Engineering *Escherichia coli* BL21(DE3) for the Production of 5-Amino-1-Pentanol

by

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ABSTRACT

Alkanolamines are useful as building blocks for a variety of applications, ranging from medical applications such as drug and gene delivery. In this work, *Escherichia coli* was investigated as a viable candidate for the production of 5-amino-1-pentanol (5-AP). Taking advantage of the existing L-lysine degradation pathway, a novel route to 5-AP was constructed by co-expressing the genes *cadA* (encoding lysine decarboxylase, responsible for the conversion of L-lysine to cadaverine) and *patA* (encoding putrescine aminotransferase, responsible for the conversion of cadaverine to 5-amino-1-pentanal), followed by the endogenous reduction of 5-amino-pentanal (5-APL) to 5-AP. To avoid the competing conversion of 5-APL to 5-amino-1-pentanoate and avoid accumulation of byproduct 1-Δ-piperideine, further host engineering was performed to delete the gene *patD* also known as *prr* (encoding 5-amino-pentanal dehydrogenase). Flask scale fermentation experiments in minimal medium of the newly constructed pathway was conducted where 62.6 mg/L 5-AP was observed to be produced. It was hypothesized that 5-AP production could be boosted by optimizing production medium to M10 media. However, change in the culture medium resulted in the production of just 51 mg/L 5-AP. Shifts observed in HPLC chromatogram peaks made it difficult to conclude exact titers of 5-AP and can be further improved by exploring different analysis methods and optimization of the method currently in place.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my family for the continued support they have given me while obtaining my degree. My brother Dr. Nicholas Brookhouser has been a major inspiration while perusing my dreams.

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INTRODUCTION

1.1 Metabolic Engineering Enables the Production of Useful Biochemicals

The ability to produce bio-based chemicals in a sustainable and eco-friendly way has been extensively researched over the past several decades. Advances in metabolic engineering of various microorganisms have opened the door for production of natural and unnatural products quickly and efficiently from renewable feedstocks [1][2]. *Escherichia coli* has become the most characterized microorganism to date and has limitless potential for industrial applications [3][4]. Ranging from production of biofuels such as isopropanol [5] and butanol [6] to synthesis of amino acids such as lysine [7] and tryptophan [8], *E. coli* has the potential to tackle many modern global issues.

1.2 5-Amino-1-Pentanol is an Alkanolamine with Diverse Applications

Amino alcohols, also classified as alkanolamines, have recently become of great interest for bioproduction due to their physiochemical properties [9]. The availability of both an amine and alcohol functional group allow for a wide variety of applications such as surfactants, cosmetics, and building block chemicals [9][10]. In this study, the amino alcohol 5-amino-1-pentanol (5AP) is synthesized through reduction of the intermediate 5-amino-pentanal (5-APL), which can be synthesized via the L-lysine degradation pathway native to *E. coli*. The main interest in 5-AP production is that it can serve as a building block for the synthesis of branched poly(5-amino-1-pentanol-*co*-1,4-butanediol diacrylate) for development of a novel gene delivery system [11][12]. Poly(5-amino-1-

pentanol-*co*-1,4-butanediol diacrylate) is classified as a linear poly(*β*-amino ester) (LPAE) (Figure 1)

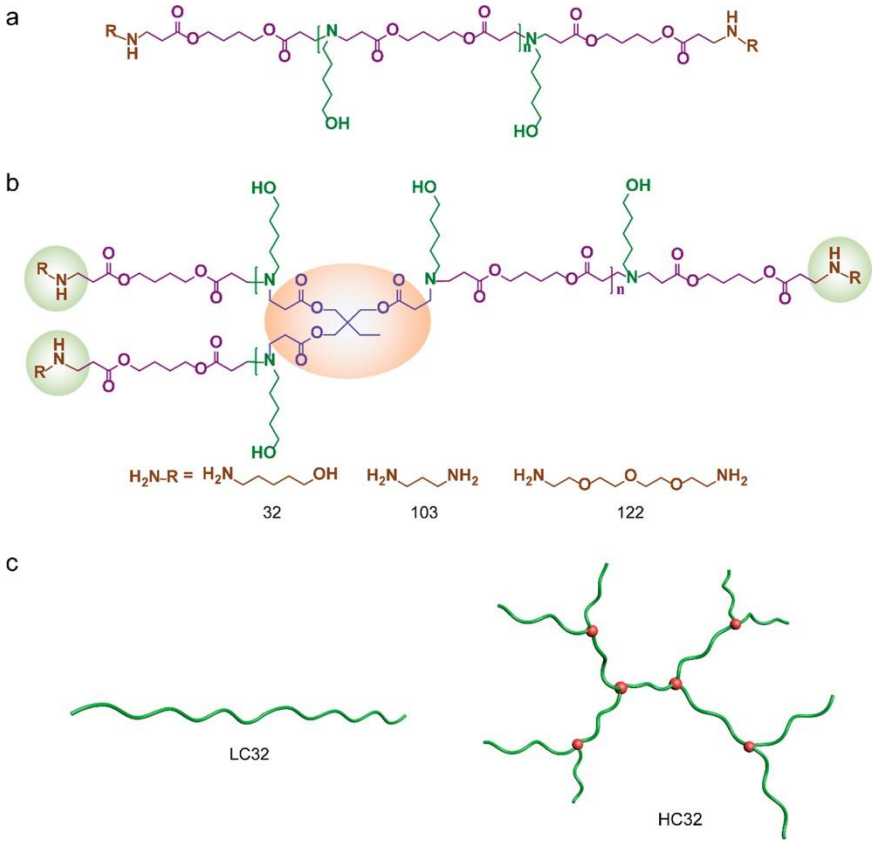

Figure 1. Linear poly(*β*-amino ester) Structures for Gene Delivery Applications (Reprinted with permission from Zhou, D., Gao, Y., O'Keeffe Ahern, J., Xu, Q., Huang, X., Greiser, U., & Wang, W. (2016). Development of branched poly (5-amino-1-pentanol-co-1, 4-butanediol diacrylate) with high gene transfection potency across diverse cell types. *ACS Applied Materials & Interfaces*, *8*(50), 34218-34226.Copyright {2016} American Chemical Society.)

To date there have been no reports of bio-based production of 5-AP as it is typically synthesized by reacting ammonia directly with epoxies; however, this remains a very challenging and expensive process [13]. Thus, this study presents an alternative strategy for the direct microbial production of 5-AP from sugar via engineered *E. coli*. It is noted that the production of 4-amino-1-butanol has recently been reported via a pathway that involves the same chemistry and analogous, 4-carbon intermediates [14]. Similarly, there

have been reports of producing 5-aminovalerate (5-AMV), a 5-carbon polyamide, utilizing similar methods of pathway construction [15]. It was hypothesized that overexpression of the L-lysine decarboxylase (encoded by *cadA*) for production of cadaverine and the putrescine aminotransferase (encoded by *patA*) for cadaverine conversion to the omega-aminoaldehyde 5-APL would be a suitable method for 5-AP synthesis. Combined with the over expression, it was also hypothesized that knock out of the γ-aminobutyraldehyde dehydrogenase (encoded by *patD*) would stop the pathway at 5-APL production allowing for natural aldehyde reductases to form the desired 5-AP (Figure 2.).

1.3 Engineering *E. coli* for L-lysine Production

L-lysine, an essential amino acid, has been successfully produced through methods of microbial fermentation [16]. Biosynthesis of L-lysine plays an incredibly important role in metabolic networks [17]. Using L-aspartate as a precursor, *E. coli* will undergo nine enzymatic reactions utilizing the diaminopimelate (DAP) and aspartate pathways for synthesis of L-lysine [17][18][19]. Previous studies have shown that engineered *Corynebacterium glutamicum* can produce 1,000,000 metric tons per year of L-lysine [19]. These strains of *C. glutamicum* have been engineered using enzymes that's are also native to *E. coli* [15][19][20] allowing for an abundance of L-lysine to be produced as a building block for production of target chemicals. Expression of both *lysC* encoding for asparate kinase III (AK-3) and *dapA* encoding for dihydrodipicolinate synthase is crucial as L-lysine overproduction is the driving force for metabolic production of 5-AP. The genes contained in the lysine overproduction plasmid carry a

feedback resistance mutant (T3521) allowing for free lysine to be generated [15][21]. Previous studies have confirmed that inhibition of AK-3 allows for a higher tolerance to L-lysine and production up to 100 mM [21][22].

1.4 Engineering *E. coli* for Cadaverine Production

Cadaverine, known as 1,5-diaminopentane, is an important platform chemical used as a building block for production of a variety of chemicals [19][23]. It has applications to produce biopolymers, chelating agents, and polyamides (PA) [24]. Consisting of repeating diamines in the backbone PAs have industrial uses for the production of clothing fibers or thermoplastics for carpets, car parts, and tire reinforcements [24][25]. An example of successful production of cadaverine from an initial literature review reported a titer of 9.6 g/L in *E. coli* [23]. A second example from literature reports production of cadaverine at a titer of 2.39 g/L also in *E. coli* [19] by overexpression of lysine decarboxylase (*cadA*) in *E. coli*. To date these are the best-known examples of successful cadaverine production. With overexpression of the upstream L-lysine biosynthesis pathway production of cadaverine can be significantly increased. Previous works confirm that *E. coli* is a suitable host for cadaverine production based on past observed titers and tolerance to cadaverine up to 0.5 M [19].

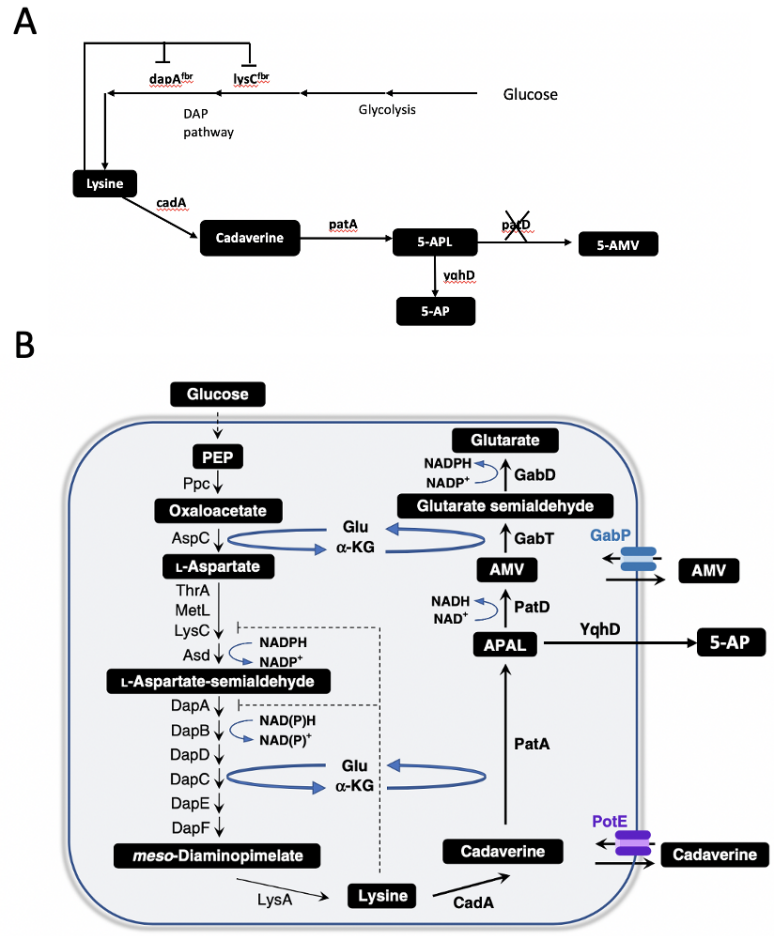

Figure 2. Overall schematic for 5-AP production from Glucose. (A) Illustration of reactions of designed pathway. (B) Metabolic pathway for Glutarate synthesis native to *E.* coli. Over expressed Genes: *lysC*^{fbr}, aspartate kinase III; *dapA*^{fbr}, 4-hydroxy-tetrahydrodipicolinate synthase; *cadA*, lysine decarboxylase; *patA*, putrescene aminotransferase. Gene knockout: *patD*, γ-aminobutyraldehyde dehydrogenase. (**Open Access:** Li, W., Ma, L., Shen, X. *et al.* Targeting metabolic driving and intermediate influx in lysine catabolism for high-level glutarate production. *Nat Commun* **10,** 3337 (2019). https://doi.org/10.1038/s41467-019-11289-4)

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

5-Amino-1-pentanol (>95%) and 1,5-diaminopentane (cadaverine; >98%) were acquired from TCI Chemicals (Portland, OR). L-Lysine was acquired from SAFC Pharma (St. Louis, MO) and α-ketoglutaric (2-KG) acid potassium salt (>=98%) was acquired from Sigma Life Science (St. Louis, MO).

2.2 Microorganisms

Strains used and engineered in this study included *E. coli* DH5α , for molecular cloning, and *E. coli* BL21(DE3) as the expression host for the newly constructed pathway n. This strain was chosen due to the nature of carrying λDE3 lysogen, allowing for suitable T7 RNA polymerase expression when coupled with inserted Duet vectors.

2.3 Culture Conditions and Culture Medium

All *E. coli* strains were cultured in liquid Luria-Bertani (LB) Broth (MiliporeSigma; Burlington, MA) at 37°C in a shaking incubator at a rate of 225 rotations per minute (RPM). *E. coli* strains were also cultured with LB agar plates in standing incubators at 37°C. All cultures and plates were supplemented with appropriate antibiotics with concentrations of ampicillin (amp) at 100 mg/L, kanamycin (kan) at 35mg/L, and/or streptomycin (strep) at 100 mg/L. Initial production testing was performed in M9 minimal media containing 0.4% Glucose. Optimized production of 5-AP was performed in M10 media with a composition of (per liter): 25 g glucose, 16 g

$(NH_4)_2SO_4$, 1 g $MgSO_4$, 1 g KH_2PO_4, 2 g yeast extract, and 30 g $CaCO_3$. M10 media has shown successful production of lysine by *E. coli* in previous studies [15][26]. Seed cultures were grown in LB overnight and inoculated into 250 mL baffled flasks containing 50 mL M10 medium at an initial OD of 0.05. Cultures were grown until reaching an OD of ~0.7 and induced with 0.25 mM IPTG. The cultures were grown for an additional 4 to 6 hours then spiked with a known concentration of respective precursor and allowed to grow for another 48 hours. Samples were taken periodically and used for further analysis.

2.4 Plasmid Construction

All strains and plasmids used for this work can be observed in Table 2.4.2. For the construction of the plasmid pETDuet-*patA*-*cadA* restriction enzyme cloning (REC) was used for metabolic cloning of the target genes for this study. High-Fidelity Restriction enzymes NedI, XhoI, NcoI, and SacI were purchased from New England Biolabs (Ipswich, MA). Primers developed for this work can be observed listed in Table 2.4.1. All primers used for this study were purchased from IDT (Coralville, IA).

Table 2.4.1 Primer Sequences

Primer Name	Primer Sequence (5'→ 3')
F_*patA*_NedI	TAACATATGAACAGGTTACCTTCGAGCGC
R_*patA*_XhoI	CTGCTCGAGTTACGCTTCTTCGACACTTACTCGC
F_*cadA*_NcoI	ATACCATGGGCGTTATTGCAATATTGAATCACATGGGGG
R_*cadA*_SacI	CGTGAGCTCTTATTTTTTGCTTTCTTCTTTCAATACCTTAACGG
*patD*_KO_F	ACTGCTGGTT ATGTTGGTAA
*patD*_KO_R	TTGATGTAGT GATTTTGCAC

*Restriction Sites Underlined

Table 2.4.2 Bacterial Strain and DNA plasmids used for this study

Strain	Function	Reference
E. coli DH5	Molecular Cloning Strain	Prather Lab (MIT)
E. coli BL21(DE3)	DE3 lysogen	Prather Lab (MIT)
E. coli MG1655	Used to obtain linear fragments of *cadA* and *patA*	Prather Lab (MIT)
E. coli BL21(DE3)Δ*prr*	Deletion of *prr* gene responsible for conversion of 5-APL to 5-Aminovalerate	This Study
E. coli JW1439-1	BW25113 ::kan^R	CGSC
Plasmids		
pETDuet-1	Expression vector, Amp^R, P_{T7}, pBR322 ColE1 Ori	Prather Lab (MIT)
pKD46	Used for expression of λ red recombinase gene knockout	Prather Lab (MIT)
pETDuet-patA	*patA* inserted between *NedI* and *XhoI*. Conversion of cadaverine to 5-AP	This Study
pETDuet-patA-cadA	*cadA* inserted between *NcoI* and *SacI*. Conversion of Lysine to cadaverine.	This Study
pCDF-$lysC^{fbr}$-$dapA^{fbr}$	Used for L-lysine overproduction in *E. coli* BL21(DE3)	Adkins et al.

Figure 3 depicts a visual representation of the restriction enzyme digestion and ligation cloning method utilized for this study.

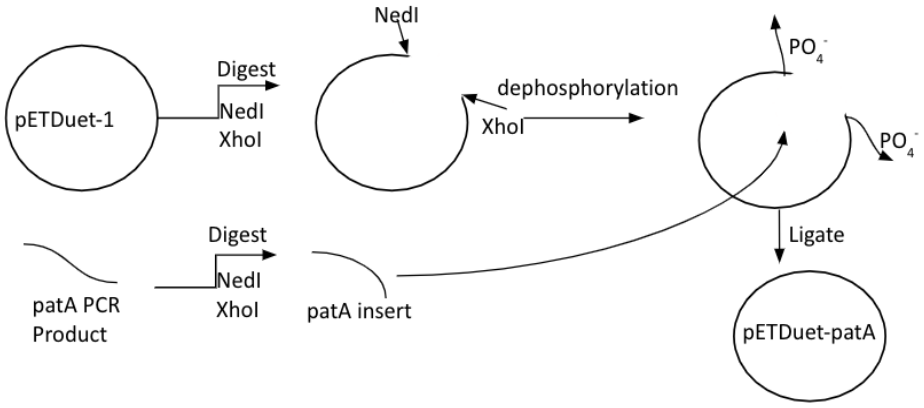

Figure 3. Restriction Digestion and Ligation Cloning Method

The primers F_*patA*_NedI and R_*patA*_XhoI were used for the amplification of the putrescine aminotransferase enzyme *patA* implementing Q5 High-Fidelity DNA Polymerase protocol acquired by New England Bio Labs (Ipswich, MA). The PCR fragment DNA was obtained through amplification of the K-12 *E. coli* strain *MG1655* and purified using a Zymo Research DNA and Concentrator purification kit. Confirmation of PCR fragment was conducted using gel electrophoresis (Figure 3). The concentration of the purified fragment was determined using a nano drop nucleotide analyzer to be 73.8 ng/μL. The backbone (*pETDuet-1*) was purified using a QIAprep Spin Miniprep Kit and similarly concentration was tested using a nano drop nucleotide analyzer and observed to be 54.1 ng/μL. Both purified PCR fragment and backbone were digested using the protocol described in Table 2.4.2.

Table 2.4.3 Digestion Protocol

Component	Volume
DNA (Vector or PCR Product)	2 μg
10x Cutsmart Buffer	2 μL
Restriction Enzyme 1 (*NedI*)	1 μL
Restriction Enzyme 2 (*XhoI*)	1 μL
DI Water	Up to 20 μL

All components were mixed together in a 20 μL reaction and placed in a non-shaking incubator overnight to ensure that both the backbone and PCR fragment are cut properly. To prevent the cut plasmid from re-circularizing, the plasmid was treated with Shrimp Alkaline Phosphatase (rSAP) through a process known as dephosphorylation described in the Table 2.4.3 below.

Table 2.4.4 Dephosphorylation Protocol

Component	Volume
DNA (plasmid)	1 μg
10x Cutsmart Buffer	2 μL
rSAP	1 μL
DI H_2O	Up to 20 μL

The dephosphorylation reaction was reacted in a volume of 20 μL and placed in the 37°C incubator overnight to allow for the removal of phosphate groups (sticky ends) preventing re-circularization. Once sticky ends are removed the *pETDuet-1* was once again digested using the protocol described in Table 2.4.2 and allowed to incubate at 37°C overnight before the ligation step can take place. The ligation protocol utilized was obtained through New England Bio Labs. Both the 10x T4 buffer and T4 DNA ligase was purchased from New England Bio Labs (Ipswich, MA).

Table 2.4.5 T4 Ligation Protocol

Component	Ligation	No Insert	Vector only
10x T4 Buffer	1 μL	1 μL	1 μL
Vector	2 μL	2 μL	2 μL
Insert	1 μL	0 μL	0 μL
T4 DNA Ligase	0.5 μL	0.5 μL	0 μL
DI H_2O	Up to 10 μL	Up to 10 μL	Up to 10 μL

As seen in Table 2.4.4 the ligation reaction occurs alongside controls one lacking the inserted PCR fragment but still has added ligase while the other control lacks both the PCR fragment and T4 ligase. Once these reactions were allowed to occur, DNA was transformed into chemically competent BL21(DE3) cells and seeded onto LB/amp agar plates to grow at 37°C overnight. Confirmation of successful cloning of *patA* was conducted using Taq colony PCR and gel electrophoresis.

2.5 5-AP Toxicity Assay

To test the toxicity limit of 5-AP coupled with the engineered *E. coli* strain was determined by observing changes in growth when supplemented with 5-AP concentrations ranging from 0.1 g/L (minimum) to 5 g/L (maximum) in an incubator at 37°C and shaking at a rate of 225 RPM as well as in a Molecular Devices Spectramax iD3 plate reader (San Jose, CA). The toxicity test was conducted in M9 medium with a starting OD_{600} of ~0.1. OD_{600} was monitored for the next 24 hours.

2.6 Gene Deletions

To preserve precursor within the production pathway the γ-aminobutyraldehyde dehydrogenase enzyme *prr* (*patD*) was knocked out using the lambda red recombinase

system expressed in the plasmid *pKD46* described in previous works by Datsanko and Wanner (Figure 4). This was done using *E. coli* BW25113 mutants from the Keio Collection that carry the single gene deletion for *prr* (JW1439-1) [27]. This strain was purchased from the Coli Genetic Stock Center (CGSC, Yale University).

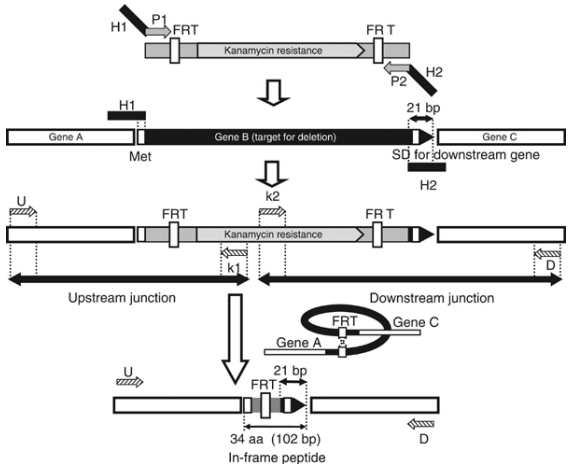

Figure 4. Gene Knockout strategy. H1 and H2 represent homology regions. P1 and P2 represent priming sites (**Open Access:** reprinted from Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., ... & Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular systems biology, 2(1), 2006-0008.)

This deletion was used to insert an FRT-Kanamycin-FRT cassette in place of *patD* in the genome. The primers *patD*_KO_F and *patD*_KO_R were used to amplify the linear gene fragment from the mutant strain JW1439-1. Once the fragment *Δprr*::kan^R fragment was obtained and purified, it was transformed via electroporation into electrocompetent BL21(DE3) cell containing the plasmid *pKD46* [28] using a Bio-Rad MicroPulser (Hercules, CA).The transformation was allowed to recover in 2x ampicillin/0.2% L-arabinose at 30°C in an incubator shaking at 225 RPM for 2 hours. Once recovered, 100 μL was streaked onto LB kan/amp plates and grown overnight at 30°C while the remainder was left to recover overnight at room temperature and plated the next day. A

taq colony PCR was performed to confirm the deletion of *patD* and the resultant host strain became BL21(DE3)*Δprr*.

2.7 Resting Cell Assay

To test the functionality of *patA* to determine conversion of cadaverine to 5-AP a resting cell assay was performed [15]. The strains BL21(DE3)*Δprr* containing empty vectors of *pETDuet* and *pCDFDuet*, BL21(DE3) *Δprr pETDuet-patA* and BL21(DE3) *Δprr pETDuet-patA-cadA pCDFDuet-lysC^{fbr}dapA^{fbr}* were inoculated into 50 mL of LB containing appropriate antibiotics and a starting OD of 0.05. Once cells had grown to an OD_{600} ~0.7 each culture was induced with 0.25 mM IPTG to allow for enzyme expression.

2.8 Analytical Methods

Optical density (OD) was used to measure cell growth using a Thermo BioMate3 spectrophotometer (Waltham, MA) at a wavelength of 600nm (OD_{600}). Concentrations of cadaverine, L-lysine, and 5-AP were determined via HPLC analysis (Agilent Series 1,100, Santa Clara, CA). All samples were analyzed using a single method for diamine derivatization using diethyl ethoxymethylmalonate (DEEM) [29]. Samples were allowed to rest at room temperature (RT) for 2 hours allowing for DEEM to fully react and derivatize samples. Derivatization was performed before injection and separation of samples using a Thermo Fisher Hypersil GOLD™ column (5 μm, 250 mm × 4.6mm; Waltham, MA) maintained at 35°C. Mobile phases (A) pH 4.8 25 mM sodium acetate and (B) 100% acetonitrile were flown through the column with a mixture of 80% A and

20% C at a flow rate of 1.00 mL/min. Ramp of (A:C) from 80:20 to 40:60 over the first 2 minutes, hold at 40:60 for 30 minutes, finally equilibrium at 80:20 for the final 8 minutes for a total run time of 40 minutes. Analytes were detected with a diode array detector (DAD) at 284 nm.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Assembly of Production Cassette

Being a part of the *Duet* vector family, pETDuet-1 offers several advantages for co-expression of genes in *E. coli* [30]. Duet vectors contain two multiple cloning sites (MCS), each with a respective T7 promoter allowing for expression of multiple target genes at once. These vectors also allow for co-expression of genes contained in different plasmids provided the origins of replication are compatible with the host [30][31]. Co-expression of target genes contained in separate plasmids work particularly well in (DE3) strains due to the λDE3 bacteriophage encoding for T7 RNA polymerase and allows for transcription of the targeted gene downstream from a T7 promoter site when induced with IPTG [32]. For this work, several restriction sites were considered in *pETDuet-1* as possibilities for insertion of the enzymes needed for this study. It was determined that NedI and XhoI were the most optimal sites for cloning of *patA*.

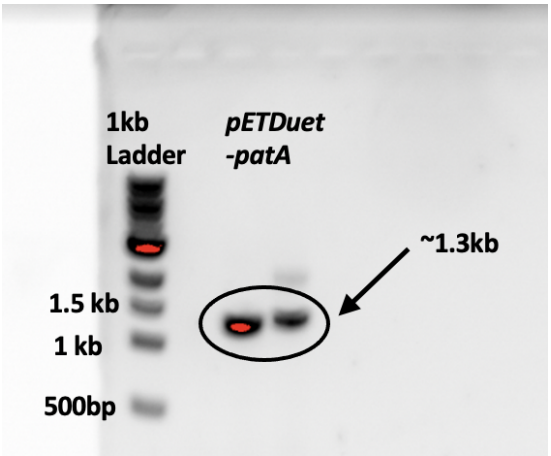

Figure 5. Confirmation of *patA* Cloning into Production Plasmid

As observed in Figure 5, the band located at approximately 1.3 kilobases (kb) signifies the successful cloning of *patA* into the chosen production plasmid pETDuet. The resultant plasmid after cloning became pETDuet-*patA*. Further validation of *patA* cloning was conducted by transforming the resultant plasmid into the host strain of BL21(DE3) and grown overnight at 37°C on LB/amp. Of the colonies observed after overnight incubation, 8 colonies picked at random were tested for *patA* through colony PCR (Figure 6.).

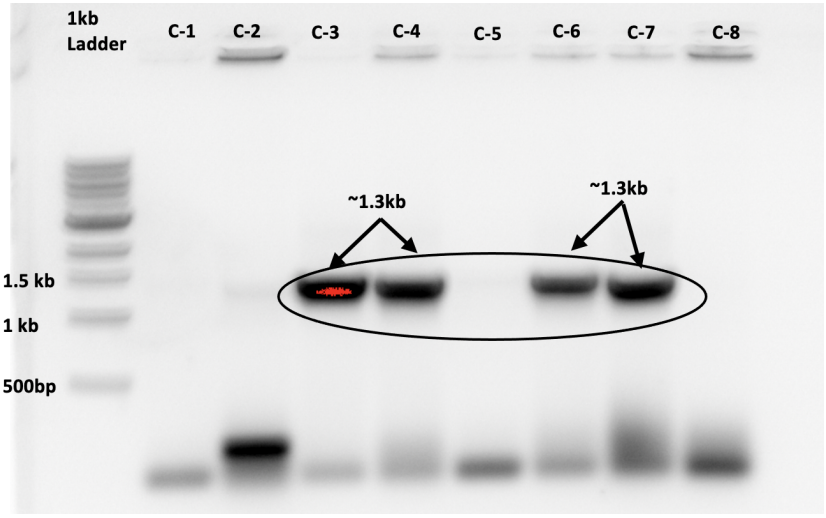

Figure 6. Gel Image of Colony PCR product

As shown in Figure 6, of the 8 colonies chosen only 4 showed bands at the appropriate size range for the gene *patA* indicating successful cloning of this gene. The cloning of the L-lysine decarboxylase gene *cadA* was performed using the same process of restriction enzyme digestion and ligation (Figure 7A). The restriction sites chosen for the cloning of this gene were NcoI and SacI.

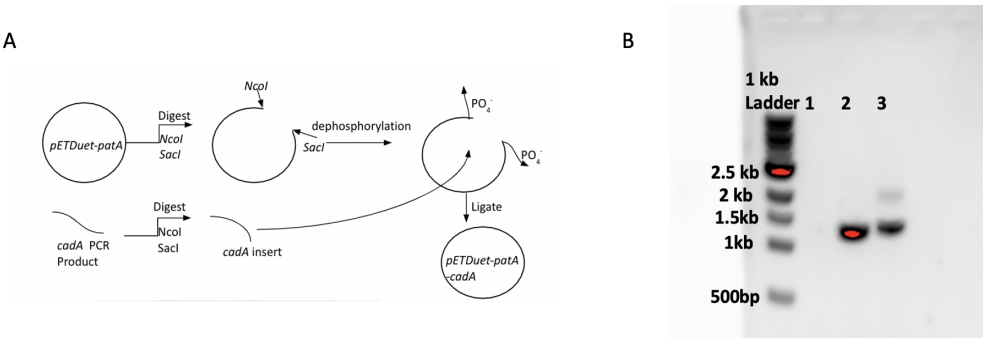

Figure 7. Restriction Enzyme Digestion and Ligation of *cadA*. (A) Restriction enzyme cloning schematic for *cadA*. (B) Gel image for *cadA* cloning confirmation; (1) *pETDuet-cadA* ,(2) *pETDuet-patA*, (3) pETDuet-*patA*-*cadA*.

As seen in Figure 7B, column 1 contains pETDuet-*cadA*, column 2 contains pETDuet-*patA*, and column 3 contains pETDuet-*patA*-*cadA*. Bands at a length of approximately 1.3kb can be observed in both columns 2 and 3 identifying *patA*. However, no bands are observed in column 1 and a very faint band is shown in column 3 at the appropriate length of 2.1kb identifying *cadA*. It was theorized that an issue with the annealing process when obtaining the linear fragment from *E. coli* MG1655 gDNA. To identify an appropriate annealing temperature range, a temperature gradient PCR was run (Figure 8).

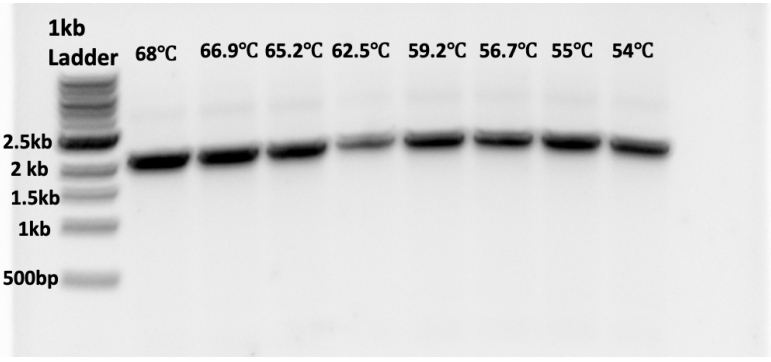

Figure 8. Temperature Gradient PCR for Determination of annealing temperature for *cadA*

A temperature range of 68°C being the maximum and 54°C being the minimum was chosen based on the melting temperatures (T_m) of *cadA* primers. The results of the

temperature gradient PCR were analyzed through gel electrophoresis and at all temperatures tested a band was observed at 2.1kb signifying the gene *cadA*. Digestion and ligation once again took place and colony PCR was used to determine successful cloning to develop the final constructed production plasmid pETDuet-*patA*-*cadA* (Figure 9).

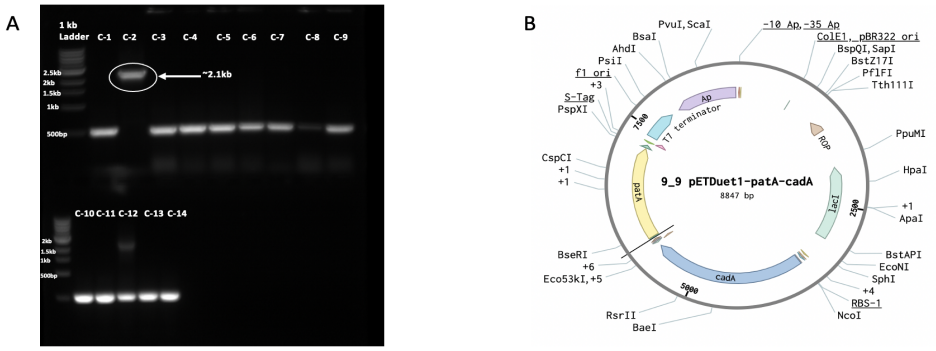

Figure 9. Validation of *cadA* Cloning and Final Plasmid Construction (A) confirmation of *cadA* via colony PCR. (B) Fully constructed production plasmid

From the overnight plating of the final ligation a colony PCR of 14 different colonies was performed and of the 14 colonies tested only one (C-2) showed a band at the desired length of 2.1 kb. The production plasmid pETDuet-*patA*-*cadA* was co-transformed into the host strain BL21(DE3) along with pCDF-$lysC^{fbr}$-$dapA^{fbr}$ used for lysine overproduction [15]. Due to L-lysine overproduction being the driving force for metabolic production of 5-AP, expression of both *lysC* encoding for asparate kinase III (AK-3) and *dapA* encoding for dihydrodipicolinate synthase is crucial. The genes contained in the lysine overproduction plasmid carry a feedback resistance mutant (T3521) allowing for free lysine to be generated [15][32]. Previous studies have confirmed that inhibition of AK-3 allows for a higher tolerance to L-lysine production up to 100 mM [32][33]. The conversion of L-lysine to cadaverine is taken on by one of the

two native L-lysine decarboxylase encoded by *cadA*. This gene has proven to be more favorable by researchers when compared to its counterpart *ldcC*. Previous works have shown *cadA* to have a higher thermostability and activity when compared to *ldcC* [26][34][35][36]. Final conversion of cadaverine to the intermediate 5-APL is driven by the putrescine aminotransferase enzyme encoded by *patA* which is then reduced to 5-AP by natural aldehyde reductase within *E. coli*.

3.2 Finalizing Production Strain via Gene Knockout

A single gene knockout (KO) was performed by replacing the γ-aminobutyraldehyde dehydrogenase encoded by *patD* (also known as *prr*) with a kanamycin cassette. Utilizing lambda red recombinase [28] described in previous works using the K-12 mutant JW1439-1 from the Keio collection [27] carrying the deletion for *prr*. The deletion of this gene was crucial to the construction of this pathway to restrict further pathway progression for synthesis of glutarate. Through electroporation, the plasmid pKD46 was transformed into the host strain BL21(DE3) and plated on LB/amp incubating at 30°C. Confirmation of successful plasmid integration was determined through colony PCR (Figure 10).

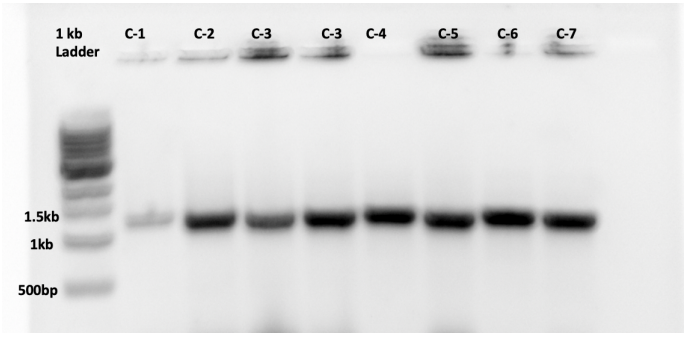

Figure 10. Confirmation of pKD46 integration via electroporation colony PCR

Of the seven colonies tested it was confirmed that all seven did show bands for the origin of replication and temperature sensitive replication encoded by repA101 approximately 1.1kb in length. Further confirmation was conducted by growing BL21(DE3) that contained pKD46 at a temperature of 42°C. Growth was not observed indicating the temperature sensitive plasmid has been successfully integrated into the host. The linear fragment obtained from the Keio collection strain JW1439-1was transformed into the host via electroporation. Confirmation of the successful gene KO was performed by plating the recovered transformation of LB plates containing kanamycin and ampicillin antibiotics.

3.3 Initial Testing for 5-AP Production

Initial fermentation testing with the constructed strain BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* was performed in M9 minimal medium containing 0.4% glucose. The optical density of all fermentation cultures was documented and compared to observe the effect the inserted plasmids had on growth (Figure A.1). Each fermentation test consisted of a control with the host strain BL21(DE3)*Δprr* pETDuet-pCDF*Duet* containing no genes for expression as a control, BL21(DE3)*Δprr* pCDF-*lysC-dapA* designed for overproduction of lysine, and BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* . Fermentation tests took place over the duration of 48 hours with each culture induced with IPTG at a concentration of 0.25 mM. Samples collected at points of induction, 24 hours, and 48 hours were analyzed through HPLC (Figure 11).

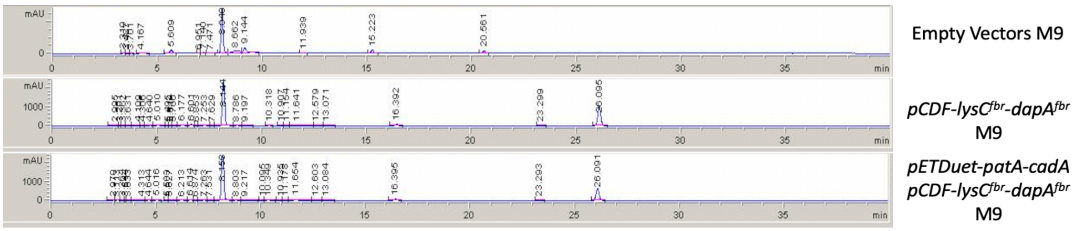

Figure 11. HPLC Chromatogram for flask fermentation in M9 minimal media after 48 hours. (Top) BL21(DE3)*Δprr*-pETDuet(empty)-pCDFDuet(empty) 48 hrs. (Middle) BL21(DE3)*Δprr* pCDF-*lysC-dapA* 48 hrs. (Bottom)BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* 48 hrs.

After 48 hours of fermentation in M9 minimal media cadaverine (~ 26 minutes) was produced by BL21(DE3)*Δprr* pCDF-*lysC-dapA* at a concentration of 6.82 mM (696.86 mg/L) and by the full constructed strain BL21(DE3)*Δprr pET-patA-cadA pCDF-lysC-dapA* at a concentration of 4.3 mM (439.37 mg/L). As expected, the full pathway carrying empty vectors showed no formation of L-lysine or cadaverine. However, no peaks were observed for L-lysine (~12 minutes) in any of the three chromatograms. Further fermentation testing of the production strain BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* was performed by the addition of precursors ~ 4 hours after induction. It was hypothesized that a higher concentration of IPTG may be needed to overproduce L-lysine as it may be used by competing pathways within the cell. To test this hypothesis, this fermentation test was performed again with an increased amount of IPTG from 0.25 mM to 0.5 mM. The results of this experiment showed inconsistencies as no L-lysine or cadaverine was observed to be produced (Figure 12).

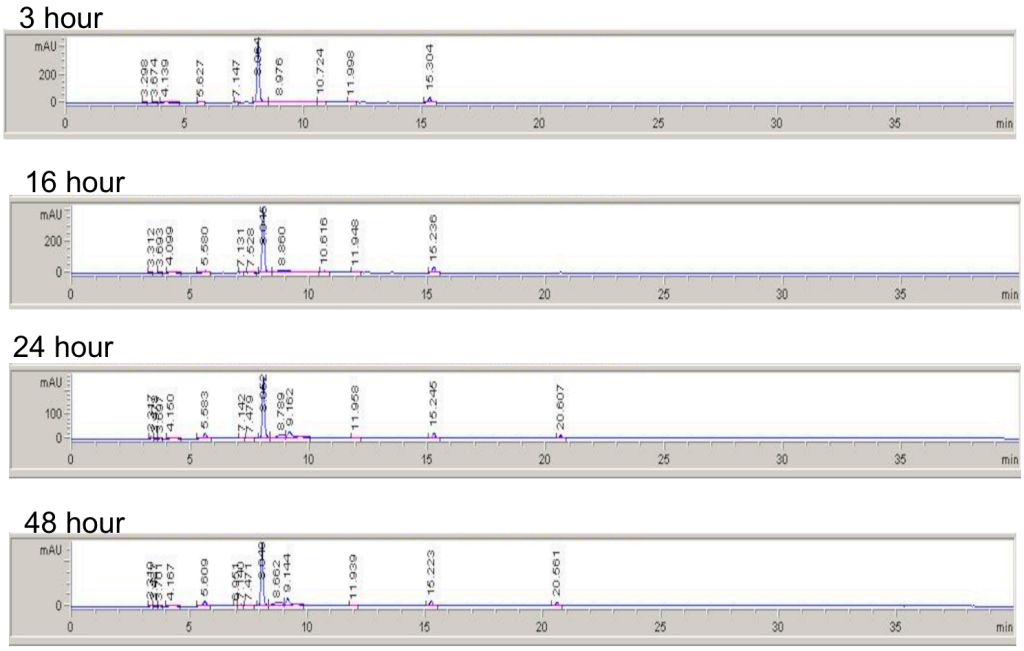

Figure 12. Flask Fermentation of BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* 0.5mM IPTG

As seen in the figure above, no L-lysine (~12 minutes) or cadaverine (~26 minutes) were produced after 48 hours of fermentation in M9 minimal medium when IPTG levels were increased. Leading to the question of gene functionality. Without proper function of *lysC* and *dapA* L-lysine will not be produced at sufficient levels to allow for downstream pathway conversion to cadaverine and finally 5-AP.

3.4 Resting Cell Assay

To test functionality of the genes within the L-lysine overproduction pathway (pCDF-*lysC-dapA*) and within the 5-AP production pathway (pETDuet-*patA-cadA*) a resting cell assay was performed [15]. Using the production strain (BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA*) and BL21(DE3)*Δprr* containing empty vectors as a control. Precursors were added to appropriate culture and allowed to run for a maximum of 16 hours in PBS (Figure 13).

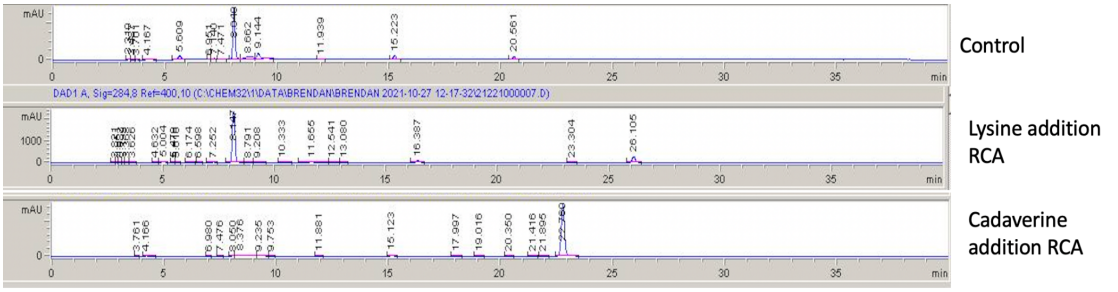

Figure 13. Resting Cell Assay HPLC Chromatogram sample at 16 hours. (Top) Control no precursor added. (Middle) BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* addition of 3 mM L-lysine. (Bottom) BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* addition of 3 mM cadaverine and 6 mM 2-KG

With a starting concentration of 3 mM (438.57 mg/L) L-lysine was consumed to a final concentration of 0.057 mM (8.46 mg/L) and produced 194.14 mg/L cadaverine as well as 62.8 mg/L 5-AP. However, with the addition of 3mM cadaverine and 6 mM 2-KG a negligible amount of cadaverine was consumed and only 23 mg/L 5-AP was observed to be produced. Indication that 5-AP is being produced confirms functionality of the genes however it was hypothesized that 5-AP may be hindering growth and as a result production. To assess the toxicity limit of 5-AP simple 15 mL culture tubes containing 5 mL M9 minimal media and the strain BL21(DE3)*Δprr* were seeded with 5-AP at concentrations of 0 g/L, .5 g/L, 1 g/L, 1.5 g/L, 2 g/L, 2.5 g/L, 3 g/L, 4 g/L, and 5 g/L. The culture tubes were allowed to grow for 24 hours with monitoring growth visually. After 24 hours it was observed that growth continued up to a maximum concentration of 3 g/L. These results confirmed that toxicity is not the issue as 5-AP has not been produced at concentrations higher than 62.8 mg/L. With toxicity not being the leading issue, it was hypothesized that the glucose concentration may not be high enough to push L-lysine overproduction.

3.5 Production of 5-AP

Testing the theory of glucose concentration being the limiting substrate, the fully constructed production strain was tested using M10 media that has proven suitable for L-lysine overproduction in previous works [15][26]. Through HPLC analysis no L-lysine production was observed; However, cultures with added L-lysine appeared to progress making the target product 5-AP at a concentration of 51.9 mg/L (Figure 14).

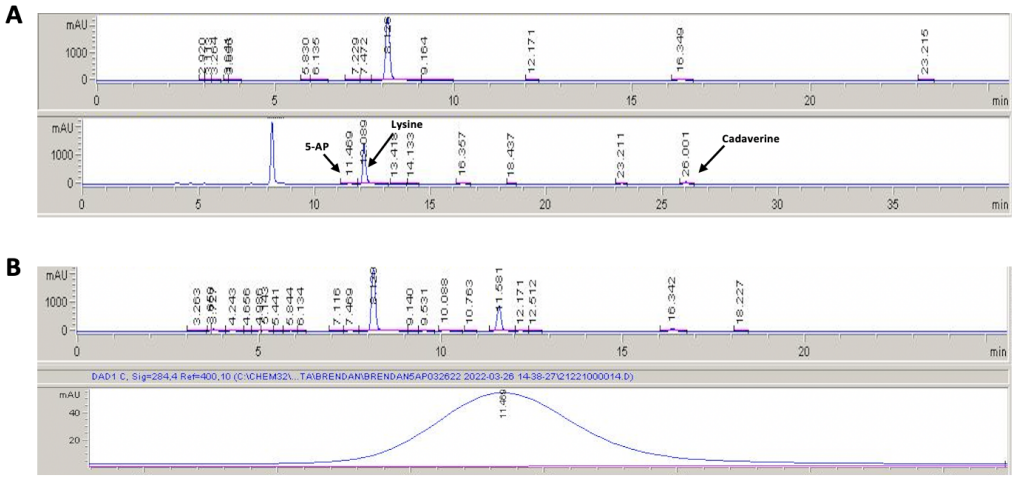

Figure 14. HPLC Chromatogram for 5-AP Production (A) Top: BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* no precursor added 48 hour fermentation. Bottom: BL21(DE3)*Δprr pET-patA-cadA pCDF-lysC-dapA* Lysine spike 48 hour fermentation. (B) Top: 5-AP standard (peak ~11.5 minutes). Bottom: 5-AP peak Lysine spike 48 hour fermentation.

The HPLC chromatogram in Figure 14A compares production between full pathway with no precursor added and full pathway with L-lysine added. It can be observed that without the addition of precursor to the culture no significant L-lysine synthesis was observed after 48 hours, resulting in no production of 5-AP as L-lysine is a necessary precursor for this pathway to properly function [14][15]. Conversely, a peak for L-lysine (~12 minutes), cadaverine (~26 minutes), and 5-AP (~11.5 minutes) were observed with the

addition of L-lysine. Figure 14B confirms the time point at which 5-AP would be seen after separation in the column by comparing an analytical standard (Top) to the results obtained from the fermentation test (Bottom). Based on the data observed it is hypothesized that the L-lysine overproduction pathway may not be functional. To further support this hypothesis, the 5-AP production plasmid pETDuet-*patA*-*cadA* was sequenced to confirm the existence of these genes (Figure 15).

Figure 15. Sanger Sequencing data for (A) *patA* and (B) *cadA*

The results shown above provide validation that both *cadA* and *patA* have been cloned into the plasmid. Confirming the existence of these genes further solidifies the hypothesis that the L-lysine overproduction plasmid may be the bottleneck of this work.

3.6 Data Inconsistency

HPLC chromatograms suggest that there are some inconsistencies observed when detecting for lysine and cadaverine. A significant shift is seen in the sample spiked with 2.5 g/L lysine (Figure 16.).

Figure 16. Inconsistent HPLC Results. (Top) BL21(DE3)*Δprr pET-patA-cadA pCDF-lysC-dapA* 2.5 g/L L-lysine added 48 hours. (Bottom) Same sample BL21(DE3)*Δprr* pETDuet-*patA-cadA* pCDF-*lysC-dapA* 2.5 g/L L-lysine added 48 hours.

Looking further into the top chromatogram there is a noticeable shift for lysine and cadaverine to 10.4 minutes and 22.8 minutes respectively. It was theorized that this shift may be due to insufficient time for the system to properly reach equilibrium while ramping to a final flowrate of 1.00 mL/min. In attempts to solve this issue, the system was allowed to reach equilibrium and stabilize for a significantly longer period of time. With these changes to the system, this same lysine spiked sample was run again shown in the bottom chromatogram. Comparing the top and bottom chromatograms lysine and cadaverine are perceived at their expected elution time of 12 minutes and 26 minutes. These results suggest that allowing the system to have sufficient time to reach equilibrium may yield more consistent results. With this observation of elution time shifting, previous HPLC results were revisited to see if similar shifts are detected. Revisiting the results of previous fermentation data in M9 minimal medium showed a similar shift in elution time (Figure 17.)

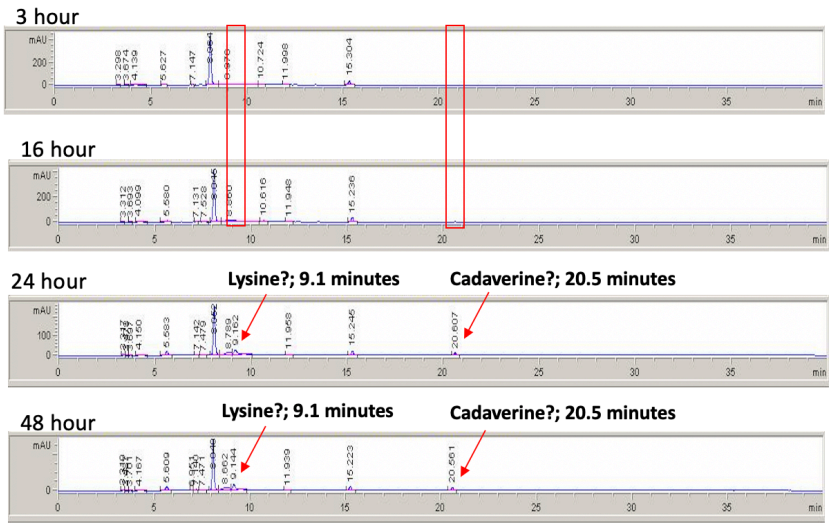

Figure 17. Additional Inconsistencies in Elution Times

After 24 hours of fermentation in M9 minimal medium peaks appear at 9.1 minutes and 20.5 minutes again indicating potential detection of lysine and cadaverine. The proposed solution may aid in clarifying the existence of lysine and cadaverine, and furthermore production of 5-AP. Additional testing to determine why these shifts are occurring and if there is consistency in the magnitude of shifts will provide a better understanding of how further improve the analytical methods used. Testing different derivatization and separation methods may provide the clarity needed to confirm what the driving force is behind these observed inconsistencies. By understanding the root cause of these inconsistencies and how to solve them will result in a more accurate representation of reproducible results.

CHAPTER 4

CONCLUSION AND FUTURE WORK

To conclude the work performed, 5-AP was shown to be produced at a titer of 51 to 62 mg/L demonstrated in M9 minimal media and M10 production media. The results obtained have shown to be lower than literature benchmarks due to insufficient conversion of cadaverine to 5-AP by the cadaverine aminotransferase *patA* or a lack of lysine overproduction within the cell leading to the question of gene functionality.

To test for gene functionality a resting cell assay was performed. Introducing induced cells to a buffer solution along with essential pathway precursors such as lysine to test for *cadA* functionality and cadaverine to test for *patA* functionality. With a spike in lysine 194.14 mg/L cadaverine and 62.8 mg/L 5-AP was produced. With a spike of cadaverine only 23 mg/L 5-AP was produced indicating a potential functionality issue with the gene *patA* as there is minimal conversion of cadaverine to 5-AP observed. However, it is unclear why such little conversion is observed.

Analytical inconsistencies were noted in HPLC chromatograms where a shift in elution times for both cadaverine and lysine were observed. These inconsistencies make it difficult to compare results obtained from HPLC analysis. However, further investigation into these inconsistencies suggest that they could be solve by allowing the system enough time to equilibrate while ramping to the desired final flowrate.

Future work for this project would be to further investigate the reasoning as to why *patA* may not be functioning at the levels expected. Testing a variety of IPTG levels to find the optimal expression levels. It is also hypothesized to express *cadA,* patA, and the aldehyde reductase *yqhD* in *Corynebacterium glutamicum* which may prove to be a more

suitable host over *E. coli* as it is extremely efficient for amino acid production. Previous works have demonstrated successful production of a primary amino alcohol in *C. glutamicum* utilizing genes from *E coli*. Additionally, literature suggests the aldehyde intermediate 5-Amino-1-pentanal can spontaneously react to form the molecule 1-Δ-piperideine[37][38]. It was hypothesized that 1-Δ-piperideine was being formed due to changes in media color to a dark brown. This change in color was a possible indicator that this molecule was being formed. However, no literature has been found to support this color change in *E. coli* calling for the need for further investigation into this hypothesis. Purchasing and testing analytical standards for this molecule may aid in determining if this product is being formed rather than the desired target 5-AP.

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APPENDIX A

SUPPORTING GROWTH CURVE DATA

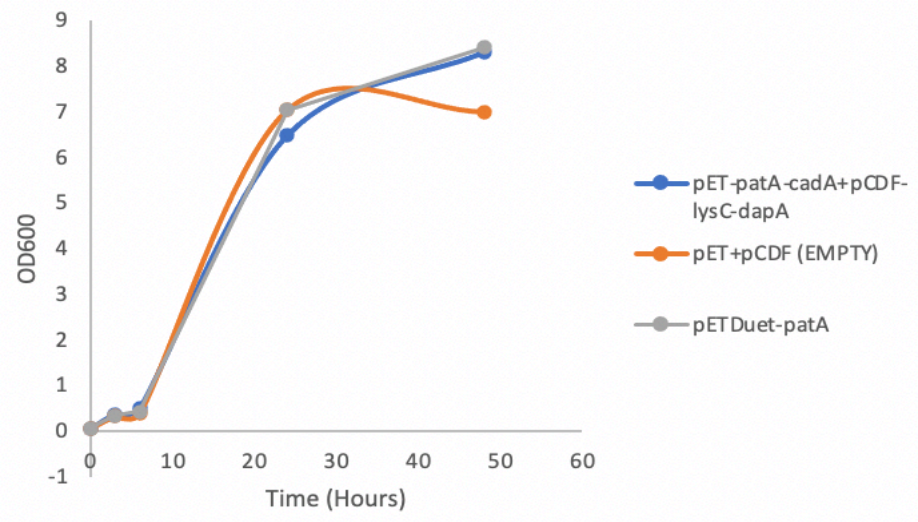

Figure A.1 Growth Comparison for Culture pairs used in this Study

Figure A.1 is a depiction of how culture pairs grew under production conditions. The host containing both production plasmids (blue), empty production plasmids (orange), and the 5-AP production plasmid with only the putrescine aminotransferase gene *patA* all grew at a similar rate for all fermentation runs. This similarity in growth shows that growth is not observed to be affected by the addition of multiple plasmids. The growth for all production samples showed a similar trend.