BIOSYNTHETIC PATHWAY FOR THE PRODUCTION

OF ACETOIN IN *ESCHERICHIA COLI*

by

RACHEL BROWN

(Under the Direction of Mark Eiteman)

ABSTRACT

Acetoin is a versatile chemical used in the cosmetic, food, and fuel industries. High yields and productivities are needed in microbial production to rival chemical synthesis from crude oil. To produce acetoin using *Escherichia coli*, two enzymes must be introduced from native acetoin producers: acetolactate synthase (*alsS* gene) and acetolactate decarboxylase (*budA* gene). Previous studies have demonstrated these two genes and the butanediol dehydrogenase gene from *Enterobacter cloacae* ssp. *dissolvens* are effective to produce the reduced form of acetoin, 2,3-butanediol, in *E. coli*. The first overall objective of this work is to determine genetic modifications that lead to the highest yield of acetoin from glucose by improving the metabolic flux of carbon towards target products. The second overall objective of this work is to utilize nutrient limitation to increase the yield of acetoin from glucose.

INDEX WORDS: *Escherichia coli*, acetoin, fermentation, knockouts, synthetic biology, metabolic engineering

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RACHEL BROWN

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RACHEL BROWN

Major Professor:	Mark Eiteman
Committee:	James Kastner
	Ross Marklein

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Acetoin is a versatile building block used as flavoring and fragrance in cosmetics and food industries, biological pest control, and an intermediate in chemical synthesis. Currently, the majority of acetoin is produced from crude oil, a nonrenewable source, and there is a desire to produce this chemical in a renewable way. Bacteria can produce acetoin with native enzymes or through the insertion of foreign genes. Glucose is the typical inexpensive starting carbon and energy source selected for microbial growth. The key intermediate in the metabolism of glucose by the Embden-Meyerhof-Parnas (EMP) pathway is pyruvate. The acetoin pathway starts with two pyruvate molecules and results in the production of one acetoin molecule (**Figure 1**). Two genes are needed to produce acetoin and the one pathway intermediate that does not normally accumulate in *Escherichia coli*. Acetoin is an intermediate to 2,3-butanediol (BDO) requiring one additional enzyme (**Figure 1**). BDO is used for a multitude of purposes including cosmetics, fuel additive, and industrial solvents. Production of BDO would be future work expanding upon the acetoin pathway. Optimization of the EMP, acetoin, and side pathways are necessary to generate either acetoin or BDO at high yields and productivities. Many different parameters can alter the performance of these pathways in bacteria including reactor conditions, genes selected for deletion, insertion or modification, and the specific genes and promoters involved.

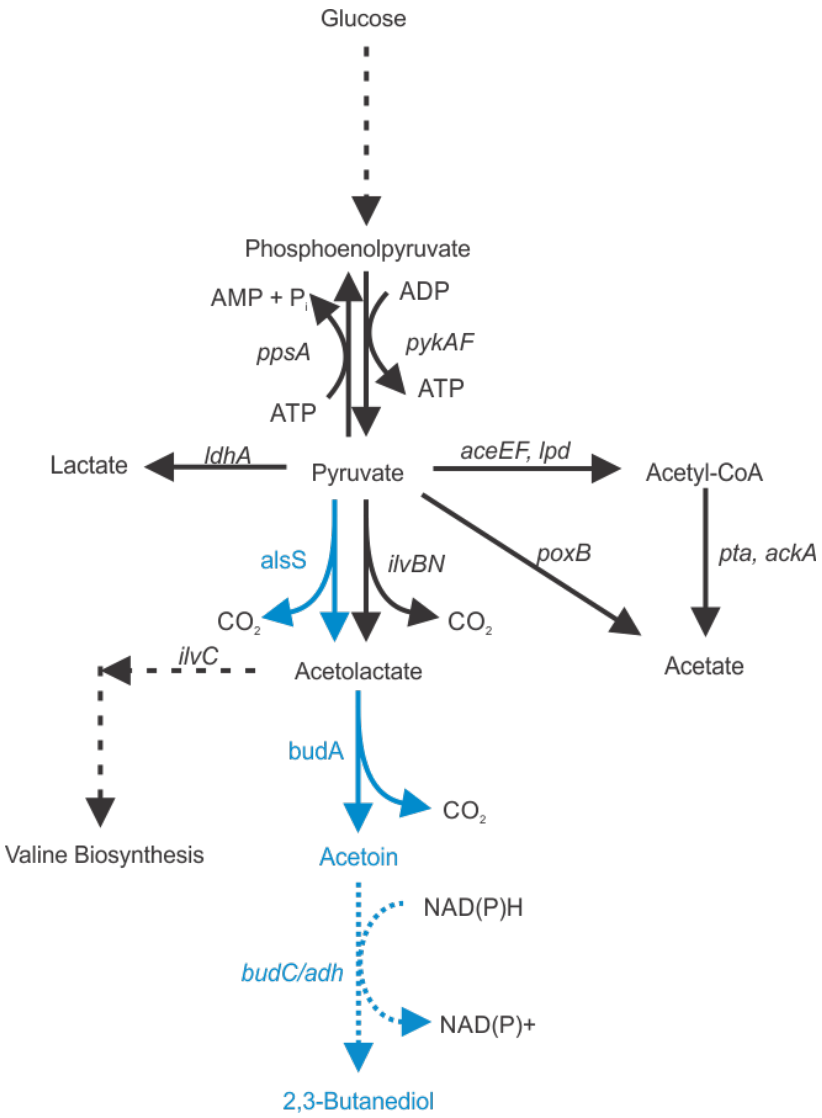

Figure 1: The acetoin pathway involves two enzymatic steps from pyruvate. One additional enzyme is needed for the production of BDO. The genes shown in blue represent the foreign genes necessary to produce acetoin and BDO: *alsS* for acetolactate synthase, *budA* for acetolactate decarboxylase, and *budC* or *adh* for butanediol dehydrogenase.

CHAPTER 2

BACKGROUND

Acetolactate

The first intermediate in the acetoin pathway is acetolactate, a five-carbon molecule which is unstable, especially in the presence of oxygen. Oxygen causes acetolactate to decarboxylate spontaneously into diacetyl (Nielsen et al., 2010). In bacteria, acetolactate is derived from pyruvate by the enzyme acetolactate synthase.

Acetolactate Synthase

Acetolactate synthase or alternatively acetohydroxyacid synthase (AHAS) is the first step in the biosynthesis of the amino acids leucine, isoleucine, and valine (Tittmann et al., 2005). AHAS belongs to a homologous family of thiamine diphosphate (ThDP)-dependent enzymes and requires the coenzyme flavin adenine dinucleotide and the Mg^{+2} or Mn^{+2} ion (Tittmann et al., 2005; Chien et al., 2010; Vyazmensky et al., 2011). Bacterial AHAS has two subunits: catalytic and regulatory. The ThDP-dependent catalytic subunits are of similar size and sequence across species (Kaplun et al., 2006). The regulatory subunit activates the catalytic subunit and responds to different inhibitors across species (Kaplun et al., 2006).

AHAS has different functions depending on species. Members of the Gram-negative bacteria family *Enterobacteriaceae*, which includes *Escherichia coli*, have three AHAS isozymes with different substrate specificities, inhibition by valine, and cofactor affinity (Vinogradov et al., 2006; Belenky et al., 2012). Other bacteria only have one AHAS enzyme known as acetolactate synthase, which is similar in sequence and properties to AHAS isozyme

III in *E. coli* (Kaplun et al., 2006). *Bacillus subtilis* and a few other bacteria have one AHAS enzyme that functions differently to *E. coli* in that its expression occurs in stationary phase (Gollop et al., 1990).

The first step in AHAS is the decarboxylation of pyruvate into hydroxyethyl-ThDP$^-$ anion/enamine (Tittmann et al., 2005). The decarboxylation of pyruvate is irreversible (Chien et al., 2010). This step is followed by the condensation of hydroxyethyl-ThDP$^-$ anion/enamine with either pyruvate or 2-ketobutyrate (2-KB) to form respectively 2-*S*-acetolactate (acetolactate) or 2-hydroxybutyrate (hydroxybutyrate) (Tittmann et al., 2005; McCourt and Duggleby, 2005). AHAS isozymes typically have differing specificity to the second substrate, and this specificity is an intrinsic property not affected by inhibitors or pH (Gollop et al., 1989). This observation provides evidence that the first steps in the reaction before the second substrate binds are rate determining (Gollop et al., 1989). Specificity towards hydroxybutyrate or acetolactate is often described by a parameter R (McCourt and Duggleby, 2005):

$$R = \frac{hydroxybutyrate\ formed/[2KB]}{acetolactate\ formed/[pyruvate]}$$

The magnitude of R does not change the amount of product formed (Tittmann et al., 2005). Varying the concentration of 2-KB with a fixed concentration of pyruvate form the same amount of total product (Gollop et al., 1989; Tittmann et al., 2005).

As noted above *E. coli* has three AHAS isozymes, and 2-KB is the preferred second substrate for isozymes II or III (Gollop et al., 1989; Steinmetz et al., 2010). This specificity is caused by a strong hydrophobic interaction between a substituent of 2-KB and the indole ring on W464. 2-KB is toxic in high concentrations (Gollop et al., 1990), and the concentration of 2-KB in a typical cell is almost two magnitudes lower than pyruvate (Gollop et al., 1990; Steinmetz et al., 2010). The greater specificity towards 2-KB in AHAS isozymes II and III also allows

balanced formation of each amino acid (Gollop et al., 1990; Steinmetz et al., 2010). Since pyruvate is in relative excess, without this specificity more acetolactate would be formed than hydroxybutyrate, resulting in excess valine and leucine. AHAS isozyme I shows little preference for the second substrate (Vinogradov et al., 2006). This low preference causes AHAS I to be the dominant producer of acetolactate with a K_M of 4.8 mM pyruvate and k_{cat} of 71 s^{-1} (Vinogradov et al., 2006).

The unique properties in AHAS I are attributed to structural differences and allow the rapid departure of the product (Belenky et al., 2012). In *E. coli*, the two subunits of AHAS isozyme I are encoded by the operon *ilvBN* (Vinogradov et al., 2006; Belenky et al., 2012). The operon is induced by a cAMP-dependent inducer protein of the catabolite repression system (Belenky et al., 2012). Two residues at the C-terminal of AHAS I are critical to the specificity of the second substrate and release of product: a conserved glutamine (Q480) and a conserved leucine (L476) (Belenky et al., 2012). These two residues in AHAS I cause a lower specificity to 2-KB and a faster product release compared to the corresponding residues in AHAS II, a conserved tryptophan (W464) and a conserved methionine (M460) (Belenky et al., 2012). In AHAS I, the L476M substitution causes a lower specificity to 2-KB compared to the wild type (Belenky et al., 2012). This L476M variant increases the K_M to 13 mM and decreases k_{cat} to 65 s^{-1} (Belenky et al., 2012). Other variants of AHAS I are listed in **Table 1**.

Table 1: Kinetic parameters for the *E. coli* AHAS I wild type and variants (Belenky et al., 2012)

	k_{cat} (s^{-1})	K_M (pyruvate) (mM)	R[a]
Wild Type	71	4.8	1.3
L476M	65	13	0.7
Q480W	15	4.7	3.2
L476M/Q480W	25	3.6	0.95
E60A	2	28	0.4
E60Q	2.1	21	0.6
M263A	62	26	1.6
R289Q	6.4	124	0.2
R289K	86	50	2.2
V391A	8.2	18	1.2
V477I	9.5	8	0.64
C83A	20	1.5	1.25
C83T	2	0.33	0.7
C83S	22	1.2	0.76

[a] R is the specificity towards producing acetolactate as a product over 2-KB. R closer to 0 means acetolactate is the preferred product.

In AHAS II certain residues affect product formation and the rate of formation similar to AHAS I. As noted above, W464 and M460 are critical to the specificity of the second substrate and release of product (Belenky et al., 2012). AHAS II shows 60-fold greater specificity towards 2-KB as the second substrate due to a stronger hydrophobic interaction of the ethyl substituent with the indole ring of W464 to ThDP (Steinmetz et al., 2010). The interaction of the pyruvate methyl group with the indole ring of W464 is less favorable (Steinmetz et al., 2010). The W464L

substitution causes a loss in preference for binding to 2-KB with R equal to 1.3, which confirms W464 is important for binding of the second substrate (McCourt and Duggleby, 2005; Steinmetz et al., 2010).

Acetoin

Acetoin is a volatile pale-yellow liquid with a yogurt odor and butter taste (Xiao and Lu, 2014). Commercially, acetoin is used as a flavor or fragrance in wide variety of products including foods, cigarettes, cosmetics, and biological pest controls (Xiao and Lu, 2014; Kandasamy et al., 2016). Acetoin is the simplest acyloin, a compound with a hydroxy group adjacent to a ketone. As the simplest acyloin, acetoin has many unique properties and is widely used in chemical synthesis (Xiao and Lu, 2014). Although acetoin is mainly produced via chemical synthesis of fossil feedstocks, acetoin derived from petroleum typically cannot be used in cosmetics and food industries as customers prefer natural acetoin in these applications (Xiao and Lu, 2014). Natural acetoin is much more expensive than chemically derived form of acetoin (Xiao and Lu, 2014). Many bacteria are capable of producing acetoin from acetolactate (Xiao and Lu, 2014, **Figure 1**). Acetoin generated microbially is generally recognized as safe by the US FDA (Xiao and Lu, 2014).

Acetoin is a neutral four-carbon molecule that is produced in bacteria by decarboxylating acetolactate. This reaction helps maintain pH homeostasis, especially during stationary phase (Renna et al., 1993; Monnet et al., 2003). As noted previously, acetolactate is unstable and spontaneously decarboxylates to diacetyl (Monnet et al., 2003; Kandasamy et al., 2016), which itself can be reduced to (3S)-acetoin by diacetyl reductase or nonspecific butanediol dehydrogenases (Kandasamy et al., 2016; Liang and Shen, 2017; Lian et al., 2014).

Acetolactate Decarboxylase

The enzyme acetolactate decarboxylase converts acetolactate into (R)-acetoin (Kandasamy et al., 2016). Because *E. coli* does not have a gene encoding acetolactate decarboxylase, a gene from other bacteria must be inserted to enable *E. coli* to accumulate acetoin.

Most organisms that produce acetoin have the genes from acetolactate synthase and acetolactate decarboxylase clustered on an operon. For example, in *Bacillus subtilis*, *alsS* (for acetolactate synthase) and *alsD* (for acetolactate decarboxylase) form the *alsSD* operon (Yan et al., 2009; Xiao and Lu, 2014). Additionally, on the operon is an essential regulatory gene encoding a LysR-type transcriptional activator (Xiao and Lu, 2014). In *B. subtilis,* this gene is *alsR* (Xu et al., 2014; de Oliveira and Nicholson, 2015).

BDO

BDO is a vicinal diol meaning that two hydroxyl groups occupy adjacent atoms to form three stereoisomers: *meso*-BDO (m-BDO), (2R,3R)-butanediol (R-BDO), and (2S,3S)-butanediol (S-BDO) (Li et al., 2012). There are many applications for BDO including as a starting material for bulk chemicals, fuel additive or liquid fuel, and antifreeze agent (Li et al., 2015). Traditionally, BDO is produced from crude oil, and microbial production could reduce the cost of BDO and be renewable (Li et al., 2015). In bacteria, BDO is formed by the reduction of acetoin (**Figure 1**).

There are many bacteria capable of generating BDO. The greatest producers of BDO are (biosafety) class 2 bacteria *Klebsiella pneumoniae* and *Klebsiella oxytoca* (Yang and Zhang, 2018). However, class 2 bacteria are unsuitable for industrial scale fermentations (Xu et al., 2014), and a class 1 microbe is desirable for industrial use (Yang and Zhang, 2018). The class 1

bacteria *B. subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens*, and several *Klebsiella* strains naturally produce BDO (Yang and Zhang, 2018; Yan et al., 2009). The enantiomeric purity of BDO produced varies from species to species (Nielsen et al., 2010). An alternative is to use the non-native producers of BDO such as *E. coli*, *Saccharomyces cerevisiae,* and *Lactococcus lactis*. These bacteria are suitable for large scale production due to their well-characterized genetics with well-known cultivation strategies (Yang and Zhang, 2018).

While *E. coli* is not a native producer of BDO, *E. coli* can form m-BDO without foreign genes from pyruvate when (S)-2-acetolactate spontaneously decomposes to diacetyl, which is then enzymatically converted to (S)-acetoin and m-BDO (Liang and Shen, 2017). As noted previously, acetolactate decarboxylates spontaneously in the presence of oxygen to form diacetyl (Liang and Shen, 2017). Diacetyl can be converted into (S)-acetoin by the enzyme diacetyl reductase (DAR) (Liang and Shen, 2017). Although *E. coli* does not form BDO under anaerobic conditions (Liang and Shen, 2017), it is capable of producing BDO. Knocking out the *ldhA* gene with extracellular feeding of acetoin led to BDO, showing that there is a native dehydrogenase capable of this reduction (Liang and Shen, 2017). The dehydrogenase responsible for the conversion of acetoin to BDO was identified to be the product of the *gldA* gene (Liang and Shen, 2017).

BDO Dehydrogenases

BDO dehydrogenases (BDHs) convert acetoin into BDO with a stereoselective reaction (Li et al., 2012). BDHs are generally NAD(H)- or NADP(H)-dependent and contain a zinc motif (Yang and Zhang, 2018). The particular stereoisomer of BDO formed depends on the specificity of the BDH (Yang and Zhang, 2018). S-BDH converts diacetyl into (3S)-acetoin, and then (3S)-acetoin into S-BDO (Li et al., 2012). (3R)-acetoin can be converted to any BDO isomer by

specific BDHs (Kandasamy et al., 2016). Using a non-native producer that expresses a specific BDO dehydrogenase enzyme would be advantageous to produce a pure enantiomer of BDO (Yan et al., 2009).

By adding the biomachinery of native producers of BDO in *E. coli*, there can be control of which specific isomer is produced. Among the typical BDO producers, *B. subtilis* 168 *bdhA, Bacillus pumilus bdh, Paenibacillus polymyxa* ATCC 12321 *adh*, *Clostridium beijerinckii adh,* and *Thermoanaerobacter brockii adh* code for proteins which produce R-BDO as the major product (Yan et al., 2009; Xu et al., 2014; Li et al., 2015). *B. licheniformis* 10-1-A produces R-BDO and m-BDO with a ratio of nearly 1:1 (Xu et al., 2014). *K. pneumoniae* and *Enterobacter cloacae* produce m-BDO and S-BDO as the major products (Xu et al., 2014). The protein coded by the *K. pneumoniae budC* gene produces only m-BDO (Yan et al., 2009). *Serratia marcescens* produces nearly enantiomerically pure m-BDO (Xu et al., 2014). The gene *bdh* from *Enterobacter cloacae* ssp. *dissolvens* SDM produces S-BDO from diacetyl (Li et al., 2012).

As acetoin producers have genes clustered into an operon, relatively superior producers of BDO do as well like *E. cloacae* ssp. *dissolvens* SDM. The operon from *E. cloacae* ssp. *dissolvens* SDM inserted into *E. coli* produced 12.8 g/L acetoin from an initial glucose concentration of 50 g/L, which was the highest concentration out of four gene clusters examined (Xu et al 2014).

To control production, different promoters can be used. The optimization of the promoter is an important step, especially when introducing foreign DNA (Xu et al., 2014). A constitutive promoter converts more acetoin into BDO than an inducible promoter (Xu et al., 2014). Under varying constitutive promoter strengths, the BDO operon from *E. cloacae* ssp. *dissolvens* SDM inserted into *E. coli* produced 20.2 and 21.8 g/L BDO (Erian et al., 2018). This BDO production

was greater using genes from *E. cloacae* ssp. *dissolvens* SDM than *Enterobacter cloacae* ssp. *cloacae* and *K. oxytoca* (Erian et al., 2018). Because the *alsS* gene encoding for acetolactate decarboxylase from *B. subtilis* was thought to produce more acetoin, this gene was used in replace of the acetolactate decarboxylase gene from *E. cloacae* ssp. *dissolvens* SDM (Erian et al., 2018). However, the native operon from *E. cloacae* ssp. *dissolvens* SDM still produced greater amounts of BDO (Erian et al., 2018).

CHAPTER 3

STRATEGIES FOR PRODUCING ACETOIN

Enzymes selected for the acetoin pathway

To produce acetoin and BDO in *E. coli*, at minimum foreign genes must be introduced to the background strain for acetolactate decarboxylase and BDH. Moreover, the basal level of acetolactate production in *E. coli* is low (Nielsen et al., 2010). In order to produce greater quantities of acetoin and BDO, more acetolactate needs to be converted from pyruvate.

Because the acetolactate production is low in wild-type *E. coli*, an approach must be used to increase the formation of acetolactate. One approach would be to insert a foreign AHAS gene that has been previously shown to increase the formation of acetolactate. Another approach would be to mutate and/or overexpress the native *E. coli* AHAS I gene to produce more acetolactate. While there have been studies that compared mutations to increase acetolactate production (Belenky et al., 2012), few studies use the native *E. coli* AHAS I gene for the production of acetoin and BDO. Many other studies use acetolactate synthase from *B. subtilis* because of its high activity (Yan et al., 2009; de Oliveira and Nicholson, 2015), but when compared to the acetolactate synthase from *E. cloacae* ssp. *dissolvens*, less BDO was produced in the strain containing acetolactate synthase from *B. subtilis* (Erian et al., 2018).

As previously mentioned, the genes in the acetoin and BDO pathway form a gene cluster. Studies using the native gene cluster to produce acetoin or BDO generally result in high yields of acetoin and BDO production (Xu et al., 2014; Erian et al., 2018). The gene cluster from *E. cloacae* ssp. *dissolvens* resulted in the greatest yield of BDO, over the gene cluster from *B.*

subtilis, *B. licheniformis*, *S. marcescens*, *K. pneumoniae*, and *K. oxytoca* (Xu et al., 2014; Erian et al., 2018). As acetoin is a precursor to BDO, the gene clusters that produce the greatest yield of BDO are likely to produce a high yields of acetoin. Specifically, the gene cluster from *E. cloacae* ssp. *dissolvens* is expected to produce the highest acetoin yield. Therefore, the acetolactate decarboxylase gene *budA* and acetolactate synthase gene *alsS* from *E. cloacae* ssp. *dissolvens* is preferred to produce acetoin. Also, the gene cluster from *B. subtilis* and *E. cloacae* ssp. *cloacae* is used to produce acetoin to determine as these gene cluster have been shown to produce adequate amounts of acetoin or BDO (Förster et al., 2017; Erian et al., 2018).

Prevention of the formation of by-products

The optimal production of acetoin necessitates pyruvate be directed to the acetoin pathway. Thus, nonessential pathways that use pyruvate should be eliminated. Other, necessary pathways might be limited to reduce the diversion of pyruvate. Most studies focus on the production of BDO without first considering acetoin. Because of this trend, most information regarding strain construction comes from studies producing BDO.

Under anaerobic and acidic conditions using glucose as the carbon source, *E. coli* generates lactate as a means to balance NAD(H) (Mat-Jan et al., 1989; Bunch et al., 1997). Lactate is still formed in aerobic conditions and is generated by allosteric activation of lactate dehydrogenase (*ldhA* gene) by pyruvate (Tarmy and Kaplan, 1968). Thus, in pyruvate-accumulating strains, lactate can be readily generated under aerobic conditions (Zelić et al., 2003; Tomar et al., 2003). Lactate is also a common byproduct of the BDO mixed-acid fermentation (Li et al., 2015). By deleting the *ldhA* gene, the availability of pyruvate and NADH increases and thus, increases the availability of NADH to reduce acetoin (Nielsen et al., 2010). Studies which examined the *ldhA* deletion have shown that this deletion leads to more acetoin

and BDO compared to strains with intact lactate dehydrogenase (Nielsen et al., 2010; Mazumdar et al., 2013; Li et al., 2015). Other studies simply include the *ldhA* deletion in their base strain without comparing the direct effect (Yan et al., 2009). The *ldhA* deletion will likely increase acetoin formation and, through an increase availability of NADH, increase the reduction of acetoin to BDO.

Acetate is a byproduct of mixed-acid fermentations. Pyruvate is converted to acetate by pyruvate oxidase encoded by the *poxB* gene (Nielsen et al., 2010). Most studies do not compare the effect of deleting *poxB* on the production of acetoin or BDO, but the deletion is included in their base strain (Nielsen et al., 2010; Erian et al., 2018). In one direct comparison, the deletion of *poxB* caused a two-fold increase in BDO formation (Shen et al., 2012) and a 71% decrease in acetate although acetate was still produced (Li et al., 2010). In addition to decreasing the amount of acetate produced, a *poxB* deletion is anticipated to increase acetoin productivity.

Acetate can also be formed by the hydrolysis of acetyl-CoA (Nielsen et al., 2010). Acetyl-CoA is formed from pyruvate through the pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex is composed of three different enzymes: the E1 component encoded by *aceE*, the E2 component encoded by *aceF*, and the E3 component encoded by *lpdA*. Strains deficient in their ability to form acetyl-CoA from pyruvate have to be supplemented with an acetyl-CoA precursor such as ethanol or acetate for survival (Langley and Guest, 1978). Previous studies have deleted pyruvate dehydrogenase (*e.g.*, knockout in *aceEF* or *lpd*) to accumulate pyruvate (Tomar et al., 2001; Zhu et al., 2008). An alternative to eliminating pyruvate dehydrogenase activity is to decrease the activity of the enzyme complex. To increase the accumulation of pyruvate, the E1 component of the pyruvate dehydrogenase complex has been altered to reduce the flux of carbon that goes to acetyl-CoA (Moxley and Eiteman, 2021).

For example, the AceE[H106V] variant accumulates more pyruvate than other variants examined and the wild type enzyme (Moxley and Eiteman, 2021). The deletion of *aceE* or integrating the AceE[H106V] substitution is anticipated to increase the productivity of acetoin.

The conversion of pyruvate to phosphoenolpyruvate is facilitated by phosphoenolpyruvate synthase encoded by the gene *ppsA* (Berman and Cohn, 1970; Nielsen et al., 2010). One ATP is needed for the conversion (Berman and Cohn, 1970). This enzyme is important for glucogenesis when pyruvate or lactate is the primary carbon source (Berman and Cohn, 1970; Niersbach et al., 1992). In a microbial process using glucose, accumulated pyruvate is consumed when the glucose is depleted. A *ppsA* deletion significantly lowers the re-assimilation of pyruvate (Moxley and Eiteman, 2021). In studies incorporating the BDO pathway, *ppsA* is rarely deleted, with only one study identified to use a background strain of *E. coli* with the *ppsA* deletion in the production of BDO (Nielsen et al., 2010). The deletion of *ppsA* should increase the acetoin productivity.

Operational Strategies

To alter the production of acetoin, various operational conditions such as limiting a nutrient, adjusting pH, or adjusting agitation can be examined. Non-carbon, nutrient-limited conditions can cause maximal generation of carbon-based products. Previous studies have used oxygen-limited conditions to increase production of BDO because of the need to reduce acetoin as a final step.

Altering the oxygen levels affects the conversion of acetoin to BDO (Xu et al., 2014; Erian et al., 2018). Lowering the oxygen levels increases the conversion of acetoin to BDO (Xu et al., 2014; Fu et al., 2016). However, the glucose consumption rate and the growth rate decrease as oxygen levels decrease, which causes less acetoin and BDO to be produced (Li et al.,

2010). The combined yield of acetoin and BDO increased only slightly with decreased oxygen (Li et al., 2010; Fu et al., 2016). For acetoin production, in which NADH or NADPH is not required as a cofactor within the pathway, oxygen-limitation would likely not be beneficial. Lower oxygen levels do not seem conducive to producing larger quantities of acetoin and BDO.

Nitrogen-limited conditions increase glucose uptake and pyruvate production rates in a pyruvate-forming strain compared to glucose-limited conditions (Zhu et al., 2008). Under nitrogen-limited conditions, *E. coli* containing *ldhA*, *poxB*, and *ppsA* knockouts and containing the AceE[H106V] substitution attained a greater yield of pyruvate than a control strain expressing the wild-type pyruvate dehydrogenase (Moxley and Eiteman, 2021). Increased pyruvate production will likely support elevated acetoin formation in a strain with the pyruvate-to-acetoin pathway.

Current Limitations

Genetic modifications examined previously to *E. coli* for the production of acetoin and BDO vary, but primarily include a deletion of *ldhA*. The combinations of the proposed knockouts have not been integrated together to see the effect on acetoin or BDO. The effect of *ppsA* or *aceE* on the production of acetoin and BDO has not been studied. Few studies include modifications to pyruvate dehydrogenase (*aceEF* and *lpd*) to eliminate its activity to increase production of acetoin and BDO (Nielsen et al., 2010). Modifications to pyruvate dehydrogenase to reduce enzymatic activity have been shown to increase the amount of pyruvate accumulated (Moxley and Eiteman, 2021). Thus, reducing the enzymatic activity of pyruvate dehydrogenase should increase the production of acetoin.

In the production of acetoin and BDO, the only nutrient-limited operational strategy which has been examined to alter production is oxygen-limited, and these conditions have not

significantly increased the productivity of acetoin and BDO. Oxygen-limited conditions increase the amount of acetoin converted to BDO, but the total amount of BDO produced is less under oxygen-limited growth than under oxygen-rich conditions. Nitrogen-limited conditions has not been explored for the production of acetoin and BDO, but nitrogen-limited conditions show promise to increase production of acetoin or BDO.

Objectives

The overall objective is to improve the formation of acetoin. Four plasmids with different combinations of gene clusters, promoter strengths, promoter types, and antibiotic resistances were tested to determine which plasmid produces the highest yield of acetoin. The four plasmids pTrc99a-*budA-alsS*, pMAL_*alsSD*, 44_ediss, and 44_ecloa used for acetoin production are listed with their detailed genotype (**Table 2**). The additional plasmids were used to construct strains and plasmid construction are listed in **Table 2**.

Table 2: Plasmids used in this study

Plasmid	Genotype	Reference
pTrc99A	Amp^R, P_{lac}	Amann et al., 1988
pTrc99A-*budA-alsS*	Amp^R, P_{lac} *budA alsS*	This Study
pMAL_*alsSD*	Amp^R, P_{lac} *alsSD*	Förster et al., 2017
445_ediss	*BB3_pUC(*Kan^R*)_LinkerA_114p_budB_114p _budA_105p_budC_TT_Ediss_LinkerD*	Erian et al., 2018
445_ecloa	*BB3_pUC(*Kan^R*)_LinkerA_114p_budB_114p _budA_105p_budC_TT_Ecloa_LinkerD*	Erian et al., 2018
44_ediss	*BB3_pUC(*Kan^R*)_LinkerA_114p_budB_114p _budA_TT_Ediss_LinkerD*	This Study
44_ecloa	*BB3_pUC(*Kan^R*)_LinkerA_114p_budB_114p _budA_TT_Ecloa_LinkerD*	This Study
pKD46	Amp^R, λ Red Recombinase expression	Datsenko and Wanner 2000
pCP20	Amp^R, Cm^R, FLP Recombinase expression	Datsenko and Wanner 2000

The pTrc99A-*budA-alsS* has ampicillin resistance and expresses the *alsS* gene from *E. cloacae* ssp. *dissolvens* coding acetolactate synthase and the *budA* gene from *E. cloacae* ssp. *dissolvens* coding acetolactate decarboxylase (Erian et al., 2018). The pMAL_*alsSD* has ampicillin resistance and expresses the *alsS* gene from *B. subtilis* coding acetolactate synthase and *alsD* gene from *B. subtilis* coding acetolactate decarboxylase (Förster et al., 2017). To test constitutive and kanamycin resistance plasmids, 445_ediss and 445_ecloa were modified so that the *alsS* gene coding acetolactate synthase and the *budA* gene coding acetolactate decarboxylase from *E. cloacae* ssp. *dissolvens* (445_ediss) and from *E. cloacae* ssp. *cloacae* (445_ecloa) are only expressed (Erian et al., 2018). The two original plasmids showed greater yields of BDO over other genes for acetolactate synthase and acetolactate decarboxylase from *B*. *subtilis*, *B*. *licheniformis*, *S*. *marcescens*, *K. pneumoniae*, and *K. oxytoca* (Xu et al., 2014; Erian et al., 2018). Because BDH has been removed from these two plasmids, they are referred to as 44_ediss and 44_ecloa.

In order to maximize the conversion of glucose to acetoin, genes associated with the formation of unwanted byproducts were knocked out. In particular, the *ldhA* gene coding lactate dehydrogenase and the *poxB* gene coding pyruvate oxidase were deleted in all strains. Thus, *E. coli* Δ*ldhA* Δ*poxB* expressing *alsS* and *budA* serves as the base strain for studies involving acetoin formation.

The first hypothesis (A) is that a deletion in the *ppsA* gene coding PEP synthase will increase the yield of acetoin. The effect of a PEP synthase knockout is readily established by comparing strains with and without a deletion in the *ppsA* gene as shown in (**Table 3**). The detailed procedure for performing knockouts and conducting this study is described in the Methodology section.

The second hypothesis (B) is that a deletion or reduction in activity of pyruvate dehydrogenase will increase the yield of acetoin. As noted previously, a key conduit which competes with acetolactate synthase under aerobic conditions is the pyruvate dehydrogenase complex. To test this hypothesis, three different strains having modifications in the *aceE* gene coding the E1 component of this complex were constructed and compared with a strain containing the wild-type *aceE* gene (MEC1023, **Table 3**). MEC1081 contains a knockout of the *aceE* gene. As noted above, a strain lacking pyruvate dehydrogenase activity necessitates supplementing the medium with acetate. MEC1101 contains the AceE[H106V] substitution which was previously shown to result in pyruvate accumulation (Moxley and Eiteman, 2021). MEC1102 contains the AceE[E401A] substitution that decreased the growth rate of *E. coli* but did not show pyruvate accumulation, and presumably has a higher pyruvate dehydrogenase activity than the AceE[H106V] substitution but a lower activity than the wild-type enzyme. The detailed procedure for performing knockouts, constructing the variants, and conducting this study is described in the Methodology section.

Table 3: Strains used in Objective I.

Strain	Relevant Characteristics	Reference
ATCC 8739	*Escherichia coli* C	Wild-type
MEC981	ATCC 8739 *poxB ldhA*	This study
MEC1023	MEC981 *ppsA*	This study
MEC1081	MEC1023 *aceE*	This study
MEC1101	MEC1023 *aceE*::*aceE*$^{[H106V]}$	This study
MEC1102	MEC1023 *aceE*::*aceE*$^{[E401A]}$	This study

The third hypothesis (C) is that growth under nitrogen-limited conditions will increase the yield and generation rate of acetoin. To test this hypothesis, the effect of nitrogen-limited growth in a fed-batch process is compared to a batch fermentation.

CHAPTER 4

METHODOLOGY

Genetic modifications

All gene knockouts were performed in *E. coli* C (ATCC 8739) using the previously described method (Datsenko and Wanner, 2000). P1 transduction was used to create strains with multiple gene knockouts (Thomason et al., 2007). Knockouts were selected using kanamycin supplemented Lysogeny Broth (LB) or TYA plates (Chang and Cronan, 1982). To insert DNA to express AceE protein variants, a chloramphenicol-*sacB* cassette was used to knockout *aceE* using previously described method (Moxley and Eiteman, 2021). Then, fragments containing specific point mutations previously created (Moxley and Eiteman, 2021) were inserted to replace of the chloramphenicol-*sacB* cassette using pKD46. Sucrose was added to TYA plates for counterselection against *sacB* to ensure only variants containing the modified *aceE* allele were able to grow. Forward and reverse primers external to the target gene were used to confirm chromosomal integration of the kanamycin cassette, chloramphenicol-*sacB* cassette, and *aceE*. If the target gene was of similar size of 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.

Plasmid Construction

Plasmids in the study are listed in **Table 2**. pMAL_*alsSD* was gifted by Johannes Gescher (Förster et al., 2017) while 445_ediss and 445_ecloa were gifted by Stefan Pflügl (Erian et al., 2018). For the acetoin pathway, pTrc99A, 445_ediss, and 445_ecloa were used as templates for plasmid construction. The genes *alsS* and *budA* from *E. cloacae* ssp. *dissolvens* were synthesized and codon optimized (GeneArt, Thermo Fisher Scientific, Waltham, MA, USA). Primers were designed to create regions of homology with pTrc99A and each gene. For 445_ediss and 445_ecloa, the butanediol dehydrogenase gene *budC* must be removed to produce acetoin. Primers were designed to remove *budC* without creating an open reading frame. Both primers were 45 base pairs long with one primer containing 20 base pairs homology. Plasmids were assembled using NEBuilder HiFi Assembly (New England Biolabs, Ipswich, MA, USA). Primers used in this study are listed in supplementary information **Table S1**. Phusion High-Fidelity Polymerase (New England Biolabs, Ipswich, MA, USA) or PrimeStar Max High-Fidelity Polymerase (Takara Bio, Mountain View, CA, USA) were used to amplify DNA. Restriction enzymes digestion or PCR were used to confirm assembly. Zyppy Plasmid Miniprep Kit was used to purify plasmid DNA (Zymo Research, Irvine, CA, USA). DNA Clean and Concentrator or Zymoclean Gel DNA Recovery Kits were used to purify PCR fragments (Zymo Research, Irvine, CA, USA). Restriction enzymes were purchased from New England Biolabs.

Media and Growth Conditions

For plasmid construction and knocking out genes, strains were grown on LB medium except strains containing a modified (or deleted) *aceE* gene which were grown on TYA medium. The final concentrations of the following antibiotics were added to the medium as needed: ampicillin (100 mg/L), kanamycin (50 or 150 mg/L), and chloramphenicol (20 mg/L).

The defined basal medium for shake flask experiments to which carbon/energy sources were added contained (per L): 3.5 g NH_4Cl, 0.29 g KH_2PO_4, 0.50 K_2HPO_4, 2.0 g K_2SO_4, 0.45 g $MgSO_4 \cdot 7H_2O$, 0.25 mg $ZnSO_4 \cdot 7H_2O$, 0.125 mg $CuCl_2 \cdot 2H_2O$, 1.25 mg $MnSO_4 \cdot H_2O$, 0.875 mg $CoCl_2 \cdot 6H_2O$, 0.06 mg H_3BO_3, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 5.5 mg $FeSO_4 \cdot 7H_2O$, 20 mg $Na_2EDTA \cdot 2H_2O$, 20 mg citric acid, 20 mg thiamine·HCl, and 20.9 g 3-[*N*-morpholino]propanesulfonic acid (100 mM MOPS). The medium was adjusted to a pH of 7.1 with 20% (w/v) NaOH. TYA medium contains 10 g/L tryptone, 1 g/L yeast extract, 5 g/L sodium chloride, and 1.33 g/L sodium acetate trihydrate. T5YA is identical to TYA except for containing 5 g/L yeast extract.

The defined basal medium for batch fermentation experiments to which carbon/energy sources were added contained (per L): 8 g NH_4Cl, 0.576g KH_2PO_4, 1.0 K_2HPO_4, 2.0 g K_2SO_4, 0.45 g $MgSO_4 \cdot 7H_2O$, 0.25 mg $ZnSO_4 \cdot 7H_2O$, 0.125 mg $CuCl_2 \cdot 2H_2O$, 1.25 mg $MnSO_4 \cdot H_2O$, 0.875 mg $CoCl_2 \cdot 6H_2O$, 0.06 mg H_3BO_3, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 5.5 mg $FeSO_4 \cdot 7H_2O$, 20 mg $Na_2EDTA \cdot 2H_2O$, 20 mg citric acid, 20 mg thiamine·HCl, and 5.23 g 3-[*N*-morpholino]propanesulfonic acid (25 mM MOPS). The medium was adjusted to a pH of 7.2 with 20% (w/v) NaOH.

Shake Flask Experiments

A single colony from an LB or TYA plate was used to inoculate 3 mL of the same medium. After 2-3 h of growth, this culture was used to inoculate, 500 μL this culture was used to inoculate three 250 mL shake flasks containing 50 mL of basal medium with 5 g/L glucose and 50 mg/L. All cultures were grown aerobically at 37°C on a rotary shaker at 250 rpm.

Batch Processes

A single colony from an LB or TYA plate was used to inoculate one 250 mL shake flask containing 125 mL LB or T5YA with 1 g/L glucose and 150 mg/L kanamycin (adapted from Erian et al., 2018). The culture was harvested at an OD of 4 and washed once with 0.9% (w/v) NaCl (2500 × g for 15 minutes at room temperature). The washed cells were all resuspended in 10 mL of 0.9% (w/v) NaCl and used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific Co., New Brunswick, NJ, USA) containing 1200 mL basal medium with 40 g/L glucose (and 8 g/L acetate for MEC1081) to an OD of ~0.4. Duplicate batch processes were performed.

Batch studies were conducted with a constant agitation of 400 rpm and at 37°C. Air and/or oxygen-supplemented air was sparged at 1.0 L/min to maintain a dissolved oxygen concentration above 40% of saturation. The pH was controlled at 7.0 using 30% (w/v) KOH and 20% (w/v) H_2SO_4. Antifoam 204 (Sigma) was used as necessary to control foaming.

Nutrient-limited Process

The same method of inoculation was used as described in *Batch Processes*, and the basal medium was the same as the batch fermentations except for 1 g/L NH_4Cl. Once the culture reached an OD of 3.5, 0.5 g NH_4Cl dissolved in 20 mL water was added continuously per hour to maintain a nitrogen-limited environment. Once the glucose concentration was below 10 g/L, an additional dose of glucose was added to reach about 40 g/L.

Studies were conducted with a constant agitation of 400 rpm and at 37°C. Air and/or oxygen-supplemented air was sparged at 1.0 L/min to maintain a dissolved oxygen concentration above 40% of saturation. The pH was controlled at 7.0 using 30% (w/v) KOH and 20% (w/v) H_2SO_4. Antifoam 204 (Sigma) was used as necessary to control foaming.

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). Liquid chromatography was used to quantify substrates and metabolites using RI detection (Eiteman and Chastain, 1997).

CHAPTER 5

RESULTS

Plasmid Screening

The principal goal of this study was to produce acetoin using plasmids carrying the genes for acetolactate synthase and acetolactate decarboxylase. In order to produce higher yields of acetoin, four different plasmids were studied in strains containing Δ*ldhA* Δ*poxB* Δ*ppsA* knockouts (MEC1023) and Δ*ldhA* Δ*poxB* knockouts (MEC981). Two plasmids (pMAL_*alsSD* and pTrc99A-*budA*-*alsS*) are ampicillin resistant and require IPTG induction for gene expression. The two plasmids 44_ediss and 44_ecloa are kanamycin resistant and have constitutive gene expression.

The plasmid pTrc99A-*budA*-*alsS* containing the genes for acetolactate synthase and acetolactate decarboxylase from *E. cloacae* ssp. *dissolvens* was used first for acetoin production. These two genes have been shown to produce the highest yields of acetoin and BDO (Erian et al., 2018). To determine if acetoin can be produced by a strain harboring this plasmid, *E. coli* C strain MEC1023 was studied in 250 mL shake flasks. A single colony from an LB plate was used to inoculate 3 mL of the same medium. After 6-10 h of growth, 500 μL of this culture was used to inoculate 3 mL of basal medium with 5 g/L glucose and 100 mg/L ampicillin. After 12-15 h of growth, this culture was used to inoculate three 250 mL shake flasks containing 50 mL of basal medium with 5 g/L glucose to an OD of 0.02. This experiment did not result in any acetoin production, which was unexpected as this gene cluster from *E. cloacae* ssp. *dissolvens* previously produced a yield of 0.40 g acetoin plus BDO/g glucose (Erian et al., 2018).

After initial attempts at using pTrc99a-*budA-alsS* plasmid to produce acetoin, little to no acetoin could be produced. Different tactics were used to determine why acetoin was not produced including potential plasmid loss and IPTG concentration. Using the same method of inoculation (from rich medium tubes to defined medium tubes to defined medium flasks), 10^6, 10^7, and 10^8 dilutions of the culture at the end of the fermentation were spread onto both ampicillin-containing Agar plates and plates containing no antibiotic. The results of this experiment demonstrated that 98% of the plasmid was lost showing that further exploration to prevent plasmid loss was needed (**Figure 2**).

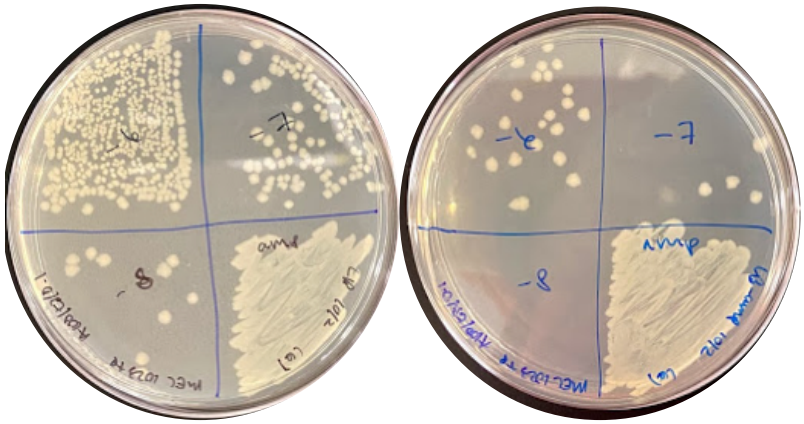

Figure 2: MEC1023/pTrc99A-*budA-alsS* culture plated on LB (left) and LB-AMP (right) to 10^6, 10^7, and 10^8 dilutions.

To reduce plasmid loss, an alternative method of inoculation was used. In this case, each passage was grown to a lower OD (0.3 to 0.6) before transfer. Additionally, the middle step using the defined medium tube was removed. Furthermore, two different concentrations of ampicillin were compared to determine the effect of ampicillin on plasmid loss. After 8 hours of

growth, cultures with an ampicillin concentration of 200 mg/L resulted in 25% more plasmid retained compared with cultures having 100 mg/L ampicillin. Therefore, new method of inoculation of rich media tube to defined media flask at an OD of ~0.4 in conjunction with ampicillin 200 mg/L resulted in higher plasmid retention and was therefore used as the principal method of inoculation.

The effect of different concentrations of IPTG and production temperatures on plasmid loss was also examined. Higher concentrations of IPTG resulted in the loss of plasmid and slower growth. Specifically, using 500 µM IPTG resulted in an OD of only 0.55 after 10 h, and the culture did not retain any plasmid nor produce acetoin. Under the same conditions except using 50 µM IPTG, about 100% of the plasmid was retained after 10 h, and the culture attained an OD of 5.4 while producing 0.22 g/L acetoin. Some anaerobic products (formate) were detected by the HPLC analysis (~ 0.1 g/L). These two experiments were not performed in triplicate but were used to determine important conditions to explore further.

While troubleshooting pTrc99a-*budA-alsS* for acetoin production, an additional plasmid pMAL_*alsSD* was donated that was previously shown to produce acetoin (Förster et al., 2017). Shake flask experiments were also conducted to learn how this plasmid behaved. One shake flask experiment with pMAL_*alsSD* was conducted to determine the effect of ampicillin concentration. Using 200 mg/L ampicillin resulted in 0.36 g/L acetoin while 400 mg/L ampicillin resulted in 0.25 g/L acetoin. At the end of the fermentation, a 10^7 dilution of the cultures were spread onto both ampicillin-containing Agar plates and plates containing no antibiotic to check for plasmid loss, but the results were inconclusive due to plating complications. Another flask experiment examined the optimal concentrations of IPTG and the effect of induction time. Specifically, 50 µM IPTG was compared to 500 µM IPTG, each added either at 0 h or at 2 h.

Using 500 μM IPTG resulted in the least amount of acetoin (0.19 g/L). Using 50 μM IPTG at either induction time resulted in about 0.35 g/L acetoin. Therefore, the induction time does not seem to have a strong effect on acetoin formation, while the concentration of IPTG does have a strong effect.

An additional experiment was conducted in triplicate to determine the effect of IPTG concentration for the production of acetoin using both inducible plasmids (pTrc99A-*budA*-*alsS* and pMAL_*alsSD*). Three concentrations of IPTG were compared (20, 40, and 80 μM), and the results are summarized in **Figure 3**. For pTrc99A-*budA*-*alsS*, 80 μM IPTG resulted in the highest yield of acetoin. Greater yields of acetoin resulted from using pMAL_*alsSD* compared to using pTrc99A-*budA*-*alsS*, and the three concentrations of IPTG examined showed yields that were within one standard deviation of each other. For pMAL_*alsSD*, although the 80 μM IPTG culture had a slightly higher yield of acetoin, only cultures containing 20 μM IPTG exhausted glucose. Thus, the ultimate acetoin yield might be different in the 40 and 80 μM IPTG cultures once glucose is depleted, particularly since less acetoin was produced towards the end of the fermentation (data not shown).

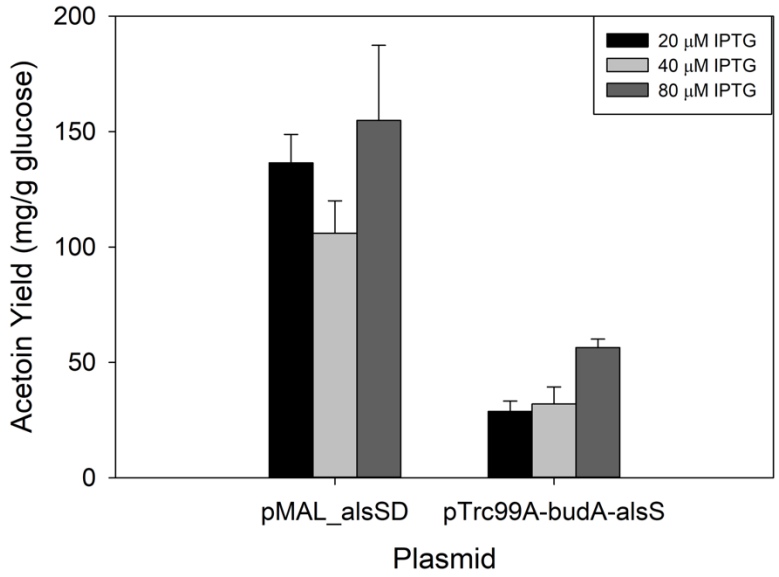

Figure 3: Acetoin yields at different concentrations of IPTG in MEC1023/pMAL_*alsSD* and MEC1023/pTr99A-*budA-alsS*

Since pMAL_*alsSD* produced higher yields of acetoin than pTrc99A-*budA-alsS*, one additional experiment was conducted with pMAL_*alsSD* to confirm the optimal concentration of IPTG for acetoin production once glucose is exhausted. In this experiment, three concentrations of IPTG (20, 50, and 100 μM) were examined in two strains MEC1023 and MEC981 (**Figure 4**). In this case, the yield was determined after glucose exhaustion. For both strains, 20 μM IPTG resulted in the highest yield of acetoin.

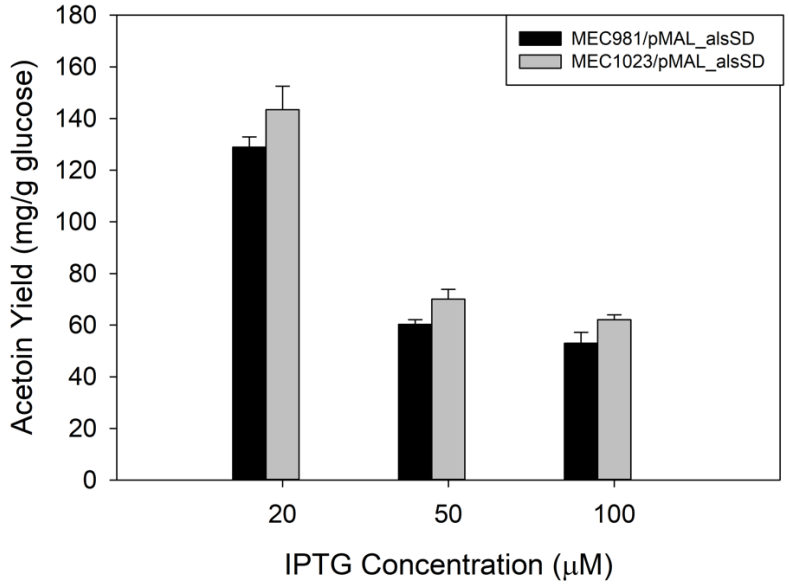

Figure 4: Acetoin yields at different concentrations of IPTG in MEC981/pMAL_*alsSD* and MEC1023/pMAL_*alsSD*

The plasmids 44_ediss and 44_ecloa containing constitutive gene expression for acetoin production were also studied in MEC1023 and MEC981. The method of inoculation was the same as the inducible plasmids. **Figure 5** compares these two constitutive plasmids in MEC981 and MEC1023 to pMAL_*alsSD* induced with 20 μM IPTG. MEC1023 produced the greatest acetoin yields containing the plasmid 44_ediss compared to MEC1023 containing 44_ecloa and pMAL_*alsSD*. MEC1023/44_ediss produced the most similar yield of product (0.18 g/g) to previous experiments utilizing the original plasmid (0.40 g/g) (Erian et al., 2018). These three plasmids in MEC981 produced a similar yield of acetoin with pMAL_*alsSD* producing the least. Of the four plasmids tested, the plasmid 44_ediss produced the highest yield of acetoin and was used in further studies.

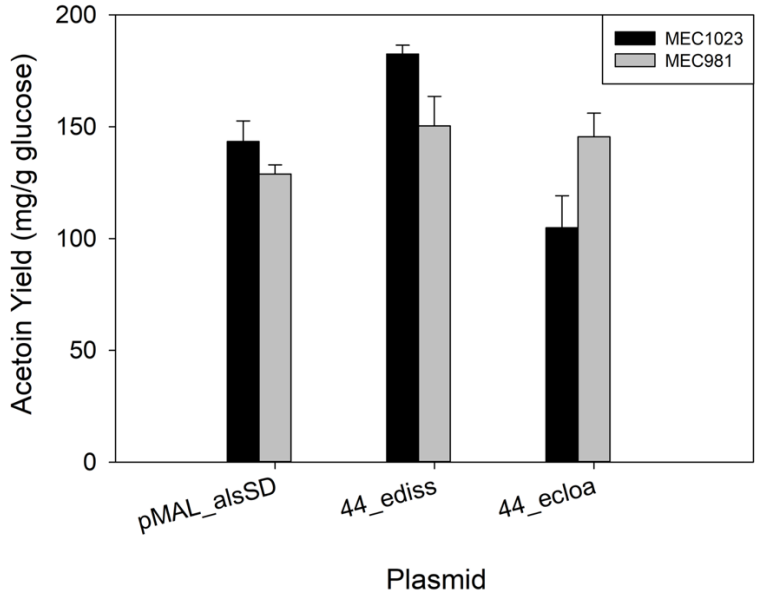

Figure 5: Acetoin yields for pMAL_*alsSD*, 44_ediss, and 44_ecloa in strains MEC1023 and MEC981

PEP synthase

The first hypothesis (A) is that a deletion in the *ppsA* gene coding PEP synthase will increase the yield of acetoin. This hypothesis was tested by comparing the strain MEC981/44_ediss to the strain MEC1023/44_ediss containing the *ppsA* deletion in triplicate shake flask experiments (**Table 4**). MEC1023/44_ediss (Δ*ppsA*) had a 22% greater yield and produced 22% more acetoin compared to MEC981/44_ediss. This increase demonstrates that the deletion of PEP synthase did positively impact the flux of carbon to acetoin.

Table 4: Acetoin produced from nominally 5 g/L glucose in shake flasks using MEC981 or MEC1023 containing the 44_ediss plasmid.

Strain	OD	Acetoin (g/L)	Acetoin Yield (mg/g)
MEC981	3.53 ± 0.09	0.70 ± 0.06	152 ± 14
MEC1023	3.37 ± 0.24	0.86 ± 0.01	186 ± 4

Results are shown as mean values of the replicates ± the standard deviation.

Pyruvate Dehydrogenase Complex

The second hypothesis (B) is that a reduction or deletion in the pyruvate dehydrogenase complex will increase the yield of acetoin. Three variations of the pyruvate dehydrogenase complex component E1 were examined. First, MEC1081 contains a deletion of the *aceE* gene coding the E1 component. Since an Δ*aceE* strain is unable to grow using glucose as the sole carbon source, growth of MEC1081 required supplementing acetate to the medium. Second, the MEC1101 chromosomally expresses the AceE[H106V] variant. This strain was previously shown to accumulate pyruvate at a yield exceeding 0.65 g/g under nitrogen-limited conditions (Moxley and Eiteman, 2021). Third, MEC1102 chromosomally expresses the AceE[E401A] variant. This protein variant results in a lower growth rate, though no pyruvate was generated under non-limiting conditions (Moxley and Eiteman, 2021). Each altered *aceE* allele was integrated in MEC1023. These three strains transformed with the 44_ediss plasmid were compared to MEC1023/44_ediss (wild-type *aceE*) in batch processes at the 1.2-liter scale using a controlled bioreactor.

Batch processes using MEC1023/44_ediss resulted in less than 0.4 g/L acetoin. The yield of acetoin was 9.5 mg/g, which is a 95% decrease from shake flask experiments. An additional experiment was conducted based on previous methodology (Erian et al., 2018). The concentration of kanamycin was increased from 50 mg/L to 150 mg/L. However, after altering the method of inoculation and the concentration of kanamycin, the production of acetoin was consistent to the first batch process of less than 0.4 g/L acetoin. Duplicate batch experiments for MEC1023/44_ediss produced small amounts of acetoin with yields of 4-8 mg/g and productivity of 11 and 21 mg/Lh (**Table 5**).

Table 5: Final product concentrations in batch processes from nominally 40 g/L glucose.

Strain	AceE type	Inoculum Medium	Time (h)	OD	Acetoin (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin Yield (mg/g)	Acetoin Productivity (mg/Lh)
MEC1023	WT AceE	LB	14.8	34.7	0.16	0.00	0.37	4	11
MEC1023	WT AceE	LB	14.8	35.0	0.31	0.00	0.41	8	21
MEC1102	AceE[E401A]	TYA	31.3	38.0	1.13	0.00	0.00	29	36
MEC1102	AceE[E401A]	TYA	30.0	40.0	0.85	0.00	0.35	21	28
MEC1102	AceE[E401A]	T5YA	18.5	33.2	0.10	0.00	0.42	3	5
MEC1102	AceE[E401A]	T5YA	18.5	36.0	0.07	0.00	0.64	2	4
MEC1101	AceE[H106V]	T5YA	22.5	14.9	7.89	0.53	0.00	210	351
MEC1101	AceE[H106V]	T5YA	22.0	20.0	6.76	0.79	0.00	190	307
MEC1081	ΔAceE	T5YA	35.0	21.5	10.4	2.36	0.00	278	324
MEC1081	ΔAceE	T5YA	37.3	16.8	12.1	2.12	0.00	330	298

The AceE[E401A] variant (MEC1102) was expected to be the most similar to the wild type AceE. One set of duplicate batches used TYA medium for the inoculum, but these cultures attained lower ODs compared to the inoculum of LB used for MEC1023/44_ediss. These MEC1102/44_ediss cultures also had a lag time of roughly 24 h followed by 7 h of exponential growth. Greater yields of acetoin were obtained in these cultures compared to MEC1023/44_ediss (**Table 5**). However, because of the long lag time, the acetoin productivity using MEC1102/44_ediss was not significantly higher than the productivity attained using MEC1023/44_ediss. Therefore, an additional duplicate batch studies were conducted with TY5A medium for the inoculum containing 5 g/L yeast extract, a concentration identical to that found in LB medium. These batch fermentations had a shorter lag time with a similar period of exponential growth. However, the final acetoin concentrations were 90% lower than the acetoin concentrations attained from the MEC1102/44_ediss processes using TYA as the medium for the inoculum. All MEC1102/44_ediss batches had a similar amount of acetate produced except one batch that at the end of the fermentation had no detectable acetate. It is possible that the absence of acetate in this one culture was simply a consequence of the culture consuming this chemical, and that the other cultures could have consumed the remaining acetate had the process time been extended.

The more significant variant of the E1 complex of pyruvate dehydrogenase complex studied is the H106V variant (MEC1101). This variant showed a similar lag time to the E401A variant. The maximum OD attained in these cultures was lower than the cell densities observed with MEC1023/44_ediss and MEC1102/44_ediss. In duplicate batch experiments, MEC1101/44_ediss converted 37.5 and 35.5 g/L glucose to 7.89 and 6.76 g/L acetoin, respectively (**Table 5**, **Figure 6**). The acetoin yield attained (210 mg/g and 190 mg/g) and the

acetoin productivities (351 mg/Lh and 307 mg/Lh) during these two batches were consistent (**Table 5**). The chromatography analysis of the medium showed pyruvate as well as a significant unknown peak with a retention time of about 19.2 min. The unknown peak attained a maximum area at 20 h and was depleted by the end of the process, while pyruvate attained a maximum concentration of 3.81 g/L at 19 h and thereafter decreasing to less than 1 g/L (**Figure 6**). The unknown peak is speculated to be acetolactate. Unfortunately, because the chemical is unstable, a purified sample of this compound cannot be purchased commercially.

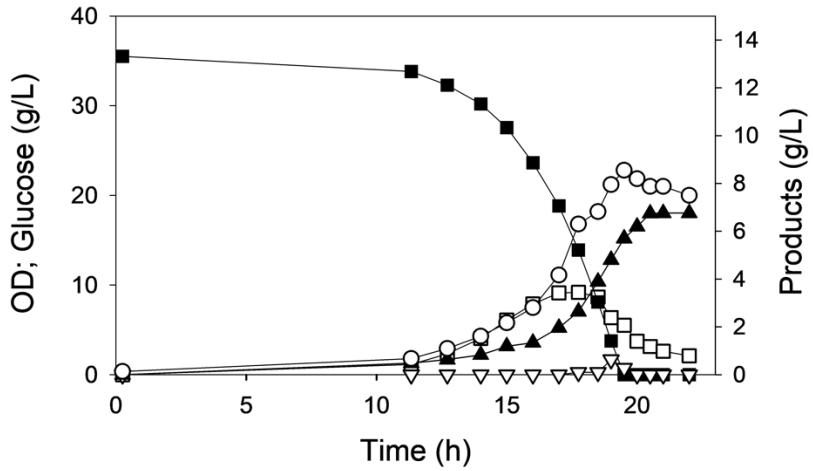

Figure 6: MEC1101/44_ediss batch fermentation time course. Glucose (■), OD (○), pyruvate (□), acetate (▽), and acetoin (▲).

The most severe "variation" of the pyruvate dehydrogenase complex was a deletion of the *aceE* gene (MEC1081), a deletion which necessitates the addition of acetate for growth. Approximately 8 g/L of acetate was supplied to the medium. The culture is anticipated to become starved when this carbon source was depleted, corresponding to the time of the maximum OD. For the cultures, the maximum OD was similar to MEC1101/44_ediss cultures,

and acetate was depleted at 19.5 h, before glucose (**Figure 7**). The culture accumulated pyruvate, and the same unknown peak (retention time of 19.2 minutes) was observed in the chromatographic analysis. The maximum pyruvate was 5.30 g/L at 25 h, and the maximum unknown peak occurred at 26.5 h (**Figure 7**). Ultimately, 10.4 g/L and 12.1 g/L acetoin was generated with yields of 278 and 330 mg/g (**Table 5**). The productivities were 324 and 298 mg/L/h in MEC1081/44_ediss (**Table 5**). While the yield of acetoin was highest in MEC1081/44_ediss, the productivity was similar to MEC1101/44_ediss.

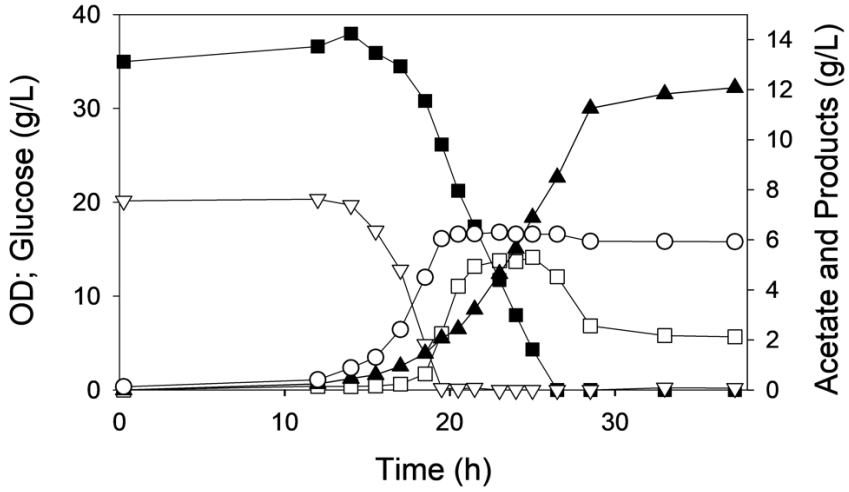

Figure 7: MEC1081/44_ediss batch fermentation time course with 8 g/L acetate. Glucose (■), OD (○), pyruvate (□), acetate (▽), and acetoin (▲).

Nitrogen-Limited Fed-Batch Process

To test the effect of nitrogen-limitation, a nitrogen-limited fed-batch process was used with MEC1101/44_ediss. This strain was chosen because of the high concentration of acetoin produced without require acetate supplementation like MEC1081/44_ediss. The culture had an

initial concentration of 1.0 g/L ammonium chloride so that nitrogen would become limited at an OD of approximately 6. This maximum OD is approximated by the following calculation: in1.0 g/L ammonium chloride, there is 0.26 g/L nitrogen; for every 1.0 g nitrogen, about 8 g dry cell weight is produced (Egli, 2015); for every 0.393 g dry cell weight produced, there is 1 OD unit (Hemshikha Rajpurohit, unpublished work). To avoid nitrogen-starvation, when the culture reached an OD of 3.5, ammonium chloride feed was pumped in at a rate to keep the culture nitrogen-limited and increase the OD of the culture by about an OD of 3 every hour. Initially, the culture was not nitrogen-limited, but quickly the exponential growth rate would cause the culture to deplete the residual nitrogen.

This culture, like the batch cultures of MEC1081/44_ediss and MEC1101/44_ediss, accumulated pyruvate and the unknown having retention time of 19.2 minutes. An additional unknown peak at retention time 22.2 was observed after 17 hours, and the area continued to increase to the end of the process. The maximum area of the unknown HPLC peak (RT = 19.4 minutes) and pyruvate concentration (5.02 g/L) was at 20.5 h (**Figure 8**).

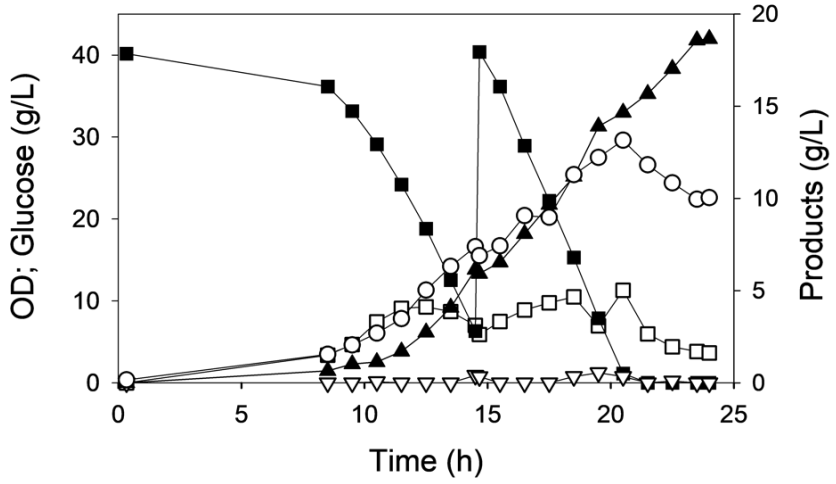

Figure 8: MEC1101/44_ediss nitrogen-limited fed-batch process with additional pulse of glucose time course. Glucose (■), OD (○), pyruvate (□), acetate (▽), and acetoin (▲).

With the addition of 22 mL/h ammonium chloride and 100 mL of 50 g/mL glucose, the fermentation was significantly diluted by water. In order to calculate the yield of acetoin, this dilution effect was considered. The total mass of glucose consumed was 99.24 g, and the total mass of acetoin generated was 29.9 g. Thus, the yield of acetoin was 300 mg/g. The yield from the nitrogen-limited fed-batch process using MEC1101/44_ediss was 50% higher than the yields attained during the batch process from this strain. Moreover, this yield was about the same as the MEC1081/44_ediss batch process.

CHAPTER 6

DISCUSSION

The goal of this study was to increase production of acetoin by increasing the flux of carbon within central metabolism to product formation. This redirection of flux was achieved by deletions (or reductions in activity) to PEP synthase (*ppsA*) and the E1 component of the pyruvate dehydrogenase complex (*aceE*). For the strain that produced a high yield of acetoin in a batch process, a nitrogen-limited fed-batch process was used to increase the yield as this nutrient-limitation has been shown to increase pyruvate production (Zhu et al., 2008). Before these alterations were studied, acetoin must be produced in our base strain MEC981.

The original pTrc99A-*budA*-*alsS* with inducible gene expression met with limited success. Even after troubleshooting expression by varying ampicillin and IPTG concentrations, the highest yield of acetoin produced was 75% lower than previous studies with the genes from the same bacterium (Erian et al., 2018). Other plasmids were examined, with 44_ediss ultimately being chosen and similar yields were produced to the previous study (Erian et al., 2018).

While troubleshooting acetoin production, the effects of ampicillin and IPTG concentration on acetoin production were studied. For pMAL_*alsSD*, the concentration of IPTG at concentrations greater than 500 μM caused minimal product formation (less than 0.2 g/L acetoin) while lower concentrations of IPTG resulted in as much as 0.68 g/L acetoin (20 μM IPTG). Previous studies with pMAL_*alsSD* used 50 μM IPTG (Förster et al., 2017), which did not result in the greatest acetoin formation in the current study (**Figure 4**). For pTrc99A, the IPTG concentration suggested is 1 mM (Amann et al., 1988). However, for pTrc99A-*budA*-*alsS*

even 500 μM resulted in minimal growth and no acetoin production. The IPTG concentration that resulted in the highest yield of acetoin (155±32 mg/g) was 80 μM. Clearly, the plasmids are very sensitive to induction, and attempting to 'over-induce' the culture by using elevated IPTG is detrimental to the culture and to acetoin formation.

PEP synthase is important in glucogenesis when the primary carbon source is pyruvate or lactate (Berman and Cohn, 1970; Niersbach et al., 1992). For strains that accumulate pyruvate, PEP synthase will reassimilate pyruvate when glucose is consumed. By deleting *ppsA*, MEC1023/44_ediss (Δ*ppsA*) produced 22% higher acetoin yield over MEC981/44_ediss. This result supports hypothesis A that the deletion of *ppsA* would increase the yield of acetoin. In batch experiments, MEC1023/44_ediss was shown not to accumulate pyruvate, but MEC1081/44_ediss and MEC1101/44_ediss did accumulate pyruvate. If *ppsA* were intact, the accumulated pyruvate from MEC1081/44_ediss and MEC1101/44_ediss cultures would likely be reassimilated after glucose was consumed as has been shown in pyruvate producing strains with and without the deletion of *ppsA* (Moxley and Eiteman, 2021).

Acetate is a common byproduct of fermentations formed by the conversion of pyruvate by pyruvate oxidase or the hydrolysis of acetyl-CoA (Nielsen et al., 2010). Pyruvate oxidase (*poxB*) was deleted in all strains studied, but acetyl-CoA is necessary for survival (Langley and Guest, 1978). To generate acetyl-CoA, either acetate or ethanol must be supplemented in the medium, or acetyl-CoA must be generated from pyruvate by the pyruvate dehydrogenase complex (Langley and Guest, 1978). The E1 component (*aceE*) had not previously been studied to determine the effect of varying this enzyme activity on acetoin or BDO production. The deletion of *aceEF* was included in the base strain of one study that was able to produce acetoin and BDO, but the effect of the deletion was not studied (Nielsen et al., 2010).

Four strains with presumably different levels of expression of the E1 component of the pyruvate dehydrogenase complex (*aceE*) were tested. MEC1023/44_ediss contains the wild type *aceE* and presumably the highest activity of the pyruvate dehydrogenase complex. The wild type strain produced the lowest concentration of acetoin of all four AceE variations by between 64-97%.

The E401A variant (MEC1102/44_ediss) was expected to be the most similar to the wild type with a slightly lower activity. Because of the long lag times (22 h) of the initial duplicate batch processes, additional duplicate batch processes with new inoculum medium T5YA were conducted ensuring that the inoculum was healthy by harvesting immediately when the culture reached an OD of 4. This batch process had a 50% shorter lag time but resulted in less than 0.1 g/L acetoin. The results using E401A variant with different inoculum cultures seems inconsistent. Moreover, the second set of batch processes using T5YA medium produced a similar amount of acetoin as MEC1023/44_ediss. MEC1102/44_ediss and MEC1023/44_ediss cultures also produced a similar amount of acetate (0.4 g/L) demonstrating the E401A variant did not materially reduce the flux of carbon through the pyruvate dehydrogenase complex compared to the wild type AceE. Both strains also attained a similar maximum OD of 35 indicating insignificant diversion of glucose to acetoin.

Because the H106V variant was previously shown to accumulate pyruvate (Moxley and Eiteman, 2021), MEC1101/44_ediss was expected to produce more acetoin than the wild type AceE and the E401A variant. In duplicate batch processes, MEC1101/44_ediss attained a 97% greater acetoin yield compared to the wild type AceE and an 86% greater yield compared to the E401A variant. MEC1101/44_ediss did not produce acetate and was able to grow without acetate supplementation. These results demonstrated that the H106V variant had a severe enough

mutation to the E1 component of the pyruvate dehydrogenase complex to limit the flux of carbon through the pyruvate dehydrogenase complex. The observation that the maximum OD of MEC1101/44_ediss was about 50% lower than either MEC1102/44_ediss and MEC1023/44_ediss further demonstrates that the flux of carbon was diverted from biomass formation toward acetoin. The elevated acetoin in the H106V variant compared to the wild type AceE and the E401A variant supports hypothesis B that a reduction in the activity of pyruvate dehydrogenase will increase the yield of acetoin.

The deletion of *aceE* requires supplementation of acetate. In Δ*aceE* strains, the "yield" calculated is based on glucose consumed, and since that acetate is converted to biomass, this yield is difficult to compare with strains in which no acetate is supplied. In MEC1081/44_ediss cultures, nominally 8 g/L acetate was supplemented which limited the maximum OD. The two batch processes had slightly differing amount of acetate, and the culture with the lesser amount of acetate had 22% lower OD and a 15% greater acetoin yield. The yield of acetoin (0.33 g/g) produced from the batch process using MEC1081/44_ediss was close to the yield reported from pulse-fed fermentations (0.38 g/g) (Erian et al., 2018).

In MEC1101/44_ediss and MEC1081/44_ediss cultures, an unknown peak was observed in the HPLC analysis. The unknown peak attained maximum area immediately after glucose was depleted, and at that time there remained about 2.0 g/L pyruvate in MEC1101/44_ediss cultures and about 4.5 g/L pyruvate in MEC1081/44_ediss cultures. Both strains fully consumed the unknown compound by the end of the fermentation. The unknown is speculated to be acetolactate, the intermediate in the conversion of pyruvate to acetoin. If the unknown is acetolactate, then the presence of the peak could suggest that conversion of acetolactate to acetoin is slower than the conversion of pyruvate to acetolactate. Once glucose is depleted, the

unknown was consumed in only 1.5 h for MEC1101/44_ediss, which could signify the presence of glucose or exponential phase inhibits conversion.

The fed-batch process with MEC1101/44_ediss maintained a linear increase in OD through nitrogen-limitation. At the end of the process, the yield of acetoin in the nitrogen-limited was 50% greater than the batch culture, supporting hypothesis C that a nitrogen-limitation will increase the yield of acetoin. The additional unknown peak (HPLC retention time 22.2 minutes) present after 17 hours of the process is speculated to be 2,3-butanediol as another study has found the occurrence of a native dehydrogenases capable reducing acetoin to 2,3-butanediol when *ldhA* is deleted (Nielsen et al., 2010).

The increase in glucose consumption rate and pyruvate production rate expected in nitrogen-limited processes seem to be present by the comparing nitrogen-limited and batch processes with MEC1101/44_ediss (Zhu et al., 2008). Initially, both processes had nominally 40 g/L glucose. After about 32 g/L glucose was consumed in both processes, the nitrogen-limited process had an OD of 16, and the batch process had an OD of 22. The OD was 27% less in the nitrogen-limited culture as a consequence of the growth being limited by the amount of nitrogen supplied. Even though the OD was lower, 32 g/L glucose was consumed 5 hours faster in the nitrogen-limited process than the batch process. Since in the nitrogen-limited process the glucose did not go to biomass, presumably glucose was redirected to acetoin production. After 32 g/L glucose was consumed in both processes, the nitrogen-limited process was 12% higher than the batch process.

CHAPTER 7

CONCLUSIONS

Overall, the goal of increasing acetoin yield by genetic modifications and nutrient-limited processes was achieved. The process took many iterations of troubleshooting and ultimately high yields of acetoin were produced. The best strains for producing acetoin were MEC1101/44_ediss (H106V variant) and MEC1081/44_ediss (Δ*aceE*). Although in batch experiments more acetoin was produced using MEC1081/44_ediss, having to supplement acetate for growth adds an expense.

One important observation that came from this study was plasmid loss. The initial attempts were to use pTrc99A and insert the genes necessary to produce acetoin. Gene expression was difficult to achieve and led to troubleshooting aspects like antibiotic concentration, IPTG concentration, and induction time. Even after troubleshooting, plasmid was still lost, and expression levels were lower than expected. One possibility is that the *E. coli* C strain contributed plasmid loss. Almost all previous studies on acetoin and butanediol formation used K-12 strains of *E. coli*. Using a different strain of *E. coli* with the same set of knockouts as MEC1101/44_ediss could improve plasmid retention and the amount of acetoin produced.

Hypothesis A was only supported by one shake flask study comparing MEC1023/44_ediss and MEC981/44_ediss. The increase of acetoin produced was significant in MEC1023/44_ediss (Δ*ppsA*). Using additional strains to compare the effect of *ppsA* deletion would provide greater evidence that this deletion improved acetoin production. Specifically,

studying strains that are known to accumulate pyruvate like MEC1081/44_ediss and MEC1101/44_ediss could provide greater evidence on the effect of PEP synthase.

In general, the greater the OD, the less acetoin seems to be produced. Specifically, for MEC1101/44_ediss and MEC1081/44_ediss, the cultures with lower ODs resulted in higher yields of acetoin. This result makes sense since the amount of carbon source is fixed, and this element can either be metabolized to generate biomass or be converted into acetoin. Additional studies would be needed for both strains to confirm this observation. For MEC1081/44_ediss, the growth is limited by acetate, but for MEC1101/44_ediss, the cause of the lower OD in one culture is harder to elucidate as the culture is limited by glucose.

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APPENDICES

APPENDIX A: PRIMERS

Table A.1: Primers used in this study

Name	Sequence
budA-HA-ptrc-F-V2	TTCACACAGGAAACAGACCATGATGCATAGCAGCGCATG
ptrc-HA-budA-R-V2	CATGCGCTGCTATGCATCATGGTCTGTTTCCTGTGTG
als-F-budA-HA	GTTGAAAACTAACAGGAGAACTACCATGAATAGC
budA-R-als-HA	GCTATTCATGGTAGTTCTCCTGTTAGTTTTCAAC
ptrc-F-HA-als	CACTATAAGGACAGAGAAGATCCTCTAGAGTCGACCTGCAGG
als-R-HA-ptrc	GGTCGACTCTAGAGGATCTTCTCTGTCCTTATAGTGAG
pKD4-REB-aceE-F	ACAGGTTCCAGAAAACTCAACGTTATTAGATAGATAAGGAAT AACCCATGGTGTAGGCTGGAGCTGCTTC
pKD4-REB-aceE-R	GATTTCGATAGCCATTATTCTTTTACCTCTTACGCCAGACGCG GGTTAACCATATGAATATCCTCCTTAG
KD4_RB_ldhA_F	TATTTTTAGTAGCTTAAATGTGATTCAACATCACTGGAGAAAG TCTTATGGTGTAGGCTGGAGCTGCTTC
KD4_RB_ldhA_R1	CTCCCCTGGGTTGCAGGGGAGCGGCAAGATTAAACCAGTTCG TTCGGGCACATATGAATATCCTCCTTAG
ediss budC fw	TTCATACCTCGCCGGGCCAGACTCTGACTACATGACTGGTCAG TC
ediss budC rev	CTGGCCCGGCGAGGTATGAAGTCTCATCGTTGTAGTCGGCGA TAG
ecloa budC fw	TTCGTATCTCGCCGGGCCAGATTCCGACTACATGACCGGCCA GTC
ecloa budC rev	CTGGCCCGGCGAGATACGAATCGTTATAGTCGGCGATAGCGA CAG
aceE_F	TGAGCGTTCTCTGCGTCGTCTGGA G
aceE_R	ATCGCCAACAGAGACTTTGATCTC
P-Kanconf_R	CTTTTCCTTACCCATGGTTGT
44_ediss REB fw	TGCTCGACTATCAGTTGGAG
44_ecloa REB FW	CAAGCTAATGATTGACCTGC
ppsA_5	CGCACAGAAGCGTAGAACGTTATG
ppsA_3	CGTTTAGGTGAACGATCATGCGC
ldhA_F	TTAAGCATTCAATACGGGTATTGTG
ldhA_R	GTCATTACTTACACATCCCGCCATC
poxB_5	CCGGTTGTCGCTGCCTGC
poxB_3	TTCAAACAGATAGTTATGCGCGGCC

APPENDIX B: BATCH DATA

Date	4/8/21
Strain	MEC 1023/44_ediss

Inoculum	
Medium	LB + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	10:00 PM
Start Date	4/7/21
End Time	8:15 AM
Duration	10 h
End OD	4.97

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10
Inoculum OD	52
Agitation	400 rpm
Start Time	9:45 am
Start Date	4/8/21
Initial OD	0.456

Table B.1: MEC1023/44_ediss Batch Data A on April 8th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
9:45 AM	0.00	6.96	0.46	39.33	0.01	0.00	0.00	0	0	0	0	98
10:45 AM	1.00	7.02	0.47	38.71	0.00	0.00	0.00	0	0	0	0	100
1:45 PM	4.00	6.99	0.60	36.67	0.05	0.00	0.00	0	0	0	0	109
3:15 PM	5.50	6.88	1.05	36.47	0.07	0.02	0.00	0	0	1	0	106
4:30 PM	6.75	6.87	1.84	37.84	0.07	0.07	0.06	0	0	3	0	96
5:45 PM	8.00	7.02	3.64	36.79	0.07	0.05	0.00	0	0	5	0	80
7:15 PM	9.50	6.90	7.96	31.39	0.12	0.15	0.08	0	0	11	0	40s
8:30 PM	10.75	6.87	15.20	25.07	0.18	0.35	0.09	0	0	20	0	40s
9:30 PM	11.75	6.83	21.41	17.95	0.20	0.56	0.12	0	0	25	0	40s
10:45 PM	13.00	6.60	30.10	9.84	0.06	0.25	0.14	0	0	28	0	40s
11:45 PM	14.00	6.74	32.56	3.80	0.10	0.37	0.16	0	0	32	0	40s
12:30 AM	14.75	-	34.74	0.07	0.00	0.37	0.16	0	0	34	0	100

Notes:

5:55 pm 100 μL 1% antifoam and turned-on oxygen flow

7:08 pm 100 μL 1% antifoam

7:55 pm 100 μL 1% antifoam

8:15 pm 200 μL 1% antifoam

8:35 pm 200 μL 1% antifoam

9:00 pm 100 μL 1% antifoam

9:20 pm 300 μL 1% antifoam

9:36 pm 400 μL 1% antifoam

10:50 pm 400 μL 1% antifoam

11:25 pm 400 μL 1% antifoam

12:12 am 250 μL 5% antifoam

Date	4/8/21
Strain	MEC 1023 44_ediss

Inoculum	
Medium	LB + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	10:00 PM
Start Date	4/7/21
End Time	8:15 AM
Duration	10 h
End OD	4.77

Fermentor	RIGHT F
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10 mL
Inoculum OD	48
Agitation	400 rpm
Start Time	9:45 AM
Start Date	4/8/21
Initial OD	0.44

Table B.2: MEC1023/44_ediss Batch Data B on April 8th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
9:45 AM	0.00	6.93	0.44	39.79	0.00	0.00	0.00	0	0	0	0	98
10:45 AM	1.00	6.99	0.46	39.00	0.04	0.00	0.00	0	0	0	0	100
1:45 PM	4.00	6.91	0.69	37.12	0.06	0.00	0.00	0	0	0	0	100
3:15 PM	5.50	6.95	1.03	36.74	0.07	0.02	0.00	0	0	1	0	97
4:30 PM	6.75	6.90	2.04	37.63	0.10	0.02	0.00	0	0	2	0	80s
5:45 PM	8.00	6.98	3.70	36.74	0.08	0.05	0.07	0	0	5	0	80s
7:15 PM	9.50	6.85	7.04	32.42	0.14	0.21	0.11	0	0	9	0	40s
8:30 PM	10.75	6.80	12.25	27.16	0.20	0.37	0.14	0	0	15	0	40s
9:30 PM	11.75	6.68	17.80	21.30	0.22	0.48	0.19	0	0	18	0	40s
10:45 PM	13.00	6.67	28.90	11.59	0.20	0.25	0.27	0	0	25	0	40s
11:45 PM	14.00	6.87	31.38	4.53	0.13	0.35	0.30	0	0	33	0	40s
12:30 AM	14.75	-	35.00	0.07	0.00	0.41	0.31	0	0	34	0	100

Notes:

6:07 pm 100 μL 1% antifoam turned on oxygen

7:30 pm 100 μL 1% antifoam

8:00 pm 200 μL 1% antifoam

8:55 pm 200 μL 1% antifoam

9:22 pm 200 μL 1% antifoam

9:42 pm 200 μL 1% antifoam

10:08 pm 200 μL 1% antifoam

10:55 pm 250 μL 1% antifoam

11:30 pm 350 μL 1% antifoam

11:40 3pm 50 μL 5% antifoam

Date	4/12/21
Strain	MEC1102/44_ediss

Inoculum	
Medium	TYA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	8:05 PM
Start Date	4/11/21
End Time	8:45 AM
Duration	12.5 hours
End OD	2.56

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10 mL
Inoculum OD	
Agitation	400 rpm
Start Time	9:50 AM
Start Date	4/11/21
Initial OD	0.25

Table B.3: MEC1102/44_ediss Batch Data A on April 12th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
10:00 AM	0.00	6.81	0.25	38.65	0.00	0.00	0.00	0	0	0	0	98
10:30 AM	24.50	6.99	1.40	38.05	0.11	0.11	0.08	0	0	1	0	-
12:30 PM	26.50	6.80	4.80	35.15	0.20	0.02	0.24	0	0	7	0	-
1:30 PM	27.50	6.91	9.10	30.81	0.14	0.02	0.42	0	0	13	0	-
2:30 PM	28.50	6.86	19.30	22.61	0.19	0.19	0.71	0	0	-	0	-
3:15 PM	29.25	-	26.50	13.28	0.21	0.42	1.02	0	0	33	0	-
4:15 PM	30.25	6.90	39.00	3.67	0.14	0.37	1.19	0	0	39	0	-
5:15 PM	31.25	-	38.00	0.01	0.15	0.00	1.13	0	0	39	0	-

Notes:

Having issues with DO probe so I did not take readings

12:30 pm 100 μL 1% antifoam and turned oxygen on

2 pm 200 μL 1% antifoam

2:30 pm 300 μL 1% antifoam

2:45 pm 200 μL 5% antifoam

3:30 pm 250 μL 5% antifoam

Date	4/12/21
Strain	MEC1102/44_ediss

Inoculum	
Medium	TYA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	8:05 PM
Start Date	4/11/21
End Time	8:45 AM
Duration	12.5 hours
End OD	2.56

Fermentor	RIGHT F
	MXWT with 25 mM
Medium	MOPS (2)
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	12
Inoculum OD	22
Agitation	400 rpm
Start Time	9:50 AM
Start Date	4/11/21
Initial OD	0.27

Table B.4: MEC1102/44_ediss Batch Data B on April 12th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
10:00 AM	0.00	6.88	0.27	39.81	0.00	0.00	0.00	0	0	0	0	100
10:30 AM	24.50	6.83	3.40	36.42	0.16	0.01	0.17	0	0	1	0	100
11:45 AM	25.75	6.86	8.59	32.74	0.14	0.04	0.34	0	0	9	0	40
12:45 PM	26.75	6.80	16.00	25.31	0.29	0.34	0.53	0	0	16	0	40
1:45 PM	27.75	6.70	27.80	13.99	0.22	0.16	0.89	0	0	30	0	40
2:45 PM	28.75	6.75	40.00	4.66	0.14	0.26	0.99	0	0	38	0	50
4:00 PM	30.00	-	40.00	0.01	0.00	0.35	0.85	0	0	38	0	70

Notes:

11:00 am Do started dropping without use of antifoam

11:30 am 100 μL 1% antifoam and turned on oxygen

12:30 pm 100 μL 1% antifoam

1:15 pm 400 μL 1% antifoam

1:40 pm 200 μL 1% antifoam

2 pm 100 μL 5% antifoam

2:30 pm 200 μL 5% antifoam

3:45 pm no longer need o2

Date	5/5/21
Strain	MEC1102/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	10:10 PM
Start Date	5/4/21
End Time	8:40 AM
Duration	10.5 h
End OD	4.89

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10
Inoculum OD	54.8
Agitation	400 rpm
Start Time	9:55 AM
Start Date	5/5/21
Initial OD	0.45

Table B.5: MEC1102/44_ediss Batch Data A on May 5th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
10:10 AM	0.25	6.81	0.45	40.62	0.00	0.00	0.00	0	0	0	0	100
3:55 PM	6.00	7.05	0.48	40.24	0.00	0.00	0.00	0	0	1	0	93
9:50 PM	12.00	7.00	1.02	39.57	0.10	0.09	0.00	0	0	2	0	96
11:55 PM	14.00	6.98	2.78	37.72	0.16	0.14	0.00	0	0	4	0	75
12:55 AM	15.00	6.96	5.01	36.27	0.14	0.16	0.00	0	0	6	0	70
1:55 AM	16.00	6.86	9.52	32.43	0.19	0.21	0.05	0	0	11	0	40
2:55 AM	17.00	6.97	19.00	23.98	0.42	0.43	0.06	0	0	23	0	40
3:55 AM	18.00	6.75	31.60	13.43	0.39	0.65	0.09	0	0	31	0	40
4:25 AM	18.50	6.79	33.20	7.93	0.34	0.42	0.10	0	0	35	0	40

Notes:

12:37 pm 100 μL 1% antifoam and turned on oxygen

2:02 pm 200 μL 1% antifoam and need a lot of oxygen

2:50 pm 200 μL 1% antifoam

3:15 pm 200 μL 1% antifoam

4:00 pm 100 μL 5% antifoam

Date	5/5/21
Strain	MEC1102/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	10:10 PM
Start Date	5/4/21
End Time	8:40 AM
Duration	10.5 h
End OD	4.56

Fermentor	RIGHT F MXWT with 25 mM
Medium	MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10
Inoculum OD	50.90
Agitation	400 rpm
Start Time	9:55 AM
Start Date	5/5/21
Initial OD	0.37
Date	4/12/21
Strain	MEC1102/44_ediss

Table B.6: MEC1102/44_ediss Batch Data B on May 5th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
10:10 AM	0.25	6.97	0.37	39.34	0.00	0.00	0.00	0	0	0	0	99
3:55 PM	6.00	6.99	0.46	38.94	0.00	0.00	0.00	0	0	1	0	99
9:50 PM	12.00	7.03	1.10	38.97	0.10	0.09	0.00	0	0	2	0	80
11:55 PM	14.00	6.93	3.47	35.73	0.19	0.14	0.00	0	0	5	0	50
12:55 AM	15.00	6.85	6.14	33.35	0.14	0.18	0.00	0	0	8	0	60
1:55 AM	16.00	6.82	12.66	27.60	0.19	0.26	0.00	0	0	15	0	40
2:55 AM	17.00	6.81	23.70	18.26	0.40	0.60	0.06	0	0	32	0	-
3:55 AM	18.00	6.75	33.30	7.94	0.42	0.72	0.31	0	0	39	0	-
4:25 AM	18.50	6.92	36.00	3.58	0.00	0.64	0.07	0	0	43	0	-

Notes:

DO probe did not work. Most likely went anaerobic

12:10 pm turned on oxygen

12:37 pm 100 μL 1% antifoam

1:25 pm 100 μL 1% antifoam

2 pm 100 μL 1% antifoam

2:05 pm 200 μL 1% antifoam

2:30 pm 100 μL 5% antifoam

2:50 pm 200 μL 1% antifoam

3:13 pm 200 μL 1% antifoam

3:22 pm 100 μL 5% antifoam

4:00 pm 100 μL 5% antifoam

I could not control DO

Date	4/26/21
Strain	MEC1101/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	2:00 PM
Start Date	4/26/21
End Time	11:15 PM
Duration	9:15
End OD	4.1

Fermentor	LEFT E2
	MXWT with 25 mM
Medium	MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	9 mL
Inoculum OD	53.5
Agitation	400 rpm
Start Time	12:15 AM
Start Date	4/27/21
Initial OD	0.345

Table B.7: MEC1101/44_ediss Batch Data A on April 27th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
12:30 AM	0.25	7.08	0.35	37.55	0.00	0.00	0.00	0	0	0	0	100
10:45 AM	10.50	7.03	1.36	35.10	0.25	0.00	0.40	0	0	2	0	100
1:15 PM	13.00	7.01	2.77	33.38	0.77	0.00	0.76	0	0	6	0	97
2:45 PM	14.50	6.95	4.44	31.16	1.56	0.00	0.95	0	0	11	0	76
4:15 PM	16.00	6.94	6.80	26.41	2.56	0.00	1.47	10336	0	17	0	70
5:45 PM	17.50	6.91	11.50	19.39	3.62	0.00	2.57	26565	0	25	0	65
7:15 PM	19.00	6.81	19.00	7.98	3.81	0.00	4.51	52058	0	36	0	50s
8:45 PM	20.50	7.02	22.70	0.00	1.59	0.00	6.53	98137	0	43	3	90s
10:40 PM	22.50	7.08	14.90	0.00	0.53	0.00	7.89	0	0	43	6	130

Notes:

DO probe did not work. Most likely went anaerobic

12:10 pm turned on oxygen

12:37 pm 100 μL 1% antifoam

1:25 pm 100 μL 1% antifoam

2 pm 100 μL 1% antifoam

2:05 pm 200 μL 1% antifoam

2:30 pm 100 μL 5% antifoam

2:50 pm 200 μL 1% antifoam

3:13 pm 200 μL 1% antifoam

3:22 pm 100 μL 5% antifoam

4:00 pm 100 μL 5% antifoam

I could not control DO

Date	4/28/21
Strain	MEC1101/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	1:10 PM
Start Date	4/28/21
End Time	11:40 PM
Duration	10.5 h
End OD	4

Fermentor	RIGHT F
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10 mL
Inoculum OD	48
Agitation	400
Start Time	12:40 AM
Start Date	4/29/21
Initial OD	0.35

Table B.8: MEC1101/44_ediss Batch Data B on April 29th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
12:55 AM	0.25	7.06	0.35	35.51	0.00	0.00	0.00	0	0	0	0	100
12:00 PM	11.33	6.95	1.80	33.84	0.43	0.00	0.483	0	0	2	0	100
1:20 PM	12.72	7.02	2.92	32.30	0.87	0.00	0.632	0	0	6	0	100
2:40 PM	14.00	7.17	4.29	30.20	1.52	0.00	0.826	0	0	10	0	100
3:40 PM	15.00	7.05	5.78	27.57	2.28	0.00	1.189	4463	0	14	0	100
4:40 PM	16.00	6.92	7.48	23.64	2.96	0.11	1.34	10455	0	19	0	100
5:40 PM	17.00	6.98	11.10	18.84	3.42	0.14	1.965	23683	0	24	0	100
6:25 PM	17.75	6.91	16.80	13.90	3.44	0.26	2.636	37167	0	30	0	70
7:10 PM	18.50	6.87	18.20	8.10	3.25	0.32	3.89	52441	0	34	0	30-70
7:40 PM	19.00	6.96	21.20	3.76	2.39	0.63	4.81	55878	0	40	0	80
8:10 PM	19.50	7.31	22.80	0.00	2.07	0.40	5.70	84801	0	42	2	100
8:40 PM	20.00	7.06	21.86	0.00	1.40	0.10	6.20	44949	0	0	3	100
9:10 PM	20.50	7.10	21.00	0.00	1.18	0.00	6.75	14748	0	0	1	100
9:40 PM	21.00	7.06	21.00	0.00	0.98	0.00	6.77	0	0	0	-	100
10:40 PM	22.00	7.04	20.00	0.00	0.79	0.00	6.76	0	0	0	2	100

Notes:

Had a hard time with the cells in the inoculum. They would just clump.

So the inoculum OD may be wrong. Also lost cells at the bottom of the falcon tube.

Having DO problems. I do not trust the reading.

2:30 pm 100 μL 1% antifoam

4:25 pm 100 μL 1% antifoam and turned on oxygen

5:53 pm 100 μL 1% antifoam

7:20 pm 300 μL 1% antifoam and DO dropped significantly

8:13 pm 150 μL 5% antifoam

8:30 pm No oxygen needed

Date	5/16/21
Strain	MEC1081/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	10:35 AM
Start Date	5/16/21
End Time	10:30 PM
Duration	12 h
End OD	3.712

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Acetate	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10
Inoculum OD	44.2
Agitation	400 rpm
Start Time	11:25 PM
Start Date	5/16/21
Initial OD	0.34

Table B.9: MEC1081/44_ediss Batch Data A on May 16th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
11:40 PM	0.25	6.50	0.34	34.99	0.00	7.562	0.00	0	0	0	0	100
11:25 AM	12.00	7.04	1.09	36.602	0.147	7.627	0.241	0	0	2	1	98
1:30 PM	14.00	6.97	2.35	37.979	0.137	7.389	0.456	0	0	2	2	95
2:55 PM	15.50	7.04	3.48	35.931	0.164	6.361	0.61	0	0	2	3	75
4:25 PM	17.00	7.03	6.44	34.505	0.232	4.8	0.95	0	0	2	4	6070
5:55 PM	18.50	6.99	12.00	30.809	0.644	1.823	1.458	0	0	2	7	50
6:55 PM	19.50	7.10	16.12	26.17	2.247	0.07	2.067	0	0	4	9	80
7:55 PM	20.50	6.88	16.60	21.23	4.148	0.046	2.434	17267	0	10	9	90
8:55 PM	21.50	6.96	16.65	17.44	4.937	0.076	3.216	45227	0	15	9	100
10:25 PM	23.00	6.98	16.81	11.71	5.162	0.00	4.644	88000	0	18	9	70
11:25 PM	24.00	6.97	16.61	7.97	5.132	0.00	5.653	109968	0	21	9	70
12:25 AM	25.00	6.98	16.60	4.33	5.301	0.00	6.894	119237	0	23	9	60
1:55 AM	26.50	6.98	16.60	0.00	4.522	0.00	8.498	122441	0	25	10	110
3:55 AM	28.50	6.96	15.83	0.00	2.561	0.00	11.257	37183	0	25	17	100
8:20 AM	33.00	7.02	15.82	0.00	2.175	0.00	11.832	0	0	25	19	120
12:40 PM	37.25	7.00	15.80	0.00	2.122	0.00	12.084	0	0	25	21	120

Notes:

turned on oxygen at 3:45 pm

5:27 pm added 75 μL 1% antifoam

6:12 pm added 100 μL 1% antifoam

7:00 pm added 100 μL 1% antifoam

8:00 pm added 200 μL 1% antifoam

turned off oxygen at 10 pm

Date	5/17/21
Strain	MEC1081/44_ediss

Inoculum	
Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	8:30 AM
Start Date	5/17/21
End Time	8:45 AM
Duration	~12h
End OD	3.766

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	8 g/L
Acetate	8 g/L
Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10
Inoculum OD	48.8
Agitation	400 rpm
Start Time	9:50 PM
Start Date	5/17/21
Initial OD	0.325

Table B.10: MEC1081/44_ediss Batch Data B on May 17th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
10:05 PM	0.25	6.66	0.33	37.40	0.00	8.12	0.00	0	0	0	0	100
11:20 PM	13.50	7.02	2.34	35.65	0.177	7.121	0.44	0	0	0	1	99
1:20 PM	15.50	7.02	4.75	34.18	0.35	5.67	0.74	0	0	0	3	95
2:50 PM	17.00	7.05	7.80	31.92	0.554	3.64	1.04	0	0	0	5	95
3:50 PM	18.00	6.99	12.70	29.11	1.17	2.06	1.62	0	0	2	7	70
4:50 PM	19.00	6.86	17.40	23.00	2.36	0.83	2.07	0	0	4	7	55
5:50 PM	20.00	6.97	19.90	16.20	4.93	0.00	2.56	24687	0	9	7	50
6:50 PM	21.00	6.98	21.10	9.83	6.63	0.10	3.37	61799	0	24	7	70
8:20 PM	22.50	6.98	21.50	2.29	6.25	0.00	5.34	128450	0	30	7	60
9:20 PM	23.50	7.04	20.60	0.00	4.20	0.00	6.87	147007	0	31	10	50
10:20 PM	24.50	7.03	20.40	0.00	2.30	0.00	8.05	97337	0	31	12	38
9:00 AM	35.00	7.03	16.60	0.00	2.36	0.00	10.41	0	0	31	21	33

Notes:

3:03 pm added 100 μL 1% antifoam and turned on oxygen

3:50 pm added 100 μL 1% antifoam

4:34 pm added 100 μL 1% antifoam

5:00 pm added 100 μL 1% antifoam

5:15 pm added 200 μL 1% antifoam

5:30 pm added 200 μL 1% antifoam

5:45 pm added 200 μL 1% antifoam

6:00 pm added 100 μL 1% antifoam

6:09 pm added 200 μL 1% antifoam

6:23 pm added 100 μL 5% antifoam

6:50 pm added 100 μL 5% antifoam

Switched to only air at 10pm

APPENDIX C: NITROGEN-LIMITATION DATA

Date	5/29/21
Strain	MEC1101/44_ediss

Inoculum

Medium	T5YA + 1 g/L glucose
Volume	125 mL
Agitation	250 rpm
Start Time	12:05 PM
Start Date	5/28/21
End Time	10:15 PM
Duration	10 h
End OD	4.01

Fermentor	LEFT E2
Medium	MXWT with 25 mM MOPS
Glucose	40 g/L
NH4Cl	1 g/L

at OD of 3.5, turn on feed in 0.5 g NH4Cl/h (0.3 ml/min flow rate)

Kanamycin	150 mg/L
Volume	1.2 L
Inoculum V	10 mL
Inoculum OD	45
Agitation	400
Start Time	11:40 PM
Start Date	5/28/21
Initial OD	0.352

Table C.1: MEC1101/44_ediss Nitrogen-Limited Fed-Batch Data on May 28th, 2021

Time (clock)	Time (h)	pH	OD	Glucose (g/L)	Pyruvate (g/L)	Acetate (g/L)	Acetoin (g/L)	Unknown at 19.4 (area)	Unknown at 22.2 (area)	Cumulative Base (mL)	Cumulative Acid (mL)	approx DO
12:00 AM	0.33	7.37	0.35	40.16	0.00	0.00	0.00	0	0	0	0	100
8:10 AM	8.50	7.30	3.47	36.15	1.50	0.00	0.65	0	0	14	0	75
9:10 AM	9.50	7.12	4.61	33.16	2.08	0.00	1.02	0	0	16	2	70
10:10 AM	10.50	6.88	6.06	29.11	3.31	0.06	1.13	10509	0	20	2	60
11:10 AM	11.50	6.93	7.82	24.19	4.04	0.00	1.71	14521	0	26	2	60
12:10 PM	12.50	6.90	11.30	18.82	4.12	0.00	2.74	35633	0	30	2	70
1:10 PM	13.50	6.91	14.20	12.53	3.86	0.00	4.09	59527	0	34	2	70
2:10 PM	14.50	6.91	16.60	6.31	3.11	0.41	6.14	73436	0	38	2	70
2:20 PM	14.67	6.77	15.50	40.38	2.62	0.33	5.93	74314	0	38	2	50
3:10 PM	15.50	6.91	16.70	36.14	3.33	0.00	6.54	86795	0	42	2	70
4:10 PM	16.50	6.91	20.40	28.93	3.95	0.00	8.08	95354	0	47	2	40-120
5:10 PM	17.50	6.91	20.20	22.21	4.34	0.00	9.67	102396	5303	51	2	60
6:10 PM	18.50	6.88	25.40	15.28	4.66	0.34	11.19	107544	6885	56	2	40-120
7:10 PM	19.50	6.90	27.50	7.87	3.08	0.54	13.91	97118	7092	61	2	40-120
8:10 PM	20.50	6.85	29.60	1.13	5.02	0.34	14.66	144357	6603	65	2	40-120
9:10 PM	21.50	7.00	26.60	0.00	2.65	0.00	15.68	120421	7405	66	7	80
10:10 PM	22.50	7.01	24.40	0.00	1.95	0.10	17.03	45457	8190	67	13	60
11:10 PM	23.50	6.96	22.40	0.00	1.70	0.00	18.58	0	15983	67	17	70
11:40 PM	24.00	6.89	22.60	0.00	1.62	0.00	18.65	0	18189	67	17	80

Notes:

	DO probe 3 didn't work. Used DO probe 4. The DO probe 4 never had time to polarize so that I could span it appropriately. By the time I got there in the morning I had to add antifoam so I had no good way of approximating what the DO should have been when I arrived.
8:10 AM	When I arrived at riverbend, the culture had foamed out of the reactor. It was not super significant maybe 20 mL. The foam out probably occurred less than 15 minutes before I arrived. Had a significantly shorter lag time than usual (took 14 h previously). Turned on pump of ammonium chloride feed. Cannot tell if the ammonium chloride is reaching the broth because the inlet stream is suspended in the foam at the top that I cannot remove
8:37 AM	Foam has dissipated and I can tell ammonium chloride is being added
8:00 AM	added 200 μL 1% antifoam
9:40 AM	added 100 μL 1% antifoam
10:00 AM	turned on oxygen (only needed less than 0.2 L/min at this point)
10:15 AM	added 100 μL 1% antifoam
10:40 AM	added 250 μL 1% antifoam
12:15 PM	added 300 μL 1% antifoam
2:15 PM	Adding dose of glucose
2:15 PM	after adding the dose of glucose. The DO was hard to control.
2:45 PM	added 100 μL 1% antifoam

3:37 PM added 100 μL 1% antifoam

3:58 PM added 250 μL 1% antifoam

4:03 PM added 200 μL 1% antifoam

5:40 PM added 300 μL 1% antifoam

5:55 PM added 150 μL 1% antifoam

6:05 PM added 200 μL 1% antifoam

8:30 PM added 350 μL 1% antifoam

8:30 PM turned off oxygen.

9:00 PM turned off NH4Cl feed pump

In total, I approximately used about 280 mL of ammonium chloride

9:50 PM added 400 μL 1% antifoam

9:54 PM added 300 μL 1% antifoam

9:57 PM added 300 μL 1% antifoam

10:03 PM added 250 μL 1% antifoam