BIOSYNTHETIC PATHWAY TO PRODUCE ESTERS

IN *ESCHERICHIA COLI*

by

JACOBY SHIPMON

(Under the Direction of Mark Eiteman)

ABSTRACT

Sustainable chemical production is a major challenge in the chemical industry. Esters are examples of bulk chemicals that have room for improvement in sustainable production. Microbial production is one avenue to meet this need, where the titer, rate, and yield must be optimized to be competitive with traditional chemical synthesis. To produce acetate esters in *Escherichia coli*, the Alcohol-O-Acetyl Transferase (Atf1) enzyme was introduced into the organism from the native ester producer *Saccharomyces cerevisiae.* In prior studies, knocking out genes associated with the overflow metabolites lactate (*ldhA)* and acetate (*pta-ackA* and *poxB*), which divert carbon away from acetyl-CoA generation and thus ester production, has been shown to facilitate an increase in the production of acetate esters in *E. coli.* Another diversion of acetyl-CoA is via citrate synthase (expressed by *gltA* gene). This work aims to explore the effect of citrate synthase variants on the production of acetate esters.

INDEX WORDS: *Escherichia coli*, Acetate Esters, Propyl Acetate, Fermentation, Knockouts, Metabolic Engineering

BIOSYNTHETIC PATHWAY TO PRODUCE ESTERS

IN *ESCHERICHIA COLI*

by

JACOBY SHIPMON

B.S., North Carolina Agricultural and Technical State University, 2017

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2023

© 2023

Jacoby Courtland Shipmon

All Rights Reserved

BIOSYNTHETIC PATHWAY TO PRODUCE ESTERS IN *ESCHERICHIA COLI*

by

JACOBY SHIPMON

Major Professor:	Mark Eiteman
Committee:	James Kastner
	William Kisaalita

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
May 2023

ACKNOWLEDGMENTS

Thank you to my wonderful wife for supporting me throughout all my schooling. Thank you to all my colleagues in Dr. Eiteman's lab. Thank you to DaniMer Scientific for your support.

Table of Contents

1.0 Introduction

1.1 Esters and Their Chemical Synthesis

Sustainable production of bulk chemicals is one of the major challenges in the chemical industry, particularly due to the low market prices of commodity chemicals made using conventional processing. Low market prices make it difficult to justify the adoption of higher cost green alternatives for the synthesis of bulk chemicals. Short and medium chain esters are examples of bulk chemicals that are currently manufactured using fossil fuels as feedstocks and have room for improvement in sustainable production. Esters are used in a wide range of applications from fragrance compounds to industrial solvents.

Acetate esters find many applications in the food and chemical industry. They are naturally produced by *Saccharomyces cerevisiae* and at low concentrations impact the unique taste and odor of fermented beverages (Saerens et al., 2010). The most important acetate esters for flavoring food and beverages are ethyl acetate (fruity, solvent aroma), isobutyl acetate (sweet, fruit), isoamyl acetate (banana) and 2-phenylethyl acetate (rose) (Van Laere et al., 2008). Several esters are also used as industrial solvents due to their biodegradability and low toxicity, or as plasticizers and polymer additives (Loser at al., 2014). They even find applications as lubricants, coatings and are explored for their potential as drop-in fuels or biodiesel. Acetate esters are truly versatile compounds.

Although acetate esters have many industrial uses and are prized for their biodegradability and low toxicity, the chemical synthesis of acetate esters has harsh environmental effects. Acetate esters are typically synthesized by Fisher-Speier esterification, a highly energy-consuming process that takes place at high temperature (200 - 250°C) using

concentrated sulfuric acid as the catalyst. These conditions cause severe corrosion and the formation of significant hazardous wastes (Zhang et al., 2020). In the Fisher-Speier esterification process, alcohols and carboxylic acids, produced from fossil fuels, are condensed in the high temperature acidic environment. Water is released in this process, which leads to the formation of the desired ester (Liu et al., 2006). Because the water released from this process inhibits the catalytic activity of the acid catalyst, water must continuously be removed from the system by energy intense distillation or adsorption steps for the reaction to proceed.

1.2 Biotechnological Acetate Ester Formation

Biosynthesis of esters offers a promising alternative to chemical synthesis, because microorganisms are able to perform hundreds of chemical conversions at ambient conditions. Microbial conversion has already received much attention for sustainable bulk chemical synthesis (Donate et al., 2014). Microbial ester production by yeast and lactic acid bacteria is well established and is associated with food and flavor production. Certain plants are also known to produce natural esters in their fruit or as a method to attract insects. Thus, enzymes clearly exist which generate esters. The four main pathways for ester biosynthesis are mediated by the following enzymes: i) esterases, ii) hemiacetal dehydrogenases (HADHs), iii) Baeyer-Villiger monooxygenases (BVMOs), and iv) alcohol acyltransferases (AATs). The reactions catalyzed by AATs and esterases are redox neutral, whereas BVMOs and HADHs require NAD(P)H or NAD(P), respectively (Kruis et al., 2019). These reactions are compared in Figure 1 (Kruis et al., 2019).

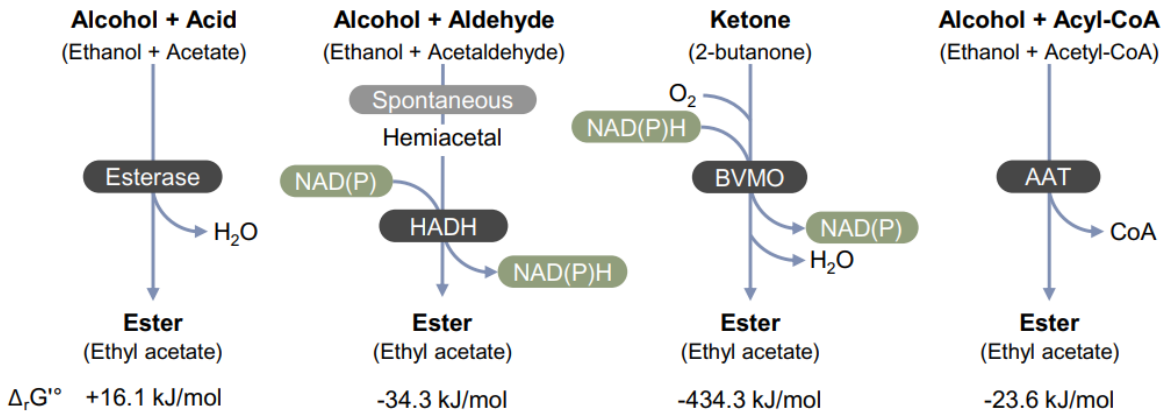

Figure 1: Enzymatic reactions that result in ester production. The $\Delta_rG°$ of the reactions were estimated using Equilibrator (Flamholz et al., 2014) under standard conditions and pH 7. Figure taken from (Kruis et al., 2019).

The two redox neutral enzymatic pathways to ester production are AATs and esterases. Redox neutral means that redox equivalents NAD(P)H and NAD(P) are not needed for the reaction to proceed. The absence of cofactors in the reaction provides an advantage in metabolically engineering a pathway using these two enzymes, since the system will not need to be engineered to produce access redox equivalents. Esterases, including lipases, are ubiquitous enzymes that have been identified in all domains of life. In aqueous environments, they catalyze the hydrolysis of ester bonds, resulting in the formation of an alcohol and a carboxylic acid. Under aqueous conditions ester formation has a positive $\Delta_rG°$, making the reaction thermodynamically unfavorable. Hence, industrial production of esters via esterases is typically performed in non-aqueous systems using organic solvents or high substrate concentrations (Kruis et al., 2019). The stoichiometric equation for ester production via esterases is as follows:

carboxylic acid + alcohol → acetate ester + H_2O.

AATs also mediate ester biosynthesis and are redox neutral. AATs are a large and diverse group of enzymes that are the primary ester producing enzymes in plants, yeast, filamentous fungi and some bacteria (Kruis et al., 2019). AATs form esters by transferring the acyl moiety

from an acyl-CoA molecule to an alcohol. They vary significantly in the specificities for their alcohol and acyl-CoA substrates (Table 1). Therefore, many different esters are produced in nature, ranging from short chain esters such as ethyl acetate, to long chain wax esters (Kruis et al., 2019). For example, when ethanol is used for AAT catalysis, the stoichiometric equation is:

Acetyl-CoA + ethanol → ethyl acetate + CoA

Table 1: Characteristics of the most studied and applied microbial AATs. Adapted from Kruis et al., 2019).

	AtfA (WS)	**Atf1**	**Atf2**	**Eht1**	**Eeb1**	**Eat1**
Source, species examples	Bacteria, *A. baylyi*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *W. anomalus K. marxianus S. cerevisiae*
Typical ester product	Wax esters and TAG	Acetate esters of various alcohols	Acetate esters of various alcohols	MCFA ethyl esters	MCFA ethyl esters	Ethyl acetate, other acetate esters
Cellular location	Lipophilic inclusion in the bacterial cytosol	ER, Cytosolic lipid droplets	ER, Cytosolic lipid droplets	ER, Mitochondrial outer membrane, lipid droplets	Mitochondria	Mitochondria
Protein family	WES acyl-transferase-like domain	AATase domain	AATase domain	α/β-hydrolase family 4	α/β-hydrolase family 4	α/β-hydrolase family 6
Side activities	Unknown	Thioesterase (in vitro)	Unknown	Esterase, thioesterase (in vitro)	Esterase (in vitro)	Esterase (in vitro, in vivo)
Alcohol specificity	Broad (C4-C20) unbranched primary alcohols (in vitro)	Broad towards primary alcohols (in vitro)	Primary alcohols (in vitro) and Sterols (in vivo)	Ethanol, Phenylethyl alcohol (in vivo)	Ethanol (in vivo)	Ethanol (in vitro) Primary alcohols (in vivo)
Acyl-CoA specificity	Preferred long acyl-CoAs (C14-C18) (in vitro)	Acetyl-CoA only (in vitro)	Acetyl-CoA only (in vivo)	MFCA-CoA (C4-C12) Caffeoyl-CoA (in vivo)	MFCA-CoA (C6-C10) (in vitro)	Acetyl-CoA (in vitro) Propionyl-CoA (in vivo)

1.3 The Ester Catalytic Enzyme Alcohol-O-Acetyltransferase

Acetyltransferases mediate the formation of volatile esters by yeast and fungi during fermentation and in plants during fruit ripening. The most studied microbial ester producing AATs are derived from yeasts (Table 1). The *S. cerevisiae* alcohol-O-acetyltransferases (EC 2.3.1.84) Atf1p and Atf2p are thought to synthesize a range of acetate esters from acetyl-CoA and various alcohols (Nancolas et al., 2017). Though Atf1p has been the focus of many research studies, thus far there are no crystal structures of Atf1 or Atf2 from *S. cerevisiae* or other yeasts, and there are no suitable templates to generate high confidence homology models (Lin et al., 2014).

Atf1p is a peripheral membrane protein in *S. cerevisiae* that is localized to the endoplasmic reticulum and lipid particles via amphipathic domains at the N- and C-termini. The enzyme adopts a mixed α/β structure, with a relative proportion of different secondary structure elements. The enzyme has a structure composed of 0.23 α-helix, 0.25 β-strand, 0.12 Turn and 0.40 Unordered domains (Nancolas et al., 2017).

When expressed recombinantly in a host organism, Atf1p does not always localize to the endoplasmic reticulum as a peripheral membrane protein. In one study, various alcohol-O-acetyltransferases from *S. cerevisiae*, other yeasts, and tomato fruit were expressed recombinantly in *S. cerevisiae* and *E. coli* (Zhu et al., 2015). All studied AATases formed aggregates with diminished enzymatic activity when expressed in *E. coli* and any membrane localization observed in *S. cerevisiae* was lost in *E. coli*. These aggregates in recombinant *E. coli* collected in the cytosol of the cell, and all cells expressing an AATase had between one and three aggregates. Fermentation and whole cell lysate activity assays of the two most active AATases, Atf1 and the tomato fruit AATase, demonstrated that the aggregates were enzymatically active,

but with lower specific activity compared to activity in *S. cerevisiae*, thus demonstrating that the proteins were not misfolded when expressed. Although enzymatic activity was slightly diminished on a specific basis, western blot analysis revealed that expression levels in *E. coli* were upwards of 100-fold higher than *S. cerevisiae* (Zhu et al., 2015).

Alcohol-O-acyltransferases function as bisubstrate enzymes that catalyze the transfer of acyl chains from an acyl-coenzyme A (CoA) donor to an acceptor alcohol. During catalysis, substrate access to the buried active site from either face of the enzyme is provided via two linked solvent channels that extend to the protein surface, and in many cases, each of the tunnels appears to be dedicated to one of the cosubstrates (Nancolas et al., 2017). The catalytic activity of Atf1 from *S. cerevisiae* has been the focus of many research papers in the hope of better understanding enzymatic kinetics.

Typically, an enzyme is characterized by kinetic parameters that include the K_M values for its substrates and the specific activity of the enzyme. Various research studies have been conducted to quantify the kinetic parameters of the alcohol-O-acyltransferase Atf1. Because Atf1 is a flexible enzyme, able to mediate the reaction of multiple short to medium chain alcohols with acetyl-CoA, kinetic parameters for this enzyme are reported for each alcohol/ester reaction it mediates. In the case of the Atf1 mediated bioproduction of isoamyl acetate, the K_M for acetyl-CoA is 0.19 mM, while the K_M for isoamyl alcohol is 29.8 mM (Minetoki et al., 1993). In this case the K_M for isoamyl alcohol is much greater than that of acetyl-CoA meaning that a higher concentration of the substrate isoamyl alcohol is needed than acetyl-CoA for the enzyme to be saturated with substrates. Thus, isoamyl alcohol is considered the rate limiting factor in the formation of isoamyl acetate in yeast cells (Minetoki et al., 1993). Stated another way, the reaction rate likely benefits the most from high concentrations of isoamyl alcohol.

In another study, using sake yeast, the kinetic parameters of Atf1 catalyzing the formation of isoamyl acetate were measured as: K_M for acetyl CoA of 25 μM and K_M for isoamyl alcohol of 25 mM (Mason et al., 2000). This same group reported K_M values for ethyl acetate production: K_M for acetyl CoA was 45 μM (0.045 mM), and K_M for ethanol was 15-80 μM (Malcorps et al., 1992; Mason et al., 2000). Another key kinetic parameter of an enzyme is its specific activity. This value is defined as the units of activity of that enzyme in units of micromoles of substrate consumed per minute per milligram of enzyme. For Atf1, the specific activity was measured at 190.4 μmol/min·mg (Akita et al., 1990).

1.4 Factors Affecting the Atf1 Enzyme

Literature highlights a few factors that affect the Atf1 enzyme's catalytic activity and ability to mediate the production of acetate esters. These factors include nitrogen conternt (typically in the form of added casamino acids or tryptone), the induction concentration of IPTG, and oxygen. These factors shown to affect the ability of the Atf1 protein to produce esters.

An important medium parameter for ester production, especially in breweries, is the carbon to nitrogen ratio of the fermentation medium. In a paper studying the parameters affecting ethyl acetate production in *S. cerevisiae,* the researchers varied the carbon to nitrogen ratio of medium. To obtain worts with a set of predetermined C/N ratios, synthetic wort was used. The nitrogen content of wort which can be "consumed" or "assimilated" by the yeast is called FAN, whereas the carbon or total sugar content of the wort is reflected in the specific gravity of the wort. Ethyl and isoamyl acetate concentrations increased when more nitrogen was available in the fermentation medium (Saerens et al. 2008). These results fit with the previous demonstration

that Atf1 gene expression also increases with increasing FAN content of the fermentation medium.

IPTG induction concentration was also shown to affect the ester productivity of the Atf1 enzyme. In a study comparing the expression and activity of various AATases, researchers found that induction concentration has a direct effect on enzymatically active protein aggregate size and expression levels in *E. coli* (Zhu et al., 2015). Increased IPTG induction concentration resulted in considerable reduction in Atf1-S.c (*S cerevisiae)* specific activity. Despite the considerable decrease in specific activity, high expression levels of Atf1-S.c resulted in increased ethyl acetate production under fermentation. For Atf1-S.c, the amount of ethyl acetate produced in 24-hours of fermentation increased from 1.35 mg/L to 4.78 mg/L and 6.74 mg/L with induction of 1 μM, 10 μM, and 100 μM IPTG, respectively (Zhu et al., 2015). These results suggest that an optimum exists in terms of IPTG induction concentration for ester formation.

A third factor important to the production of acetate esters using the *S. cerevisiae* enzyme Atf1 is oxygen. The *ATF1* gene encodes an alcohol acetyl transferase (AATase), that catalyzes the synthesis of acetate esters from acetyl CoA and several kinds of alcohols. *ATF1* transcription is negatively regulated by unsaturated fatty acids and oxygen (Fujiwara et al., 1999).

1.5 Central Metabolism to Acetyl-CoA

Acetyl-CoA occupies a central position in metabolism, and serves as a precursor of anabolic reactions, as an allosteric regulator of enzymatic activities, and as a key determinant of protein acetylation. The largest flux of acetyl-CoA is entering the tricarboxylic acid (TCA) cycle via a reaction with oxaloacetate mediated by citrate synthase (Hua et al., 2004) (Figure 2). Acetyl-CoA is impermeable to the cell membrane and chemically is an acetyl moiety (CH_3CO)

linked to coenzyme A (CoA), a derivative of vitamin B5 (pantothenate) and cysteine, through a high energy thioester bond (Pietrocola et al., 2015). Because thioester bonds are energy rich, acetyl-CoA facilitates the transfer of the acetyl moiety to a variety of acceptor molecules, including amino groups on proteins. In eukaryotes, such as yeast and humans, acetyl-CoA is generated in the mitochondria, the membrane bound organelle that functions as the energy center of the cell. Here, because acetyl-CoA is membrane impermeable, only after the reaction to form citrate can these atoms be transported across the membrane into the cytosol, where it can be converted back to acetyl-CoA (Martinez-Reyes et al., 2020). In prokaryotes such as *E. coli*, which do not have membrane bound organelles, acetyl-CoA can accumulate directly in the cytosol.

Acetyl-CoA is not only the product of multiple catabolic reactions, but also one of the central substrates for anabolic metabolism. Acetyl-CoA is involved in numerous essential central metabolic pathways such as the TCA cycle, fatty acid biosynthesis, and amino acid biosynthesis. Acetyl-CoA is also a key metabolite precursor for the biosynthesis of lipids, polyketides, isoprenoids, amino acids, and numerous other bioproducts which are used in various industries (Ku et al., 2020).

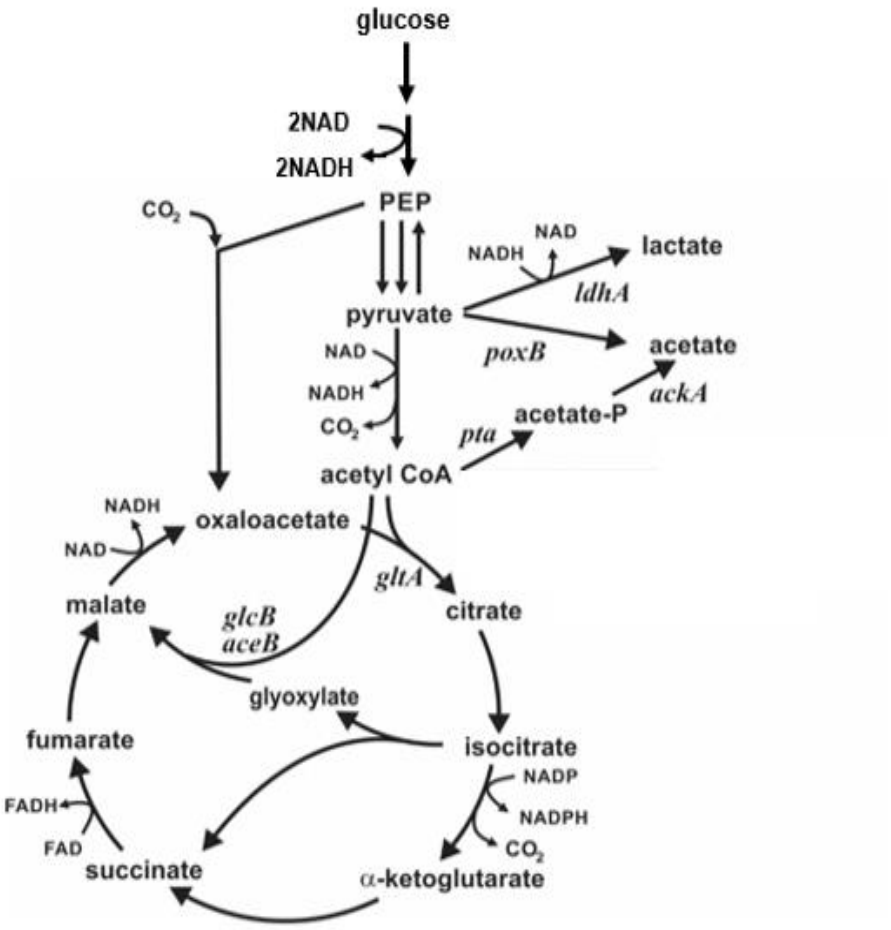

Figure 2: Central carbon metabolism in *E. coli* leading to acetyl-CoA and the TCA cycle.

Pyruvate dehydrogenase (Pdh) is responsible for acetyl-CoA biosynthesis. This enzyme complex is allosterically regulated with pyruvate as an activator, while acetyl-CoA and NADH are inhibitors (Ku et al., 2020). Intracellular flux and concentration of acetyl-CoA are highly regulated to avoid potential metabolic burdens. The intracellular concentration of acetyl-CoA in *E. coli* is 0.05-1.5 nmol/mg cell dry weight, corresponding to 20 – 600 μM (Takamura et al., 1988). The carbon source consumed by cells also affects intracellular acetyl-CoA concentration: acetyl CoA concentration is 0.82, 0.62, and 0.37 nmol/mg cell dry weight for growth on glucose,

glycerol, and succinate, respectively (Ku et al., 2020). The low intracellular concentration of acetyl-CoA presents challenges to produce biochemicals via pathways that utilize enzymes having high K_M values for acetyl-CoA. To overcome the regulation of native acetyl-CoA biosynthesis, numerous metabolic engineering strategies have been applied to increase the intracellular availability of acetyl-CoA.

1.6 Citrate Synthase (gltA)

Disrupting the tricarboxylic acid (TCA) cycle is one of the strategies employed by metabolic engineers to increase acetyl-CoA availability within the cell. The TCA cycle represents a large drain of acetyl-CoA especially during aerobic metabolism (Ku et al., 2020). As noted above, citrate synthase is the key enzyme which mediates the entry of acetyl-CoA into the TCA cycle. During aerobic steady-state growth of *E. coli* on glucose, 62% of the acetyl-CoA flows through citrate synthase (Hua et al., 2004). Thus, disrupting the function of citrate synthase (GltA) presents an opportunity to create a 'bottleneck' and possibly increase intracellular concentration of acetyl-CoA towards products that rely on acetyl-CoA dependent enzymes. A complete citrate synthase knockout has been shown to increase acetyl-CoA availability for citramalate synthesis, which require acetyl-CoA as the direct precursor (Ku et al., 2020). However, a strain with a citrate synthase deletion requires supplementing the medium with glutamate or another TCA cycle intermediate for growth.

An alternative to a citrate synthase deletion is to reduce the expression or the activity of this enzyme. A reduction in citrate synthase activity results in increased formation of acetate, an example compounds derived from acetyl-CoA using glucose as the sole carbon source (Tovilla-Coutiño et al., 2020). In contrast to growth of cells without citrate synthase activity, this strategy

would not require glutamate supplementation. Twenty-eight *E. coli* GltA variants were constructed harboring point mutations with the goal of reducing but not eliminating the activity of citrate synthase. Mutations in citrate synthase at residues W260, A267 and V361 resulted in the greatest acetate yields at approximately 0.24 g/g glucose, nearly five-fold greater than acetate formed by the strain expressing the native citrate synthase (0.05 g/g). These results demonstrated that constricting flux at citrate synthase can leads to increased products derived from acetyl-CoA (Tovilla-Coutiño et al., 2020).

1.7 Metabolic Engineering Approaches to Ester Formation

Sustainable production of bulk chemicals is one of the major challenges in the chemical industry. Short and medium chain esters are used in a wide range of applications, for example fragrance compounds, solvents, lubricants, or biofuels. However, these esters are produced mainly through unsustainable, energy intensive processes. Microbial conversion of biomass derived sugars into esters may provide a sustainable alternative. To achieve ester formation using microorganisms, one strategy is to express yeast AAT. The amount of esters naturally produced by microorganisms is generally too low to be cost competitive, therefore considerable metabolic engineering efforts have been invested in enhancing ester production (Kruis et al., 2019).

The AAT reaction determines the efficiency of the final catalytic step in ester formation. However, the reaction also depends on the supply of the alcohol and acyl-CoA substrates. Metabolites such as phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA lie at the core of acyl-CoA, alcohol, and consequently ester synthesis (Kruis et al., 2019). Since the availability of these metabolites increases the microbial production of esters, most metabolic engineering approaches have focused on improving the supply of alcohols and acyl-CoAs (Table 3). A

common strategy is increasing the availability of basic cellular building blocks, such as acetyl-CoA. Some of the strategies that have been employed include channeling flux away from competing pathways such as lactate and acetate in *E. coli*, increasing cytosolic acetyl-CoA by expressing the Xpk-Pta pathway in *S. cerevisiae*, or increasing the availability of the cofactor CoA by overexpressing the Pdh complex in *E. coli.* The last two of these strategies increase the production of esters in these organisms 1.6 and 6.3-fold respectively (Kruis et al., 2019).

Table 2: Metabolic engineering approaches for increasing ester production. Taken from Kruis et al., 2019 and Lee and Trinh, 2020.

Target ester	**Organism**	**Goal / Strategy**	**AAT catalyst**	**Acyl – CoA supply**	**Alcohol supply**	**Effect / Titer**	**Notes**	**References**
Ethyl acetate	*S. cerevisiae*	Pathway colocalization	Atf1	Ald-Acs targeted to Atf1 (lipid droplet)	Native ethanol	2X increase		Lin et al., 2017
Propyl Acetate	*E. coli*	Acetate ester production	Atf1	Native Acetyl-CoA	2 g/L propanol supplied	802 mg/L	Shake flask	Lee et al., 2019
Isobutyl acetate	*E. coli*	Isobutyl acetate production	Atf1	Native Acetyl-CoA	Overexpression of 2-Keto pathway genes for isobutanol	17 g/L	80% theor. max yield (Batch)	Rodriguez et al., 2014
Isobutyl acetate	*E. coli*	Isobutyl acetate production	Atf1	Native Acetyl-CoA	Overexpression of 2-Keto pathway genes for isobutanol	36 g/L	42 % theor. Max yield (Batch)	Tai et al., 2015
Isoamyl acetate	*E. coli*	Isoamyl acetate production	Atf1	Native Acetyl-CoA	Overexpression of 2-Keto pathway genes for isoamyl alcohol	386 mg/L	11% theor. Max yield (Shakeflask)	Tai et al., 2015
Isoamyl acetate	*E. coli*	Isoamyl acetate production	Atf2	(1) Increased CoA production (2) Combined with Pdh overexpression	Isoamyl alcohol supplementation	(1) 2.3X increase (2) 6.2X increase	Suppl. Of pantothenic acid needed (CoA precursor)	Vadali et al., 2004
Butyl Acetate	*E. coli*	Butyl acetate production	Atf1	Native Acetyl-CoA	Modified *Clostridium* CoA-dependent butanol pathway	22.8 ± 1.8 g/L	Bench top Bioreactor	Ku et al., 2022

2.0 Objectives

This research effort consists of two objectives. The primary objective (Objective 1) is to determine the effect that GltA variants have on production of several acetate esters. The hypothesis is that a reduction in citrate synthase activity will lead to greater ester titer and yield. The *S. cerevisiae* alcohol-O-acetyltransferase Atf1 can condense various alcohols and acetyl-CoA to produce acetate esters (see Figure 3 for pathway in *E. coli*). This enzyme will be expressed (heterologously in *E. coli*) using a plasmid in an *E. coli* strain having knockouts in genes associated with pathways competing for acetyl-CoA including *ldhA*, *poxB*, and *pta-ackA*. The medium will be supplied with glucose as the carbon and energy source and one of several alcohols in excess as the source of alcohol for ester synthesis via Atf1.

In order to meet Objective 1, *E. coli ldhA poxB pta-ackA* will be transformed with pTrc99A-*ATF1* and analyzed for the production of various acetate esters including ethyl acetate, propyl acetate, n-butyl acetate, sec-butyl acetate, and isobutyl acetate. This *E. coli* strain will be compared for ester formation with strains containing the A267T and F383M amino acid substitutions in citrate synthase.

The second objective will focus on the *E. coli* strain observed to have the optimal GltA for the production of esters. In this objective I will employ various engineering techniques to optimize the fermentation of esters. The hypothesis is that slowly adding alcohol in a fed or repeated batch fashion will minimize alcohol toxicity while increasing the titer and yield of the ester.

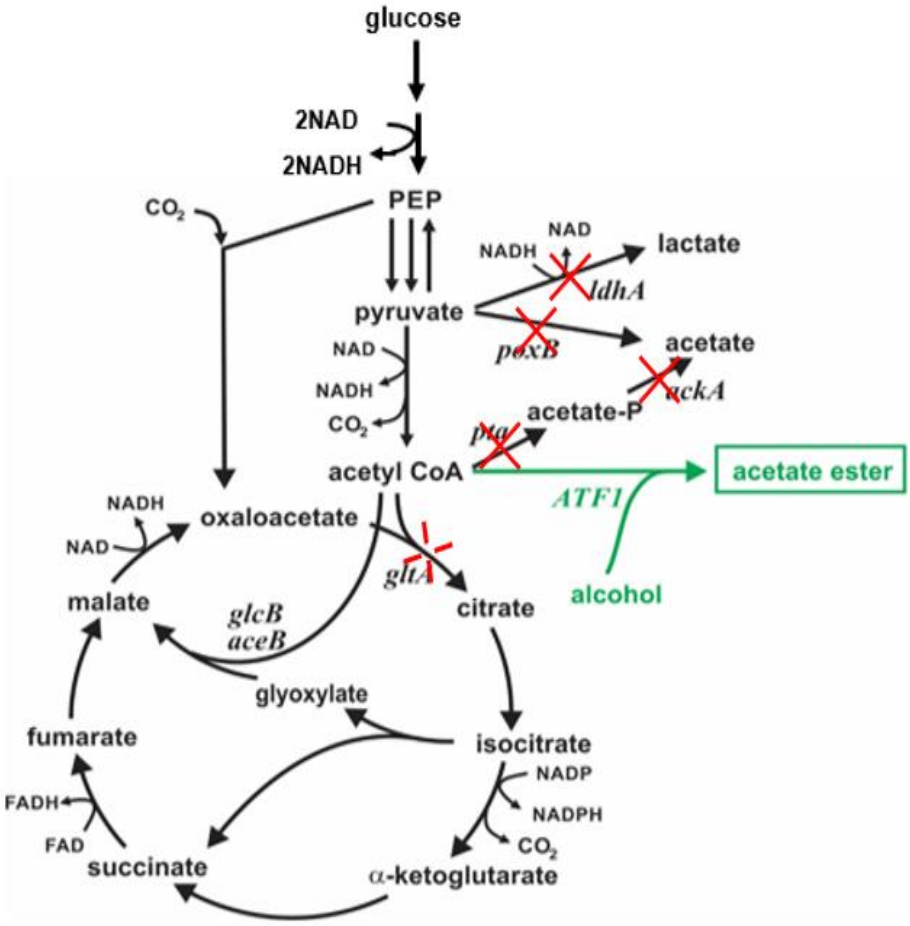

Figure 3*:* Central carbon metabolism in *E. coli* containing knockouts in *ldhA, poxB, pta* and *ackA* genes and expressing heterologous *ATF1* coding alcohol-O-acetyltransferase from *S. cerevisiae*. Strains each contained a point mutation in the *gltA* gene coding citrate synthase.

3.0 Materials and Methods

3.1 Strains

Table 3 is a list of all strains of *E. coli* generated during this research effort. All strains were created from wild-type *E. coli* W by sequentially knocking out genes in the chromosome.

Table 3: Strains generated in this project.

Name	Genotype
	Wild-type *E. coli* W
MEC1352	*E. coli* W Δ*gltA*::Kan
MEC1316	*E. coli* W Δ*ldhA* Δ*poxB*
MEC1364	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA*::Kan
MEC1365	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA*
MEC1380	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA* Δ*gltA*::Kan
MEC1381	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA* Δ*gltA*
MEC1394	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA* Δ*gltA*::*gltA*[A267T]
MEC1410	*E. coli* W Δ*ldhA* Δ*poxB* Δ*pta-ackA* Δ*gltA*::*gltA*[F383M]

3.2 Knockouts

Gene knockouts were performed in *E. coli* W using lambda red recombination. Knockouts were selected using kanamycin supplemented Lysogeny Broth (LB) while the integration of plasmids was selected using ampicillin supplemented LB. Forward and reverse primers external to the target gene were used to confirm chromosomal integration of the kanamycin cassette. If the target gene was of similar size to the kanamycin cassette, one internal

primer within the kanamycin cassette and one primer external to the target gene was used instead to confirm integration. To remove the kanamycin marker, the strain was transformed with pCP20, a plasmid that expresses FLP recombinase (Datsenko and Wanner, 2000). Genetic modifications were verified using PCR.

First, strains containing a single gene knockout were constructed. Then, genomic DNA of a single knockout strain was used as a template for PCR amplification with external primers greater than 100 base pairs upstream and downstream of the kanamycin cassette. Thus, a fragment with greater than 200 base pairs of homology would be created. This fragment was used to perform lambda red recombination and subsequent FLP recombination. This method, by increasing the amount of homology of the desired insert to the target region of the *E. coli* genome, decreased "false positives" and increased transformation efficiency. To create strains with multiple gene knockouts, first a single knockout was made in the *E. coli* W background. This single knockout was used as a "genetic template" which would be used to create multi-knockout strains. Once the single knockout strain was created, gDNA was extracted using Zymo Quick DNA miniprep kit (Genesee Scientific, San Diego, CA) to isolate the genomic material of the single knockout strain. A DNA fragment containing the knockout was created using PCR, with primers further than 100 base pairs upstream and downstream of the kanamycin cassette. This DNA fragment was processed using a Zymo Clean and Concentrate kit (Research Products International, Mount Prospect, IL) to obtain the PCR product for integration into the desired strain. This product was used to transform multiple knockout strain utilizing the same method for lambda recombination as described above.

3.3 Medium Preparation

All shake flask experiments were conducted in 125 mL flasks containing a defined medium (MMB) adapted from Zhu et al. 2008. Specifically, the medium was composed of the following components: 8 g/L glucose, 3.50 g/L NH_4Cl, 0.288 g/L KH_2PO_4, 0.502 g/L $K_2HPO_4 \cdot 3H_2O$, 2 g/L K_2SO_4, 20 mg/L $Na_2(EDTA) \cdot 2H_2O$, 0.45 g/L $MgSO_4 \cdot 7H_2O$, 0.25 mg/L $ZnSO_4 \cdot 7H_2O$, 0.125 mg/L $CuCl_2 \cdot 2H_2O$, 1.25 mg/L $MnSO_4 \cdot H_2O$, 0.875 mg/L $CoCl_2 \cdot 6H_2O$, 0.06 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 5.5 mg/L $FeSO_4 \cdot 7H_2O$, 20 mg/L citric acid, 20 mg/L thiamine HCl, and 10.46 g/L MOPS. In these shake flask experiments, this medium was supplemented with 0, 2, 4, or 6 g/L casamino acids.

All batch experiments were conducted in 2.5 L bioreactors containing a defined medium (MMB) adapted from Zhu et al. 2008. Specifically, the medium was composed of the following components: 20 g/L glucose, 8 g/L NH_4Cl, 1.2 g/L KH_2PO_4, 2 g/L $K_2HPO_4 \cdot 3H_2O$, 2 g/L K_2SO_4, 20 mg/L $Na_2(EDTA) \cdot 2H_2O$, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.25 mg/L $ZnSO_4 \cdot 7H_2O$, 0.125 mg/L $CuCl_2 \cdot 2H_2O$, 1.25 mg/L $MnSO_4 \cdot H_2O$, 0.875 mg/L $CoCl_2 \cdot 6H_2O$, 0.06 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 5.5 mg/L $FeSO_4 \cdot 7H_2O$, 50 mg/L citric acid, 20 mg/L thiamine HCl, and 5.23 g/L MOPS. In this batch experiments, this medium was supplemented with 2 or 6 g/L casamino acids.

3.4 Shake flask Experiments

A single colony from an LB plate was used to inoculate 4 mL of the medium described in the section above, describing how shake flask medium was composed. After 12-16 h (an overnight) of growth, this culture was used to inoculate, this culture was used to inoculate a 125 mL shake flask containing 25 mL of LB medium to and OD of 0.1. After 6-8 hr. of growth this

culture was used to inoculate a 125 mL shake flask containing 25 mL of MMB medium to an OD of 0.1. After 6-8 hours this shake flask reached an OD from 3-5. Culture was taken from this shake flask to inoculate three 125 mL shake flasks containing minimal medium broth with 8 g/L glucose and 5 g/L of any one of the following alcohols: a) ethanol, b) propanol, c) butanol, d) sec-butanol, e) isobutanol. All cultures were grown aerobically on a rotary shaker at 250 rpm. The general flow of the experiments is shown in Figure 4.

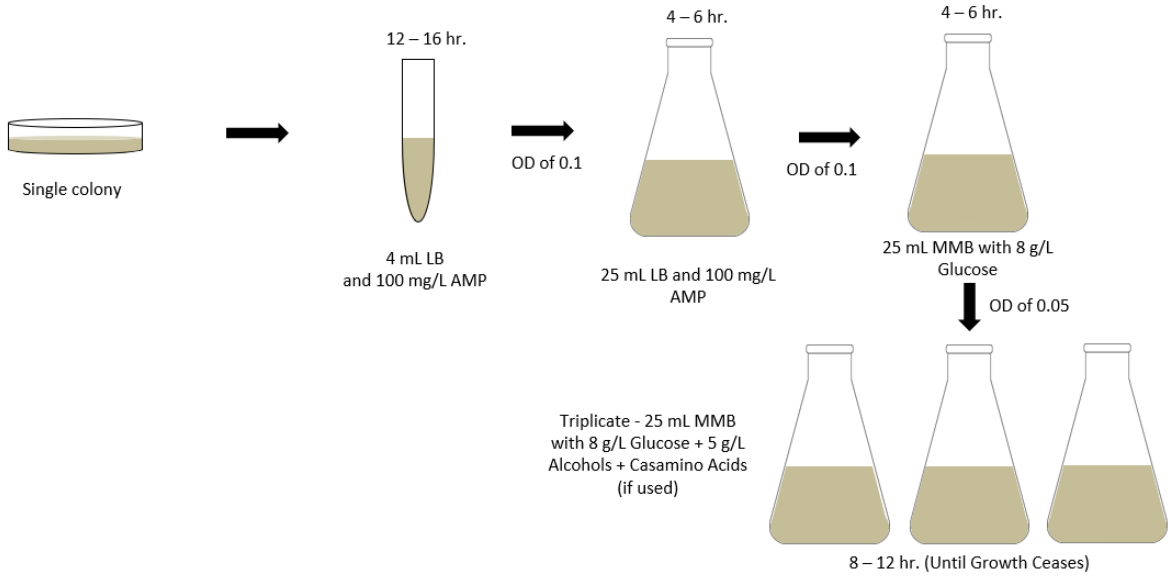

Figure 4: Inoculation train graphic for shake flask experiments.

For the first set of experiments, cells were grown in shake flasks at differing conditions in order to determine the optimal growth conditions for producing esters. To complete this task, strain MEC1365 pTrc99A-*ATF1*, containing the wild-type *gltA* (no point mutations) was grown under various conditions in 125 mL shake flasks containing 25 mL of MMB as growth medium. The conditions varied were temperature (30°C and 37°C) and the presence of casamino acids (2 g/L casamino acids added or no casamino acids added). Alcohols were added to each shake flask in the manner described above.

For the second set of experiments, two GltA variants (MEC1394 and MEC1410) and wild-type GltA strain, each expressing pTrc99A-*ATF1*, were each grown in shake flasks containing initially 5 g/L of either propanol, butanol, or isobutanol at 30°C with 2 g/L casamino acids and 8 g/L glucose.

For a third set of experiments, the effect of casamino acid concentration on propyl acetate production was examined by varying the concentration of this medium component (0, 2, 4, and 6 g/L). These shake flask experiments examined MEC1365 and MEC1410 expressing pTrc99A-*ATF1* using initially 5 g/L propanol at 30°C with 8 g/L glucose.

The fourth set of experiment, the effect of IPTG concentration on propyl acetate production was examined using MEC1365 and MEC1410, each expressing pTrc99A-*ATF1*. The IPTG concentration was varied to be either 5 µM, 50 µM, or 200 µM. These shake flasks contained initially 5 g/L propanol at 30°C with 8 g/L glucose.

A fifth experiment examined the effect of aeration on propyl acetate production using MEC1365 and MEC1410, each expressing pTrc99A-*ATF1*. Two different agitation rates were examined in shake flask culture: 100 rpm and 250 rpm. These shake flasks contained initially 5 g/L propanol at 30°C with 8 g/L glucose and 50 µM IPTG.

During these shake flask experiments, samples were withdrawn at the beginning and the end (approximately 8-12 hours) for measurement of ester production and glucose consumption.

3.5 Batch Processes

A single colony from an LB plate was used to inoculate a test tube containing 4 mL MMB with 20 g/L glucose and 150 mg/L ampicillin, and this culture was grown for 12-16 h. This culture was used to inoculate a 125 mL shake flask containing 25 mL of MMB medium

containing 20 g/L of glucose to an initial OD of 0.1. Once the culture reached an OD of 4-5, the contents of the shake flask was used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1250 mL MMB with 20 g/L glucose and 2 g/L of casamino acids to an initial OD of ~ 0.2. Duplicate batch processes were performed. The general flow of the experiments is shown in Figure 5.

Batch studies were conducted with a constant agitation of 400 rpm and a constant temperature of 30°C. Air and/or pure oxygen-supplemented air was sparged at 1.25 L/min to maintain a dissolved oxygen concentration above 40% of saturation. The pH was controlled at 7.0 using 30% (w/v) KOH. Antifoam 204 (Sigma) was used as necessary to control foaming.

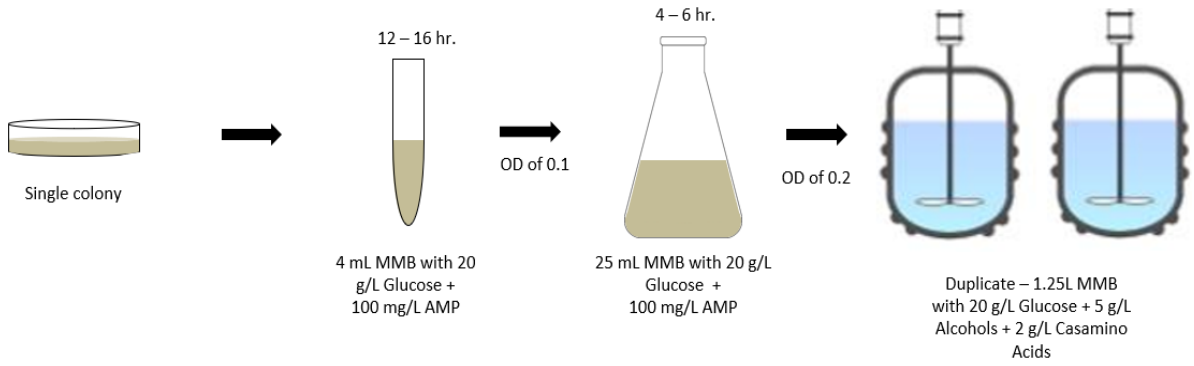

Figure 5: Inoculation train graphic for batch experiments.

3.6 Batch Reactor Off Gas Stripping

Acetate esters are volatile compounds which are easily volatilized from aqueous medium. Our batch experiments involve flowing air and pure oxygen to maintain DO above a set value. By flowing these gases through the fermentation medium containing the esters produced by our strains of *E. coli* there is the potential for loss of ester due to volatilization. To address this, we employed gas stripping. Acetate esters in the batch reactor off-gas were collected by connecting the off-gas line to a 250 mL gas washing bottle. The gas washing bottle was filled with 250 mL

of water and submerged in ice water to create a cold-trap where esters in the off gas were collected. The gas washing apparatus was sampled periodically corresponding to the sampling interval of the batch reactor. The concentration of ester reported in the batch reactor was normalized to consider the additional volume of the 250 mL gas washing bottle.

3.7 Analytical Methods

The optical density at 600 nm (OD) (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA, USA) was used to monitor cell growth. Samples were routinely frozen at -20°C for further analysis, and thawed samples were centrifuged (4°C, 10000 × g for 10 min), and filtered (0.45 μm nylon, Acrodisc, Pall Corporation, Port Washington, NY). High performance liquid chromatograph (HPLC) was used to measure sugars, acetic acid and pyruvic acid using RI detection (Eiteman and Chastain, 1997).

Gas chromatography (GC) was used to quantify acetate esters. A capillary column (Restek, Crossbond 5% diphenyl / 95% dimethyl polysiloxane, L = 30 m, ID = 0.32 mm) was used with nitrogen (20 mL/min). The oven temperature was held at 30°C for 2 minutes, increased to 210°C at 15°C/min, then held at 210°C for 10 minutes. The detector was FID, and the injection volume was 5 μL. GC samples were extracted from the cultures using isopropyl myristate as the extractant at a 1:1 ratio.

3.8 Statistical Analysis

The Student's t-test was applied to the dataset as appropriate, using $p < 0.05$ as the criterion for significance.

4.0 Results

4.1 Ester Production Condition Optimization – Shake Flasks

The overarching goal of this work is to use *E. coli* expressing the *ATF1* allele to produce acetate esters. To facilitate this goal, *E. coli* Δ*ldhA* Δ*poxB* Δ*pta-ackA* (MEC 1365) containing knockouts of the genes coding for lactate dehydrogenase, pyruvate oxidase, phosphotransacetylase and acetate kinase was transformed with the IPTG inducible plasmid pTrc99A-*ATF1*. The purpose of the first experiment was to determine the effects of temperature (30°C and 37°C) and casamino acid supplement (no casamino acids and 2 g/L casamino acids) to the otherwise defined medium. For this experiment we chose the nominal IPTG concentration of 200 μM for induction. MEC1365 *E. coli* cells expressing the *ATF1* allele were inoculated into shake flasks containing MMB with 8 g/L glucose and one of five alcohols at 5 g/L. The triplicate flasks were grown for 10-13 h at which point growth of the cultures ceased. The results are summarized in Figure 6.

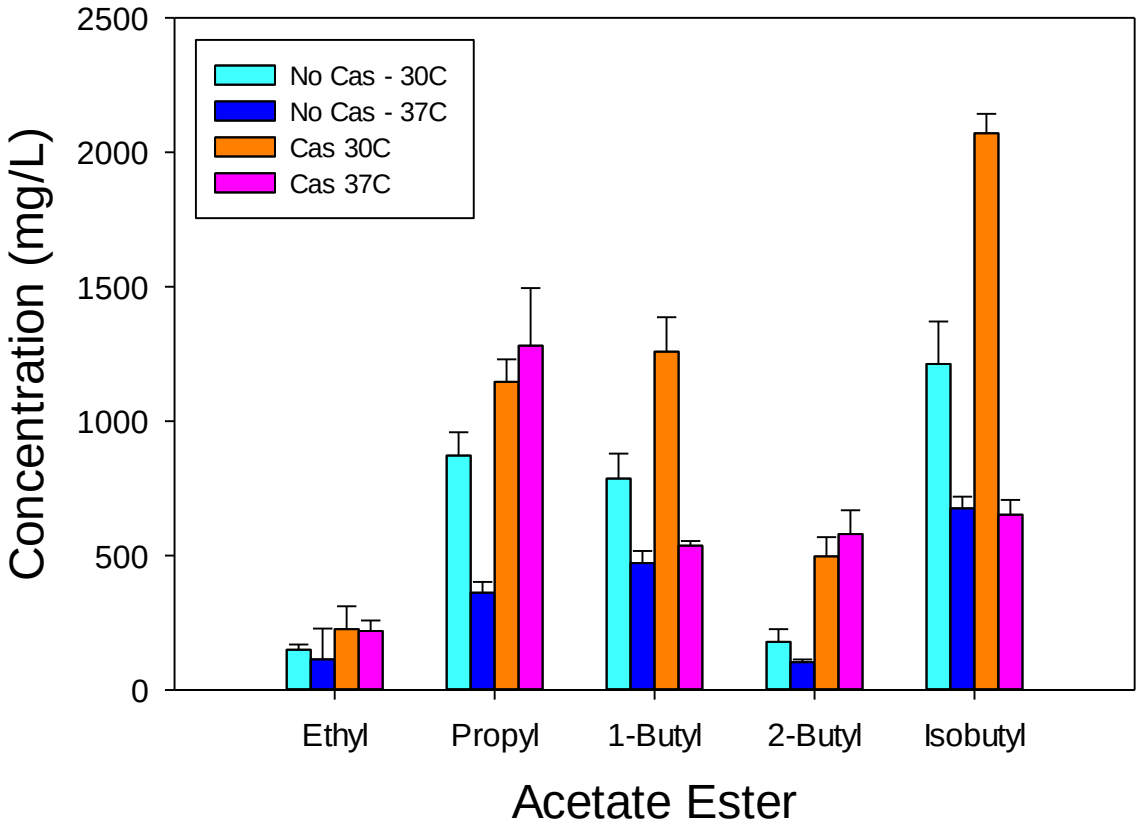

Figure 6: Final acetate ester titer at 30°C or 37°C and with or without 2 g/L added casamino acids.

Across both temperature conditions (30°C and 37°C) and regardless of whether casamino acids were added, the addition of ethanol to the culture medium produced significantly ($p < 0.05$) lower amounts of ethyl acetate than other esters, excluding 2-butyl acetate. No single set of conditions led to the greatest ester titer for all five of the acetate esters (Figure 6). Generally, the presence of casamino acids in the medium increased ester titer at the same conditions of temperature (for ethyl, propyl and 2-butyl, presence of casamino acids in the medium supported greater ester formation at either temperature). 30°C generally favored ester formation,

particularly for cultures without casamino acids. Based on these results, subsequent experiments used 2 g/L casamino acids and were conducted at 30°C.

4.2 GltA Variants – Shake Flasks

The next experiment examined the effect of substitutions in citrate synthase (GltA) on the production of acetate esters. The hypothesis stated that GltA variants would increase the concentration of esters by cells expressing the Atf1 protein, by virtue of increased availability of acetyl-CoA, a co-substrate for Atf1. To test this hypothesis, we selected propyl alcohol, 1-butyl alcohol, and isobutyl alcohol. These three alcohols showed the greatest titers in the experiment examining the effect of temperature and the presence of casamino acids. Shake flask experiments were conducted using *E. coli* Δ*ldhA* Δ*poxB* Δ*pta-ackA* containing the wild-type *gltA* allele, and two variant alleles: A267T (MEC1394) and F383M (MEC1410).

GltA variant strains (MEC1394 and MEC1410) produced a greater titer of acetate ester compared to the wild-type GltA (MEC1365) strain (Figure 7). For the production of propyl acetate, MEC1394 (2100 ± 200 mg/L) and MEC1410 (1900 ± 100 mg/L) accumulated significantly greater ($p<0.05$) propyl acetate compared to the wild-type GltA strain MEC1365 (1100 ± 80 mg/L). There was not a significant difference in the amount of propyl acetate accumulated by the two variant strains MEC1394 (A267T) and MEC1410 (F383M). For the production of 1-butyl acetate, the same trend was apparent. GltA variant strains MEC1394 (2200 ± 300 mg/L) and MEC1410 (2200 ± 200 mg/L) produced significantly greater concentration of butyl acetate ester ($p<0.05$) compared to the wild-type GltA strain MEC1365 (1300 ± 100 mg/L). As observed for propyl acetate production, no significant difference was observed between the two variant strains. For isobutyl acetate, there was no significant

difference between either of the variants and the wild-type GltA stain for the concentration of isobutyl acetate.

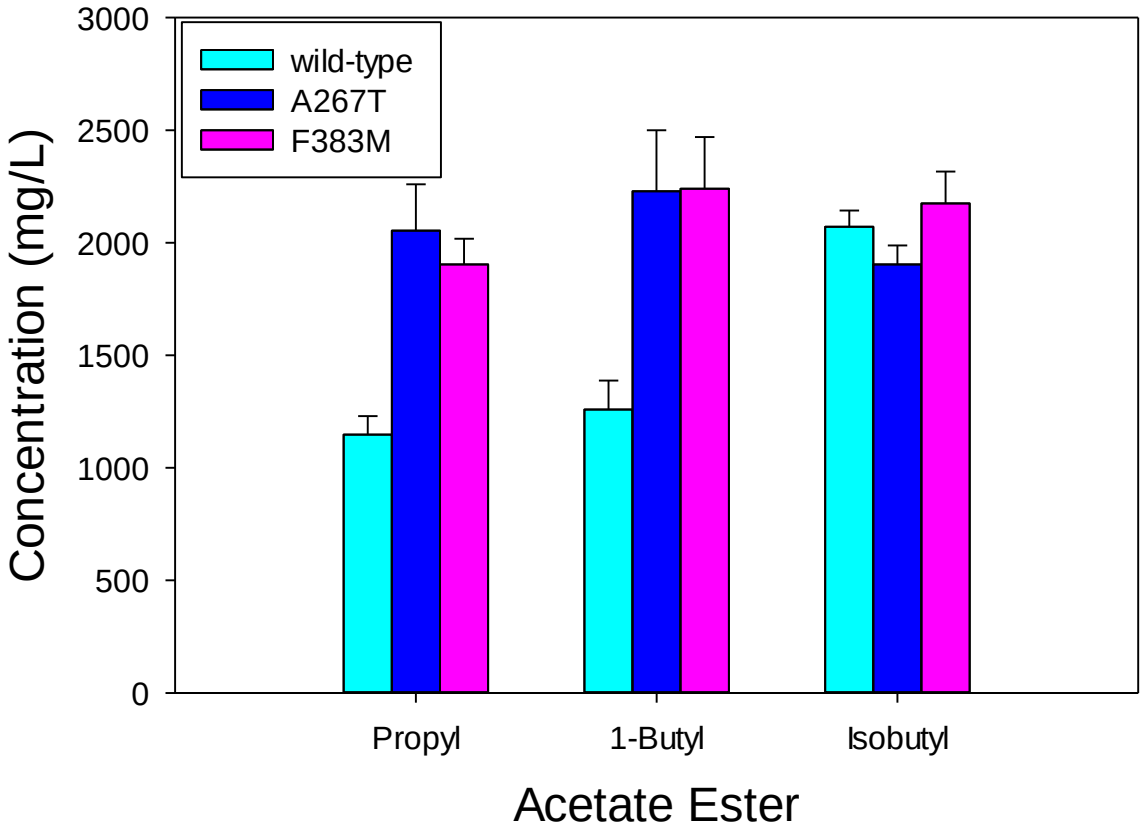

Figure 7: Final acetate ester titer at 30°C and with 2 g/L added casamino acids. Three different GltA variant strains were compared: Wild-type GltA (MEC1365), the A267T substitution (MEC1394) and the F383M substitution (MEC1410). Three alcohols were examined at 5 g/L: propyl alcohol, 1-butyl alcohol, or isobutyl alcohol.

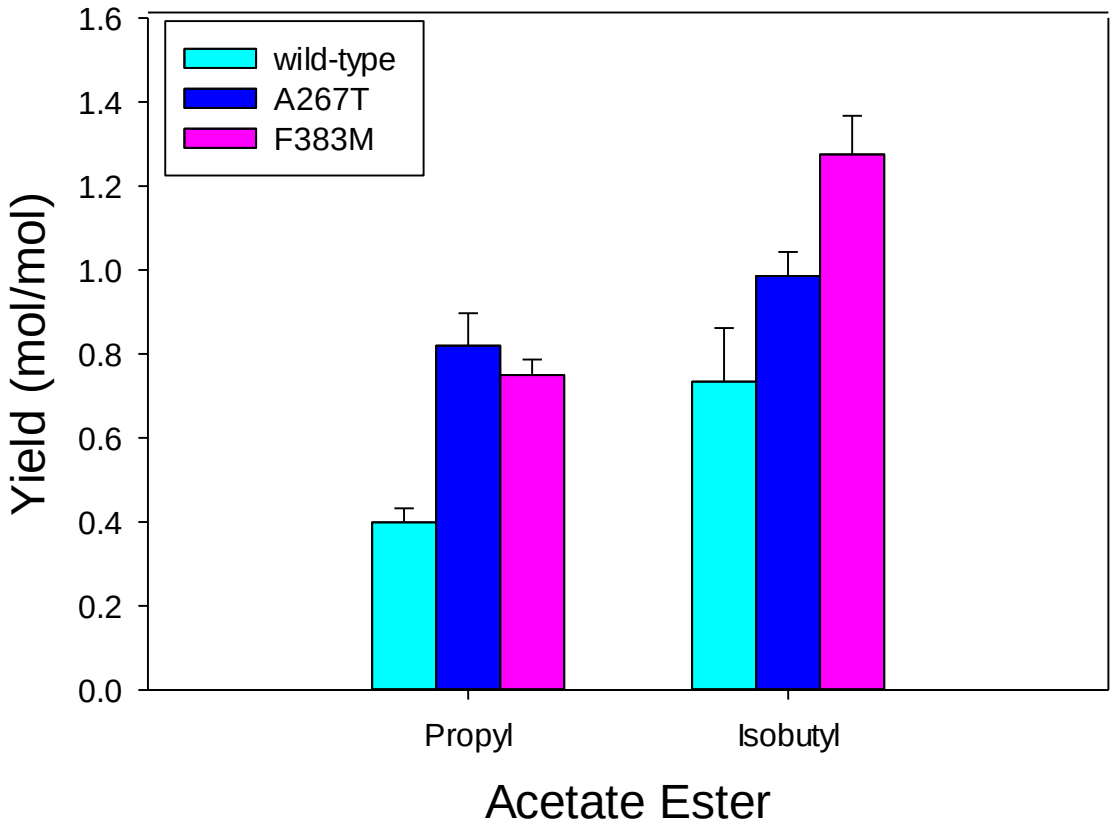

Figure 8: Final acetate ester yield (mol ester formed/mol glucose consumed) at 30°C and with 2 g/L added casamino acids. Three different GltA variant strains were compared: Wild-type GltA (MEC1365), the A267T substitution (MEC1394) and the F383M substitution (MEC1410). Two alcohols were examined at 5 g/L: propyl alcohol or isobutyl alcohol. Molar yield was not calculated for 1-butyl acetate because negligible glucose was consumed.

In addition to calculating the final titer of acetate ester produced by these strains, we also determined the molar yield of acetate ester with respect to glucose (Figure 8). For the formation of propyl acetate, GltA variant strains MEC1394 (0.80 ± 0.10 mol/mol) and MEC1410 (0.75 ±

0.04 mol/mol) had a significantly greater molar yield of propyl acetate ($p<0.05$) compared to the wild-type GltA strain MEC1365 (0.40 ± 0.03 mol/mol). No significant difference existed between the two variant strains. For isobutyl acetate production, MEC1394 (0.99 ± 0.06 mol/mol) and MEC1410 (1.3 ± 0.09 mol/mol) each showed a significantly greater molar yield ($p<0.05$) than the wild-type GltA strain MEC1365 (0.73 ± 0.13 mol/mol). Furthermore, MEC1410 showed a significantly greater molar yield than MEC1394 for isobutyl acetate formation ($p>0.05$). In the conversion of 1-butanol to 1 butyl-acetate cells consumed less than 1 g/L glucose, but were still able to produce ~ 2 g/L 1-butyl acetate, resulting in a molar yield greater than 2.

4.3 Casamino Acids Effects on Propyl Acetate Production

The observation that the molar yield of 1-butyl acetate was greater than 2 mol/mol suggested some of this product was derived from casamino acids. We speculated that the addition of greater initial concentration of casamino acids into the medium would facilitate the generation of more acetate esters. We therefore examined the affect that casamino acids have on the production of propyl acetate.

Triplicate shake flask experiments were again conducted with 8 g/L glucose and using 5 g/L propanol, but several different concentrations of casamino acids were compared: 0 g/L, 2 g/L, 4 g/L, or 6 g/L. These shake flasks were inoculated with either MEC1365 or MEC1410.

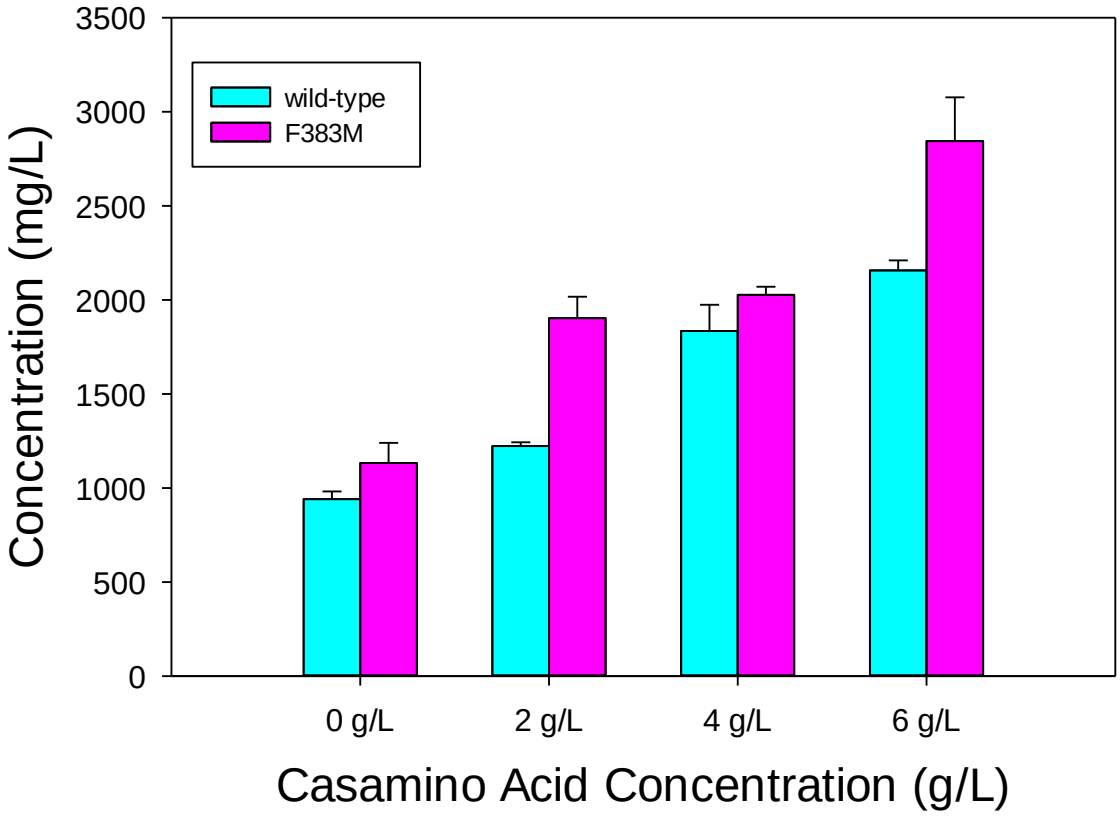

Figure 9: Final acetate ester titer at 30°C and with varying concentrations of (0, 2, 4, and 6 g/L) casamino acids added. Two different GltA strains were compared: Wild-type GltA (MEC1365) and the F383M substitution (MEC1410). Propanol was examined at 5 g/L.

Increasing casamino acid concentration in the medium increased the final titer of propyl acetate (Figure 9). The greatest titer of propyl acetate was produced when 6 g/L casamino acids were added to the medium. At each concentration of casamino acids added to the medium, the variant strain MEC1410 (F383M) generated more propyl acetate than the wild-type GltA strain (MEC1365). Even at the highest concentration of casamino acids added to the medium (6 g/L casamino acids), the GltA variant strain, MEC1410 (2800 ± 200 mg/L) accumulated a

significantly greater ($p<0.05$) concentration of propyl acetate than the wild-type GltA strain, MEC1365 (2200 ± 50 mg/L).

4.4 IPTG and Aeration Effects on Propyl Acetate Production

Prior research highlighted two additional factors that may affect the Atf1 protein catalytic activity. These factors are oxygen (O_2) and the IPTG concentration. The effect of oxygen exposure was examined by comparing two agitation rates, 100 rpm and 250 rpm. The effect of IPTG concentration was examined by comparing three levels: 5 µM, 50 µM, and 200 µM.

In the experiment on the effect of IPTG concentration, triplicate shake flask experiments were conducted using medium with 8 g/L glucose, 6 g/L casamino acids and 5 /L propanol. These shake flasks were inoculated with either MEC1365 or MEC1410, and the results are shown in Figure 10. For the experiment on the effects of agitation, the same process was used, and triplicate shake flasks were induced with 50 µM IPTG, the results of this experiment can be seen in Figure 11.

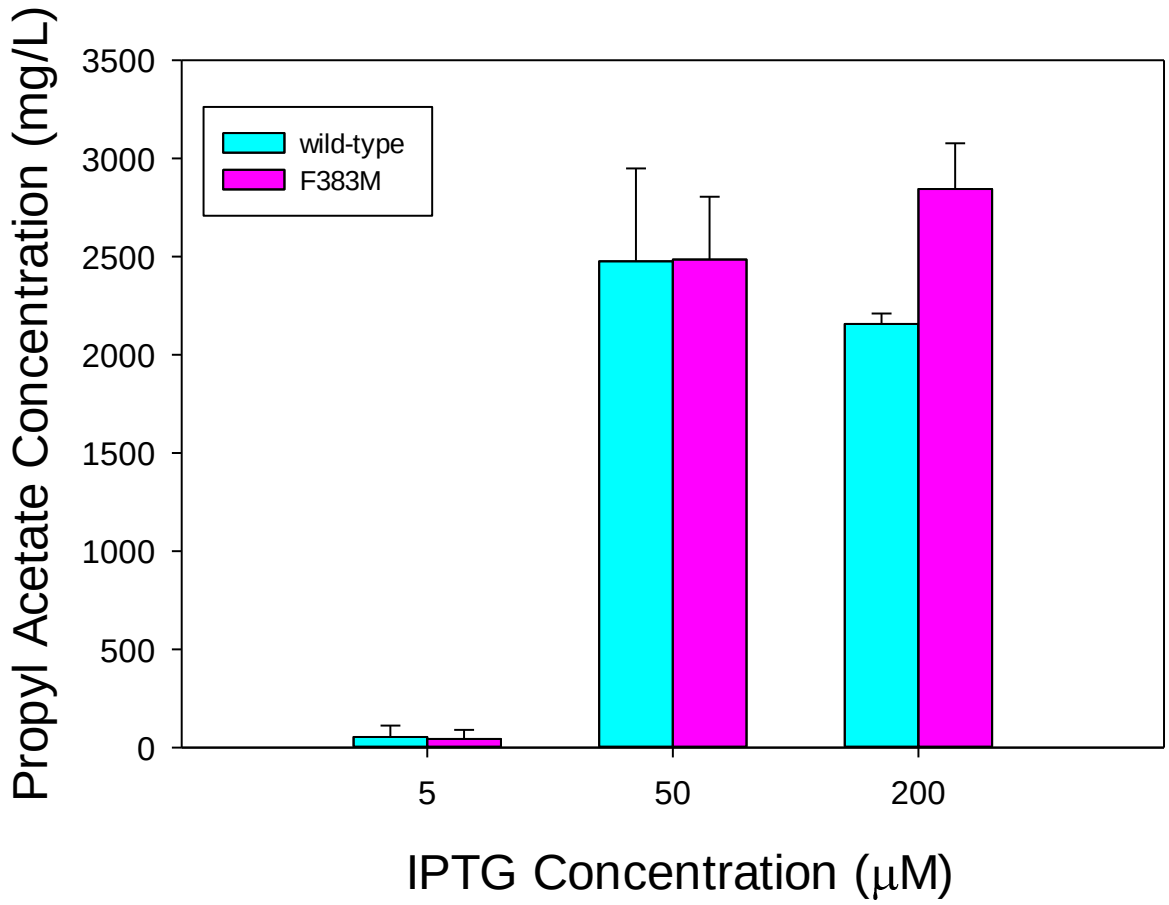

Figure 10: Final acetate ester titer at 30°C and with 6 g/L added casamino acids. Propanol was added initially at 5 g/L. Three levels of IPTG were compared: 5 µM, 50 µM, and 200 µM. Two different GltA variant strains were compared: Wild-type GltA (MEC1365) and the F383M substitution (MEC1410).

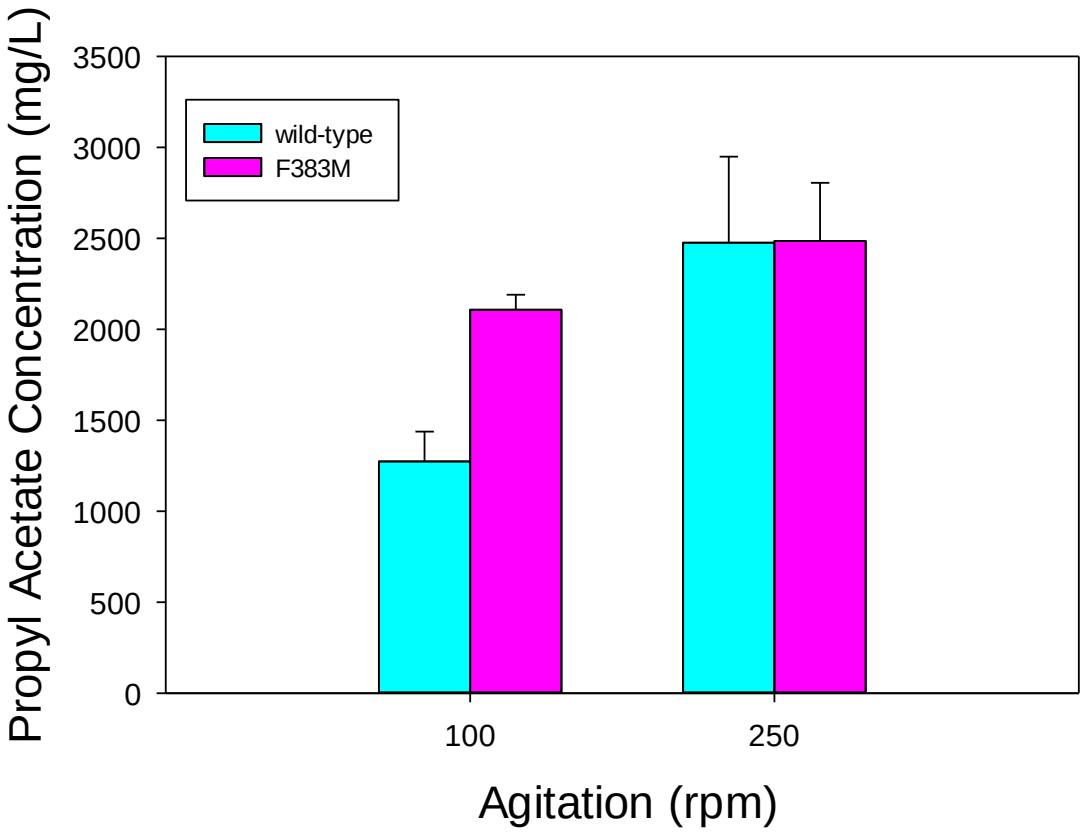

Figure 11: Final acetate ester titer at 30°C and with 6 g/L added casamino acids. Propanol was added initially at 5 g/L, and the IPTG concentration was 50 µM IPTG. Two levels of agitation rate were compared: 100 rpm and 250 rpm. Two different GltA variant strains were compared: Wild-type GltA (MEC1365) and the F383M (MEC1410).

The concentration of IPTG used for induction had an effect on the amount of propyl acetate produced. At 5 µM IPTG, negligible titers of propyl acetate were generated for both MEC1365 and MEC1410. At 50 µM IPTG both strains accumulated more propyl acetate: MEC1365 generated 2500 ± 500 mg/L and MEC1410 generated 2500 ± 300 mg/L, with no significant difference between the two strains. At 200 µM, there was a significant difference in propyl acetate formation between the wild type GltA strain (2200 ± 50 mg/L) and the variant strain (2800 ± 200 mg/L).

The experiment using an agitation rate of 100 rpm showed a significant difference ($p<0.05$) in the accumulation of propyl acetate of the wild type GltA strain MEC1365 (1300 ± 200 mg/L) compared to the variant strain MEC1410 (2100 ± 80 mg/L). At an agitation rate of 250 rpm, no significant difference was observed between the strains. A change in agitation rate affected the strains differently: Increasing the agitation rate (from 100 rpm to 250 rpm) led to a significant increase in titer for the wild-type GltA strain (MEC1365), while increasing the agitation rate had no effect on propyl acetate formation for the variant MEC1410.

4.5 Ester Production Fermentations – Batch

We performed batch experiments using propanol in a controlled bioreactor to optimize ester production. Propanol was chosen because of its limited toxicity in comparison to the other alcohols used in this study.

Two strains were compared in this experiment: MEC1365 (wild type GltA) and MEC1410 (F383M) each expressing pTrc99A-*ATF1*. The batch processes were conducted using MMB containing 20 g/L glucose and 2 g/L casamino acids. All batch experiments were conducted at a volume of 1.25 L, pH was controlled at 7.0 using 30% KOH, and DO was maintained above 45% by flowing 1 vvm (1.25 L/min) air with the intermittent addition of pure oxygen as needed. The temperature was 30°C. 5 g/L propanol and 200 μM IPTG were added two hours after inoculation. Figures 12 and 13 show the results of batch experiments.

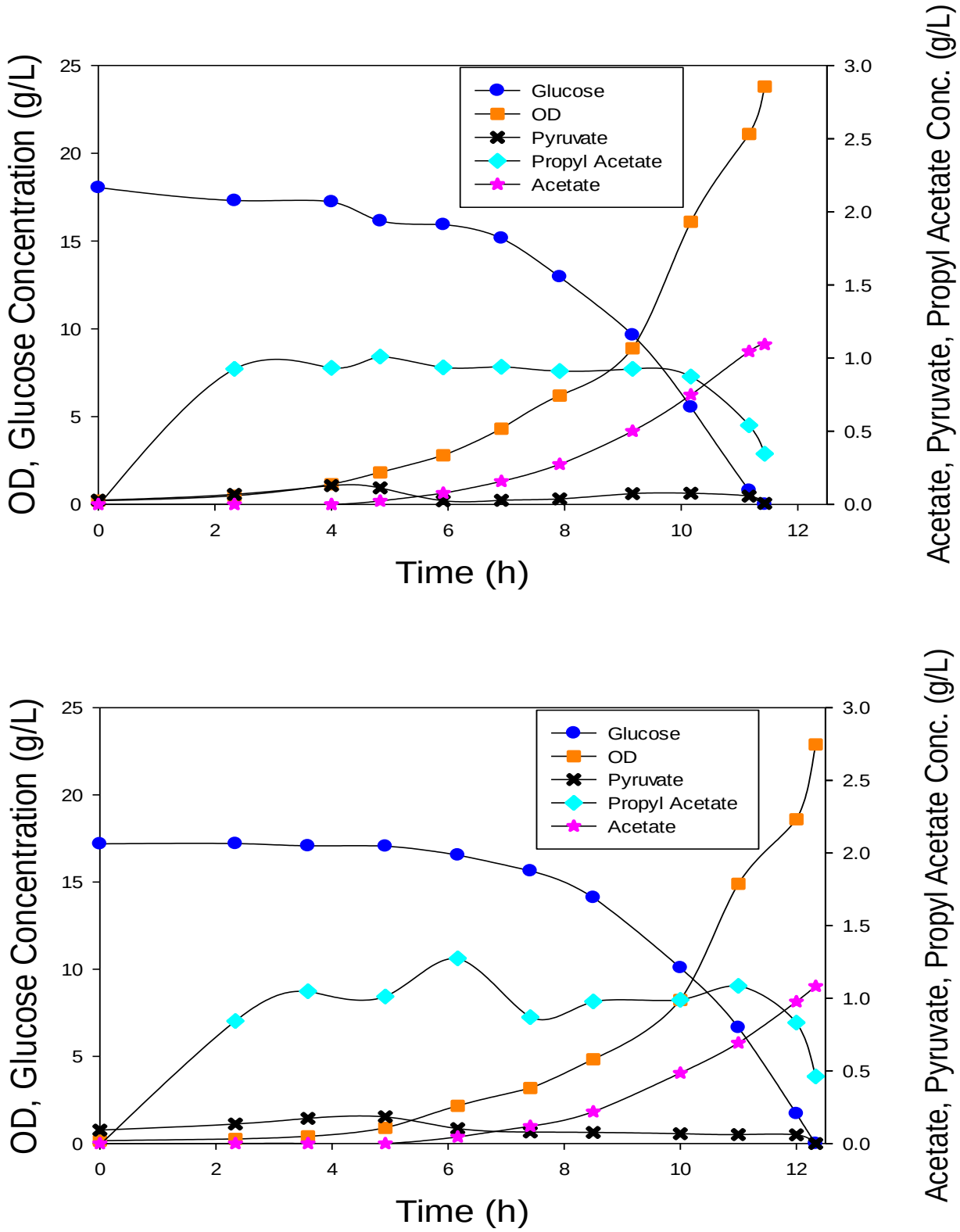

Figure 12: Duplicate batch fermentation results growing MEC1365 (wt GltA) on MMB containing 8 g/L glucose, 2 g/L casamino acids, 5 g/L propanol, and 200 µM IPTG. Alcohol and IPTG were added two hours post inoculation.

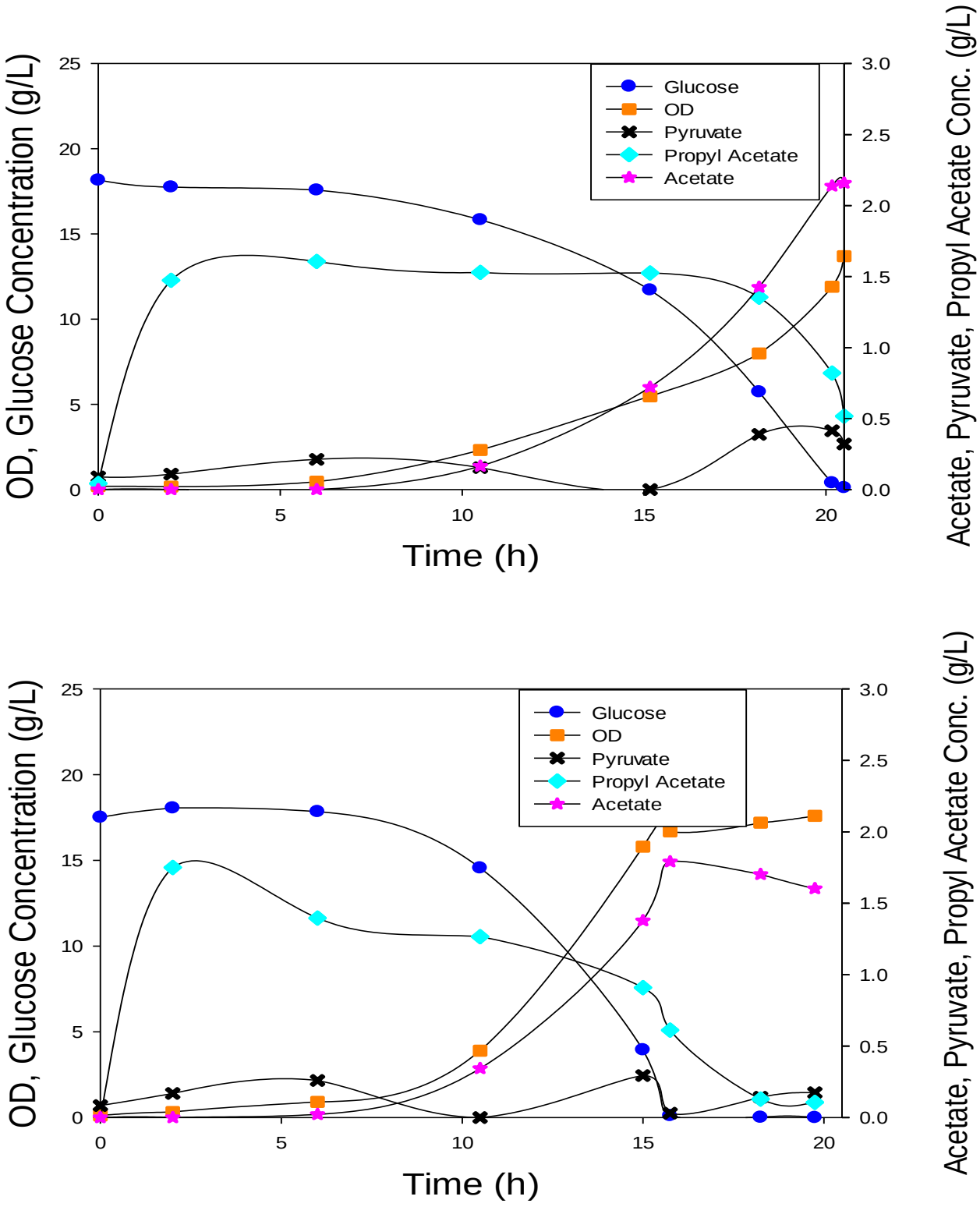

Figure 13: Duplicate batch fermentation growing MEC1410 (F383M) on MMB containing 8 g/L glucose, 2 g/L casamino acids, 5 g/L propanol, and 200 µM IPTG. Alcohol and IPTG were added two hours post inoculation.

5.0 Discussion

Small to medium chain volatile esters have extensive applications in the flavor, fragrance, cosmetic, solvent, paint and coating industries. Achieving high titer and yield is paramount to the commercialization of whole-cell biological product. To accomplish this, careful consideration of pathway construction, enzyme mechanisms, and enzymatic activity are needed (Rodriguez et al., 2014).

By channeling the flux of carbon within the central metabolism of *E. coli* to product formation, an increase in the production of acetate esters was realized. This redirection of flux was achieved by knocking out genes associated with the by-products lactate (*ldhA*) and acetate (*pta-ackA* and *poxB*), and introducing substitutions into citrate synthase, the key entry point to the tricarboxylic acid (TCA) cycle. The primary focus of this research effort was to examine the effect that these citrate synthase substitutions have on the titer, rate, and yield of acetate esters. Several preliminary studies were conducted using the strain with wild-type GltA, MEC1365.

Interpretation of Ester Production Condition Optimization Experiments

Shake flask experiments were conducted at either 30°C or 37°C with or without casamino acids in minimal medium. In this set of experiments, we used our base strain MEC1365 (wild-type GltA) expressing the plasmid pTrC99A-*ATF1* which is induced by IPTG. The addition of ethanol to the culture medium produced lower amounts of ethyl acetate than other esters, excluding 2-butyl acetate. This is consistent with literature that states that Atf1 has lower affinity to ethanol than other longer chain alcohols. Although Atf1 produces ethyl acetate, it does so to a lesser extent than Atf2, another AATase also found in yeast (Rodriguez et al., 2014). Previous research showed that a rather concentrated ethanol solution was necessary to produce ethyl-

acetate. In a set of experiments where *E. coli* expressing *ATF1* was used to generate ethyl, butyl, and isoamyl acetate, researchers found that *E. coli* produces approximately the same amount of ester using 200 mM ethanol (0.14 mM ethyl acetate) as is produced using 15 mM butanol (0.31 mM butyl acetate) or 15 mM isoamyl alcohol (1.40 mM isoamyl acetate) (Horton et al. 2006).

2-butyl acetate also accumulated to a much lower titer than other esters regardless of fermentation conditions. Though this result is similar to that seen with the production of ethyl acetate, we believe the low amount of 2-butyl acetate production to be due to toxicity of 2-butanol. A study analyzing the toxic effects of fermentative metabolites measured the toxicity of various compounds such as alcohols, acids, and esters on *E. coli* growth. This study measured the growth rate and OD of *E. coli* (MG1655) when various concentrations of alcohols, acids, or esters were added to the fermentation medium at concentrations of 0, 2.5, 5.0, 7.5, 10, 12.5 and 15 g/L. The growth rate and OD of *E. coli* grown in medium with each concentration of acid, ester, or alcohol was compared to the growth rate and OD of *E. coli* (MG1655) without the addition of acid, ester, or alcohol. These researchers found butanol to be the most toxic of the alcohols they studied, excluding pentanol (ethanol, propanol, isopropanol, butanol, isobutanol, and pentanol). Butanol was the first alcohol to display strong toxic effects before 10 g/L. At 7.5 g/L, growth rate (0.29 ± 0.03 1/h) and OD (0.50 ± 0.05) were reduced more than 50% as compared to the reference. Growth was entirely inhibited in butanol at 15 g/L (Wilbanks et al. 2017). The addition of propanol or isobutanol to the culture medium resulted in the accumulation of a much greater titer of their respective esters. These observations correspond to other research which found product and substrate toxicity often prevents reaching sufficiently high titers during microbial production of chemicals, especially regarding alcohol, acids, and esters (Wilbanks et al. 2017). The presence of 4 g/L (50 mM) 2-butanol negatively affected the growth rate of *S.*

cerevisiae, *E. coli* and *Bacillus subtilis*, while 16 g/L butanol inhibits growth and continuation of the fermentation in solventogenic Clostridia (Kruis et al., 2019).

Cultures grown in minimal medium at 30°C (without casamino acids) consistently accumulated greater titers of acetate ester than cultures grown at 37°C. In another study on the production of isoamyl acetate, researchers studied three cultivation temperatures: 25°C, 30°C, and 37°C. Under these conditions the greatest ester production was observed at 25°C. At 37°C the lowest yields were observed for moles of isoamyl acetate produced (Singh et al., 2008). These authors speculated as to why lower temperatures (25°C and 30°C) resulted in more efficient ester formation: 1) the Atf1 enzyme is most active at temperatures close to the optimal temperature for *S. cerevisiae* growth (28 °C – 33°C), or 2) Lac operon/IPTG induction or protein expression happens most efficiently at 30 ºC. Our studies are consistent with the previous observation, but do not shed light on why the lower temperature improves ester formation.

The addition of 2 g/L of casamino acids had a positive effect on the amount of ester accumulated by our cells. The highest titers of esters were produced with the addition of 2 g/L of casamino acid to the culture medium. These results coincide with previous studies that show the addition of a nitrogen source increased microbial acetate ester production (Saerens et al., 2008). Of course, addition of casamino acids increased the carbon source, too, so that one explanation is simply that more acetyl CoA is present from which to derive the acetate esters. This explanation is further supported by the observation that 1) further increase in casamino acid increased ester formation, and 2) the yield of 1-butyl acetate was greater than theoretically possible from glucose alone. Casamino acids is acid hydrolyzed casein, and 2 g/L would contain 1.7 mM glutamate and 1.2 mM proline as the amino acids present in greatest concentration. Most of the

other amino acids are present in less amounts, as well as trace amounts of unsaturated fatty acids such as oleic acid (Nolan, 1971).

GltA Variants

Acetyl-CoA is one of many cofactors which are required by some enzymes for full activity. Cofactor manipulation has been utilized as a strategy by metabolic engineers to increase production of industrially useful compounds such as alcohols and esters (aerobic production of isoamyl acetate). Our method of choice to drive the accumulation of acetyl-CoA within the cell is to employ citrate synthase (GltA) variants. Amino acid substitutions of citrate synthase which reduce the activity of this enzyme restrict the flux of carbon into the TCA cycle, resulting in accumulation of acetyl-CoA within the cell (Tovilla-Coutiño et al., 2020). A complete GltA knockout cannot grow on glucose as the sole carbon source without the supply of glutamate or other metabolizable carbon sources in the TCA cycle. In contrast, GltA variants allow enough carbon flux to flow through the TCA cycle for cell growth to occur without supplementation. Another method of modulating the activity of citrate synthase involves employing conditional knockout. Researchers constructed a metabolic toggle switch in *Escherichia coli* as a novel conditional knockout approach and applied it to isopropanol production. The resulting redirection of excess carbon flux caused by interruption of the TCA cycle via switching *gltA* off improved both titer and yield over three-fold (Soma et al., 2014).

Our results show that GltA variant strains containing the A267T (MEC1394) or F383M (MEC1410) substitutions were able to accumulate significantly greater titers of propyl and 1-butyl acetate ($p < 0.05$) than the wild-type GltA strain. Both variant strains also were able to generated propyl acetate and isobutyl acetate at a significantly greater yield ($p < 0.05$) than the

wild type GltA strain (MEC1365). These results support the hypothesis that GltA substitutions facilitate the formation of acetate esters in *E. coli* (as measured by titer and yield) compared to a wild-type GltA strain.

In these experiments the acetyl CoA was invariably the limiting reactant for ester formation. For example, in the case of 8 g/L glucose and 5 g/L propanol in the medium, the concentration of propanol is 83 mM, while the concentration of glucose is 44 mM. Theoretically, if all glucose were consumed and converted to acetyl-CoA, the cells would generate an intracellular concentration of 88 mM acetyl-CoA. In actuality the intracellular flux and concentration of acetyl-CoA are highly regulated to avoid potential metabolic burdens to the cell. Intracellular acetyl-CoA in *E. coli* is 0.05-1.5 nmol/mg cell dry weight, corresponding to 20 – 600 µM (Takamura et al., 1988). Thus, the maximum intracellular concentration of wild-type *E. coli* is 0.60 mM. This low intracellular concentration of acetyl-CoA (0.60 mM) causes acetyl-CoA to be limiting in the Atf1 mediated biochemical reaction that produces esters. This corresponds with our results which showcase that our strains that have point mutations in *gltA* (MEC1394 and MEC1410), by manipulating the intracellular acetyl-CoA pool, can produce greater titers of acetate esters than *E. coli* strains with a wild-type citrate synthase (MEC1365).

Analysis of Factors that Affect Aft1 Function in E. coli

Several factors affect the production of esters using Atf1. An important medium parameter, especially in breweries, is the carbon to nitrogen ratio of the fermentation medium. In the brewing industry, the nitrogen content of wort which can be “consumed” or “assimilated” by the yeast is called free amino acid nitrogen (FAN). One study showed that ethyl and isoamyl acetate concentrations increased when more FAN, in the form of peptone, was available in the

fermentation medium (Saerens et al., 2008). This study investigated the influence of several factors such as nitrogen content, carbon / nitrogen ratio in the medium, temperature, and concentration of unsaturated or medium chain fatty acids on the production of ethyl and isoamyl acetate in *S. cerevisiae* fermentations. In one of their experiments, the medium carbon content was fixed (8 % maltose), while the nitrogen content was varied from 50 to 250 mg/L FAN. Increasing the FAN content resulted in an increase in the production of both ethyl and isoamyl acetate (Saerens et al., 2008).

In this study, increasing the concentration of casamino acids from 2 g/L to 6 g/L resulted in an increase in titer of 49% for MEC1410 and 77% for MEC1365. The formation of acetate esters by Atf1 expressed in *E. coli* also benefits from greater concentrations of nitrogen source in the medium.

A challenge in interpreting these results is that casamino acids constitute both a nitrogen and a carbon source. Thus, we cannot conclusively establish whether the increased ester formation is merely due to an increase in carbon source, due to the added nitrogen, or a consequence of some other factor. Future experiments studying this trend could vary the concentration of a simpler nitrogen source such as ammonium or carbon source to eliminate the confounding effects of complex nitrogen sources such as casamino acids being used as both carbon and nitrogen sources by the cells.

The concentration of IPTG is known to affect the activity of expressed proteins. IPTG concentration affects the expression pattern of the Atf1 protein in cells expressing the *ATF1* gene. For example, a study quantified the expression of the Atf1 in *E. coli* by hybridizing a green fluorescence protein (GFP) tag to the Atf1 protein, then imaging the cells under a microscope (Zhu et al., 2015). The study demonstrated that the Atf1 protein aggregated, and the aggregate

size increased with increasing IPTG concentration. At 1 μM IPTG, intracellular aggregate volumes were 0.11 μm^3, at 10 μM IPTG the volume was 0.19 μm^3, while at 100 μM IPTG the aggregate volume was 0.44 μm^3 (Zhu et al., 2015). The authors also measured the effect of IPTG induction concentration on the activity of Atf1, and found that increased concentration of IPTG reduced the specific activity of the enzyme by 72.6 ± 13.1% for 10 μM IPTG added to the culture medium and 84.2 ± 2.7% for 100 μM IPTG added to the culture medium as compared to the specific activity of Atf1 when 1 μM IPTG was added to the culture medium. Despite the considerable decrease in specific activity, the greater IPTG concentration showed increased ethyl acetate formation. Ethyl acetate accumulation in 24-hours increased from 1.35 mg/L (1 μM) to 4.78 mg/L (10 μM) and 6.74 mg/L (100 μM) (Zhu et al., 2015). Experimentally we did not observe a significant difference in propyl acetate generation in cells induced with 50 μM IPTG or 200 μM IPTG.

Agitation rate did not affect the formation of esters in shake flask fermentation. Previous studies showed that *ATF1* is expressed in yeast under anaerobic conditions, and oxygen or unsaturated fatty acid rapidly represses the transcription of *ATF1* (Fujiwara et al., 1999). Our results using Atf1 expressed in *E. coli* showed that a greater agitation rate (a proxy for oxygenation) resulted in a lower concentration of propyl acetate. This result suggests that in *E. coli*, where Atf1 is heterologously expressed, oxygenation does not similarly repress *ATF1* transcription or activity. The current study was limited, and more sophisticated experimentation could be conducted using controlled oxygenation conditions.

Interpretation of Batch Results

Bioreactors have distinct advantages over shake flasks because the oxygenation, pH, and nutrient supply can be controlled. These factors allow growth to greater ODs which can result in greater titers for growth associated products. The final titer in the batch processes was lower than those from the shake flask experiments. This surprising result led to a study of additional casamino acids, induction concentration of IPTG, and agitation (a proxy for oxygenation). Though the amount of esters produced in the batch experiments were less than what was expected considering the high titer accumulated in shake flask experiments, the trend of the F383M variant (MEC1410) generating more acetate ester than the wild-type GltA strain was maintained.

Results Overview and Summary

The maximum titer of ester generated occurred in a shake flask culture using 6 g/L casamino acids added to the medium, grown at an agitation rate of 250 rpm and 30°C. Under these conditions, a titer of 2800 ± 200 mg/L propyl acetate was obtained using the F383M variant strain (MEC1410). Comparing this titer to the maximum propyl acetate production of 802 mg/L (Lee et al., 2019), MEC1410 produced a 3.5 greater titer in shake flasks than previously obtained by metabolically engineered *E. coli*. The titer of 802 mg/L propyl acetate represents the maximum and only titer referenced in literature where metabolically engineered *E. coli* expressing an AATase enzyme was used to produce propyl acetate. The titer achieved in our experiments (2800 ± 200 mg/L propyl acetate) represents 33% of the theoretical maximum 1-propyl acetate that could be produced if 8 g/L glucose was completely converted to acetyl-CoA.

Appreciable titers (nearly 2 g/L) were also achieved in batch reactors though additional experimentation is needed to optimize that process.

Many opportunities exist for optimizing the batch production of propyl acetate in *E. coli* expressing the Atf1 protein. One avenue to improve the production of acetate esters in *E. coli* is to incorporate the 2-keto acid pathway into the cells to produce alcohols *in vivo* rather than supplementing the medium with alcohols. Some alcohols, such as 1-propanol, isobutanol, isoamyl alcohol, 1-butanol and 2-phenylethanol can be produced from 2-ketoacids derived from the amino acid metabolism (also referred to as the 2-keto pathway). This pathway is the source of higher alcohol synthesis in yeast such as *S. cerevisiae*. The specific 2-ketoacids are first decarboxylated to an aldehyde by a 2-ketoacid decarboxylase (2-KDC) and then reduced to an alcohol by alcohol dehydrogenase. The reactions are analogous to the conversion of pyruvate to ethanol in *E. coli*. Typical approaches for increasing amino acid-derived alcohol production are the disruption of by-product formation, overexpression of 2-ketoacid biosynthetic genes, and the introduction of appropriate 2-KDC and alcohol dehydrogenase (Kruis et al., 2019). This approach enabled the production of 22 g/L isobutanol from glucose at 86% of the maximum yield (Atsumi et al., 2008). Employing this technique, researchers were able to produce 36 g/L of isobutyl acetate in a batch process (Tai et al., 2015).

Additionally, the availability of acetyl-CoA in *E. coli* could also be further manipulated by upregulating the production of the cofactor CoA. Overexpression of pantothenate kinase, which limits CoA synthesis in *E. coli*, resulted in improved production of CoA as well as acetyl-CoA (Kruis et al., 2019).

6.0 References

1. Akita, O., Suzuki, S., Obata, T., Hara, S. (2014). Purification and Some Properties of Alcohol Acetyltransferase from Sake Yeast. *Agric. Biol. Chem.* 54(6), 1485-1490. https://doi.org/10.1080/00021369.1990.10870152
2. Atsumi, S., Hanai, T., Liao, J.C., 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89. https://doi.org/10.1038/nature06450.
3. Datsenko, K. A., Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Nat. Acad. Sci. USA* 97(12), 6640–6645. https://doi.org/10.1073/pnas.120163297
4. Donate, P.M., 2014. Green synthesis from biomass. *Chem. Biol. Technol. Agric.* 1. https://doi.org/10.1186/s40538-014-0004-2.
5. Flamholz, A., Noor, E., Milo, R., Bar-Even, A., 2014. eQuilibrator [WWW Document].
6. Fujiwara, D., Kobayashi, O., Yoshimoto, H., Harashima, S., & Tamai, Y. (1999). Molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae ATF1* gene encoding alcohol acetyltransferase. *Yeast* (Chichester, England), 15(12), 1183–1197. https://doi.org/10.1002/(SICI)1097-0061(19990915)15:12<1183::AID-YEA444>3.0.CO;2-J
7. Catherine Emily Horton, George N. Bennett, Ester production in *E. coli* and *C. acetobutylicum*, *Enzyme and Microbial Technology*, Volume 38, Issue 7, 2006, Pages 937-943, ISSN 0141-0229, https://doi.org/10.1016/j.enzmictec.2005.08.025.
8. Hua, Q., Yang, C., Oshima, T., Mori, H., Shimizu, K. (2004). Analysis of gene expression in *Escherichia coli* in response to changes of growth-limiting nutrient in

chemostat cultures. *Appl. Environ. Microbiol.* 70(4), 2354–2366. https://doi.org/10.1128/AEM.70.4.2354-2366.2004

9. Koffas, M., Lee, K., Roberge, C., Stephanopoulos, G. (1999). Metabolic Engineering. *Annu. Rev. Biomed. Eng.* 1999 1:1, 535-557
10. Kruis, A. J., Bohnenkamp, A. C., Patinios, C., van Nuland, Y. M., Levisson, M., Mars, A. E., van den Berg, C., Kengen, S. W. M., Weusthuis, R. A. (2019). Microbial production of short and medium chain esters: Enzymes, pathways, and applications. *Biotechnol. Adv.* 37(7), 107407. https://doi.org/10.1016/j.biotechadv.2019.06.006
11. Ku, J. T., Chen, A. Y., Lan, E. I. (2020). Metabolic Engineering Design Strategies for Increasing Acetyl-CoA Flux. *Metabolites* 10(4). https://doi.org/10.3390/metabo10040166
12. Ku, J.T., Chen, A.Y. & Lan, E.I. Metabolic engineering of *Escherichia coli* for efficient biosynthesis of butyl acetate. *Microb Cell Fact* 21, 28 (2022). https://doi.org/10.1186/s12934-022-01755-y
13. Lee, JW., Trinh, C.T. Microbial biosynthesis of lactate esters. *Biotechnol Biofuels* 12, 226 (2019). https://doi.org/10.1186/s13068-019-1563-z
14. Jong-Won Lee, Cong T Trinh, Towards renewable flavors, fragrances, and beyond, *Current Opinion in Biotechnology*, Volume 61, 2020, Pages 168-180, ISSN 0958-1669, https://doi.org/10.1016/j.copbio.2019.12.017.
15. Lin, J. L., Wheeldon, I. (2014). Dual N- and C-terminal helices are required for endoplasmic reticulum and lipid droplet association of alcohol acetyltransferases in *Saccharomyces cerevisiae*. *PLoS One*, 9(8), e104141. https://doi.org/10.1371/journal.pone.0104141

16. Lin, J.L., Zhu, J., Wheeldon, I., 2017. Synthetic Protein Scaffolds for Biosynthetic Pathway Colocalization on Lipid Droplet Membranes. *ACS Synth. Biol. 6*, 1534–1544. https://doi.org/10.1021/acssynbio.7b00041.

17. Liu, Y., Lotero, E., Goodwin, J.G., 2006. Effect of water on sulfuric acid catalyzed esterification. *J. Mol. Catal. A Chem.* 245, 132–140. https://doi.org/10.1016/J.MOLCATA.2005.09.049.

18. Jin Luo, Elena Efimova, Pauli Losoi, Ville Santala, Suvi Santala, Wax ester production in nitrogen-rich conditions by metabolically engineered *Acinetobacter baylyi* ADP1, *Metabolic Engineering Communications*, Volume 10, 2020, e00128, ISSN 2214-0301, https://doi.org/10.1016/j.mec.2020.e00128.

19. Malcorps, P. and Dufour, J.-P. (1992), Short-chain and medium-chain aliphatic-ester synthesis in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 210: 1015-1022. https://doi.org/10.1111/j.1432-1033.1992.tb17507.x

20. Martinez-Reyes, I., Chandel, N. S. (2020). Mitochondrial TCA cycle metabolites control physiology and disease. *Nat. Commun.* 11(1), 102. https://doi.org/10.1038/s41467-019-13668-3

21. Mason, A. B., Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* (Chichester, England), 16(14), 1287–1298. https://doi.org/10.1002/1097-0061(200010)16:14<1287::AID-YEA613>3.0.CO;2-I

22. Minetoki, T. (1993). The purification, properties, and internal peptide sequences of alcohol acetyltransferase isolated from *Saccharomyces cerevisiae* Kyokai No. 7. *Biosci. Biotechnol. Biochem.* 7, 2095 – 2099.

23. Nancolas, B., Bull, I. D., Stenner, R., Dufour, V., Curnow, P. (2017). *Saccharomyces cerevisiae* Atf1p is an alcohol acetyltransferase and a thioesterase *in vitro*. *Yeast*, 34(6), 239-251. https://doi.org/10.1002/yea.3229

24. Nolan, R. A. (1971). Amino acids and growth factors in vitamin-free casamino acids. *Mycologia* 63(6), 1231-1234. https://doi.org/10.2307/3757997

25. Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J. M., Madeo, F., Kroemer, G. (2015). Acetyl coenzyme A: a central metabolite and second messenger. *Cell Metabol*. 21(6), 805-821. https://doi.org/10.1016/j.cmet.2015.05.014

26. Ravishankar V. Vadali, George N. Bennett, Ka-Yiu San., 2004. Applicability of CoA/acetyl-CoA manipulation system to enhance isoamyl acetate production in *Escherichia coli*, *Metabolic Engineering*, Volume 6, Issue 4, Pages 294-299. ISSN 1096-7176. https://doi.org/10.1016/j.ymben.2004.02.006.

27. Rodriguez, G.M., Tashiro, Y., Atsumi, S., 2014. Expanding ester biosynthesis in *Escherichia coli*. *Nat Chem Biol* 10, 259–265. https://doi.org/10.1038/nchembio.1476.

28. Saerens, S. M., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and environmental microbiology*, 74(2), 454–461. https://doi.org/10.1128/AEM.01616-07

29. Saerens, S. M., Delvaux, F. R., Verstrepen, K. J., Thevelein, J. M. (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microbial Biotechnol.* 3(2), 165–177. https://doi.org/10.1111/j.1751-7915.2009.00106.x

30. Singh, R., Vadlani, P. V., Harrison, M. L., Bennett, G. N., & San, K. Y. (2008). Aerobic production of isoamyl acetate by overexpression of the yeast alcohol acetyl-transferases

AFT1 and AFT2 in Escherichia coli and using low-cost fermentation ingredients. *Bioprocess and biosystems engineering*, 31(4), 299–306. https://doi.org/10.1007/s00449-007-0159-3

31. Yuki Soma, Keigo Tsuruno, Masaru Wada, Atsushi Yokota, Taizo Hanai, Metabolic flux redirection from a central metabolic pathway toward a synthetic pathway using a metabolic toggle switch, *Metabolic Engineering*, Volume 23, 2014, Pages 175-184, ISSN 1096-7176, https://doi.org/10.1016/j.ymben.2014.02.008.
32. Tai, Y.S., Xiong, M., Zhang, K., 2015. Engineered biosynthesis of medium-chain esters in *Escherichia coli*. *Metab. Eng*. 27. https://doi.org/10.1016/j.ymben.2014.10.004.
33. Takamura, Y., Nomura, G. (1988). Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. *J. Gen. Microbiol.* 134(8), 2249–2253. https://doi.org/10.1099/00221287-134-8-2249
34. Tovilla-Coutiño, D. B., Momany, C., Eiteman, M. A. (2020). Engineered citrate synthase alters acetate accumulation in *Escherichia coli*. *Metabol. Eng.* 61, 171-180. https://doi.org/10.1016/j.ymben.2020.06.006
35. Van Laere, S. D., Saerens, S. M., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., Delvaux, F. R. (2008). Flavour formation in fungi: characterisation of KlAtf, the *Kluyveromyces lactis* orthologue of the *Saccharomyces cerevisiae* alcohol acetyltransferases Atf1 and Atf2. *Appl. Microbiol. Biotechnol.* 78(5), 783–792. https://doi.org/10.1007/s00253-008-1366-9

36. Wilbanks, B., Trinh, C.T. Comprehensive characterization of toxicity of fermentative metabolites on microbial growth. *Biotechnol Biofuels* 10, 262 (2017). https://doi.org/10.1186/s13068-017-0952-4

37. Zhang, S., Guo, F., Yan, W., Dong, W., Zhou, J., Zhang, W., Xin, F., Jiang, M. (2020). Perspectives for the microbial production of ethyl acetate. *Appl. Microbiol. Biotechnol.* 104(17), 7239-7245. https://doi.org/10.1007/s00253-020-10756-z

38. Zhu, J., Lin, J. L., Palomec, L., & Wheeldon, I. (2015). Microbial host selection affects intracellular localization and activity of alcohol-O-acetyltransferase. *Microb. Cell Fact.* 14, 35. https://doi.org/10.1186/s12934-015-0221-9

7.0 Appendices

APPENDIX A-1: SHAKE FLASK DATA (Ester Production Condition Optimization – Selected Portion)

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	1A	0.34	336.7558528	114	114
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	1B	0.13	130.5685619		
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	2A	0.07	70.81382386		
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	2B	0.06	56		
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	3A	0.06	57.7703456		
MEC 1365	WT gltA	37	No Cas	Ethanol	Ethyl Acetate	3B	0.03	33.4671126		
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	1A	0.44	441.6631266	362	40
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	1B	0.35	349.2672048		
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	2A	0.35	349		
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	2B	0.34	339		
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	3A	0.36	358.1173888		
MEC 1365	WT gltA	37	No Cas	Propanol	Propyl Acetate	3B	0.34	336.0627301		
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	1A	0.10	100.9819771	103	10
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	1B	0.11	107.837854		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	2A	0.11	110		
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	2B	0.12	116		
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	3A	0.09	88.46775642		
MEC 1365	WT gltA	37	No Cas	2-Butanol	Sec-Butyl acetate	3B	0.09	94.66499012		
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	1A	0.53	526.7392741	472	45
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	1B	0.45	445.0127877		
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	2A	0.45	447		
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	2B	0.48	476		
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	3A	0.52	522.2563867		
MEC 1365	WT gltA	37	No Cas	Butanol	Butyl acetate	3B	0.42	415.9889652		
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	1A	0.62	618.705258	675	44
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	1B	0.62	623.9817329		
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	2A	0.72	720		
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	2B	0.71	711		
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	3A	0.69	693.686744		
MEC 1365	WT gltA	37	No Cas	isobutanol	isobutyl acetate	3B	0.68	681.6526784		
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	1A	0.15	147.9598662	150	19

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	1B	0.15	148.5172798		
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	2A	0.13	126		
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	2B	0.15	150		
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	3A	0.14	143.3890747		
MEC 1365	WT gltA	30	No Cas	Ethanol	Ethyl Acetate	3B	0.18	184.0245262		
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	1A	0.78	777.4745115	872	86
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	1B	0.97	966.3728406		
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	2A	0.77	770		
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	2B	0.96	963		
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	3A	0.86	860.4184367		
MEC 1365	WT gltA	30	No Cas	Propanol	Propyl Acetate	3B	0.89	893.7305296		
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	1A	0.18	183.5818214	179	47
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	1B	0.26	260.1041854		
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	2A	0.12	124		
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	2B	0.16	161		
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	3A	0.15	147.9252739		
MEC 1365	WT gltA	30	No Cas	2-Butanol	Sec-Butyl acetate	3B	0.19	194.8086941		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	1A	0.75	749.935343	786	93
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	1B	0.80	795.2814736		
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	2A	0.68	680		
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	2B	0.70	701		
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	3A	0.91	914.3940918		
MEC 1365	WT gltA	30	No Cas	Butanol	Butyl acetate	3B	0.87	872.1514986		
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	1A	1.33	1327.851148	1213	158
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	1B	1.20	1200.876327		
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	2A	1.05	1049		
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	2B	1.04	1041		
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	3A	1.21	1207.109356		
MEC 1365	WT gltA	30	No Cas	isobutanol	isobutyl acetate	3B	1.45	1449.179215		
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	1A	0.39	388.7625418	226	85
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	1B	0.22	219.0858417		
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	2A	0.19	187.703456		
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	2B	0.23	232.0178372		
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	3A	0.15	154.8717949		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	Cas	Ethanol	Ethyl Acetate	3B	0.17	172.6532887		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	1A	1.08	1075.265506	1147	83
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	1B	1.23	1228.550694		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	2A	1.07	1074.06188		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	2B	1.20	1199.769895		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	3A	1.07	1068.716369		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	3B	1.24	1238.392099		
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	1A	0.46	457.2181307	497	71
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	1B	0.59	590.6831926		
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	2A	0.47	470.1215496		
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	2B	0.57	572.0316149		
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	3A	0.41	405.8439614		
MEC 1365	WT gltA	30	Cas	2-Butanol	Sec-Butyl acetate	3B	0.48	484.3123166		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	1A	1.20	1203.885169	1259	128
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	1B	1.43	1426.362827		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	2A	1.40	1399.752866		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	2B	1.11	1108.71002		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	3A	1.17	1168.453116		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	3B	1.25	1246.300181		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	1A	2.09	2086.521846	2071	72
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	1B	1.94	1941.835349		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	2A	2.05	2045.328314		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	2B	2.11	2112.749938		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	3A	2.09	2088.712664		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	3B	2.15	2149.345841		
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	1A	0.22	220.6465998	219	39
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	1B	0.27	273.4336678		
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	2A	0.18	177.8372352		
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	2B	0.21	207.0457079		
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	3A	0.18	179.0635452		
MEC 1365	WT gltA	37	Cas	Ethanol	Ethyl Acetate	3B	0.25	253.4782609		
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	1A	1.28	1284.342254	1281	214
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	1B	1.60	1603.975503		
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	2A	1.01	1014.482441		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	2B	1.24	1240.586944		
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	3A	1.11	1110.383036		
MEC 1365	WT gltA	37	Cas	Propanol	Propyl Acetate	3B	1.43	1434.193571		
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	1A	0.50	504.8200707	580	88
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	1B	0.65	653.5536794		
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	2A	0.52	521.7651638		
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	2B	0.68	683.0728699		
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	3A	0.48	478.9533561		
MEC 1365	WT gltA	37	Cas	2-Butanol	Sec-Butyl acetate	3B	0.64	639.4227891		
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	1A	0.55	548.6077186	537	17
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	1B	0.55	552.8319779		
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	2A	0.55	550.3606425		
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	2B	0.54	537.4579729		
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	3A	0.51	510.6468577		
MEC 1365	WT gltA	37	Cas	Butanol	Butyl acetate	3B	0.52	522.6299606		
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	1A	0.63	634.6889657	652	55
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	1B	0.61	609.5099975		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	2A	0.60	602.6289805		
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	2B	0.62	623.9200197		
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	3A	0.72	716.829178		
MEC 1365	WT gltA	37	Cas	Isobutanol	Isobutyl acetate	3B	0.73	725.1913108		

*See supplemental excel documents for additional data

APPENDIX A-2: SHAKE FLASK DATA (GltA variants – Selected Portion)

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	1A	1.08	1075.265506	1147	83
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	1B	1.23	1228.550694		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	2A	1.07	1074.06188		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	2B	1.20	1199.769895		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	3A	1.07	1068.716369		
MEC 1365	WT gltA	30	Cas	Propanol	Propyl Acetate	3B	1.24	1238.392099		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	1A	1.20	1203.885169	1259	128
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	1B	1.43	1426.362827		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	2A	1.40	1399.752866		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	2B	1.11	1108.71002		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	3A	1.17	1168.453116		
MEC 1365	WT gltA	30	Cas	Butanol	Butyl acetate	3B	1.25	1246.300181		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	1A	2.09	2086.521846	2071	72
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	1B	1.94	1941.835349		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	2A	2.05	2045.328314		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	2B	2.11	2112.749938		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	3A	2.09	2088.712664		
MEC 1365	WT gltA	30	Cas	Isobutanol	Isobutyl acetate	3B	2.15	2149.345841		
MEC 1394	gltA::gltA[267T]	30	Cas	Propanol	Propyl Acetate	1A	2.11	2108.081988	2054	206
MEC 1394	gltA::gltA[267T]	30	Cas	Propanol	Propyl Acetate	2A	2.23	2226.568253		
MEC 1394	gltA::gltA[267T]	30	Cas	Propanol	Propyl Acetate	3A	1.83	1825.867318		
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	1A	1.84	1839.794247	2229	271
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	1B	2.58	2582.43053		
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	2A	2.35	2346.303055		
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	2B	2.17	2166.64272		
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	3A	2.21	2208.425529		
MEC 1394	gltA::gltA[267T]	30	Cas	Butanol	Butyl acetate	3B	2.24	2242.765597		
MEC 1394	gltA::gltA[267T]	30	Cas	Isobutanol	Isobutyl acetate	1A	1.85	1850.129598	1904	84
MEC 1394	gltA::gltA[267T]	30	Cas	Isobutanol	Isobutyl acetate	2A	1.86	1860.960257		
MEC 1394	gltA::gltA[267T]	30	Cas	Isobutanol	Isobutyl acetate	3A	2.00	2000.246853		

Strain	Genotype	Temperature (°C)	Casamino Acids	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1410	gltA::gltA[F383M]	30	Cas	Propanol	Propyl Acetate	1A	1.79	1792.944633	1904	114
MEC 1410	gltA::gltA[F383M]	30	Cas	Propanol	Propyl Acetate	2A	2.02	2020.67757		
MEC 1410	gltA::gltA[F383M]	30	Cas	Propanol	Propyl Acetate	3A	1.90	1897.943217		
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	1A	2.24	2239.777005	2240	230
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	1B	2.44	2438.029828		
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	2A	1.85	1847.093307		
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	2B	2.33	2333.084284		
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	3A	2.34	2340.728182		
MEC 1410	gltA::gltA[F383M]	30	Cas	Butanol	Butyl acetate	3B	2.49	2486.508233		
MEC 1410	gltA::gltA[F383M]	30	Cas	Isobutanol	Isobutyl acetate	1A	2.05	2054.647001	2175	141
MEC 1410	gltA::gltA[F383M]	30	Cas	Isobutanol	Isobutyl acetate	2A	2.14	2139.502592		
MEC 1410	gltA::gltA[F383M]	30	Cas	Isobutanol	Isobutyl acetate	3A	2.33	2330.53567		

*See supplemental excel documents for additional data

APPENDIX A-3: SHAKE FLASK DATA (Casamino Acids Effects on Propyl Acetate Production)

Strain	Genotype	Temperature (°C)	Casamino Acids (g/L)	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	30	0	Propanol	Propyl Acetate	1	0.97	966.3728406	941	41
MEC 1365	WT gltA	30	0	Propanol	Propyl Acetate	2	0.96	962.691164		
MEC 1365	WT gltA	30	0	Propanol	Propyl Acetate	3	0.89	894		
MEC 1365	WT gltA	30	2	Propanol	Propyl Acetate	1	1.23	1228.550694	1222	20
MEC 1365	WT gltA	30	2	Propanol	Propyl Acetate	2	1.20	1199.769895		
MEC 1365	WT gltA	30	2	Propanol	Propyl Acetate	3	1.24	1238		
MEC 1365	WT gltA	30	4	Propanol	Propyl Acetate	1	1.68	1678.635656	1835	140
MEC 1365	WT gltA	30	4	Propanol	Propyl Acetate	2	1.95	1947.008638		
MEC 1365	WT gltA	30	4	Propanol	Propyl Acetate	3	1.88	1880		
MEC 1365	WT gltA	30	6	Propanol	Propyl Acetate	1	2.12	2116.613566	2158	53
MEC 1365	WT gltA	30	6	Propanol	Propyl Acetate	2	2.14	2139.305438		
MEC 1365	WT gltA	30	6	Propanol	Propyl Acetate	3	2.22	2218		
MEC 1410	gltA::gltA[F383M]	30	0	Propanol	Propyl Acetate	1	1.01	1012.110592	1132	107

Strain	Genotype	Temperature (°C)	Casamino Acids (g/L)	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1410	gltA::gltA[F383M]	30	0	Propanol	Propyl Acetate	2	1.17	1166.599405		
MEC 1410	gltA::gltA[F383M]	30	0	Propanol	Propyl Acetate	3	1.22	1218		
MEC 1410	gltA::gltA[F383M]	30	2	Propanol	Propyl Acetate	1	1.79	1792.944633	1904	114
MEC 1410	gltA::gltA[F383M]	30	2	Propanol	Propyl Acetate	2	2.02	2020.67757		
MEC 1410	gltA::gltA[F383M]	30	2	Propanol	Propyl Acetate	3	1.90	1898		
MEC 1410	gltA::gltA[F383M]	30	4	Propanol	Propyl Acetate	1	2.04	2039.864769	2028	42
MEC 1410	gltA::gltA[F383M]	30	4	Propanol	Propyl Acetate	2	2.06	2062.804446		
MEC 1410	gltA::gltA[F383M]	30	4	Propanol	Propyl Acetate	3	1.98	1981		
MEC 1410	gltA::gltA[F383M]	30	6	Propanol	Propyl Acetate	1	2.99	2992.498584	2845	232
MEC 1410	gltA::gltA[F383M]	30	6	Propanol	Propyl Acetate	2	2.96	2964.956811		
MEC 1410	gltA::gltA[F383M]	30	6	Propanol	Propyl Acetate	3	2.58	2577		

APPENDIX A-4: SHAKE FLASK DATA (IPTG and Aeration Effects on Propyl Acetate Production)

Strain	Genotype	Agitation (rpm)	IPTG (µM)	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1365	WT gltA	250	200	Propanol	Propyl Acetate	1	2.12	2116.6	2158	53
MEC 1365	WT gltA	250	200	Propanol	Propyl Acetate	2	2.14	2139.3		
MEC 1365	WT gltA	250	200	Propanol	Propyl Acetate	3	2.22	2217.7		
MEC 1365	WT gltA	250	50	Propanol	Propyl Acetate	1	3.00	3002.4	2476	473
MEC 1365	WT gltA	250	50	Propanol	Propyl Acetate	2	2.34	2339.5		
MEC 1365	WT gltA	250	50	Propanol	Propyl Acetate	3	2.09	2086.3		
MEC 1365	WT gltA	250	5	Propanol	Propyl Acetate	1	-0.01	-7.4	53	58
MEC 1365	WT gltA	250	5	Propanol	Propyl Acetate	2	0.06	59.7		
MEC 1365	WT gltA	250	5	Propanol	Propyl Acetate	3	0.11	107.9		
MEC 1365	WT gltA	100	50	Propanol	Propyl Acetate	1	1.19	1190.5	1274	164
MEC 1365	WT gltA	100	50	Propanol	Propyl Acetate	2	1.46	1463.4		
MEC 1365	WT gltA	100	50	Propanol	Propyl Acetate	3	1.17	1168.7		
MEC 1410	gltA::gltA[F383M]	250	200	Propanol	Propyl Acetate	1	2.99	2992.5	2845	232
MEC 1410	gltA::gltA[F383M]	250	200	Propanol	Propyl Acetate	2	2.96	2965.0		
MEC 1410	gltA::gltA[F383M]	250	200	Propanol	Propyl Acetate	3	2.58	2576.9		

Strain	Genotype	Agitation (rpm)	IPTG (µM)	Alcohol	Ester	Triplicate	Concentration (g/L)	Concentration (mg/L)	Mean (mg/L)	Standard Dev (mg/L)
MEC 1410	gltA::gltA[F383M]	250	50	Propanol	Propyl Acetate	1	2.74	2743.1	2485	320
MEC 1410	gltA::gltA[F383M]	250	50	Propanol	Propyl Acetate	2	2.13	2127.5		
MEC 1410	gltA::gltA[F383M]	250	50	Propanol	Propyl Acetate	3	2.59	2585.3		
MEC 1410	gltA::gltA[F383M]	250	5	Propanol	Propyl Acetate	1	0.08	83.1	44	46
MEC 1410	gltA::gltA[F383M]	250	5	Propanol	Propyl Acetate	2	-0.01	-6.5		
MEC 1410	gltA::gltA[F383M]	250	5	Propanol	Propyl Acetate	3	0.06	55.4		
MEC 1410	gltA::gltA[F383M]	100	50	Propanol	Propyl Acetate	1	2.17	2168.8	2108	82
MEC 1410	gltA::gltA[F383M]	100	50	Propanol	Propyl Acetate	2	2.14	2141.3		
MEC 1410	gltA::gltA[F383M]	100	50	Propanol	Propyl Acetate	3	2.01	2014.8		

APPENDIX B-1: BATCH DATA (MEC1365 (1) – Propyl Acetate)

Batch Run Data											
Sample	Total Time (h)	Propanol Time (h)	OD	Glucose (g/L)	Glucose Error	Acetate (g/L)	Acetate Error	Pyruvate (g/L)	Pyruvate Error	peak area (area)	Propyl Acetate (g/L)
I	0.00		0.21	18.05	0.13	0.00	0.00	0.03	0.00	25	-0.01
0	2.33	0.00	0.49	17.31	0.17	0.00	0.00	0.07	0.00	26412	0.93
1	4.00	1.67	1.15	17.24	0.05	0.00	0.00	0.13	0.00	26623	0.93
2	4.83	2.50	1.82	16.15	0.63	0.02	0.01	0.11	0.00	28784	1.01
3	5.92	3.59	2.80	15.94	0.11	0.08	0.00	0.02	0.00	26700	0.94
4	6.92	4.59	4.31	15.17	0.13	0.16	0.00	0.03	0.00	26817	0.94
5	7.92	5.59	6.20	12.97	0.30	0.27	0.00	0.04	0.00	25994	0.91
6	9.17	6.84	8.89	9.65	0.09	0.50	0.00	0.07	0.00	26411	0.93
7	10.17	7.84	16.10	5.54	0.04	0.75	0.00	0.08	0.00	24951	0.87
8	11.17	8.84	21.10	0.80	0.00	1.05	0.00	0.06	0.00	15533	0.54
9	11.43	9.10	23.8	0.01	0.00	1.09	0.02	0.01	0.01	10031	0.35

Batch Run Data
*Run Notes
Sample = I (Directly after innoculation)
Sample = 0 (t = 0, IPTG [200mM - 0.06g] and Propanol [5 g/L - 7.783 mL] added)
Sample = 1
Sample = 2
Sample = 3 (Started O_2 flow - 0.2 L/ min - Final DO after addition - 81.4)
Sample = 4 (*Increased O_2 flow @ 1825 - 0.3 L/min - Final DO after addition - 85.7)
Sample = 5 (*Increased O_2 flow @ 1905 - 0.4 L/min and @ 1952 - 0.6, Final DO - 79.3)
Sample = 6 (*@ 2012 DO shot up to 116 during sample, having to frequently adjust up/down)
Sample = 7
Sample = 8 (*Glucose seems to be depleted @ 2215, decreased O_2 to stabilize, DO - 94.0)
Sample = 9 (*Decreased O_2 to 0 L/min - maintained at 90 DO)

APPENDIX B-2: BATCH DATA (MEC1365 (2) – Propyl Acetate)

Batch Run Data											
Sample	Total Time (h)	Propanol Time (h)	OD	Glucose (g/L)	Glucose Error	Acetate (g/L)	Acetate Error	Pyruvate (g/L)	Pyruvate Error	peak area (area)	Corrected reactor conc. (g/L)
I	0.00		0.16	17.20	0.19	0.00	0.00	0.09	0.00	0	-0.01
0	2.33	0.00	0.26	17.21	0.04	0.00	0.00	0.13	0.00	24039.00	0.84
1	3.58	1.25	0.41	17.08	0.12	0.00	0.00	0.17	0.00	29832.00	1.05
2	4.92	2.59	0.90	17.06	0.08	0.00	0.00	0.18	0.00	28801.00	1.01
3	6.17	3.84	2.16	16.56	0.00	0.05	0.01	0.10	0.00	36256.00	1.28
4	7.42	5.09	3.18	15.64	0.13	0.12	0.00	0.08	0.00	24788.00	0.87
5	8.50	6.17	4.83	14.12	0.09	0.22	0.00	0.08	0.00	27877.00	0.98
6	10.00	7.67	8.23	10.09	0.03	0.48	0.00	0.07	0.00	27235.00	0.99
7	11.00	8.67	14.90	6.66	0.10	0.69	0.02	0.06	0.00	29811.00	1.08
8	12.00	9.67	18.60	1.71	0.01	0.98	0.00	0.06	0.00	22733.00	0.83
9	12.33	10.00	22.9	0.00	0.00	1.08	0.02	0.00	0.00	12140	0.46

Batch Run Data
*Run Notes
Sample = I (Directly after inoculation)
Sample = 0 (t = 0, IPTG [200mM - 0.06g] and Propanol [5 g/L - 7.783 mL] added)
Sample = 1
Sample = 2
Sample = 3 (*Started O_2 flow - 0.2 L/min @ 1842 - Final DO after addition - 90.2)
Sample = 4
Sample = 5 (*Increased O_2 flow - 0.4L/min @ 2043 - Final DO - 98.2)
Sample = 6 (*Increased O2 flow - 0.5L/min @ 2206 - Final DO - 86)
Sample = 7
Sample = 8 (*Increased O2 flow - 0.8L/min @ 2325 - Final DO - 67)
Sample = 9 (*Decreased O_2 to 0 L/min - maintained at 90 DO)

APPENDIX B-3: BATCH DATA (MEC1410 (1) – Propyl Acetate)

Batch Run Data											
Sample	Total Time (h)	Propanol Time (h)	OD	Glucose (g/L)	Glucose Error	Acetate (g/L)	Acetate Error	Pyruvate (g/L)	Pyruvate Error	peak area (area)	Corrected reactor conc. (g/L)
I	0.00		0.19	18.16	0.07	0.00	0.00	0.09	0.00	1428	0.04
0	2.00	0.00	0.17	17.75	0.22	0.00	0.00	0.11	0.00	41925.00	1.47
1	6.00	4.00	0.46	17.58	0.01	0.00	0.00	0.21	0.00	45669.00	1.61
2	10.50	8.50	2.32	15.84	0.10	0.16	0.00	0.15	0.15	43477.00	1.53
3	15.17	13.17	5.47	11.71	0.06	0.72	0.00	0.00	0.00	43363.80	1.53
4	18.17	16.17	7.99	5.72	0.10	1.43	0.02	0.39	0.00	38545.00	1.35
5	20.17	18.17	11.90	0.38	0.01	2.14	0.02	0.41	0.00	23436.00	0.82
6	20.50	18.50	13.70	0.11	0.00	2.16	0.01	0.32	0.01	14846.00	0.52

Batch Run Data
*Run Notes
Sample = I (Directly after inoculation)
Sample = 0 (t = 0, IPTG [200mM - 0.06g] and Propanol [5 g/L - 7.783 mL] added)
Sample = 1
Sample = 2 (* Increased O_2 flow pre-emptively to 0.1 L/min @ 0415 DO went to 124)
Sample = 3 (* Increased O_2 flow to 0.2 L/min @ 1135 DO went to 100.5)
Sample = 4
Sample = 5
Sample = 6 (* DO remaining stable while only flowing 1.25 L/min air - Glucose = Consumed)

APPENDIX B-4: BATCH DATA (MEC1410 (2) – Propyl Acetate)

Batch Run Data											
Sample	Total Time (h)	Propanol Time (h)	OD	Glucose (g/L)	Glucose Error	Acetate (g/L)	Acetate Error	Pyruvate (g/L)	Pyruvate Error	peak area (area)	Corrected reactor conc. (g/L)
I	0.00		0.12	17.52	0.34	0.00	0.00	0.08	0.00	0	-0.01
0	2.00	0.00	0.32	18.06	0.13	0.00	0.00	0.17	0.00	49742.00	1.75
1	6.00	3.67	0.91	17.84	0.04	0.02	0.00	0.26	0.00	39721.00	1.40
2	10.50	8.17	3.90	14.56	0.26	0.34	0.01	0.00	0.00	35695.00	1.27
3	15.00	12.67	15.80	3.95	0.04	1.38	0.01	0.29	0.00	25763.00	0.91
4	15.75	13.42	16.70	0.11	0.00	1.79	0.01	0.03	0.00	15895.00	0.61
5	18.25	15.92	17.20	0.01	0.01	1.70	0.04	0.14	0.05	1745.00	0.13
6	19.75	17.42	17.60	0.00	0.00	1.60	0.00	0.17	0.00	811.50	0.11

Batch Run Data
*Run Notes
Sample = I (Directly after inoculation)
Sample = 0 (t = 0, IPTG [200mM - 0.06g] and Propanol [5 g/L - 7.783 mL] added)
Sample = 1 (* increased O_2 to 0.2 L/min @ 0414 DO increased to 96)
Sample = 2
Sample = 3 (* Reactor found foamed out @ 0815 and DO = 20, Switched to pure O_2 @ 0900 DO went to 93)
Sample = 4 (* DO uncontrollably increasing, done? *Having to adjust O_2 /air ratio up and down a lot)
Sample = 5
Sample = 6 (*DO no longer drops 1.25 L/min air only flowing)