pDL017. PDC5_{Sc} was amplified from *S. cerevisiae* cDNA using primers DL_0038/ DL_0039 and inserted into the pETite* backbone, generating pDL018. PDC6_{Sc} was amplified from *S. cerevisiae* cDNA using primers DL_0040/ DL_0041 and inserted into the pETite* backbone, generating pDL019. PDC_{Ppa} was amplified from *Pichia pastoris* cDNA using primers DL_0042/ DL_0043 and inserted into the pETite* backbone, generating pDL020. PDC_{Yli} was amplified from *Yarrowia lipolytica* cDNA using primers DL_0044/ DL_0045 and inserted into the pETite* backbone, generating pDL021.

The *Z. mobilis* PDC was assembled into the pETite* vector by using the BglBrick Gene Assembly method using 2 DNA pieces (Anderson et al., 2010). The *Z. mobilis* pdc

was amplified from pCT24 using the primers P006_f/P006_r and digested with NdeI/BamHI and the vector backbone pETite* doubly digested with NdeI/BamHI.

5.3.2.2 Construction of the ethanol production modules.

The plasmid pCT24 was previously constructed (Layton and Trinh, 2014). Briefly, pCT24 contains the *Z. mobilis* ethanol pathway encompassing PDC and adhB. pAY1 and pAY3 were constructed from pCT24 by changing the T7 promoter to two different Anderson promoters (Kelly et al., 2009). pAY1 contains the BBa_J23100 promoter whereas pAY3 contains the BBa_J23108 promoter. pAY1 was constructed by amplifying pCT24 using primers AY6.R/AY.7F, digested with BglII and ligated together. Similarly, pAY3 was constructed using primers AY6.R/AY10.F, digested with BglII and ligated together.

5.3.3 Medium and cell culturing techniques

5.3.3.1 Culture media.

For molecular cloning, Luria-Bertani (LB) was used with supplementation of antibiotic where applicable. Antibiotics at working concentrations of 50 µg/mL kanamycin (kan) was used to maintain the selection of the desired plasmids.

For growth coupling experiments, M9 medium (pH~7) was used, consisting of 100 mL/L of 10X M9 salts, 1 mL/L of 1 M MgSO₄, 100 µL/L of 1M CaCl₂, 1 mL/L of stock thiamine HCl solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), and appropriate antibiotics. Unless specified, 10 g/L glucose was used in the M9 medium.

The stock 10X M9 salt solution contained 67.8 g/L Na_2HPO_4 , 30 g/L KH_2PO_4 , 5 g/L NaCl , and 10 g/L NH_4Cl .

5.3.3.2 Strain characterization.

Strain characterization experiments were performed by growing cells overnight at 37°C in 15mL culture tubes containing LB and appropriate antibiotics, then subculturing into fresh M9 medium to adapt the cells to a defined environment. Cells were then grown until exponential phase ($\text{OD}_{600\text{nm}} \sim 1.0$, 1 OD ~ 0.5 g DCW/L). Next, cells (except the modular strain, in this case, TCS083 DE3) were again subcultured into a nitrogen sparged and pressured tube to create a complete anaerobic environment to an initial $\text{OD}_{600\text{nm}} \sim 0.10$ -0.20 at a working volume of 20 mL. The strains were allowed to adapt (at least 2 doublings) overnight to the anaerobic environment and then transferred into pre-warmed 20 mL tubes dispersed of oxygen containing M9 and appropriate antibiotics for characterization with an initial $\text{OD}_{600\text{nm}}$ of ~ 0.030 .

Cells were grown on a 75° angled platform in a New Brunswick Excella E25 at 37°C and 175 rpm with OD measured on a Thermo Scientific Genysys 30 Visible Spectrophotometer with a proper adapter to measure growth kinetics directly. Whole-cells and cell supernatants were collected and stored at -20°C for subsequent metabolite analysis. All experiments were performed with at least three biological replicates.

5.3.3.3 Evolution characterization.

Strain evolution experiments were prepared and grown in an identical way to the method described above for strain characterization experiments. Samples for metabolite

analysis were also taken in a similar manner as described in the strain characterization method. Upon preparation of adapted anaerobic strains, cultures were grown in duplicate from OD₆₀₀ of ~0.050 until exponential phase was reached (OD₆₀₀ of 0.5-1.0). A 1.5 mL sample of each replicate was collected for stock. Each replicate was then diluted to OD₆₀₀ ~0.050 and grown again to an OD₆₀₀ of 0.5-1.0, where samples were collected as before. This process was repeated until a consistent maximum growth rate was reached. Evolution was then tested for irreversibility.

When the consistent maximum growth rate was reached, cells were plated on LB plates with antibiotic as needed. A single colony was selected and streaked out on a new plate and repeated 3 times in order to ensure a single cell colony was isolated. Isolated evolved colonies were tested for irreversibility. Cells were first grown and stocked at -80°C before conducting the experiment to allow for complete metabolic interruption and recovery. Irreversibility of the adapted host strain and adapted plasmid were carried out in the same fashion as before in the strain characterization method. Plasmids were extracted from the isolated colonies and transformation into the unevolved parent strain (TCS083 DE3) for plasmid irreversibility test. The evolved plasmid in the unevolved host was also characterized in the same method of the strain coupling studies.

5.3.3.4 *Random mutagenesis.*

Random mutagenesis was performed using the GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) by amplifying the *P. pastoris* PDC insert from pDL020 using primers DL_0042/ DL_0043 with 20 ng of

targeted insert. The backbone piece was amplified from pETite* using the primers DL_0001/DL_0002. Using the NEB biocalculator (<https://nebiocalculator.neb.com>), we calculated a PDC library of 8.69×10^9 and used a calculated backbone library of 8.73×10^9 . The two pieces were assembled together using the Gibson assembly protocol (Gibson, 2009). After Gibson assembly the plasmid was cleaned and concentrated using the Zymo Research Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) to remove the salts that are present in the Gibson assembly mixture.

The cleaned plasmid library was then transformed into TOP10, for plasmid methylation, using an electroporation method. The cells were recovered for 1 hour in LB medium at 37 C and then diluted in fresh LB medium containing kanamycin for selection and grown for 1.5 hours. After selection the cells were miniprep using the ZR Plasmid Miniprep Classic (Zymo Research, Irvine, CA, USA) and cleaned and concentrated using the Zymo Research Clean and Concentrator kit (Zymo Research, Irvine, CA, USA) to concentrate the plasmid library. The plasmid library was then transformed into the modular chassis cell, TCS083 DE3, using electroporation. The cells were recovered and selected in the same manner as before however, after selection the cells were spun down and washed with M9 characterization medium to remove any residual amino acids in the LB medium. The cells were then directly added to anaerobic tubes and allowed for growth until exponential phase was reached. Once exponential phase was reached, the cells were diluted and grown to exponential phase again. This process was repeated for a total of four transfers (~20 generations). Library size for selection was estimated at a 50% efficiency at each step after initial Gibson assembly for an overall estimated library of 2.71×10^8 .

5.3.4 2.4. Analytical methods

5.3.4.1 *High performance liquid chromatography (HPLC).*

Cell culture metabolites were quantified by first filtering through 0.2- μ m filter unit and then analyzed using a Shimadzu HPLC system equipped with a RID and UV-Vis detector (Shimadzu Inc., Columbia, MD, USA) equipped with Aminex HPX-87H cation exchange column (BioRad Inc., Hercules, CA, USA) at 50°C. Samples were eluted with a flow rate of 0.6 mL/min using a 10 mN H₂SO₄ mobile phase (Trinh 2011).

5.4 Results

5.4.1 Establishing production modules

5.4.1.1 *Modular cell cannot function without an exchangeable production module.*

The modular chassis cell was designed, using elementary mode analysis and the minimal metabolic functionality algorithm to be auxotrophic under anaerobic conditions and to only be able to sustain growth if an effective and balanced redox sink is available (Trinh et al 2015). This phenotype is highly useful as a selection platform; i.e. if a library of production modules is available, the most efficient module tightly couples with the modular cell and will grow the fastest. Likewise, if a production module is not present, cell growth is not maintained due to a redox imbalance and an insufficient supply of biomass precursor for growth. This phenotype was previously demonstrated for the necessity of an ester production module to be present for the cell to couple both growth and product formation, and without a production module the modular chassis cell did not

sustain growth (Layton and Trinh 2014). However, the modular chassis cell is designed to allow for a great number of products beyond esters, such as alcohols, e.g. ethanol.

5.4.1.2 Engineering PDC modules for growth-based selection.

In order for the cell to maintain growth, an effective production module must be available to balance redox and supply metabolites for cell growth. Thus, we engineered the production module to contain a pyruvate decarboxylase, PDC, for the conversion of pyruvate to acetaldehyde, a precursor to ethanol. However, acetaldehyde alone does not allow for direct coupling between product and growth due to the lack of not being a cofactor redox sink. We relied on the endogenous alcohol/aldehyde dehydrogenase, adhE, for conversion of acetaldehyde to ethanol by turning over the redox cofactor NADH. NADH turnover from adhE provides an electron sink for the cell and allows for biomass synthesis but only from the PDC ethanol pathway. The endogenous ethanol pathway does not have a redox balance sufficient for cell growth using the modular chassis cell (Figure 5-1) due to requirement of two NADHs. We constructed a total of 6 different PDC modules from laboratory workhorses *Z. mobilis*, *S. cerevisiae*, *P. pastoris*, and *Y. lipolytica*. Specifically, pCT15 encodes PDC from *Z. mobilis*, whereas pDL017, pDL018, and pDL019 encodes the *S. cerevisiae* PDC1, PDC5, and PDC6, respectively, pDL020 encodes the PDC from *P. pastoris*, and pDL021 encodes for a hypothetical PDC from *Y. lipolytica*. The constructed PDC modules were transformed into the modular chassis cell to create EcDL107 (carrying pCT15), EcDL108 (carrying pDL017), EcDL109 (carrying pDL018),

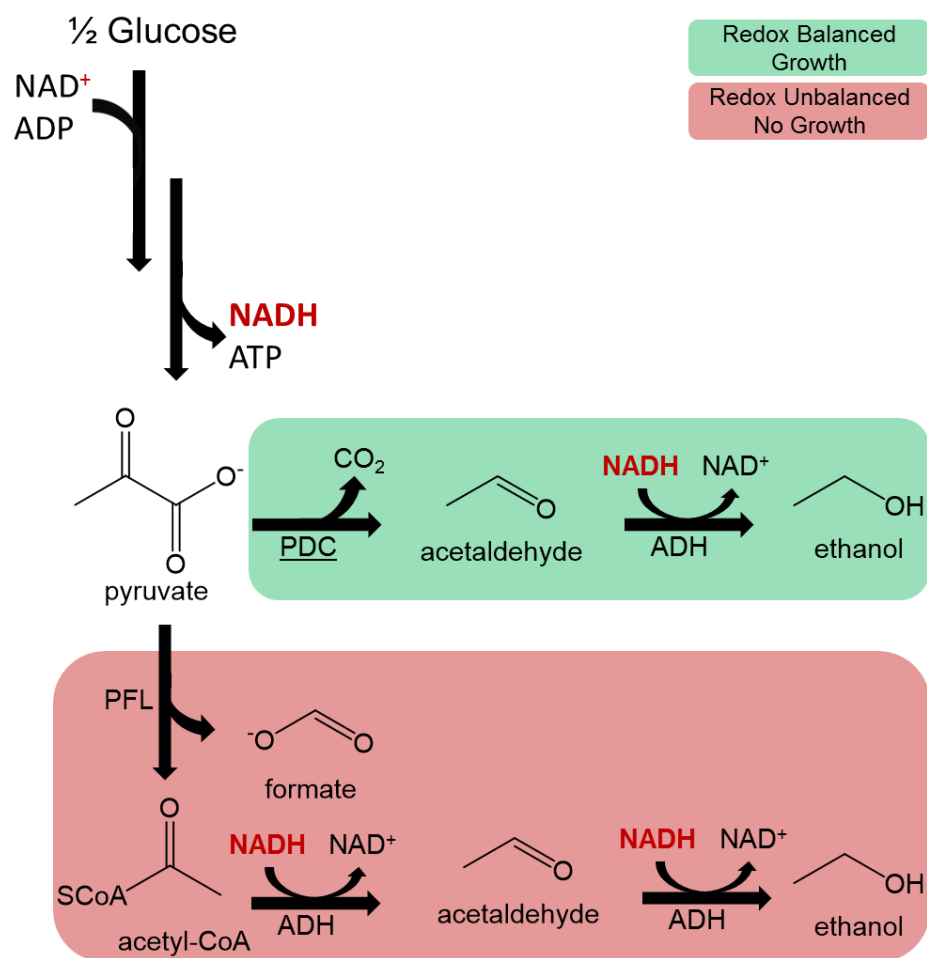


Figure 5-1: Homoethanol pathway for modular cell growth selection. The pathway highlighted in green is redox balanced which enables growth under anaerobic conditions using the modular chassis cell. The pathway highlighted in red is redox unbalanced which does not enable growth under anaerobic conditions using the modular chassis cell.

EcDL110 (carrying pDL019), EcDL111 (carrying pDL020) and EcDL112 (carrying pDL021) and were evaluated for their growth product coupling.

5.4.2 Demonstrating pathway selection by modular cell

We tested the modular chassis cell for growth product coupling by varying the PDC module for ethanol production. The degree of coupling, i.e. growth rate, provides a platform for screening and selection of an optimal pathway. The results show that each PDC was able to couple growth and product but demonstrated variability amongst the library (Figure 5-2). EcDL107, carrying pCT15 (PDC from *Z. mobilis*), demonstrated the tightest product coupling among all strains tested with the highest growth rate of $0.176 \pm 0.010 \text{ hr}^{-1}$ and an ethanol production rate of $0.209 \pm 0.012 \text{ g/gDCW/hr}$. The *Z. mobilis* PDC is known to have high activity for pyruvate to acetaldehyde formation. It was evident from the growth and ethanol production data, *adhE* was capable of rapid acetaldehyde to ethanol conversion (Figure 5-2). EcDL107 reached a maximum OD_{600nm} over 1.0 and consumed the initial 10 g/L glucose with an ethanol titer of 4.53 g/L, nearly 90% of theoretical maximum (Figure 5-2). The eukaryotic PDCs derived from *P. pastoris*, *S. cerevisiae*, and *Y. lipolytica* had distinctly different maximum growth rates and ethanol production rates compared to the *Z. mobilis* PDC. The three *S. cerevisiae* PDCs had similar growth rates and ethanol production rates. EcDL108, carrying PDC1 from *S. cerevisiae*, had a growth rate of $0.056 \pm 0.001 \text{ hr}^{-1}$ and an ethanol production rate of $0.060 \pm 0.002 \text{ g/gDCW/hr}$, a 3.17-fold and 3.48-fold decrease in maximum growth rate and ethanol

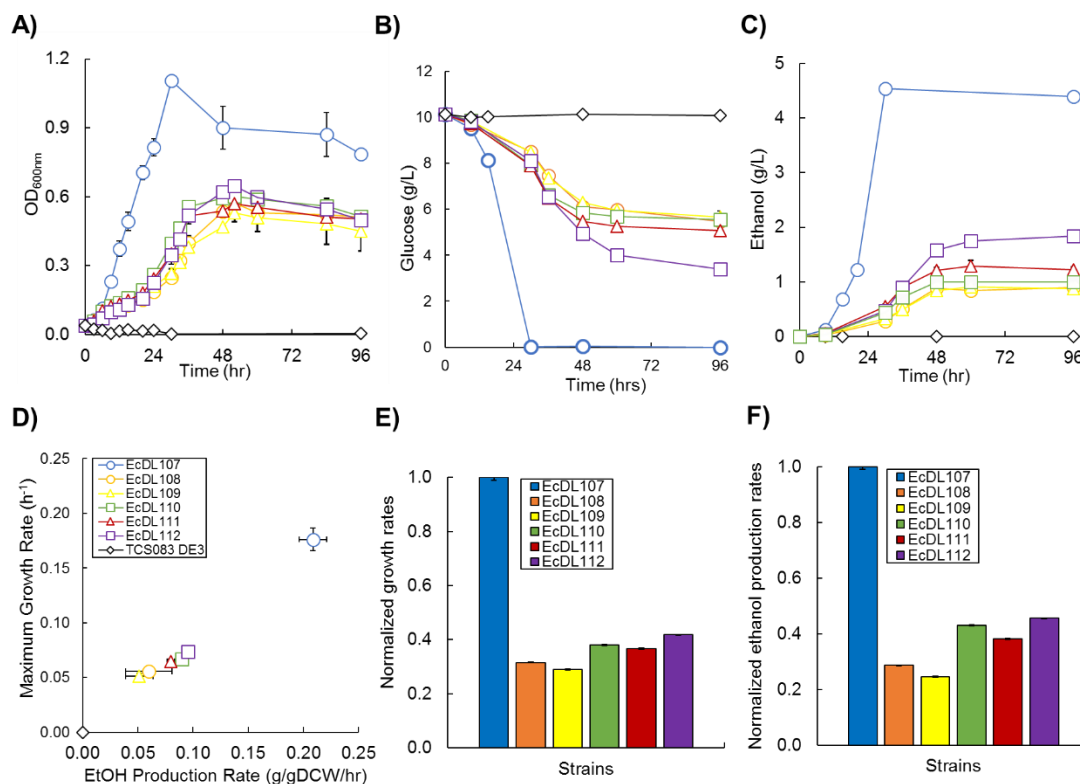


Figure 5-2: Fermentation kinetics for EcDL107-EcDL112. (A) Cell growth. (B) Glucose consumption. (C) Ethanol Production. (D) Correlation of specific growth rate with specific ethanol production rate. (E) Normalized growth rate. (F) Normalized ethanol production rate.

production rate, respectively, compared to EcDL107. EcDL109, carrying, PDC5 from *S. cerevisiae*, had the lowest growth rate and ethanol production rate of all PDCs with a growth rate of $0.051 \pm 0.000 \text{ hr}^{-1}$ and an ethanol production rate of $0.051 \pm 0.002 \text{ g/gDCW/hr}$ a 3.44-fold and 4.07-fold decrease, respectively, compared to EcDL107. EcDL110, carrying PDC6 from *S. cerevisiae*, had both a maximum growth rate and ethanol production rate of $0.067 \pm 0.002 \text{ hr}^{-1}$ and $0.090 \pm 0.006 \text{ g/gDCW/hr}$, respectively, a 2.67-fold and 2.33-fold decrease in maximum growth rate and ethanol production rate, respectively, compared to EcDL107. It is noteworthy that, EcDL108-EcDL110 did not reach an OD_{600nm} greater

than 0.65, did not consume more than 5 g/L glucose, or produce more than 1 g/L ethanol (Figure 5-2), unlike EcDL107.

P. pastoris and *Y. lipolytica*, had different growth coupling performance between each other, and from *S. cerevisiae*. EcDL111 carrying pDL020 (PDC from *P. pastoris*) had a maximum growth rate of $0.065 \pm 0.002 \text{ hr}^{-1}$ with an ethanol production rate of $0.080 \pm 0.003 \text{ g/gDCW/hr}$, a 2.72-fold and 2.62-fold decrease in maximum growth rate and ethanol production rate, respectively, compared to EcDL107. EcDL112 carrying pDL021 (PDC from *Y. lipolytica*) had the highest maximum growth rate and ethanol production rate of $0.074 \pm 0.000 \text{ hr}^{-1}$, and $0.095 \pm 0.005 \text{ g/gDCW/hr}$, respectively, a 2.39-fold and 2.19-fold decrease in maximum growth rate and ethanol production rate, respectively, compared to EcDL107. EcDL111 did not have an OD_{600nm} greater than 0.65, and did not consume more than 5 g/L glucose. Additionally, EcDL112 did not have an OD_{600nm} greater than 0.65, however, did consume nearly 7 g/L glucose. Contrary to the *S. cerevisiae* derived PDCs, the *P. pastoris* PDC enabled production of nearly 1.3 g/L ethanol and the *Y. lipolytica* PDC enabled production of nearly 1.9 g/L ethanol (Figure 5-2.) The ethanol production from *Y. lipolytica* is the first time this hypothetical protein has demonstrated PDC activity.

Each of the studied eukaryotic PDCs performed similarly to each other, but were distinctly different from the *Z. mobilis* PDC. Figure 5-2d shows a linear relationship between growth rate and production rate for all 6 PDCs investigated. In order of maximum growth rate, PDC_{Zm}>>PDC_{Yli}> PDC_{6Sc}>PDC_{Ppa}>PDC_{1Sc}>PDC_{5Sc} where PDC_{Zm} had a 4.07-fold increase of growth rate compared to PDC_{5Sc}. The ethanol production rate mimicked the growth rate trend with PDC_{Zm}>>PDC_{Yli} >PDC_{6Sc}>PDC_{Ppa}>PDC_{1Sc}

>PDC5_{Sc} where PDC_{Zm} had a 3.44-fold increase in ethanol production compared to PDC5_{Sc}. This trend demonstrates the modular cell as a capable selection chassis for the most optimal PDC for ethanol production.

5.4.3 Enabling directed evolution by modular cell

5.4.3.1 Directed evolution of EcDL108-EcDL112.

It was evident the production modules resulted in a correlation of growth rate and ethanol production rate (Figure 5-2d). We hypothesized a directed evolution approach for enhancing the growth product coupling between the modular chassis cell and the production module could be accomplished by balancing the production pathway and/or modular chassis cell fluxes either by mutation in the chassis or the production module. We tested our hypothesis by subtransferring EcDL108-EcDL112 during logarithmic cell growth for 150 generations. The results show an increase of growth rate was observed for EcDL108-EcDL112 (Figure 5-3). We used the maximum growth rate of EcDL107 ($0.176 \pm 0.010 \text{ hr}^{-1}$) as a benchmark because it accomplished the highest growth rate of all six PDCs tested initially. EcDL108 demonstrated an initial maximum specific growth rate of $0.049 \pm 0.002 \text{ hr}^{-1}$ and after 150 generations, the maximum specific growth rate increased to $0.233 \pm 0.010 \text{ hr}^{-1}$. Interestingly, by generation 60, the cells had an increased growth rate of $0.180 \pm 0.025 \text{ hr}^{-1}$ which eclipsed our initial benchmark (Figure 5-3a). EcDL109 initially demonstrated a maximum specific growth rate of $0.026 \pm 0.005 \text{ hr}^{-1}$ with a maximum specific growth rate of $0.253 \pm 0.005 \text{ hr}^{-1}$ after 150 generations. However, by generation 40, EcDL109 had an increased growth rate of $0.179 \pm 0.016 \text{ hr}^{-1}$ (Figure 5-3b). EcDL110

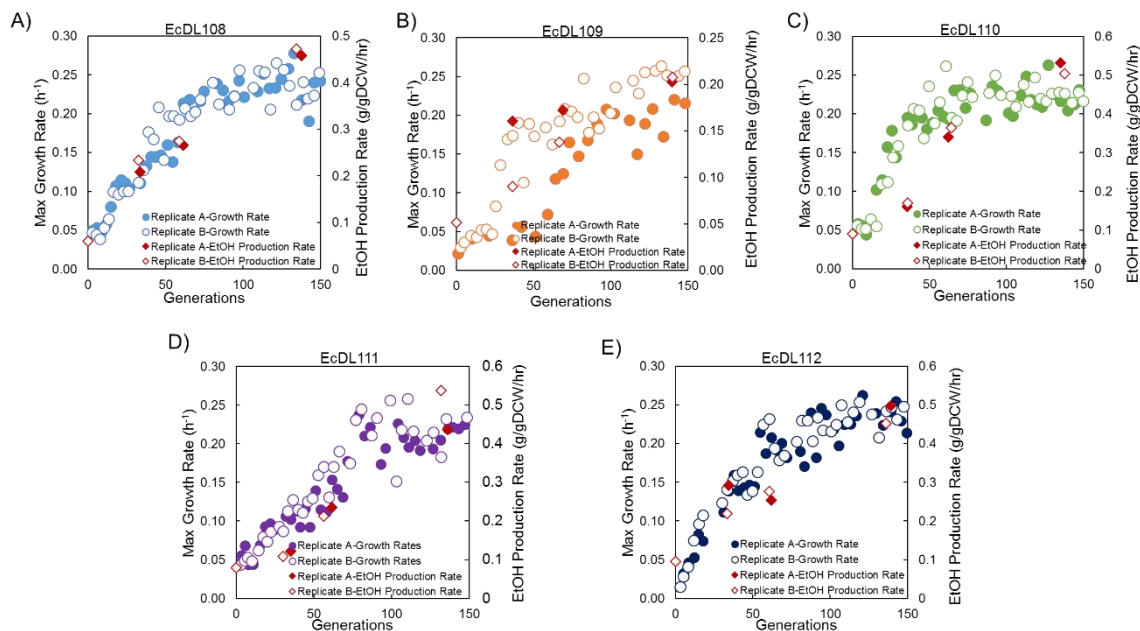


Figure 5-3: Directed evolution data for EcDL108-EcDL112 for 150 generations. (A) EcDL108. (B) EcDL109. (C) EcDL110. (D) EcDL111. (E) EcDL112

had an initial maximum specific growth rate of $0.057 \pm 0.002 \text{ hr}^{-1}$ and after 150 generations had a maximum specific growth rate of $0.237 \pm 0.025 \text{ hr}^{-1}$. EcDL110 eclipsed our benchmark growth rate at generation 36 with a growth rate of $0.191 \pm 0.007 \text{ hr}^{-1}$ (Figure 5-3c). EcDL111 initially demonstrated a maximum specific growth rate of $0.050 \pm 0.008 \text{ hr}^{-1}$ and had a growth rate of $0.233 \pm 0.010 \text{ hr}^{-1}$ after 150 generations. EcDL111 overtook our benchmark with an increased growth rate of $0.184 \pm 0.009 \text{ hr}^{-1}$ (Figure 5-3d) after 67 generations. Lastly, EcDL112 had an initial maximum specific growth rate of $0.015 \pm 0.000 \text{ hr}^{-1}$ and after 150 generations had a maximum specific growth rate of $0.231 \pm 0.024 \text{ hr}^{-1}$. After 54 generations, EcDL112 overtook EcDL107's growth rate with a growth rate of $0.189 \pm 0.0036 \text{ hr}^{-1}$ (Figure 5-3e). Each strain was tested for irreversibility of kinetic growth rate at generation 75 and was verified for growth irreversibility with near identical growth

rates (Figure 5-4). However, it was unclear if the host or the production module mutated for enhance growth rate and ethanol production rate.

5.4.3.2 Characterization of evolved *EcDL108-EcDL112*.

To understand the driving force behind the increased growth rate of evolved *EcDL108-EcDL112* (henceforth *EcDL108*-EcDL112**), extraction of the plasmid from *EcDL108*-EcDL112** was performed and transformed back into the original, unevolved, host strain, TCS083 DE3, and characterized. The results show an initial or decrease in growth rate as previously characterized (Figure 5-5). This lead us to believe the modular chassis cell likely did not enforce mutation on the production modules but instead adapted to reach higher growth and ethanol production rates. Thus, we rejected the plasmid from the evolved host for characterization. After several rounds of strain curation, we were able to successfully reject pDL020 plasmid from *EcDL111** first, and transformed the original production modules into the new host for characterization. Figure 5-6a shows the cell and production module regained the enhanced growth product coupling phenotype. Each strain was able to achieve maximum OD_{600nm} by or before 24 hours, whereas in the initial study, it took over 48 hours. This observation leads to us to believe the evolved host cell adapted to the production module. Figure 5-6b shows the maximum growth rates for pDL017-pDL021 with the evolved host and the unevolved host, TCS083 DE3. Most production modules were able to achieve much higher growth rates compared to the unevolved study. The fastest growing strain was the evolved host carrying pCT15 with a growth rate of $0.235 \pm 0.011 \text{ hr}^{-1}$ which is a 1.34-fold increase over the unevolved host chassis, a marginal

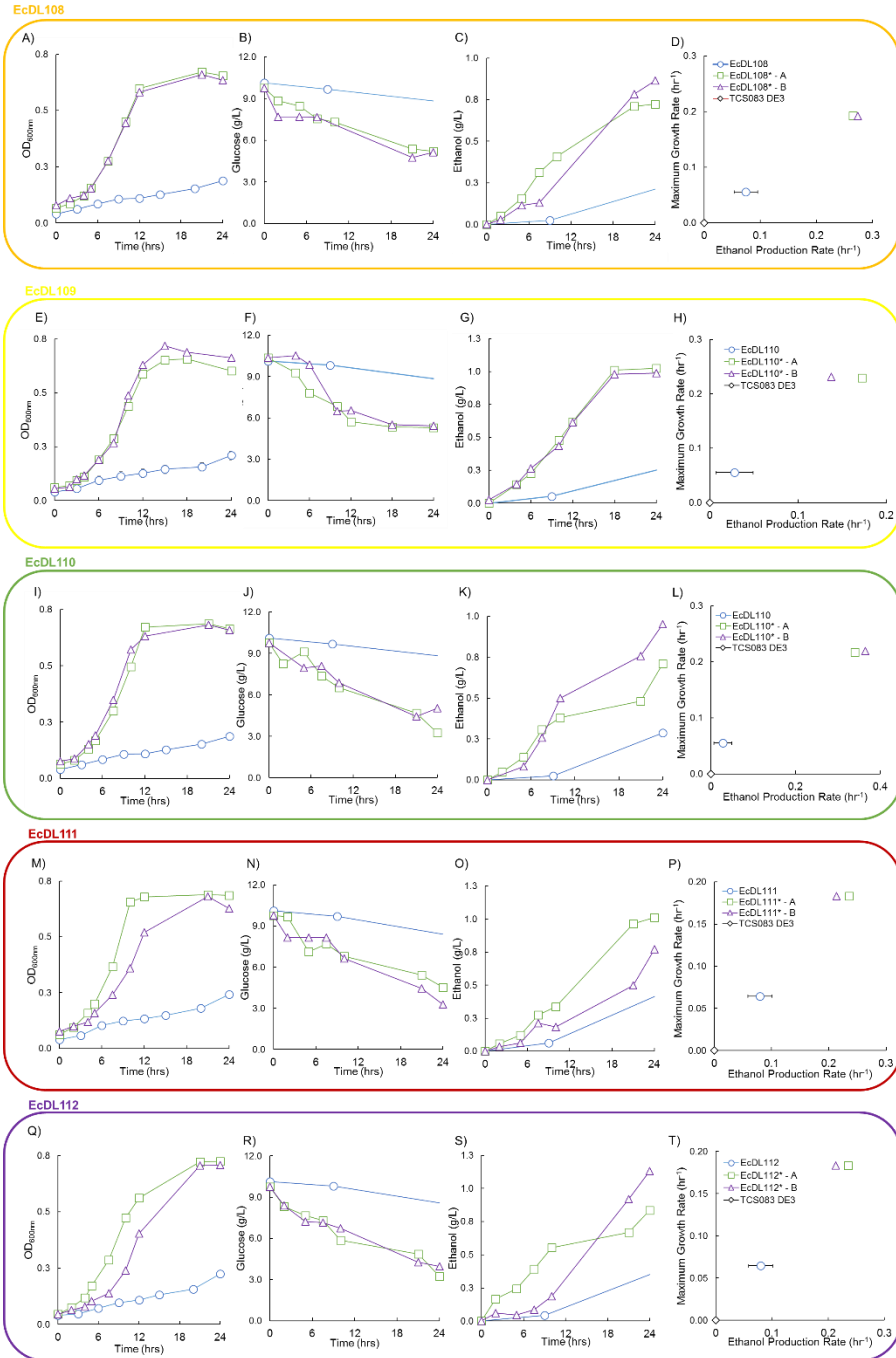


Figure 5-4: Fermentation kinetics, including cell growth, glucose consumption, ethanol production, and specific growth rate with specific ethanol production rate for EcDL109-EcDL112 at generation 75, compared to initial fermentation kinetics. (A-D) EcDL108. (E-H) EcDL109. (I-L) EcDL110. (M-P) EcDL111. (Q-T) EcDL112.

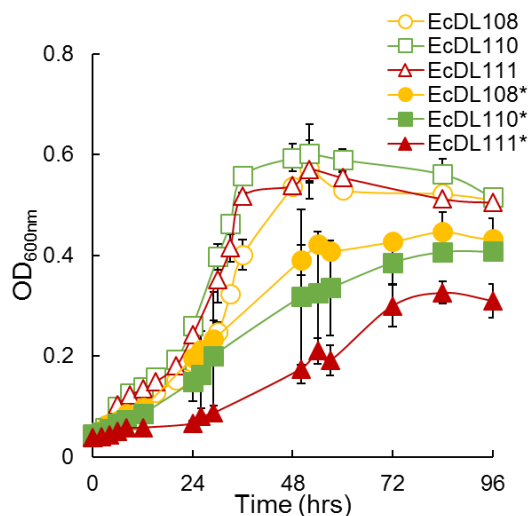


Figure 5-5: Growth kinetics of the evolved plasmid in the unevolved chassis host compared to the initial growth kinetics for EcDL108, EcDL110, EcDL111. * denotes the evolved plasmid in the unevolved modular chassis cell host.

increase. Interestingly, the *S. cerevisiae* PDC5 had the greatest increase in growth rate in the evolved host with a 3.69-fold increase over the unevolved host. PDC5 in the unevolved host showed the lowest growth and ethanol production rates of the six PDCs investigated. PDC1 and PDC6 from *S. cerevisiae* had a 3.64-fold, 2.61-fold increase, respectively, in growth rates and the *Y. lipolytica* PDC had a 2.89-fold increase in growth rate in the evolved host compared to the unevolved host. However, pDL020, PDC_{Ppa}, had a similar growth rate to the non-evolved host with only a 1.34-fold increase and was about 3-fold less than the highest maximum growth rate when the host and plasmid were evolved together. This difference points to a potential mutualistic relationship between evolved host and evolved plasmid for enhanced coupling; beneficial mutations in both the evolved *P. pastoris* PDC and the evolved host for enhanced growth rate. However, further investigation is needed.

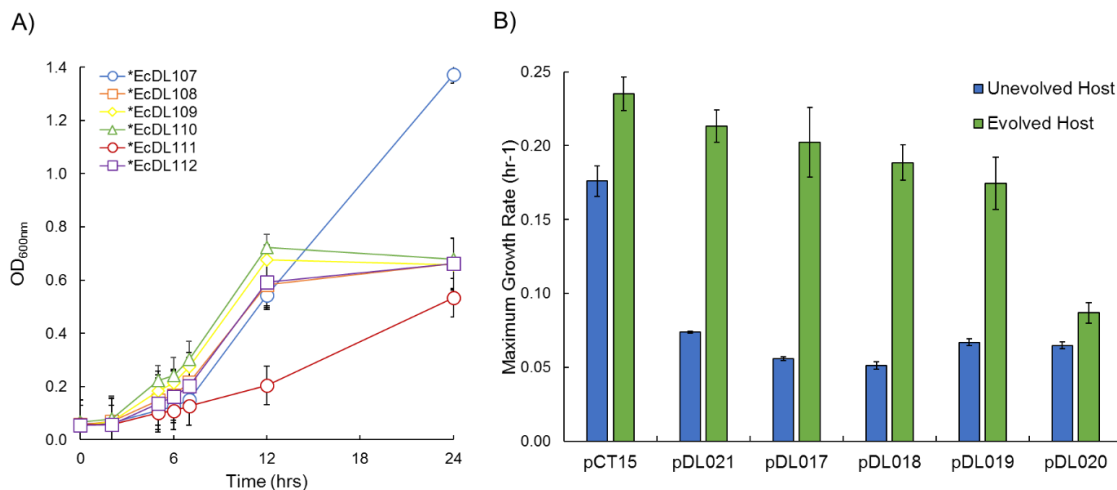


Figure 5-6: Growth kinetics of the evolved host chassis with unevolved pCT15 and pDL017-pDL021. (A) Cell growth. (B) Growth rates of evolved and unevolved chassis host with unevolved pCT15, pDL017, pDL018, pDL019, pDL020, and pDL021.

5.4.3.3 Random mutagenesis for selection of an optimal PDC.

Random mutagenesis is a powerful tool when studying an enzyme's function and has the capability to create novel enzymes. We applied random mutagenesis to demonstrate the selection power of the modular chassis cell to select an optimal PDC for turning over pyruvate to acetaldehyde. We chose the *P. pastoris* PDC for investigation because it was the median PDC for growth and ethanol production, allowing for an increase or decrease in growth rate. We generated an estimated library size of 2.71×10^8 of randomly mutated *P. pastoris* PDCs for selection.

The results show an enhanced coupling effect immediately after transformation with the mutagenized PDC outperforming the plasmid control (Figure 5-7). Upon serial dilutions of the mutagen and the plasmid control, it was evident that a tight coupling or

selection had occurred. After 72 hours, the mutagenized pDL020 (pDL020 – M) was in exponential phase whereas it took the unmutagenized plasmid 144 hours before reaching its exponential phase for subsequent transfer (Figure 5-8a-b). After four rounds of cell culture transfer, the mutagenized plasmid had reached a specific growth rate of $0.179 \pm 0.003 \text{ hr}^{-1}$, nearly 20 generations, whereas the unmutated plasmid control reached a specific growth rate of $0.073 \pm 0.016 \text{ hr}^{-1}$ which is similar to the initial unevolved study (Figure 5-8c). The mutated PDC growth rate achieved the targeted growth rate of pCT15 carrying the *Z. mobilis* PDC. Interestingly it took the directed evolution method nearly 67 generations to achieve the same growth rate of pCT15, demonstrating the modular chassis cell capable for screening novel enzymes and selecting for the most efficient pathway.

After 20 generations, the cells were single colony isolated for validation of the selected phenotype, two colonies were selected for characterization. It was seen that the

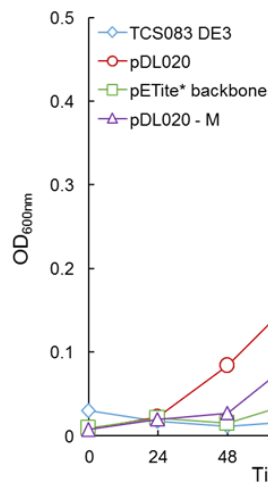


Figure 5-7: Initial kinetic growth rates of random mutagenesis study before splitting anaerobic growth into triplicates. M denotes randomly mutated PDC.

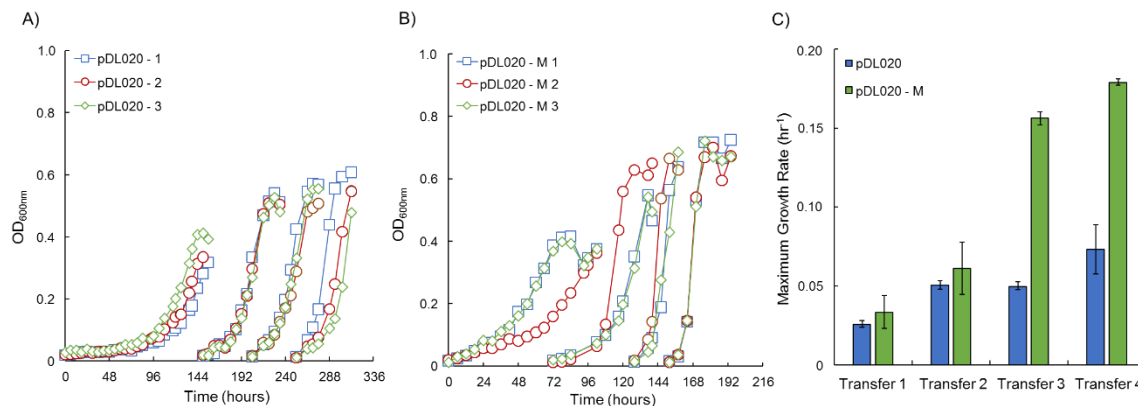


Figure 5-8: Growth kinetics for pDL020 and randomly mutated pDL020 in the host chassis cell. (A) pDL020, (B) randomly mutated pDL020, (C) growth rates of pDL020 and randomly mutated pDL020.

selection for the most efficient PDC was accomplished with a recovered phenotype and enhanced ethanol production rate (Figure 5-9). EcDL111-M1 (M denotes mutagenized PDC) had a specific growth rate of $0.207 \pm 0.012 \text{ hr}^{-1}$ and an ethanol production rate of $.229 \pm 0.002 \text{ g/gDCW/hre}$ much higher than that of EcDL111. EcDL111 – M2 had a specific growth rate of $0.234 \pm 0.002 \text{ hr}^{-1}$ and an ethanol production rate of $.192 \pm 0.010 \text{ g/gDCW/hr}$ (Figure 5-9). The differences between the two colonies could be due to the chassis cell still imposing selection or due to sampling variability. However, further validation is needed.

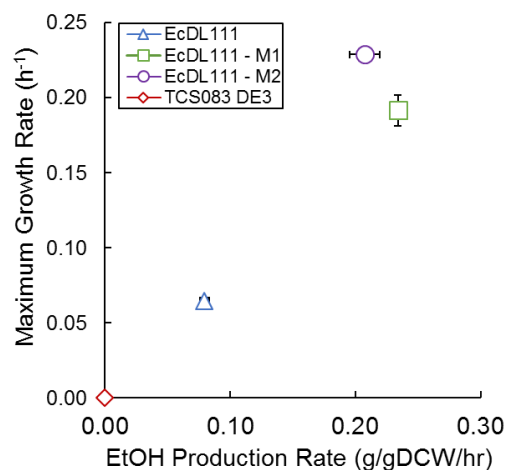


Figure 5-9: Maximum growth rate and ethanol production rates of random mutagenesis plasmids. M1 and M2 denote colony one and colony two of single colony isolates from random mutagenesis selection experiments.

5.4.4 Demonstrating modulation of genetic parts to enable degree of coupling between modular cell and production module

Coupling between the modular cell and the production module has been evident based on open reading frame modulation, i.e. varying PDC. However, each heterologous production pathway carries more genetic parts than the open reading frame, e.g. promoter, ribosome binding site, terminator, and origin of replication. We aimed to investigate the effect of genetic part variation for pathway selection. We chose the promoter for investigation using the ethanol pathway and transcriptional variance. We chose the complete ethanol pathway from *Z. mobilis* and TCS095 DE3, (the modular chassis cell $\Delta adhE$) to validate the necessity of *adhE* for effective ethanol formation. We first transformed the most effective PDC module, pCT15, into TCS095 DE3 (EcDL116) and investigated growth product coupling. The results show the main alcohol dehydrogenase

in *E. coli*, *adhE*, is necessary for turnover of acetaldehyde to ethanol and for balancing cellular redox which is necessary for cellular growth and product formation (Figure 5-10). This result was positive as it allows for further investigation of alcohol dehydrogenases or any other single NADH cofactor utilization pathway.

To investigate promoter strength based selection on the complete ethanol pathway PDC and *adhB*, we used the standard T7 promoter found on pETite* as well as used two different lac derived constitutive promoters from the iGEM Andersen promoters (Kelly et al., 2009). We chose BBa_J23100 as well as BBa_J23108. J23100 has the highest strength in the iGEM Andersen library and J23108 has 51% activity of J23100. These promoters were cloned in front of the *Z. mobilis* ethanol pathway and characterized. The T7 promoter

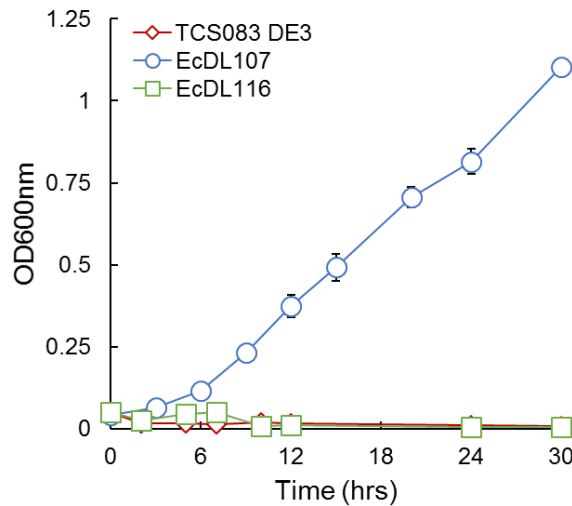


Figure 5-10: Growth kinetics of TCS083, EcDL107 EcDL116. The lack of *adhE* in EcDL116 demonstrates the necessity for an efficient redox sink driven by *adhE* in EcDL107 for growth.

had the greatest strength among all characterized promoters and thus facilitated the highest growth rate and highest ethanol production rate (Figure 5-11a-d) of $0.188 \pm 0.008 \text{ hr}^{-1}$, and $0.270 \pm 0.004 \text{ g/g DCW/hr}$, respectively. J23100 had the second highest growth rate and ethanol production rate of $0.149 \pm 0.013 \text{ hr}^{-1}$ and $0.206 \pm 0.003 \text{ g/g DCW/hr}$, respectively and J23108 had the lowest growth rate and ethanol production rate of $0.086 \pm 0.001 \text{ hr}^{-1}$ and $0.119 \pm 0.010 \text{ g/g DCW/hr}$, respectively. It is interesting to note that ethanol production rate correlated between each characterized promoter (Figure 5-11d). J23100 and J23108 correlated very closely with J23108 having a normalized 0.57 and 0.58 growth rate and ethanol production rate compared to J23100, which is very similar to the normalized 0.51 activity reported. The T7 promoter had a 1.35-fold increase of activity compared to J23100 and a 3.14-fold increase of activity compared to J23108. The linear trend of growth rate and ethanol production rate demonstrates that the most efficient genetic part, beyond the open reading frame, can also be selected using the modular chassis cell.

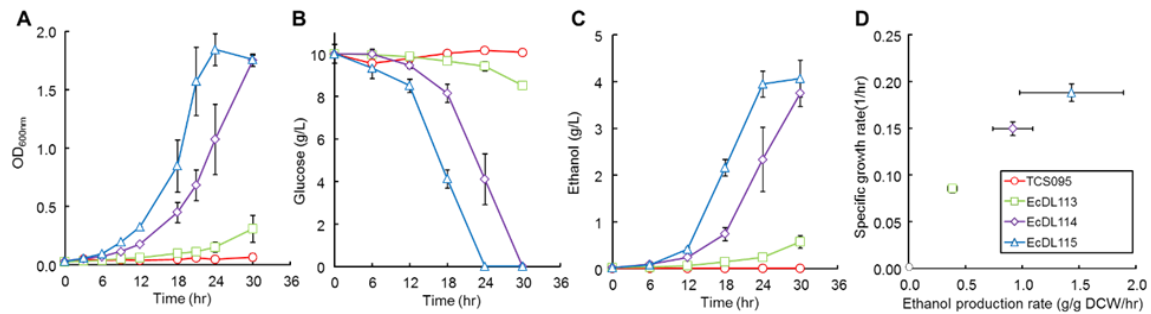


Figure 5-11: Analysis of degrees of coupling of the modular chassis cell with ethanol production modules including pCT24, pAY1, and pAY3. (A) Cell growth. (B) Glucose consumption. (C) Ethanol production. (D) Correlation of specific growth rate with specific ethanol production rate.

5.5 Discussion

In this study, we used ethanol production as a testbed for validating the modular cell theory for growth product coupling and selection. The endogenous ethanol pathway was demonstrated to be insufficient for maintaining cellular growth because the pathway requires two NADHs per $\frac{1}{2}$ glucose. This is due to an imbalance of cellular redox since glycolysis only produces half of the necessary cofactors in the modular chassis cell. Additionally, deletion of the alcohol dehydrogenase in the modular chassis cell, the first step in the ethanol pathway, pyruvate to acetaldehyde, was demonstrated as being insufficient to maintain cellular growth alone due to underutilization of the NADH cofactor generated from glycolysis. However, when available, the main alcohol dehydrogenase, adhE, utilized the NADH cofactor and demonstrated the necessity for conversion of acetaldehyde to ethanol for biomass synthesis.

Ethanol selection was conducted using a library of PDCs from *P. pastoris*, *S. cerevisiae*, *Y. lipolytica*, and *Z. mobilis*. Each PDC demonstrated tight coupling between cell growth and product formation using the chassis cell derived from the modular cell theory which allowed for selection of an optimal PDC. From this work, we demonstrated the hypothetical PDC protein from *Y. lipolytica* to have PDC activity with a specific growth rate of $0.074 \pm 0.000 \text{ hr}^{-1}$ and an ethanol production rate of $0.095 \pm 0.003 \text{ g/gDCW/hr}$ when hosted by the modular chassis cell. This is the first time the hypothetical PDC protein from *Y. lipolytica* has been demonstrated for activity, demonstrating the screening and selection platform the modular chassis cell offers for novel enzyme discovery and/or engineering.

The *Z. mobilis* PDC was the most effective PDC from the library screened. Interestingly, the *Z. mobilis* PDC with the endogenous AdhE had a growth rate of $0.176 \pm 0.010 \text{ hr}^{-1}$ and an ethanol production rate of $0.209 \pm 0.012 \text{ g/g DCW/hr}$, whereas the full ethanol production pathway had a slightly higher growth rate of $0.188 \pm 0.008 \text{ hr}^{-1}$, but a much higher ethanol production rate of $0.270 \pm 0.004 \text{ g/g DCW/hr}$. The ethanol production rate difference could be due to the overexpression of the alcohol dehydrogenase, AdhB, from *Z. mobilis*, or due to AdhB having a higher activity for acetaldehyde and NADH for ethanol production. These differences could be answered by fixing the PDC and screening alcohol dehydrogenases for activity and substrate preference using the modular chassis cell. This question will be addressed in future studies.

Directed evolution is a powerful technique for generating a desired phenotype. However, unexpected results can occur and mutation rates of plasmids during directed evolution are known to be extremely low in some scenarios (Antonovsky et al., 2016). Interestingly, after evolving EcDL108-EcDL112, we demonstrated the modular chassis cell likely adapted or evolved for increased growth. We observed that the specific growth rate did indeed increase, as well as the increase of ethanol production rate, however, overall titer of product formation was either the same or slightly diminished compared to the initial study (Figure 5-2, Figure 5-4). The cell evolved to have a faster growth rate, but did not evolve to produce more ethanol or consume more glucose. This observation leads towards several questions in regards to biomass synthesis pathways. We hypothesize the PDC production module to be limiting and the chassis cell increases fluxes through pyruvate to acetyl-CoA for faster biomass synthesis. This leads towards future study for understanding

fundamental cellular adaptation or mutation to a product pathway and can be further investigated with genome sequencing and metabolomics.

Random mutagenesis can generate novel enzymes that have desired or undesired functions. We applied random mutagenesis to the *P. pastoris* PDC to generate a large library and selected an optimal PDC that couples tighter to the modular chassis cell compared to the wildtype PDC. In only 20 generations, we were able to select for our targeted phenotype, a growth rate greater than the *Z. mobilis* PDC of 0.17 hr^{-1} . In our initial studies, it took 67 generations to generate the same phenotype. Upon single colony isolation we were able to recover the higher growth phenotype, with slightly enhanced growth rate, as well as show an increased ethanol production rate over the non-mutated PDC. Achieving both growth and ethanol rate in 47 less generations lends the ability of the modular chassis cell to select for an optimal PDC among a large library of PDCs. Future studies on the PDC library mutations and the selection made by the modular chassis cell will shed light on correlating PDC sequence to function activity. However, this demonstration points toward selection of an optimal pathway from combinatorial libraries of product pathways using the modular cell theory and chassis cell.

Selection of the most optimal product pathway can be time consuming and costly. Most methodologies for generating high performance production pathways utilize a design-build-test cycle which can lead to several iterations and take several years. The rise of combinatorial part generation and pathway engineering has added additional layers to the design-build-test cycle. Using a rational approach to engineering product formation is essential for rapid product synthesis. By using the modular cell theory for generating a

modular chassis cell to couple with plug-and-play production modules, we demonstrated and streamlined the selection process for producing an example molecule, ethanol, by simply selecting for the highest growth rate. We were able to effectively demonstrate selection of an optimal PDC from a small library, a large estimated library of 2.71×10^8 mutants, as well as demonstrate selection of an optimal ethanol pathway by varying promoter strength. Demonstration of the modular cell theory allows for high throughput *in vivo* screening and selection without the need for *in vitro* assays or *in silico* simulations, reducing the time necessary for developing optimal microbial cell factories. We envision the modular cell theory will be used and applied for rapidly advancing biochemical production.

5.6 References

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6 Conclusion

Biochemical production will reduce our dependence on non-sustainable nor renewable petroleum derived chemicals. The use of modular cells will allow for rapid and efficient construction of optimal microbial cell factories for biochemical production. The modular chassis cell was used to demonstrate enhanced production of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate by 27-fold, 24-fold and 48-fold over the wildtype cell using a developed *in situ* fermentation and extraction technique as well as demonstrated tight ester product growth coupling with the production modules. The modular cell was also used for production of platform esters from carboxylates, including, ethyl acetate, propyl acetate, ethyl propionate, propyl propionate, isopropyl propionate, butyl acetate, ethyl butyrate, butyl butyrate, isobutyl butyrate, pentyl acetate, ethyl pentanoate, isobutyl pentanoate, pentyl pentanoate, hexyl acetate, ethyl hexanoate, and isobutyl hexanoate from acetic acid, propionic acid, butyric acid, pentanoic acid, hexanoic acid and glucose and was used for *in vivo* screening of AAT substrate preference and activity. Lastly, the modular cell theory was further validated by using ethanol as an example molecule using growth-based selection of an optimal PDC for ethanol production, selection from a 2×10^8 library and selection for an optimal ethanol pathway by modulating genetic parts. Overall this work contributes to validating the modular cell theory that will enable optimal microbial cell factory construction for rapid advancement of biochemical production.

Vita

Donovan Layton was born to Mitch and Sherry Layton on September 4, 1987 in Claremore, Oklahoma. He has an older sister, Shannon, and a younger sister, Allyson. Donovan graduated from Kearney High School in 2006. He attended Iowa State University, earning a Bachelor's of Science degree in Chemical and Biological Engineering in 2011. During his time at Iowa State, he worked with Laura Jarboe researching the conversion of levoglucosan, derived from biomass pyrolysis, to ethanol using a microbial biocatalyst. He pursued his passion for research by starting his PhD in the Fall of 2011 at the University of Tennessee, Knoxville under the guidance of Dr. Cong T. Trinh. He plans to continue his career in the field of metabolic engineering and synthetic biology by pursuit of an industry position and then returning to education to teach upcoming chemical and biomolecular engineers with emphasis on his experience and practice in industry.