BALLENTINE, BRITTNEY R. M.S. Developing an Enzymatic Synthesis for Acetoacetyl-CoA Using Acetyl-CoA Acetyltransferase (AtoB) From *Escherichia coli.* (2022)
Directed by Dr. Jason Reddick. 50 pp.

Polyketides are natural products that can be found in clinical drugs that are used as antibiotics, parasiticides, immunomodulators, and many others.[1] Polyketide biosynthesis involves a complex pathway catalyzed by polyketide synthases. The Reddick laboratory at UNC-Greensboro has been studying the biosynthesis of the β-branch from bacilliene and difficidin, both from *Bacillus* species. All the steps for the biosynthesis of the β-branch of difficidin have been reconstituted except for the key C-C bond forming step catalyzed by the enzyme DfnL, which is a homolog of hydroxymethylglutaryl (HMG)-coenzyme A (CoA) synthase. Homologs of this enzyme adds an acetyl group to the nascent β-ketoacyl intermediate to form an HMG-like intermediate which is dehydrated and decarboxylated to produce the β-branch of difficidin. Since the true β-ketoacyl substrate used during difficidin biosynthesis is a long and complex structure, the Reddick lab has used acetoacetyl-CoA as a shortened model of this β-ketoacyl intermediate. For a while, many well-known science and technology companies, such as Sigma-Aldrich and Fisher, stopped producing acetoacetyl-CoA. Without access to acetoacetyl-CoA, we could not continue with experiments designed to complete the reconstitution of the biosynthetic pathway of the β-branch for difficidin. To solve this ongoing issue of supply availability, we are attempting to develop our own method for producing acetoacetyl-CoA using enzymatic synthesis rather than organic synthesis; due to slow reaction rate and the difficulty with breaking carbon-carbon bonds in organic synthesis. In doing so we will be able to continue our research in completing the biosynthesis of the β-branch of bacilliene and difficidin. The purpose of this project is to develop an enzymatic procedure preparing acetoacetyl-CoA using the enzyme

encoded by the *atoB* gene from *Escherichia coli* (*E. coli*). The central hypothesis is that AtoB synthesizes acetoacetyl-CoA from acetyl-CoA. The rationale that underlies the research proposed here is that we will have access to acetoacetyl-CoA without the need to depend on chemical companies for a commercial supply. This will also be a benefit to any biochemistry project needing acetoacetyl-CoA. We tested this hypothesis by first cloning *atoB* into a vector for expression and purification of the enzyme, purified AtoB by Nickel-Affinity Column Chromatography, and then measured the activity using UV-Vis Spectrophotometry, while also attempting Liquid Chromatography-Mass Spectrometry (LC-MS). Acetoacetyl-CoA formation and activity has been detected by a UV-based assay.

DEVELOPING AN ENYMATIC SYNTHESIS FOR ACETOACETYL-COA USING ACETYL-COA ACETYLTRANSFERASE (ATOB) F ROM *ESCHERICHIA COLI*

by

Brittney R. Ballentine

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Dr. Jason Reddick
Committee Chair

APPROVAL PAGE

This thesis written by Brittney R. Ballentine has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair

Dr. Jason Reddick

Committee Members

Dr. Kimberly Petersen

Dr. Jonathan Chekan

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May 26, 2022

Date of Final Oral Examination

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CHAPTER I: INTRODUCTION

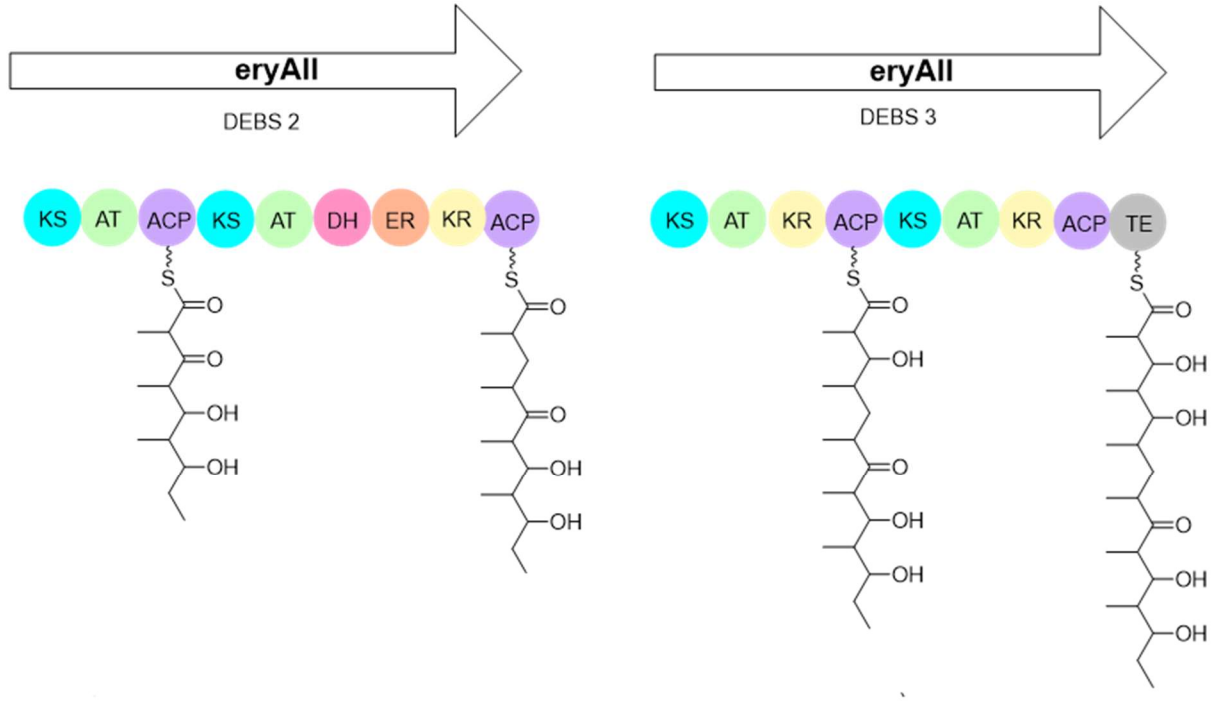
I.A. BACKGROUND

I.A.1. Polyketide Biosynthesis

Polyketide synthases, also known as PKSs, make secondary metabolites that can be found in plants, fungi, and bacteria. Polyketides that are produced by bacteria often have antibiotic properties.[1] Polyketide biosynthesis involves a sequence of enzymes that condense carbon units into a structure similar to fatty acid chains but are considered polyketides. Polyketide synthases consist of many individual catalytic enzymes domains that assemble and modify a polyketide chain.[2] Polyketide synthases fall into 3 categories, type I, type II, and type III. With type I and type II polyketides synthases, the acyl-CoA precursor is tethered to the acyl carrier protein (ACP), while type III polyketide synthases act directly on CoA thioester substrates and intermediates.[2] Type I polyketide synthases can be divided into two subgroups – *Cis*-AT PKSs and *Trans*-AT PKSs. *Cis*-AT PKSs contain acyltransferase (AT) domains, which load the acyl-CoA precursor (such as malonyl-CoA) onto the acyl carrier protein. The AT enzyme contributes to structural diversity of polyketides by selecting which type of malonyl-CoA precursor (typically, malonyl-CoA or methylmalonyl-CoA) is incorporated at each point along the polyketide chain.[3] In Dr. Reddick's lab, we focus mainly on *Trans*-AT PKSs, in which the AT unit is not a part of the polyketide synthase, but instead encoded as a separate gene in the polyketide biosynthetic gene cluster.[3] There are other enzyme domains that play a role in both *Cis*- and *Trans*-AT PKSs. Ketoacyl synthase (KS) is the enzyme that grows the polyketide chain by condensing malonyl-CoA and combining it with acyl-CoA. The ketoreductase (KR) then reduces the previous ketone to an alcohol. Dehydratase (DH) catalyzes the elimination of water,

forming a double bond. Enol reductase (ER) will reduce the double bond to an alkane. Although we see KR, DH, and ER play certain roles, they are optional domains. If the biosynthesis lacks a KR, the module will leave the ketone. If KR is present but lacks DH, then the module leaves an alcohol. If KR and DH are present but lacks the ER, then an alkene double bond is made. Thioesterase finishes the synthesis by simple hydrolysis of the compound from the polyketide from the synthase, or by catalyzing some sort of cyclization that also removes it from the synthase. The substrate and β-carbons in polyketides are determined by the biosynthesis.[4] Figure 1 Shows an example of the enzymes and the structure of polyketide biosynthesis.[1]

Figure 1. Polyketide Biosynthesis

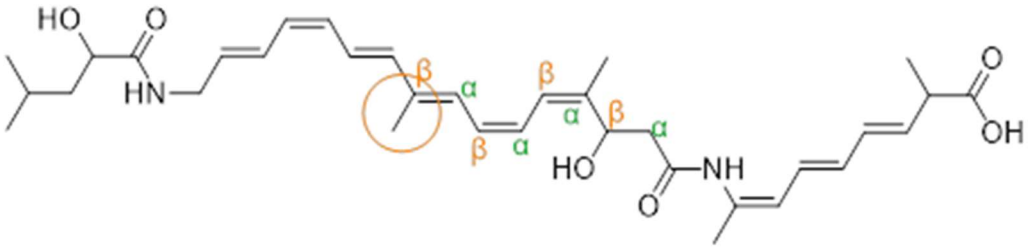


This is an example of the biosynthetic pathway that type I polyketides follow. Each enzyme shown can be used in a different sequence to allow polyketide diversity.

I.A.2. β-Branching

In chemistry, carbon chains are labeled by indicating each carbon using the Greek alphabet, starting with alpha (α). The α-carbon is the first carbon next to the carbonyl carbon, followed by beta (β), etc., with omega (ω) being the last carbon in the chain. The carbons in a carbon chain can have different groups branching off by something as simple as a methyl group to something more complex.[5] When the β-carbon has a group branching off it is considered a β-branch. A polyketide has one β-position relative to a carbonyl. But in polyketides, every two carbons along the chain are labeled as "β-positions" since they were once actually β-positions during the course of developing the biosynthesis chain. The β-position circled in Figure 2 is not a β-position in the compound due to it being 8 carbons away from the carbonyl, but it was a β-position earlier in the biosynthesis of the chain. β-branches in polyketides are installed by additional enzymes working with the biosynthetic pathway described above, which contributes to the structural diversity of polyketide natural products.[6]

Figure 2. Bacilliene β-branch



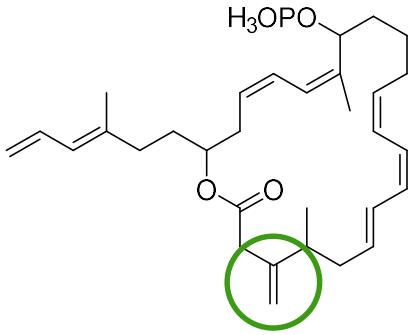
The polyketide shown above is known as bacilliene.[7] The α-positions are labeled in green and the β in orange. The circled portion shows the β-branch that is important for bacilliene. β-position in polyketides usually has groups such as a ketone, alcohol, etc. Alkylation at the β-branch position isn't as common as methylation at the α-position.[8] β-branching is an additional structural motif that is found in polyketides of diverse structures like bacillaene, jamaicamide,

mycalamide, and difficidin.[9] Polyketides, specifically type I polyketides, use β-branching as a way to introduce multiple varieties of side chains at the β-carbon position.[6]

I.A.3. Difficidin

Difficidin is a type I trans-AT polyketide that is produced by *Bacillus amyloliquefaciens* FZB42. It has been shown that difficidin possess antibacterial activity against rice pathogens that are caused by bacteria.[10] The pathogens cause bacterial blight and bacterial leaf streak disease in plants.[10] The antibacterial activity that is shown by difficidin is one thing that makes it interesting, but the unusual alkene β-branch also draws attention to the polyketide.

Figure 3. Difficidin



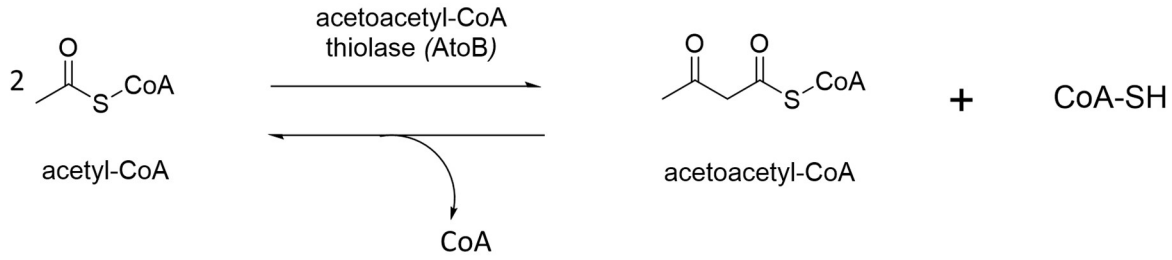
As mentioned before, β-branching at the β-position can take place in polyketides and provide a variety of structures for polyketides. In difficidin, there is an unusual β-branch involving an *exo*, disubstituted alkene, whereas in other polyketides like bacillaene, the β-branch is an isomer involving a trisubstituted alkene with an *endo* double bond situated in the main chain of the polyketide.[11] Due to the difficidin polyketide synthetase being large in size we did not work with the full PKS protein. The final intermediate on the PKS that cyclizes to form difficidin is too long and complex to produce, purify, and keep soluble. To overcome this, the polyketide can be replaced with a methyl group for simple production and analysis. Acetoacetyl-CoA is the model used since has a similar structure as the beginning of the difficidin polyketide

chain, a β-keto group, and provides a simple methyl group in place of the complex polyketide chain. Because of its structure and simplicity, acetoacetyl-CoA is a good replacement for analysis.

I.A.4. Acetoacetyl-CoA

Acetoacetyl-CoA is a well-known metabolite that is derived from the metabolite acetyl-CoA. This precursor is necessary for biosynthetic pathways like fatty acid biosynthesis (and degradation), ketone bodies, particularly the HMG-CoA intermediate, and poly-β-hydroxybutyrate.[12] Acetoacetyl-CoA thiolase (AtoB) is expected to catalyze the Claisen condensation of two acetyl-CoA molecules.[13]

Figure 4. Acetoacetyl-CoA produced from acetyl-CoA



I.A.5. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is the technique used to isolate the gene of interest/DNA fragment from its genome and amplify multiple copies of this specific fragment.[14] The amplification of the fragment arises from the repeated DNA synthesis cycles from upstream and downstream primers that are designed to flank the gene of interest's nucleotide sequence; one for each DNA strand. The two primers will each prime a strand producing two single stranded DNA templates that will be used for the DNA synthesis reaction of the isolated fragment. For this DNA synthesis reaction to occur, it will need DNA polymerase, PCR Master

Mix that consist of a large quantity of dNTPs to serve as building blocks for the new DNA fragment, and the template DNA, which is the genomic DNA isolated from *E. coli*. It will also need a high temperature to denature the DNA strands. Performing this PCR cycle repeatedly after 30 rounds generates a 2-fold increase in each round of the DNA fragment known as the gene of interest. PCR is commonly used to generate a large quantity of a single fragment but can cause mis-assemblies and unintended hybridizations during repeated cycles.

I.A.6. High-Fidelity (HiFi) Gibson Assembly

High-fidelity Gibson Assembly can be used to assemble DNA fragments rather than Restriction Digest assembly methods. The use of high-fidelity (HiFi) polymerase induces the accuracy of DNA fragment assembly because HiFi provides the ability to bridge the two DNA fragments, created by PCR with a single stranded DNA oligonucleotide, more accurately and efficiently.[15] The DNA fragments are assembled with the use of a Master Mix that consist of a 5' exonuclease, a DNA polymerase, and a DNA ligase. The Restriction Digest assembly methods involves incorporating restriction sites in the primers so the PCR product can be ligated to the same restriction sites in the plasmids. The main things that make HiFi a preferred technique over a PCR assembly restriction digestion is the shorter step process and it is not limited by the need for restriction sites. Restriction digestion requires multiple steps that uses restriction enzymes for the digestion process and DNA ligase for ligation which can take up to 2 days to complete, while HiFi can be completed in an hour and does not depend on restriction enzyme recognition sites due to all enzymes and DNA fragments being mixed at once.[16]

I.A.7. Plasmid Vectors for DNA Cloning

Plasmid vectors are non-chromosomal, double stranded molecules that exist as circular DNA and have the ability to replicate in bacterial cells.[17] Plasmids are known to consist of

components that provide bacteria with genetic advantages that are needed for survival. There are now many plasmids that were developed in research laboratories that incorporate many other features such as the T7 promoter combined with the *E. coli lac* operator. The T7 structure is included due to T7 RNA polymerase being extremely efficient and its ability to produce plenty of mRNA. Controlling this T7 system with the *lac* operator and isopropyl β-D-1-thiogalactopyranoside (IPTG) allows us to selectively induce expression of genes placed under control of the T7 promoter. The origin of replication is essential for making multiple copies of the inserted DNA in the plasmid, and the selection marker, which is an antibiotic resistance gene that allows for selection of cells with plasmids and prevent growth of bacterial cells that have lost the plasmid. Plasmids acquiring antibiotic resistance is a natural function and can be considered a form of mating between bacteria by passing genetic material between cells. Essentially, antibiotic resistance is used to introduce a selection to the culture. It ensures that every cell in the culture contains the plasmid. Some daughter cells may not receive the plasmid during cell division and will be killed off by the antibiotic. Successful growth means the cells are resistant to the antibiotic and are still replicating the plasmid.[18]

Circular plasmid DNA ability to transcribe easily is due to their small size.[19] Certain plasmids known as vectors are used to clone, transfer, and manipulate genes.[20] Due to the ability to insert genes or DNA fragments into plasmid vectors, the use of these plasmid vectors allows for a large quantity of copies of the gene/DNA fragments inserted.[21] The procedure used to create this recombinant DNA molecule requires a gene or DNA fragment of interest to be isolated, amplified, and then chemically inserted into the plasmid vector for DNA cloning. *E. coli* plasmids are the most common vectors used for cloning due to their variety of antibiotic resistance, and the ability to self-replicate the plasmid DNA and the inserted gene.

I.A.8. pET-28a Plasmid Vector

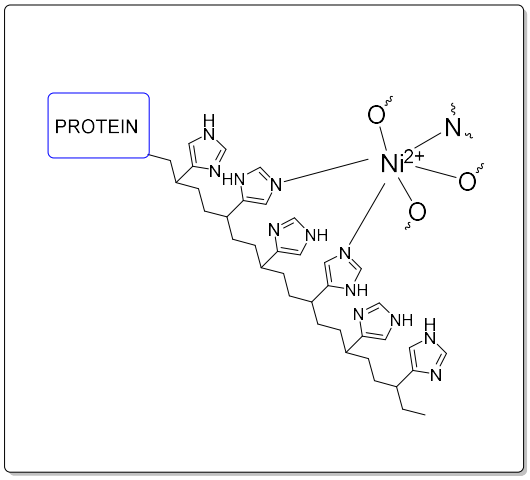
pET-28a is a plasmid vector from the series of pET expression plasmids that are well known for recombinant protein production in *E. coli*. pET expression plasmids were first developed over 30 years ago. Their ability to do high level transcription in *E. coli* is due to the T7 promoter, which promotes transcription and T7 terminator which terminates transcription - these are key features that make this series of plasmids more favorable for expression by T7 RNA polymerase. It contains the T7 promoter and T7 terminator sequences just as other pET plasmids. But there are other features that make pET-28a more favorable such as the *lac* operon sequence which is used to temporarily suppress transcription, and a Shine-Dalgarno sequence which ensures the ribosome is aligned correctly with the start codon. Although the *lac* operon suppresses transcription, it is only done temporarily and can be de-suppressed with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG is a molecular compound that binds to the *lac* repressor protein and inhibits its ability to suppress transcription.[22] A poly-histidine purification tag, also known as a His-tag, is also included to allow for protein purification.[23] pET-28a contains two sequences for his-tags, one on the N-terminus and another on the C-terminus giving the option to utilize one or both His-tags. Although the plasmid consists of both, only one is needed for protein purification. The pET-28a plasmid also uses kanamycin resistance which helps detect the bacteria that contains the plasmid and forces the bacteria to keep the plasmid, during antibiotic selection.

I.A.9. Nickel-Affinity Column Chromatography

The his-tag from the pET-28a plasmid vector will assist with purifying the protein by use of nickel-affinity column chromatography. Nickel is one of the transition metal ions that can be used in immobilized metal-affinity chromatography, which is a chromatography method used to

purify recombinant proteins that consists of an affinity tag such as a his-tag.[24] The reason that nickel-affinity columns work so well with his-tags is due to interactions histidine has with immobilized metals. The electron donor groups on the histidine form coordination bonds with the nickel.[24] Nickel tends to be the most common metal used because it generally gives the highest yield and a hexahistidine tag (His^6-tag), which is the his-tag used in pET-28a, has a high affinity for nickel.[25] Figure 5 displays an example of how the Histidine residues bind to the nickel in the column.

Figure 5. His^6-Tag Attached to the Nickel-Affinity Column[26]



For a nickel-affinity column to obtain purified protein a standard protocol requires several washes with increasing levels of imidazole. The first set of washes are completed to equilibrate the column. The purpose of equilibrating the column is so the protein will be adsorbed onto the solid matrix. The next set of washes is done to add the protein to the column and allow it to bind, wash away unwanted products, and the desired protein is eluted from the column by high levels of imidazole. Because of the high binding affinity of the his-tag to the nickel, a reagent such as imidazole is needed to elute the protein from the column. Imidazole is a

compound that mimics a histidine ring. When imidazole is added to the column, it reduces the binding of the proteins, releasing the protein from the column, increasing the final purity.[27]

I.A.10. Acetoacetyl-CoA Thiolase

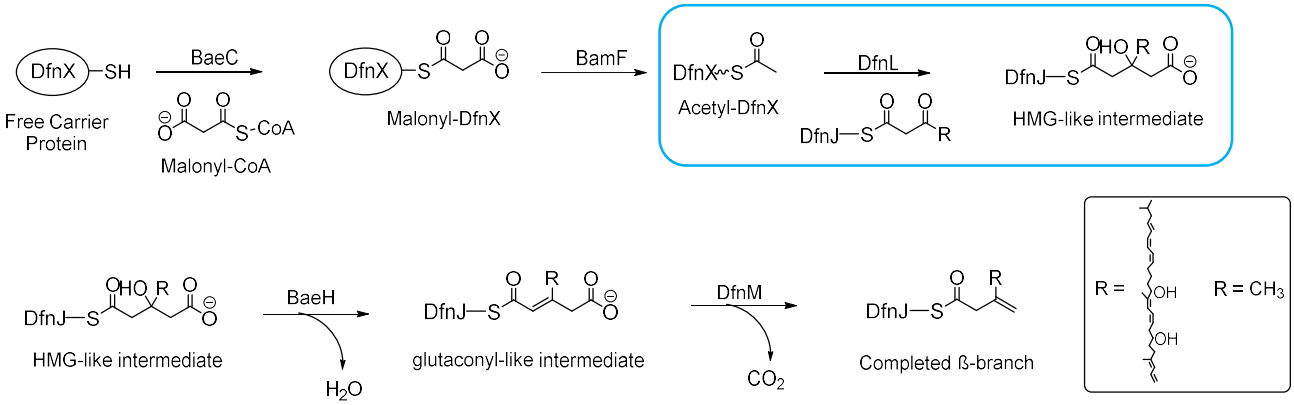
Condensing enzymes such as acetoacetyl-CoA thiolases catalyze the reaction of two acetyl-CoA molecules to produce acetoacetyl-CoA.[28] The covalent bond formed from thiolase is done by a Claisen condensation reaction[13]. This report follows the accepted, but often misunderstood, formatting convention using names derived from bacterial gene codes. The italicized form *atoB* (first letter lowercase, last letter uppercase) indicates a gene, whereas the unitalicized form AtoB (first and last letter uppercase) represents the protein encoded by the *atoB* gene. The *atoB* gene is found in many bacterial species such as *Shigella sonnei, E. coli,* etc., encodes a protein, AtoB, which can act as an acetoacetyl-CoA thiolase. A good source for this gene is *E. coli* due to its availability and abundancy in nature.

I.A.11. Previous Works from Dr. Reddick's Research Group

Polyketide synthesis is a prevalent topic of interest in Dr. Reddick's research group. An extensive range of understanding for polyketides is quite beneficial for drug development seeing many drug molecules, such as antibiotics, are polyketide natural products. Many individuals from this lab have studied the development and importance of biosynthetic pathways of polyketides such as bacilliene and difficidin. Dr. Brittany Kiel, a former PhD student in Dr. Reddick's group focused on the biosynthetic pathway of difficidin. Difficidin has an unusual alkene group on a β-branch position that is not yet fully understood. Dr. Kiel wanted to elucidate the pathway of this unusual β-branch to better understand how the alkene is formed. She was able to prove every step in the pathway except one. The secondary metabolite, acetoacetyl-CoA, needed to complete her investigation was no longer readily available. How this β-branch is

formed and why it forms has yet to be proven. Dr. Kiel showed that the final decarboxylation step shown in Figure 6 likely controls the position of the double bond.[11]

Figure 6. Using acetoacetyl-CoA to Investigate How Difficidin produces the Unusual Alkene



Dr. Kiel focused on how difficidin biosynthesis produced this alkene at the β-branch position. She did so by substituting the side chain in difficidin with a methyl group shown in Figure 6. The portion highlighted in blue shows the one step remaining to show how the alkene is produced. She used acetoacetyl-CoA to substitute for the fully elaborated β-keto acyl biosynthetic intermediate (see the R group in Figure 6). With this replacement, she was able to use HMG-CoA to study the final 2 steps of the pathway above. She had difficulty using acetoacetyl-CoA when studying the DfnL step because it degraded in the reaction conditions. Once the degradation issues were solved, scientific companies stopped producing acetoacetyl-CoA.

I.A.12. Goal and Hypothesis

Acetoacetyl-CoA is a common metabolite for certain metabolic pathways such as fatty acid β-oxidation and ketogenesis. For this specific research, we are using acetoacetyl-CoA as a shortened model substrate used in place of the actual polyketide β-keto intermediate substrate for

the β-branching enzymes we expect to be involved in the biosynthesis of difficidin. Developing an enzymatic method for producing acetoacetyl-CoA would provide us with the product needed to complete the HMG synthase step of bacilliene and difficidin biosynthesis. We hypothesize that acetyl-CoA produces acetoacetyl-CoA through a Claisen condensation reaction catalyzed by AtoB. The goal is to purify AtoB from the *atoB* gene found in *E. coli*, and to produce acetoacetyl-CoA which can then be used for biochemistry research projects.

CHAPTER II: EXPERIMENTAL

II.A. Protein Preparation

II.A.1. Cloning of The Gene

To further the study of difficidin biosynthesis, we need to gain access to acetoacetyl-CoA. We can do so with the use of the *atoB* gene from *E. coli*. The *atoB* gene is used to produce the protein, AtoB, which is then used to produce the acetoacetyl-CoA. The *atoB* gene needs to be cloned to obtain a large quantity of the DNA for future protein expression. Being able to produce a large amount of the protein will allow us to access acetoacetyl-CoA for the use of finishing the β-branch production of difficidin.

II.A.2. Gene Isolation

To isolate a specific gene from genomic DNA, we needed to design primers that recognize the specific gene and use PCR for amplification. The purpose of primers is to enable a starting point for DNA synthesis. The gene we wanted to isolate is known as *atoB*, which is found in *E. coli*. Forward and reverse primers were designed using BioEdit. Specific enzymatic sites, BspHI and XhoI sites respectively, are included in the forward and reverse primers needed to amplify the *atoB* gene. To begin, 25µL of Phusion High-Fidelity PCR Master Mix with HF buffer was added to a thin-walled PCR tube, followed by 19µL of ddH_2O (20µL for negative control), 2.5µL of both the 15.3 mmol forward primer and 27.7 mmol reverse primer, and 1µL of the template genome DNA from *E. coli*, respectively. This combined to make a PCR mixture of 50µL.

The genomic DNA from *E. coli* was isolated using the Promega Wizard Genomic DNA Purification kit, which is used as the template DNA for PCR experiments required for *atoB*. The

atoB gene was isolated from the genomic *E. coli* DNA using the upstream and downstream primers and then amplified using polymerase chain reactions. The table below shows the sequence of the primers created for ligation with pET-28a using restriction cloning. The bolded portion of the sequence shows the mutations to incorporate BspHI and XhoI restriction sites into the primers.

Table 1. Primer Sequences Used for Gene Isolation

Primer	Sequence (5' → 3')
Forward (BspHI)	GAA TAT A**TC ATG A**AA AAT TGT GTC ATC G
Reverse (XhoI)	TTT TTA T**CT CGA G**AT TCA ACC G
atoB – Forward	GCC ATC ATC ATC ATC ATC ACA TGA AAA ATT GTG TCA TCG TCA G
atoB – Reverse	TTG TTA GCA GCC GGA TCT CAA TTC AAC CGT TCA ATC ACC ATC
pET-28a – forward	TGA GAT CCG GCT GCT AAC AAA G
pET-28a – Reverse	GTG ATG ATG ATG ATG ATG GCT G

New primers for the isolated *atoB* gene were designed for ligation with the pET-28a plasmid vector using Gibson cloning. Primers were also designed for the pET-28a plasmid vector to isolate a certain portion of the plasmid vector to allow for the insertion of the *atoB* gene. Table 1 provides the forward and reverse primer sequences for both the *atoB* gene and the pET-28a plasmid vector for Gibson cloning. PCR was also used to amplify the *atoB* gene and the region of pET-28a needed for cloning. The primers provide regions where the two PCR fragments will anneal and be ligated together in the Gibson cloning.

For the polymerase chain reactions, a thermocycler was used. The thermocycler was set for an initial denaturation at 98℃ for 30 seconds. The following primer extension steps ran for 30 cycles consisting of 5 second denaturation at 98℃, annealing for 10 seconds at 55℃, and extension for 3 minutes at 72℃, finishing with a 72℃ 10-minute extension at the end of the last cycle. At the end of the program, the PCR products are held at 4℃. The thermocycler conditions to amplify the pET-28a plasmid were the same as thermocycler conditions to amplify the *atoB*, but the annealing temperature was set to 64℃ for pET-28a instead of 55℃.

II.A.3. Agarose Gel Electrophoresis for The Analysis of PCR Reactions

While the genes were being amplified in the thermocycler, a 1% agarose gel was prepared to confirm success in amplifying the *atoB* gene. Gel preparation consisted of 0.3g of agarose, 30mL of 1X Tris-acetate-EDTA (TAE) buffer, and 2µL of ethidium bromide (10 mg/mL). The gel was placed in an electrophoresis chamber with 1X TAE buffer. To prepare the DNA to be loaded into the wells, 4µL of 6X gel loading dye was added to 16µL of the PCR product. To compare the bands and make sure they consist of the correct number of base pairs, a DNA ladder is needed. The DNA ladder is made up of 1µL of 1 kb DNA plus ladder, 1µL of 6X gel loading dye, and 6µL of ddH_2O. The gel was then electrophoresed at 110V for 45 minutes. The ethidium bromide-stained gels were visualized with short UV light using the Bio Rad Gel Doc XR+ Gel Documentation System.

The gel showed an isolated product band close to 1,200 base pairs (bp), indicating that *atoB* was likely amplified successfully isolated from the genomic DNA and was ready to be cloned into the plasmid vector. The original pET-28a plasmid vector consists of 5369 bp, but the primers created for pET-28a isolation remove a portion of the plasmid vector to allow for the *atoB* gene to be placed into the vector in its place. Once that portion is removed, we should see a

band in our agarose gel at roughly 5,000 bp. Since we had the correct portion of our plasmid vector for cloning, we were able to move forward with the High-Fidelity Gibson Assembly to ligate the *atoB* and pET-28a fragments together.

II.A.4. Restriction Enzymes – Cloning

Once the PCR was confirmed through gel electrophoresis, the PCR product was purified using the QIAquick PCR Purification Kit by Qiagen. The purification kit allowed removal of excess DNA and primers. At the same time, pET28a is being purified using the Qiagen QIAprep Spin Miniprep Kit. Once the PCR product and vector were both purified, a restriction digest was done to cleave the DNA at the desired cutting sites to allow the compatible sticky ends to piece the insert DNA inside the vector DNA. The restriction digest takes roughly 3 hours to be completed. Once completed, heat deactivation at 80℃ for 20 minutes is needed to stop the endonuclease reaction. In order for these sticky ends to be joined together, they have to go through ligation. To do so, reactions of different ratios of vector: insert were mixed to increase the chances of proper ligation. The ligation reaction was completed using 2µL of T4 DNA ligase buffer (10X), 20µL of ddH_2O, 1µL of T4 DNA ligase, and the vector and insert volume depended on the ratio used. The reactions were incubated at 16℃ overnight.

The ligation reaction mixtures were directly transformed into *E. coli* TOP10 using the $CaCl_2$ competent cells procedure. Competent cell transformation allows for foreign DNA to be taken up at a higher efficiency. The cell membrane is made permeable so the plasmid can enter the bacterial cell. After cell transformation, the cells were plated on 50µg/mL kanamycin LB agar plates and incubated at 37℃ overnight. The plate produced a plethora of colonies. Six of the transformed colonies were selected to find a colony that contained the cloned insert inside the vector. Each colony was added to 5mL of LB broth with 5µL of 50 ug/mL kanamycin solution.

Before adding the colony to the starter culture, the sterile wooden applicator stick was used to streak a fresh kanamycin plate just in case the colony was successful. The cultures were shaken in an incubator at 37°C overnight. After being centrifuged at 13,000 RPM, the plasmid DNA was isolated with the Qiagen QIAprep miniprep kit. This purified plasmid should contain the *atoB* gene insert. To check for that, a PCR was performed using the *atoB* up and down primers in table 2, with a negative control lacking template DNA and a positive control using the *E. coli* genomic DNA.

Table 2. *atoB*-pET-28a Testing Primers for Restriction Enzymes

Primer	Sequence (5' → 3')
atoB-pET-28a Check UP	CAC CAT GAA AAA TTG TGT CAT CGT CAG TGC
atoB-pET-28a Check DOWN	TTA ATT CAA CCG TTC AAT CAC CAT CGC

The primers were hydrated to obtain a 25,000 nM stock concentration. From the successful plate, we grew six colonies overnight in starter cultures and purified the plasmids from them using the Qiagen QIAprep Spin Miniprep Kit. Before inserting the colonies into the starter cultures, kanamycin plates were streaked with the colony to ensure we preserved the colony if it was a successful one. The primers and purified plasmids were used to generate a PCR for ligation testing. We obtained 5 thin-walled PCR tubes and added the following: 25µL of Phusion High Fidelity Master Mix, 22µL of ddH_2O, 1µL of the check-up primer, 1µL of the check-down primer, and 1µL of the 5 purified *atoB*-pET-28a DNA were each added to a tube, respectively. The control was prepared the same way - the only difference is 1µL of ddH_2O is used in place of the DNA. The PCR reactions were analyzed by agarose gel electrophoresis as described above.

II.A.5. HiFi Gibson Assembly – Cloning

Once both the *atoB* (using new HiFi primers) and pET-28a PCR products were confirmed through gel electrophoresis, the PCR products were purified using the QIAquick PCR Purification Kit by Qiagen. The purified PCR product was also confirmed through gel electrophoresis. The concentration of the purified PCR products was measured using the Nanodrop ND-1000 spectrophotometer. The PCR products were stored overnight in a freezer at -20℃. At the same time, a starter culture of TOP10 competent *E. coli* cells using LB media broth was incubated overnight at 37℃, shaking at 220 rpm for the transformation procedure. The next day the *atoB* and pET-28a was ligated together using HiFi Gibson Assembly to later be transformed into TOP10 chemically competent *E. coli* cells or NEB 5α competent *E. coli* cells.

While the starter culture was incubating, the Hi-Fi reaction was prepared by determining the amount of *atoB* and pET-28a needed, based on the concentrations, to give a 1:2 (vector:insert) ratio, with the insert being the *atoB* PCR product. For the HiFi reaction the following was added to a thin-walled PCR tube; 10µL of HiFi Master Mix, 2.9µL of ddH_2O, 3.2µL of pET-28a, and 1.3µL of *atoB* PCR products, respectively, totaling a reaction volume of 20µL. The HiFi reaction mixture incubated in the thermocycler at 50℃ for 60 minutes. Once the incubation for the HiFi reaction mixture was complete, the ligated *atoB*-pET-28a was transformed into competent *E. coli* TOP10 using the calcium chloride competent cells procedure or the NEB 5α competent cells that were already competent from New England Biolabs (NEB).

To determine if the ligation was successful, we designed testing primers to test for the desired *atoB*-pET-28a product. This new set of primers was designed specifically to help identify if we successfully ligated the *atoB* and pET-28a. The new primers helped to prevent false positives caused by contaminating *E. coli* DNA which already has *atoB*. One primer targeted

atoB while the other primer targeted pET-28a. PCR with these primers would only work if the 2 fragments were ligated together.

Table 3. *atoB*-pET-28a Testing Primers for HiFi Cloning

Primer	Sequence (5' → 3')
atoB-pET-28a Check UP	GCT CTC CCT TAT GCG ACT CC
atoB-pET-28a Check DOWN	TTT TGC ACG TTC AAT GGC GG

The primers were hydrated to obtain a 25,000 nM stock concentration. From the successful plate, we grew six colonies overnight in starter cultures and purified the plasmids from them using the Qiagen QIAprep Spin Miniprep Kit. Before inserting the colonies into the starter cultures, kanamycin plates were streaked with the colony to ensure we preserved the colony if it was a successful one. The primers and purified plasmids were used to generate a PCR for ligation testing. We obtained 6 thin-walled PCR tubes and added the following: 25µL of Phusion High Fidelity Master Mix, 22µL of ddH_2O, 1µL of the check-up primer, 1µL of the check-down primer, and 1µL of the 6 purified *atoB*-pET-28a DNA were each added to a tube, respectively. The control was prepared the same way - the only difference is 1µL of ddH_2O is used in place of the DNA. The PCR reactions were analyzed by agarose gel electrophoresis as described above. Based on the primers designed in table 3, the expected size of the product would be 500 bp.

II.A.6. Competent *E. coli* TOP10 Cell Transformation

To make the cells competent a starter culture of TOP10 that was prepared overnight was used to create a new starter culture so it wouldn't be overgrown. The new starter culture was

incubated at 37°C, shaking at 220 rpm until slightly cloudy. The cells were then centrifuged at maximum speed. The supernatant was discarded, and the pellet was resuspended in 3mL of cold 50mM $CaCl_2$, vortexed briefly, then placed on ice for 30 minutes. This process was repeated, but this time 1mL of cold 50mM $CaCl_2$ was added to resuspend the pellet. Then, 100µL of the competent cells and 20µL of the ligation mixture with the restriction enzymes method were placed in a centrifuge tube, vortexed briefly, and incubated it on ice for 30 minutes. The cells were heat shocked at 37°C for 2 minutes and then incubated at room temperature for 10 minutes. We added 1mL of LB broth to the tube and then incubated the cells at 37°C, 220 rpm for 1.5 hours. Using a centrifuge, the cells were centrifuged at 8,000 rpm for 5 minutes. We discarded 1mL of the supernatant and resuspended the cells in the remaining supernatant. After cell transformation, 100µL the cells were spread on 50µg/mL kanamycin LB agar plates and incubated at 37°C overnight.

II.A.7. Competent *E. coli* NEB 5α Cell Transformation

Chemically competent cells from the NEBuilder HiFi DNA Assembly Cloning Kit were thawed on ice. We then added 2µL of the *atoB*-pET-28a product to the competent cells and mixed them gently, which was then iced for 30 minutes. The cells were heat shocked at 42°C for 30 seconds and transferred back to ice for 2 additional minutes. Then 950µL of room-temperature SOC Media was added to the tube of cells. The tube was incubated at 37°C, 220 rpm for 1 hour. While the cells were incubating, 50µg/mL kanamycin LB agar plates were heated to 37°C. After cell transformation, 100µL the cells were spread on 50µg/mL kanamycin LB agar plates and incubated at 37°C overnight. The presence of colonies was first indication of possible success, and these were analyzed according to the following procedures.

II.A.8. Sequencing

We prepared samples that were confirmed to be successful to be sent to Eurofins to be sequenced. To prepare the samples to be sent to Eurofins, we measured the concentration of each purified sample by using the Nanodrop spectrophotometer. The samples were then dehydrated using a vacuum and rehydrated with the amount of ddH_2O necessary to achieve a concentration of 200ng/µL.

A plasmid having the correct sequence was chosen to move forward with the BL21 Star transformation. Colonies from the re-streaked sample 1 plate were grown in a starter culture overnight to prepare for the transformation. A BL21 Star starter culture was prepared using 5mL of LB media broth and BL21 Star cells, and incubated overnight at 37℃, shaking at 220 rpm. The *atoB*-pET-28a sample was transformed into BL21 Star using the BL21 Star transformation procedures and spread on a kanamycin plate, incubated at 37℃ overnight.

II.A.9. Large Culture

To begin developing the desired AtoB protein, we needed to grow a large culture of *atoB*-pET-28a. We took a colony from the *atoB*-pET-28a BL21 Star transformation plate and created a 5mL starter culture and incubated it overnight at 37℃, shaking at 220 rpm. The next day a 1000mL culture was prepared using 1000mL of LB media broth, 1mL of 50 mg/mL kanamycin, and the 5mL overnight culture. The large culture was incubated at 37℃, shaking at 220 rpm until the desired absorbance was reached. The absorbance of the large culture needs to reach 0.5-0.6 abs, induced at 595 nm. To do so, a UV-Visible Spectrophotometer was blanked with 1mL of LB media broth at 595 nm. The absorbance of the large culture was checked every hour until it reached ~0.2 absorbance. From here on the absorbance was checked more frequently to ensure

the absorbance did not exceed 0.6. Once the absorbance reached the desired range, 238 mg of IPTG was added to induce transcription. The culture is then left to incubate and shake overnight.

II.A.10. Protein Purification Using Nickel-Affinity Column Chromatography

After transcription was induced and the culture had grown overnight, it was dispensed into two 500mL centrifuge bottles and centrifuged at 4℃, 6,500 rpm, for 30 minutes using a JA-10 rotor. The supernatant was discarded, and the pellets were stored at -80℃.

When ready, the pellet was resuspended and dissolved in 15mL of 1X binding buffer (4M NaCl, 160mM Tris, 40mM imidazole, pH=7.9) and transferred to two 50 mL round-bottom JA-20 centrifuge tubes, which were then placed on ice. The resuspended cells were lysed by sonicating the cells on ice at 20KHz. To prevent the cells from getting too hot, the cells were sonicated in 30 second intervals, and the given 30 seconds for the cells to cool. The sonicator ran for 3 minutes in total. The sonicated cells were centrifuged at 4℃, 11,500 rpm, for 30 minutes using a JA-20 rotor. The supernatant was decanted into the barrel of a 10mL syringe with a 0.45µm sterile syringe filter and loaded into a nickel-affinity column for protein purification.

The supernatant from the sonicated cells was added to a nickel-affinity column that was already equilibrated. The 2mL column was loaded with the syringe filtered extract. Once the extract reached the top of the bed in the column, the column was washed with 20mL of 1X binding buffer (0.5M NaCl, 20mM Tris, 5mM imidazole, pH=7.9), followed by 12mL of 1X wash buffer (0.4M NaCl, 20mM Tris, 60mM imidazole). To elute the bound protein off the column, the column was washed with 12mL of elution buffer (0.25M NaCl, 10mM Tris-HCl, 0.5M imidazole, pH=7.9). The flow-through from the crude extract, binding buffer, and wash buffer were each collected in separate tubes. The elute stage was collected in 1mL fractions in microcentrifuge tubes.

Protein detection was preformed using a Bradford reagent. We analyzed 33µL samples of each fraction by adding 1mL of Bradford reagent to visually check for a blue color indicating the presence of protein. Each tube was inverted 10-12 times to ensure a mixture of the elute and reagent. Each fraction was observed for a color change from brown to a brilliant blue. The fractions that exhibited the greatest color change were kept and added to a 15mL tube.

Dialysis was completed to remove any impurities from the protein and to do buffer exchange. The dialysis buffer was made by adding 25mM Tris-HCl to 4000mL of ddH_2O, pH=8. The protein fractions that were added to the 15mL tube were added to dialysis tubing (SnakeSkin, 7,000 MWCO), placed in the dialysis buffer, and stored at 4℃ overnight. The protein in the dialysis tubing was placed in a fresh 15mL tube. Aliquots of the dialyzed protein was stored at -80℃ in 10% glycerol.

II.A.11. SDS-PAGE

The purified protein was analyzed on an SDS-PAGE gel to check for purity and to measure the molecular weight, which will help confirm that the desired AtoB protein was purified. Each sample was combined with an equal amount of NEB 6X gel loading dye. Each sample was heated at 80℃ for 5 minutes. A 12% SDS-PAGE gel from Bio-Rad was obtained and 7µL of each sample beginning with the ladder was loaded into the gel, skipping a lane between each sample. The ladder used was the unstained protein standard (10-200 KDa) from New England Biolabs. The gel was set to run at 140 volts for 35 minutes. Once the gel electrophoresis was complete, the gel was removed from the chamber, placed in a staining container with SDS-Stain buffer (10% acetic acid, 50% methanol, ddH_2O, and Coomassie blue), and was left to shake overnight.

The next day, the gel was rinsed with water and washed with SDS-Destain buffer and was left to shake for 3 hours. This SDS-Destaining (10% acetic acid, 50% methanol, ddH_2O) step was repeated twice until the stain buffer was removed enough to see the dyed bands. The gel was added to a bowl containing water to prevent it from drying out an image of the gel was obtained using visual light.

II.B. Analysis of the Protein

II.B.1. Bradford Assay for Protein Concentration

After measuring the concentration of purified AtoB protein, we needed to determine the amount of AtoB needed for each reaction based on the concentration. To do so, we created 5 different concentrated stock solutions with 2mg/mL BSA. The absorbance of each BSA concentration was observed on the UV-Vis spectrophotometer at 595 nm using Simple Reads. The absorbance of each stock solution was obtained and plotted against the concentration and the data was fit using linear regression. We then obtained the absorbance of the AtoB protein treated with the Bradford reagent and determined the concentration using the equation provided by the BSA calibration plot.

II.B.2. Activity Assay

UV-Vis spectrophotometry was used to measure the expected enzyme activity of the AtoB enzyme. The acetyl-CoA → acetoacetyl-CoA reaction consisted of a buffer, acetyl-CoA, and the AtoB enzyme, added respectively. The buffer was made using Trizma Base with HCl and Magnesium Chloride ($MgCl_2$), pH = 8.1. The $MgCl_2$ helps provide the chromophore needed for this assay – this assay is dependent on the Mg-enolate of acetoacetyl-CoA at 300nm. If acetoacetyl-CoA is being formed, then the absorbance at 300nm will increase[29]. The enzyme is the AtoB protein we obtained from the *atoB* gene.

The concentrations of acetyl-CoA and the AtoB enzyme were varied in the reactions. The total amount of each reaction should equal out to 500µL. The UV-Vis spectrophotometer was set to read absorbances at 300 nm. The acetyl-CoA stock solution (20mM) was used to make smaller concentrations of acetyl-CoA solutions for different trials ranging from 0.50-1.0 mM. The same trials were completed, but with an increase of AtoB to 1.9 µM. Through both sets of trials, we took the best one and altered the trial by increasing the amount of AtoB to double the amount of acetyl-CoA. We continued trying different combinations until we got a trial that provided good assay results.

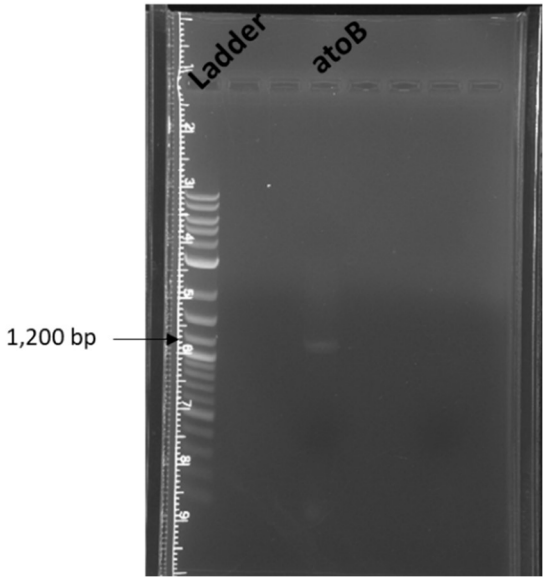
CHAPTER III: RESULTS AND DISCUSSION

III.A. Restriction Enzymes Results

III.A.1. Gene Isolation

The gel image in Figure 7 shows the isolated *atoB* product band at 1,200 bp using the primers from table 1 to create the correct amplified regions for ligation.

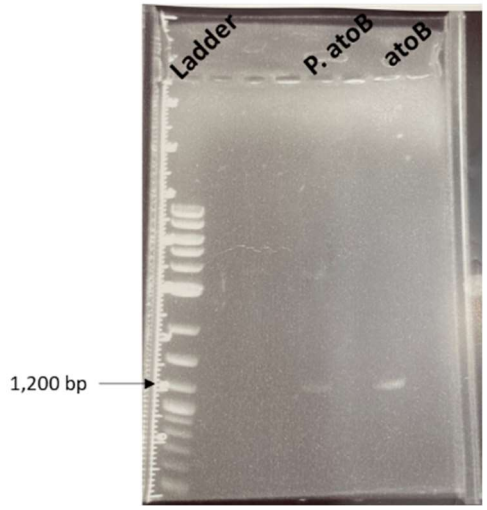
Figure 7. Isolated *atoB* Product Using Restriction Enzyme Methods



III.A.2. Cloning

After PCR purification, another gel was used for PCR product confirmation. The products should still appear at the 1,200 bp range for *atoB*. Figure 8 shows the gel images for the purified PCR products, confirming that the product was not lost during PCR purification.

Figure 8. Purified *atoB* PCR Product

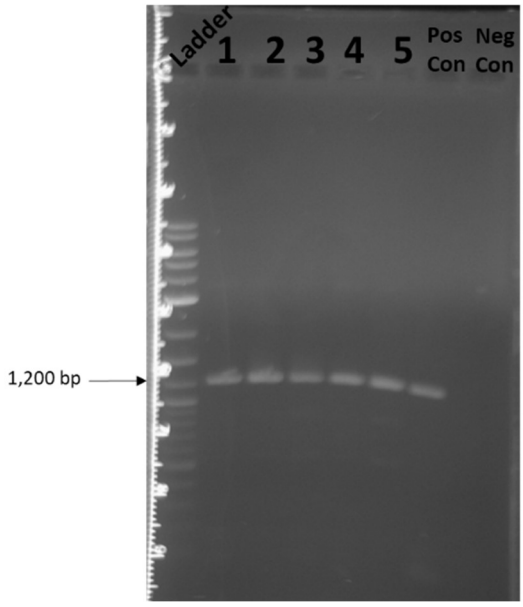


The *atoB* PCR product was digested with BspHI and XhoI enzymes, and the pET-28a was digested with NcoI and XhoI enzymes. BspHI and NcoI generate compatible overhangs. Using BspHI for the *atoB* PCR product was done to avoid digesting the *atoB* product with NcoI due to the gene consisting of an internal NcoI site. These digested products were mixed and ligated using T4 DNA ligase. The ligase reaction mixtures were transformed into competent *E. coli* TOP10 cells.

III.A.3. Post-Transformation

Once the target genes were cloned into the pET28a vector, they were transformed into *E. coli* TOP10 using the $CaCl_2$ competent cells procedure. The competent cell transformation allowed for foreign DNA to be taken up at a higher efficiency. The cell membrane is made permeable so the plasmid can enter the bacterial cell. Five of the transformed colonies were selected to find a successful colony that contained the cloned insert inside the vector. This purified plasmid should contain the *atoB* gene insert. To check for that, a PCR was performed with a negative control and positive control using the *E. coli* genomic DNA.

Figure 9. *atoB*-pET-28a Ligated Product



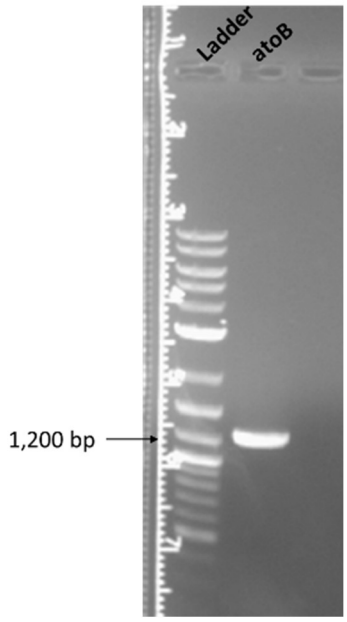
III.A.4. Sequencing

All five samples were prepared and sent to Eurofins for sequencing. However, all of the sequencing data indicated that the *atoB* gene was not cloned into the vector, and instead the samples contained only the original pET-28a sequence. After a few attempts, we realized that our initial screening assays could possibly be giving us false positives due to the cloning methods. We are using *E. coli* as the insert DNA source and also as the competent transformation cells, meaning the target *atoB* gene and vector was being cloned into a cell that already contained *atoB*. After using *E. coli* for the transformations and plasmid purifications, contaminating cells would introduce genomic DNA to the PCR reactions that are targeting the plasmids. Since the genomic DNA from *E. coli* naturally contains *atoB*, the contamination likely generated positive PCR reactions even though the plasmid in the sample did not include the *atoB* gene.

III.B. Hi-Fi Gibson Assembly Results

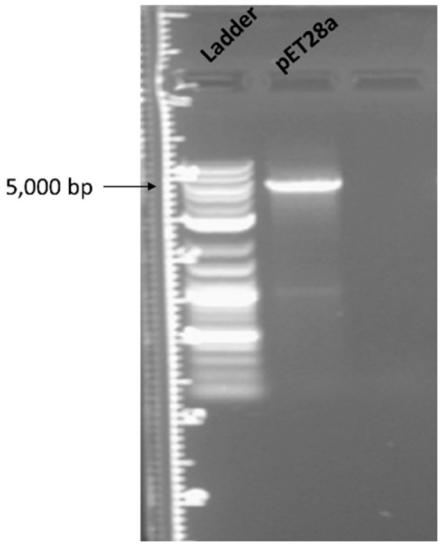
III.B.1. Gene Isolation

Figure 10. Isolated *atoB* Product Using *atoB* Forward and Reverse Primers



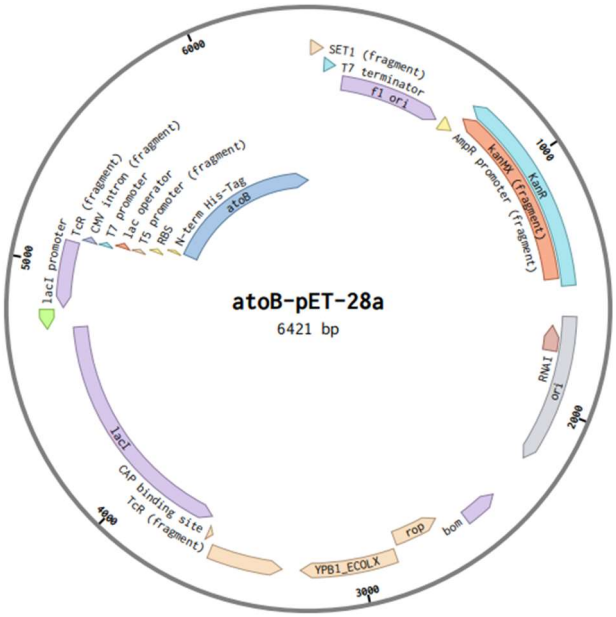
The gel image in Figure 10 shows the isolated *atoB* product band at 1,200 bp using the primers from table 1 to create the correct amplified regions for ligation. The results from the gel image in Figure 11 shows that we obtained the correct portion of the plasmid vector needed for cloning.

Figure 11. Isolated pET-28a Plasmid Using pET-28a Forward and Reverse Primers



Using the primers created for the pET-28a removed a portion of the plasmid that was replaced by the gene of interest as shown below in Figure 12.

Figure 12. Plasmid showing *atoB* gene inserted into the pET-28a Plasmid



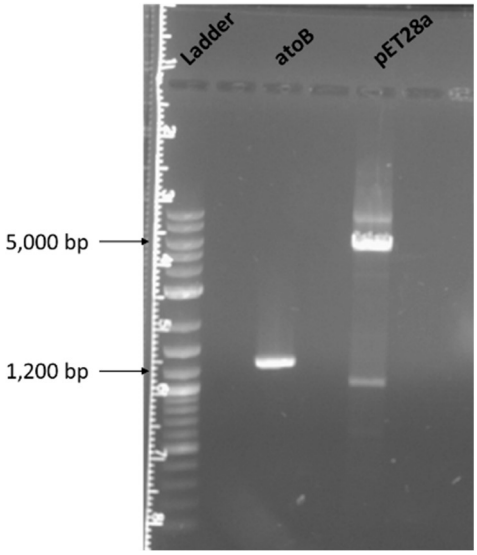
The primers were created to specifically eliminate the additional C-terminal his-tag, leaving the N-terminal his-tag since only one is needed for purification and having two could

potentially causes problems. Removing a portion of the plasmid vector changes the size from 5,369 bp to 5,000 bp.

III.B.2. Cloning

After PCR purification, another gel was used for PCR product confirmation. The products should still appear at 1,200 and 5,000 bp bands for *atoB* and pET-28a, respectively. Figure 13 shows the gel images for the purified PCR products, confirming the correct products were obtained.

Figure 13. Purified *atoB* and pET-28a PCR Products



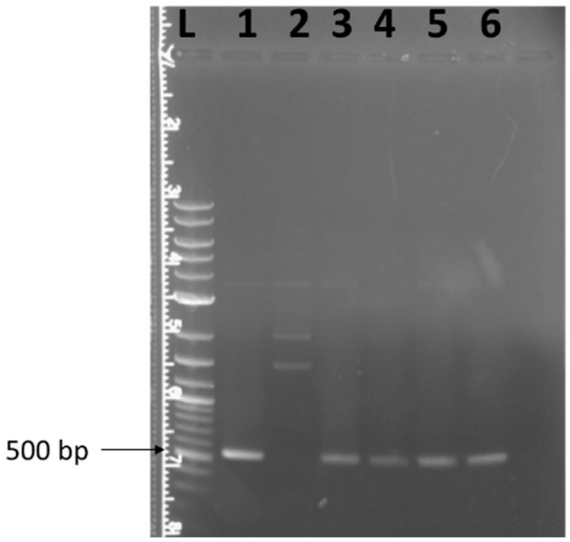
The Nanodrop spectrophotometer measured an *atoB* concentration of 36.5ng/µL and a pET-28a concentration of 32.4ng/µL. The calculated amount of *atoB* (0.64µL) and pET-28a (3.23µL), and the ratio used were recommended based on the manufacturer's instructions for the HiFi Gibson Assembly kit. Since it was a 1:2 ratio, the *atoB* was doubled to 1.28µL.

III.B.3. Post-Transformation

After transforming the *atoB*-pET-28a product into 5α competent cells, another gel electrophoresis was performed with 6 different colonies to confirm that the *atoB*-pET-28a

plasmid was assembled correctly using the "check" primers. Figure 14 displays the gel image for the *atoB*-pET-28a. We can see bands at ~500 bp for all samples except for sample 2.

Figure 14. *atoB*-pET-28a Ligated Product



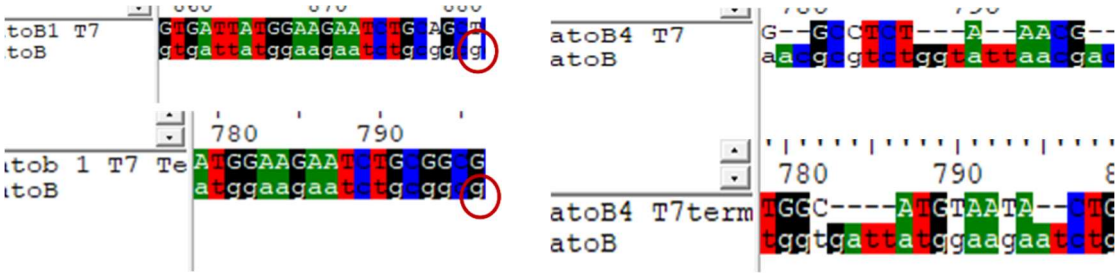
The concentration of the purified plasmids was measured, evaporated, and redissolved to achieve the final concentration needed for sequencing, 200ng/µL.

III.B.4. Sequencing

All five samples were prepared and sent to Eurofins for sequencing. Samples 1, 3, and 5 were verified by making sure all the bases for the *atoB*-pET-28a product matched the bases of the original *atoB* gene and pET-28a. Samples 4 and 6 had a lot of mismatching so the two samples were not considered for use. Figure 15 show portions of the sequencing results for the two of the samples. Figure 15a shows a portion of the sequencing results for sample 1 and 15b shows a portion of the results for sample 4. Since samples 1, 3, and 5 produced the correct sequence, it was fine to use either sample. We worked with sample 1.

Figure 15. Verified Sequences for Sample 1 and 4. The top rows labeled with *atoB*1 and *atoB*4 are the results of the sequencing. The bottom rows labeled as just *atoB* are the theoretical sequences for *atoB* from the genome database

a. **b.**

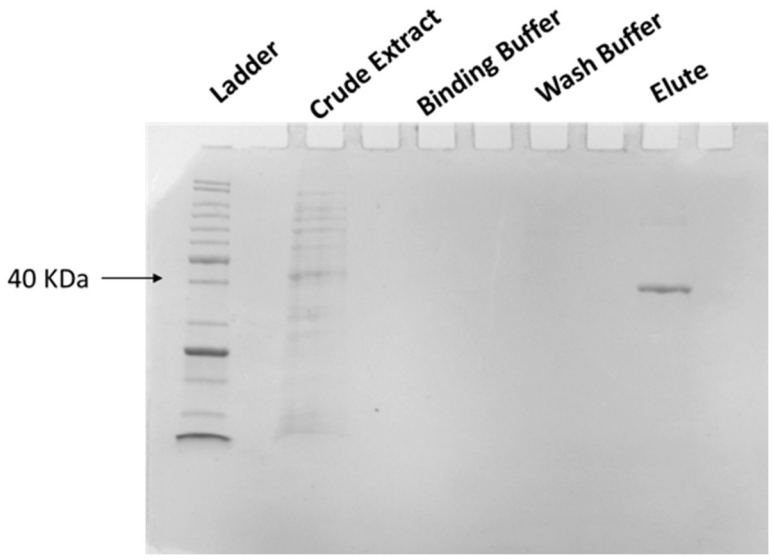


In Figure 15a, although only a portion is shown, the full sequence assembled from the *atoB*-pET-28a aligned with zero errors compared to the genome sequence data. The "g" that is circled indicates that the mismatch that happens in the beginning of the T7 sequence can be confirmed as correct with the T7 terminator sequence. Figure 15b shows many mismatches that cannot be confirmed.

III.B.5. Purification of the AtoB Protein

Now that the protein was overexpressed in *E. coli*, the protein needed to be purified by Nickel-Affinity chromatography. As each buffer ran through the column, each wash was collected separately to assist with protein detection. The final elution phase involving high concentrations of imidazole (4M) was collected in 1mL portions and tested using a Bradford assay. The Uniprot database showed AtoB to have a molecular weight of 40.3KDa. The SDS-PAGE gel (Figure 16) showed that the product eluted from the column as a single band near the 40KDa ladder band.

Figure 16. SDS-PAGE Gel



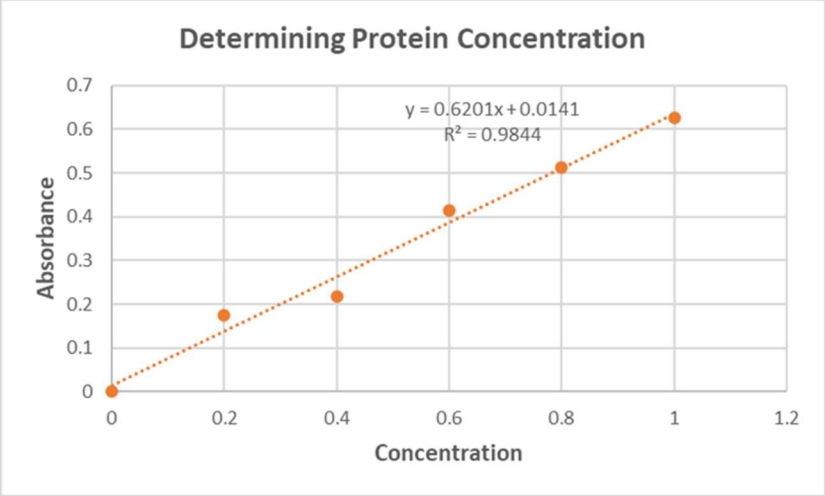
The 8mL of eluted protein was collected in 1mL fractions and were tested using Bradford reagent. The Bradford assay showed that five of them had high amounts of protein and those five fractions were combined for dialysis.

III.B.6. BSA Standard Curve Assay

Bovine serum albumin (BSA) protein was used to help us determine the concentration of our AtoB protein. We made stock solutions with different concentrations of BSA (0.0-1.0mg/mL).

Each stock solution was made to 500µL using ddH_2O. the absorbance of each stock solution was collected using UV-Vis spectrophotometry, which is also shown in table 3. The absorbance and concentration of each stock was used to create the graph shown in Figure 17.

Figure 17. Bradford Assay Calibration Plot of BSA Standards



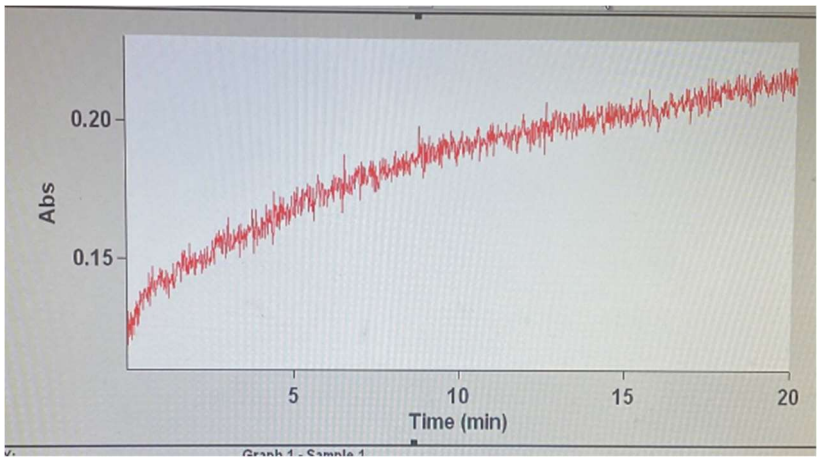
The Bradford assay and standard plot used in Figure 17 was used to determine that the enzyme was 0.19mg/mL after dialysis.

III.B.7. UV-Vis Spectrophotometry

With the kinetic results from the UV-Vis spectrophotometry, we wanted to observe an increase in absorbance that eventually stopped changing when equilibrium was reached, which favors the acetyl-CoA. We attempted 13 trials with different conditions to help determine the optimal concentration of acetyl-CoA.

When we reached our 5th trial, we noticed that it yielded better results than the previous 4 trials, but it still wasn't optimal. Trials 6-10 were repeats of 1-5 with a different amount of AtoB to possibly better the results. We saw an improvement with trial 10 but continued to try and yield a better result. Trial 11 was a continuation from trial 10, just changing the amount of acetyl-CoA. Trial 12 and 13 were also replicas of trial 5 with increases in AtoB. In trial 13 (Figure 18), we see a plateau with 0.05mM acetyl-CoA. Based on the plateau shown in the plot, we expect an effective reaction of AtoB catalyzing acetyl-CoA, forming acetoacetyl-CoA.

Figure 18. Trial 13-0.5 mM Acetyl-CoA



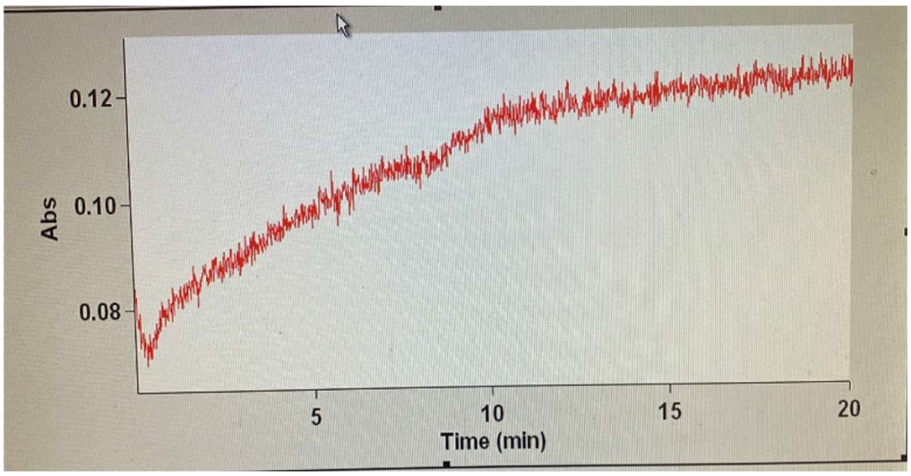
We attempted to better trial 13 by altering some of the conditions. We completed 15 more trials. Trials 1-3 had increasing acetyl-CoA concentrations with a consistent amount of AtoB. The trials 4-6 had a consistent acetyl-CoA concentration while increasing AtoB amounts. Trials 7-15 were combinations of the new acetyl-CoA concentrations with the different AtoB amounts (table 4).

Table 4. Extended Trials

Trial Number	Final Concentration of Acetyl-CoA (mM)	Amount of AtoB (mg)
1	0.75	0.13
2	0.90	0.13
3	1.0	0.13
4	0.5	0.17
5	0.5	0.20
6	0.5	0.

Out of the 15 trials, only one gave us similar results to our original trial 13, which was trial 2 with 0.9mM acetyl-CoA with 0.19mg of AtoB (Figure 19).

Figure 19. 0.9mM Acetyl-CoA



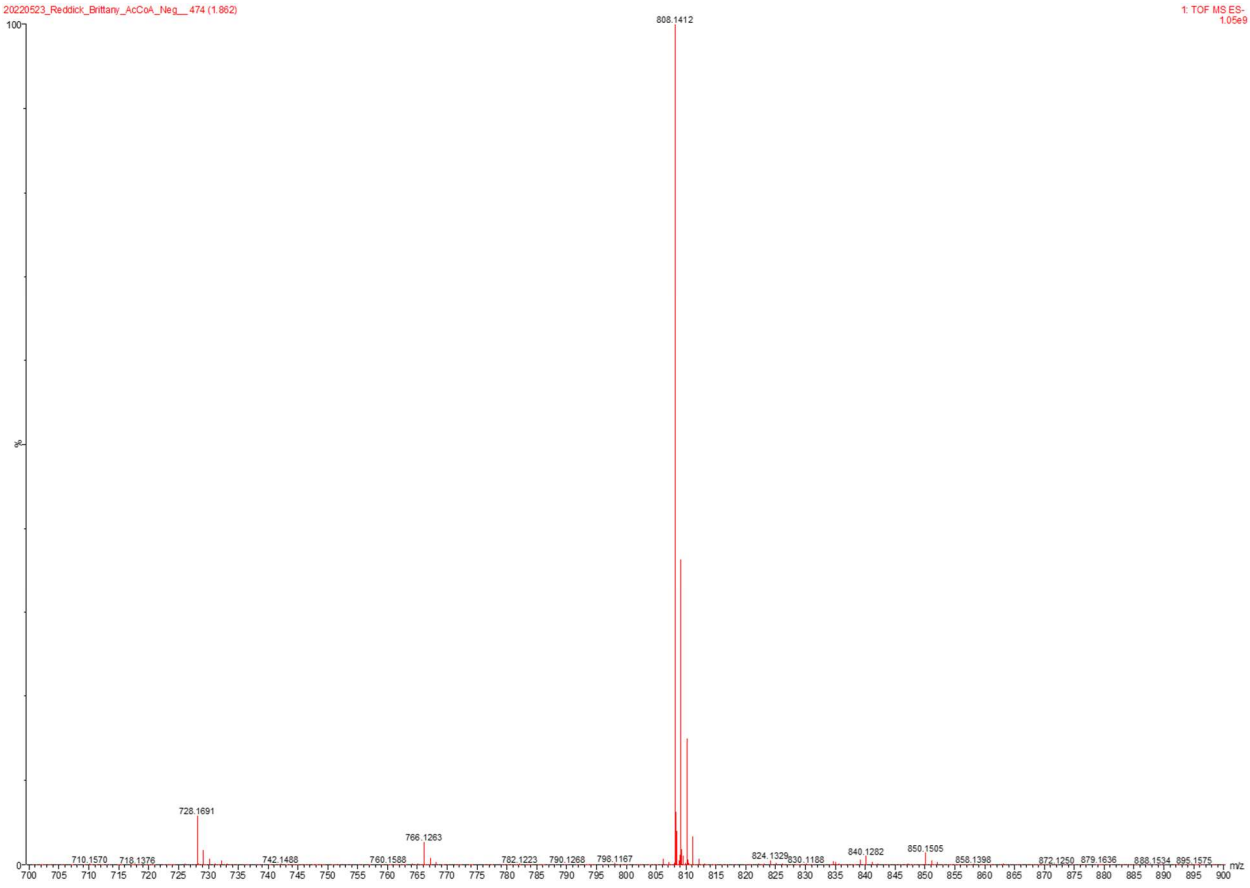
This set of reactions were completed using acetyl-CoA that was not detectable by LC-MS. It is possible that the acetyl-CoA used degraded over time, which would explain the low levels conversion analyzed by UV-Vis Spectrophotometry.

CHAPTER IV: CHARACTERIZATION OF ACETOACETYL-COA

IV.A. Mass Spectrometry Work

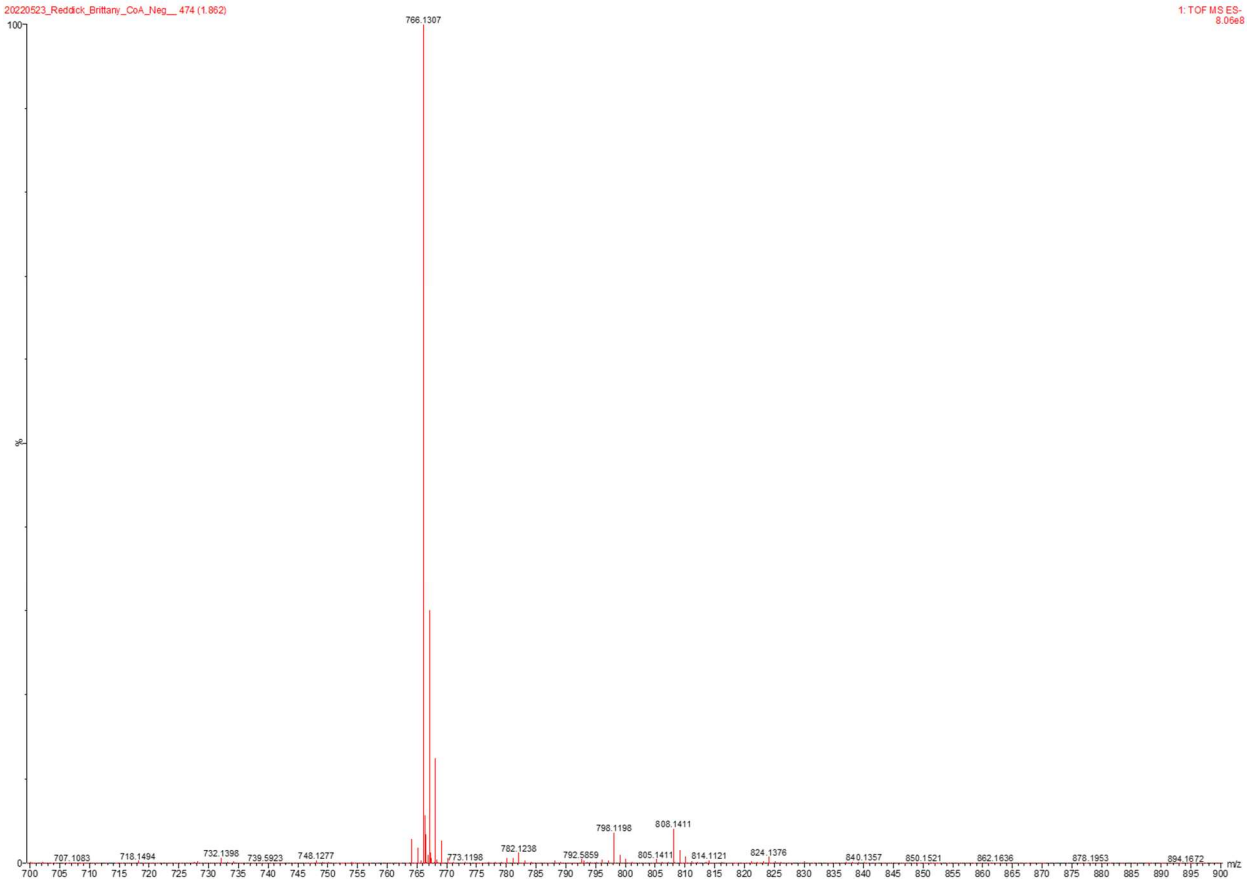
Mass spectrometry was used to analyze the formation of acetoacetyl-Co and check for activity and synthesis of the reaction. Using this instrumentation, we aim to visualize acetyl-CoA, acetoacetyl-CoA, and a single Coenzyme-A (CoA) molecule which is also a product of the Claisen condensation reaction. The first goal was to identify both acetyl-CoA and CoA separately so we could know exactly where we would see those peaks. Acetyl-CoA has a mass to charge ratio of 809.13. In Figure 20 the spectra for acetyl-CoA shows a peak at 808.14 with the instrumentation being in negative mode, meaning the molecule was deprotonated so the mass to charge would be one less than the actual value.

Figure 20. Mass Spectra Showing Acetyl-CoA



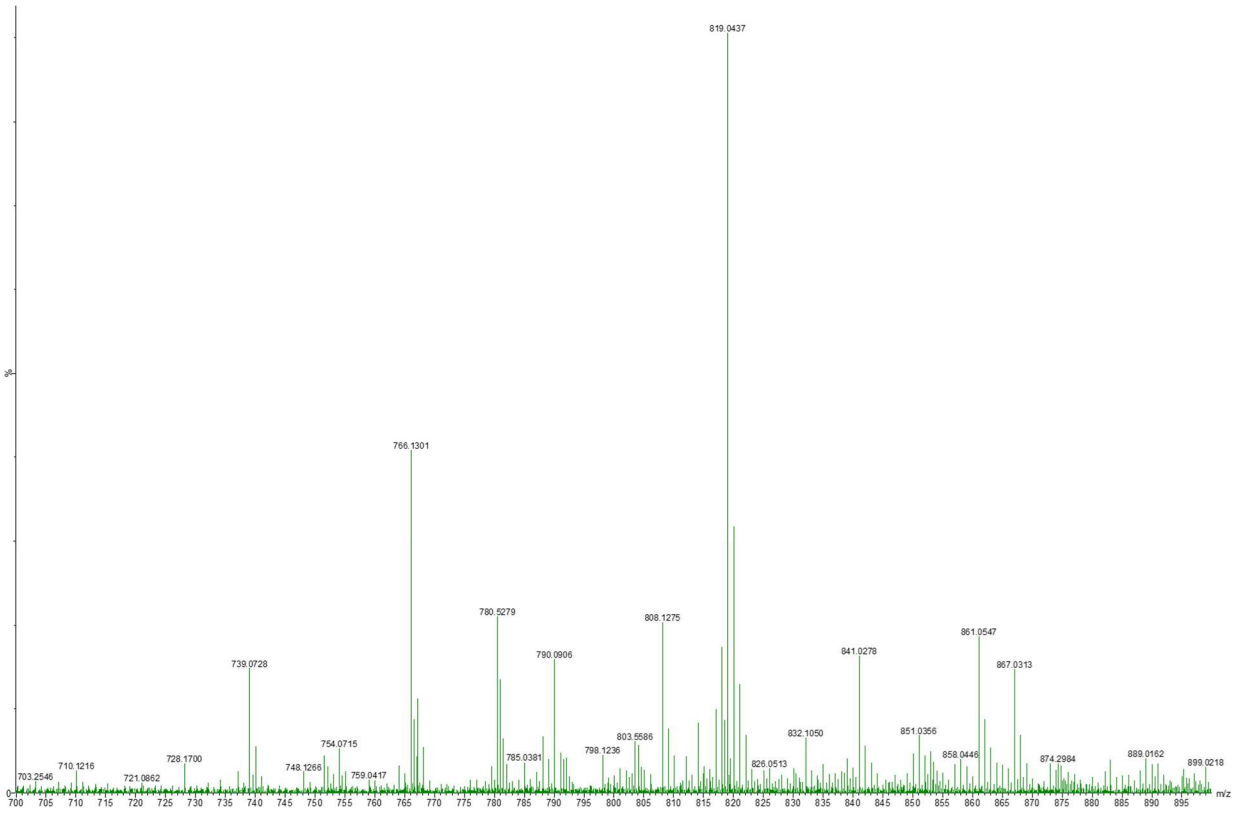
The CoA molecule has a mass to charge ratio of 767.12. A peak was analyzed at 766.13 in negative mode. The spectra result for CoA is shown in Figure 21.

Figure 21. Mass Spectra Showing CoA



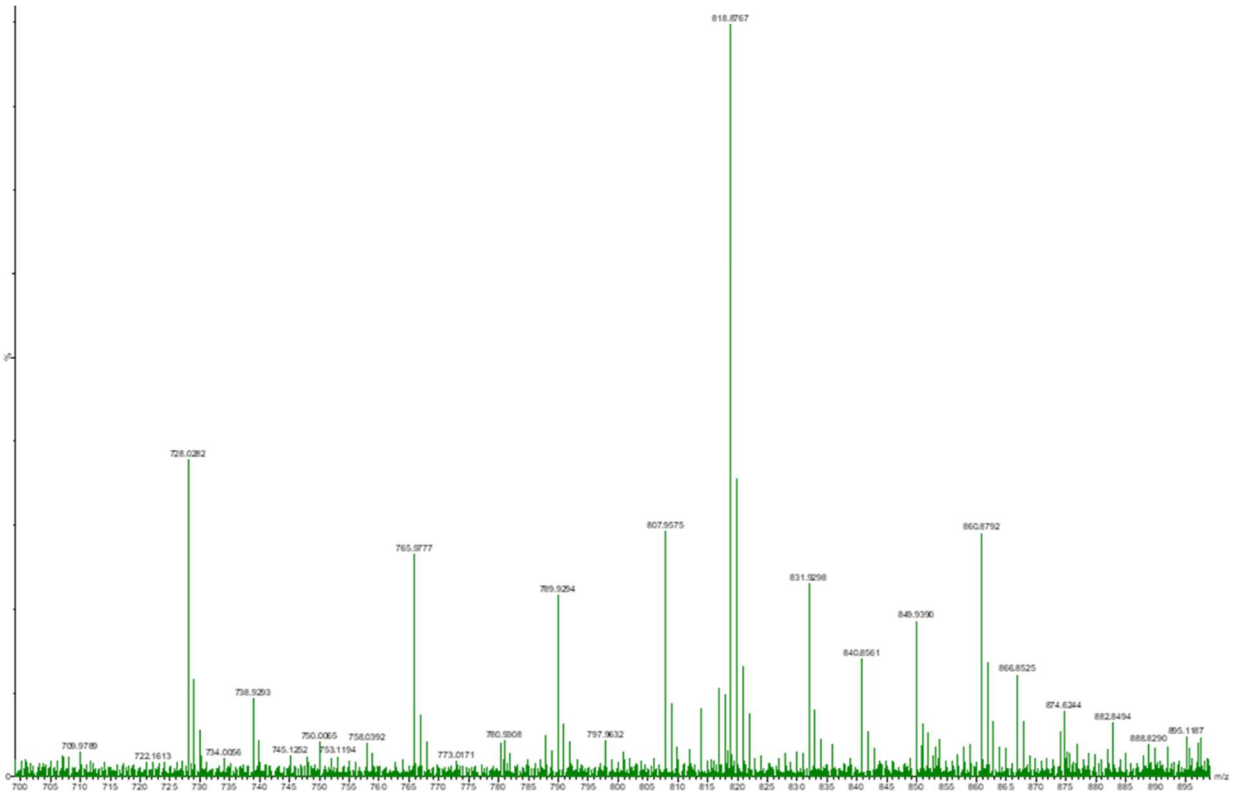
Knowing where we should see peaks for acetyl-CoA and CoA, the full reaction was then analyzed after an incubation of 20 minutes. In the spectra shown in Figure 22, we see peaks at 766.13 representing CoA, and at 808.12 representing Acetyl-CoA. Acetoacetyl-CoA has a mass to charge ratio of 851.14 so in negative mode we should see a peak at ~850. At 851.05, a small peak is shown in Figure 22.

Figure 22. Mass Spectra Observing the Reaction after 20 Minutes



Another reaction was analyzed after incubating overnight. The results from the spectra shown in Figure 23 are similar to those in Figure 22. The two spectra show differences in the size of the peaks for the three molecules being observed. In Figure 23, a peak is observed at 765.97 for CoA rather than 766.13 as seen in Figure 22. Peaks for acetyl-CoA and acetoacetyl-CoA are observed at 807.95 and 849.93 rather than 808.12 and 851.14, respectively.

Figure 23. Mass Spectra Observing the Overnight Reaction



Spectra for just the buffer used in the reaction (100mM Tris-HCl, 25mM Magnesium Chloride, at pH 8.1) and ddH_2O were analyzed as a control. The results from both the buffer (Figure 24) and the water (Figure 25) were giving similar peaks as the reaction spectra.

Figure 24. Mass Spectra Observing the Buffer

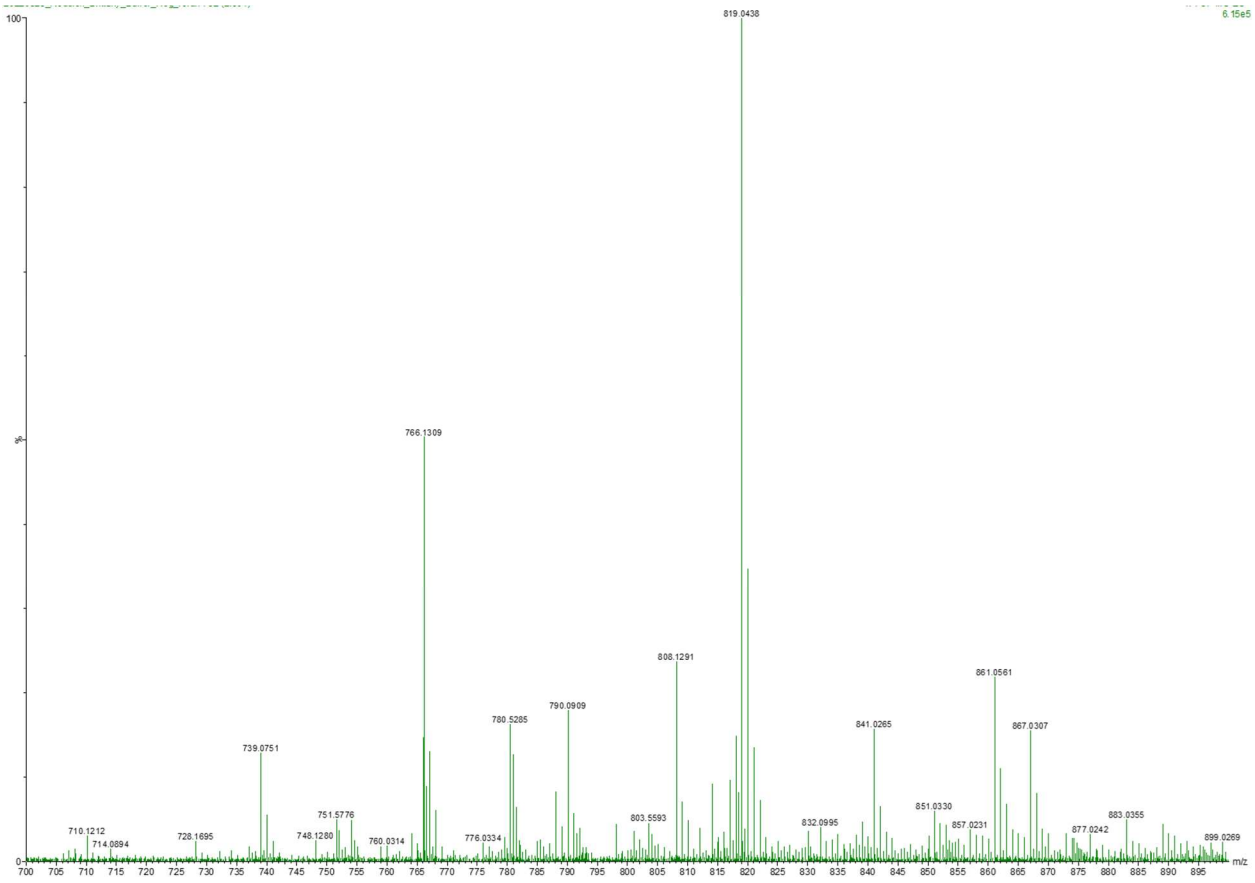
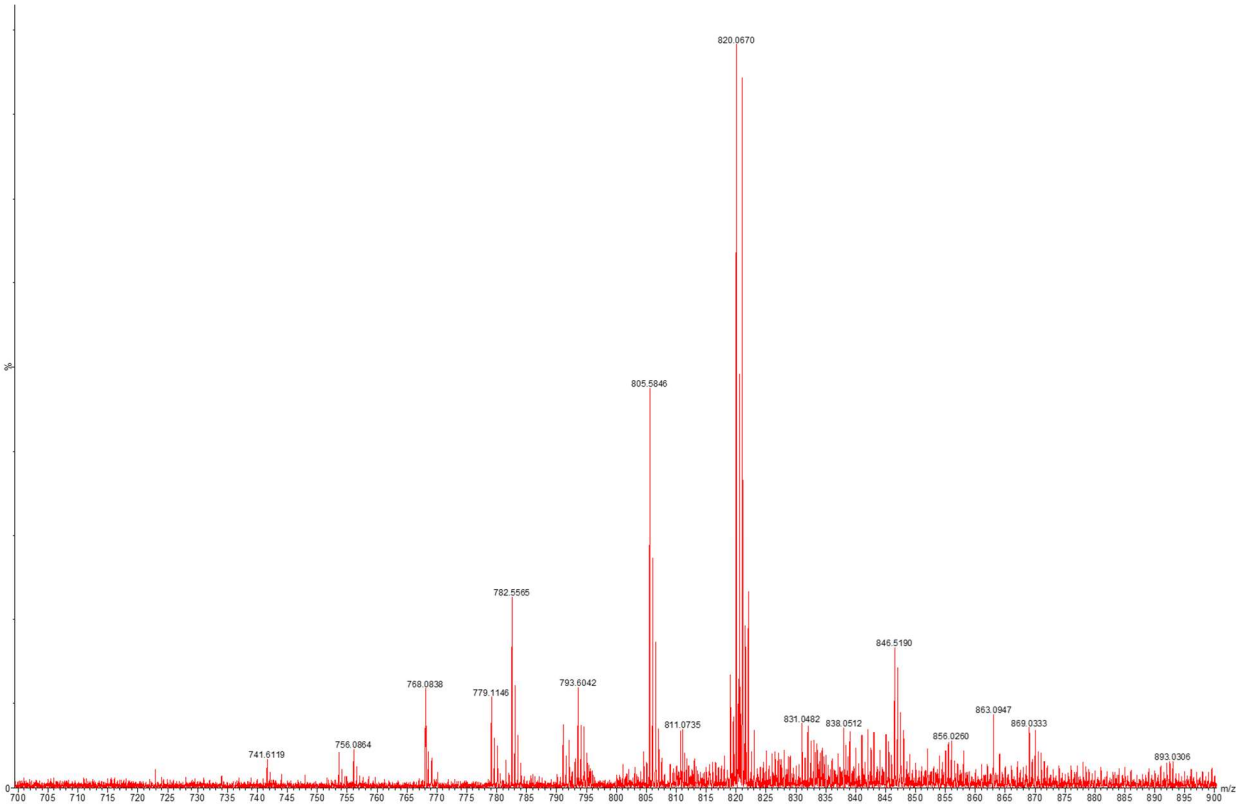


Figure 25. Mass Spectra Observing Water



Due the size of the peak, further analyses and optimizations would need to be conducted to visualize the activity and formation of acetoacetyl-CoA. Based on the results of the water spectra being similar to the reaction spectra, it is quite possible there is some contamination in the column or other components used in the instrumentation, leaving the current results to be inconclusive.

CHAPTER V: CONCLUSIONS

V.A. Restriction enzymes Vs. HiFi

At the beginning of this research, we were using the Restriction Digest assembly methods with the use of restriction enzymes. This method required multiple steps with separate steps for restriction digest, heat deactivation, and ligation in order to ligate the *atoB* gene into pET-28a. The restriction digest for the purified *atoB* gene and pET-28a plasmid required different restriction enzymes due to one of the restriction enzymes having multiple cutting sites in *atoB*. If we were to use the same enzymes for *atoB* that were used for pET-28a, we would end up having 2 different *atoB* fragments due to it being cut twice. After ligating with T4 DNA ligase, we would transform the ligated product into TOP10 competent *E. coli* cells. We would then confirm transformation with gel electrophoresis. The gel would always give us a band at the correct position, but every time we got the sequencing results back from Eurofins, the sequence was incorrect and consisted of many mismatched base pairs. We soon realized that we were getting false positive PCR bands because of the cloning method. Usually in Dr. Reddick's group, we are cloning genes from *Bacillus* bacterial genes, inserting them into a plasmid, and then transforming it into TOP10 Competent *E. coli* cells. Due to the gene being produced from *E. coli*, and then transforming into TOP10 Competent *E. coli* cells, we were essentially transforming an *E. coli* gene back into *E. coli*. Using Restriction Digest assembly methods introduced a lot of issues with cloning this specific gene.

To overcome this issue, we switched cloning methods and began to use HiFi Gibson Assembly. The use of this method left less room for errors and allowed for easier, successful ligation. After ligating the *atoB* and pET-28a fragments, we moved on to transformations. From

here, we attempted to use the TOP10 $CaCl_2$ competent cells again, while also transforming with already competent NEB 5α Competent Cells. The TOP10 cell transformation plate did not grow any colonies, but the NEB 5α Competent Cells transformation plate produced a plethora of colonies. Using the 5α competent cells rather than TOP10 $CaCl_2$ competent cells led to successful DNA sequencing confirming the *atoB*-pET-28a product as well as successful protein overexpression and purification.

V.B. Conclusions and Future Works

The main goals that needed to be achieved before characterizing the protein were reached. We were able to successfully clone the *atoB* gene and purify the encoded protein. There has been some success with detecting the desired activity of the synthesis of acetoacetyl-CoA using UV-Vis spectrophotometry. The experiments conducted with UV-Vis spectrophotometry showed some promising results and possible production of the desired product, acetoacetyl-CoA. The mass spectrometry experiments gave inconclusive data not showing any appreciable production of acetoacetyl-CoA. From here the next steps would be to characterize the enzyme reaction using mass spectrometry and see if our reaction is actually producing acetoacetyl-CoA.

For future work, the goal is to continue to analyze the reactions using mass spectrometry for the formation of acetoacetyl-CoA. Once the formation is confirmed, the reaction will be scaled, and the acetoacetyl-CoA product will be purified using anion exchange chromatography which can then be used in Dr. Kiel's β-branch work.

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