Renewable Production of 5-Carbon Polyamide Monomers Using Engineered *Escherichia coli*

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ABSTRACT

The diversity of industrially important chemicals that can be produced biocatalytically from renewable resources continues to expand with the aid of metabolic and pathway engineering. In addition to biofuels, these chemicals also include a number of monomers with utility in conventional and novel plastic materials production. Monomers used for polyamide production are no exception, as evidenced by the recent engineering of microbial biocatalysts to produce cadaverine, putrescine, and succinate. In this thesis the repertoire and depth of these renewable polyamide precursors is expanded upon through the engineering of a novel pathway that enables *Escherichia coli* to produce, as individual products, both δ-aminovaleric acid (AMV) and glutaric acid when grown in glucose mineral salt medium. δ-Aminovaleric acid is the monomeric subunit of nylon-5 homopolymer, whereas glutaric acid is a dicarboxylic acid used to produce copolymers such as nylon-5,5. These feats were achieved by increasing endogenous production of the required pathway precursor, L-lysine. *E. coli* was engineered for L-lysine over-production through the introduction and expression of metabolically deregulated pathway genes, namely aspartate kinase III and dihydrodipicolinate synthase, encoded by the feedback resistant mutants $lysC^{fbr}$ and $dapA^{fbr}$, respectively. After deleting a natural L-lysine decarboxylase, up to 1.6 g/L L-lysine could be produced from glucose in shake flasks as a result. The natural L-lysine degradation pathway of numerous *Pseudomonas sp.*, which passes from L-lysine through both δ-aminovaleric acid and glutaric acid, was then

functionally reconstructed in a piecewise manner in the *E. coli* L-lysine over-producer. Expression of *davBA* alone resulted in the production of over 0.86 g/L AMV in 48 h. Expression of *davBADT* resulted in the production of over 0.82 g/L glutaric acid under the same conditions. These production titers were achieved with yields of 69.5 and 68.4 mmol/mol of AMV and glutarate, respectively. Future improvements to the ability to synthesize both products will likely come from the ability to eliminate cadaverine by-product formation through the deletion of *cadA* and *ldcC*, genes involved in *E. coli*'s native lysine degradation pathway. Nevertheless, through metabolic and pathway engineering, it is now possible produce the polyamide monomers of δ-aminovaleric acid and glutaric acid from renewable resources.

DEDICATION

This work is dedicated to my parents, John and Lori, and my brothers and sisters: Jordan, Jesse, Tiffany, and Amber. Without your support this extension of myself would not have been a possibility.

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BACKGROUND AND MOTIVATION

1.1 ***Introduction***

In a global economy with a strong and continuing dependence on petroleum, long-term sustainability, environmental, and national security concerns provide compelling motivation for the development of renewable sources of fuels, chemicals, and materials. Through the rational re-engineering of microbial metabolism 'microbial chemical factories' can be constructed to convert biomass feedstocks (e.g., glucose, sucrose, fructose) into chemical products of high value and/or utility as greener and more sustainable alternatives to petrochemicals (Carothers et al., 2009;Martin et al., 2009). These biocatalytic processes, in general, are positive technologies that operate at near ambient temperature and pressure in aqueous solutions with reduced energetic costs, and neutral or even positive environmental impacts. This approach has already led to significant and recent improvements to the production of renewable fuels (Peralta-Yahya and Keasling;Atsumi et al., 2008;Atsumi and Liao, 2008;Schirmer et al., 2010;Steen et al.;Mendez-Perez et al., 2011;Rude et al., 2011), chemicals (Wierckx et al., 2005;Qian et al., 2009b;Kind et al., 2010a;Schirmer et al., 2010;Whited et al., 2010), and active pharmaceutical ingredients (Ro et al., 2006;Leonard et al., 2009;Tsuruta et al., 2009;Campbell and Vederas, 2010). However, as roughly 331 million barrels of liquid petroleum gases and natural gas liquids, and close to 11

billion ft^3 of natural gas were used to produce plastics in the United States alone in 2006 (U.S. Energy Information Administration, 2011), interest in developing renewable and 'greener' plastic products is also increasing. Key accomplishments wherein metabolic and pathway engineering has been applied to develop renewable 'biomonomers' will now be briefly highlighted to provide a framework for the research presented herein.

1.2 ***Metabolic Engineering for Naturally-Occurring Biopolymers and Biomonomers***

In addition to re-engineering cellular metabolism to produce useful fuels and chemicals, the same strategies have also lead to the production of natural and non-natural biopolymers and biomonomers. Bioplastics production can positively impact the carbon cycle through the consumption of atmospheric CO_2 during feedstock cultivation. Since the global demand for bioplastics is predicted to triple by 2015, reaching over 1 million metric tons per year and a $2.9 billion market (Freedonia Group, 2009), the field is ripe for development.

1.2.1 ***Naturally-Occurring Biopolymers***

A number of naturally-occurring biopolymers are known to be produced by microorganisms from renewable resources, most notably polyhydroxyalkanoates or PHAs, which have been extensively reviewed (Braunegg et al., 1998;Chen, 2009;Keshavarz and Roy, 2010). PHAs, which are

biodegradable polyesters composed primarily of monomer subunits of (*R*)-3-hydroxybutyrate (3HB), are produced as carbon and energy storage molecules by numerous different microbes. Well-studied examples of bacterial PHA producers include species of *Ralstonia* (Reinecke and Steinbuchel, 2009), *Bacillus* (Singh et al., 2009), and *Pseudomonas* (Rojas-Rosas et al., 2007), as well as phototrophic cyanobacteria (Asada et al., 1999). While PHAs are produced efficiently by microbes via naturally evolved pathways, expressing the requisite pathway enzymes from various natural producers has also enabled their production at high levels in recombinant *Escherichia coli*, a more tractable host platform (Schubert et al., 1988). All told, bacteria have been engineered to achieve PHA biosynthesis at up to 80% of cell dry weight and at productivities as high as 4 g/L-h from substrates such as glucose (Lee et al., 1999).

Despite their numerous advantages, however, PHA production also suffers from numerous inherent shortcomings which may ultimately impede their long-term and wide-spread utility. For instance, while their biodegradable nature can help reduce municipal waste, this attribute renders PHAs unsuitable for use in applications requiring long term durability and/or environmental exposure. As all PHAs are thermoplastic polyesters the achievable range of physicochemical and material properties are limited. PHA chain length and purity are difficult to tune and control in an *in vivo* setting. Lastly, because they are macromolecules, PHAs do not diffuse out from cells, but rather accumulate only intracellularly as cytosolic inclusion bodies. Thus, since PHAs can only be harvested after the

microbial biocatalyst is lysed, continuous bioprocessing of PHAs remains unviable.

1.2.2 ***Naturally-Occurring Biomonomers***

Limitations associated with naturally-occurring bioplastics such as PHAs have motivated a recent shift in focus to the alternative production of 'biomonomers'. Biomonomers are small molecules (i.e., monomeric subunits) that can undergo *ex situ* chemocatalytic polymerization to produce plastics. This strategy offers several distinct advantages. First, since most biomonomers are often naturally excreted from microorganisms the need for cell collection and lysis is eliminated which greatly reduces operating expenditures and facilitates downstream product recovery. Polymerization of biomonomers in highly controlled chemocatalytic environments leads to bioplastics with finely tuned and predictable properties and at high purities to satisfy quality control specifications. Lastly, the co-polymerization of biomonomers together with other monomers (of biological origin or otherwise) increases the diversity of renewable plastics to achieve a wider possible range of chemistries and properties.

In general, there are two classes of biomonomers of industrial interest. The first are produced from natural metabolites with the aid of simple catalytic post-processing. For example, the natural fermentation product ethanol can undergo dehydration to ethylene over a solid acid catalyst (Takahara et al., 2005;Hu et al., 2010), before later being polymerized to poly(ethylene) or its co-

polymers. The second class of biomonomers, which will be the specific focus of this review, are those metabolites that are themselves directly suitable for polymerization into bioplastics. Microbial production of biomonomers as 'drop in compatible' replacements to their petroleum-derived counterparts is advantageous as it provides a seamless interface between emerging biotechnologies and the existing polymer industry. This strategy ensures that decades of technological expertise and existing process infrastructure can be efficiently leveraged, and effectively enables conventional chemical and plastics processing to 'pick up where biology leaves off'.

The most prominent example of a naturally-occurring biomonomer is perhaps *L*(+)-lactic acid, whose polymerization leads to the polyester poly(lactic acid), or PLA. *L*(+)-Lactic acid is a natural fermentation product of numerous microbes, of which prominent examples include lactic acid bacteria (including numerous *Lactobacilli* sp.) and filamentous fungi (Kosakai et al., 1997;Zhou et al., 1999). Microbial lactic acid production has been extensively reviewed in the past (Wee et al., 2006;Reddy et al., 2008). Meanwhile, *E. coli* has also been engineered to over-produce both *L*(+)- (Zhou et al., 1999) and *D*(-)- (Zhou et al., 2003;Mazumdar et al., 2010) stereroisomers of lactic acid as optically pure products. This is important because lactic acid stereochemistry greatly controls the physical properties of PLA including, for example, crystallinity and in turn melting point (Lunt, 1998;Sodergard and Stolt, 2002).

A second naturally-occurring biomonomer that has been extensively investigated to date is succinic acid. This 4-carbon diacid is a natural fermentation product of numerous bacteria, including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Mannheimia succiniciproducens, which have been reviewed elsewhere (Song and Lee, 2006). Moreover, E. coli's metabolism* has again also been extensively engineered to over-produce this minor fermentation product to near theoretical yields (Thakker et al., 2012). As microbial succinic acid production is now becoming a mature technology, several producers, including BioAmber (Plymouth, MN) and Myriant (Quincy, MA), are now developing commercial scale fermentation processes. Succinic acid is particularly useful for producing both polyesters and polyamides, and the first commercial bioplastics derived from this biomonomer will likely be poly(butylene-*co*-succinate) (PBS) (Bechthold et al., 2008) and nylon-5,4 (Qian et al., 2009b).

1.3 ***Metabolic Engineering for Novel Biomonomers***

Despite the potential advantages of using biomonomers to produce renewable plastics, a limited pool of useful naturally-occurring metabolites ultimately constricts the diversity of bioplastics that can be produced by this approach. However, by applying metabolic engineering tools and strategies, *de novo* metabolic pathway engineering provides the potential to produce non-natural biomonomers, and novel bioplastics, that were never before possible.

For example, styrenics are a family of polymers and co-polymers that use styrene or substituted styrene as a key monomer building-block. Today, nearly 60% of styrene's global annual production (about 6 million tons in the U.S. manufacturers and 24 million tons globally), for example, supports plastics production (SRI, 2010). The recent engineering of novel enzymatic routes to both p-hydroxystyrene (pHS) and styrene and from glucose (Figure 1), however, may soon enable their renewable production.

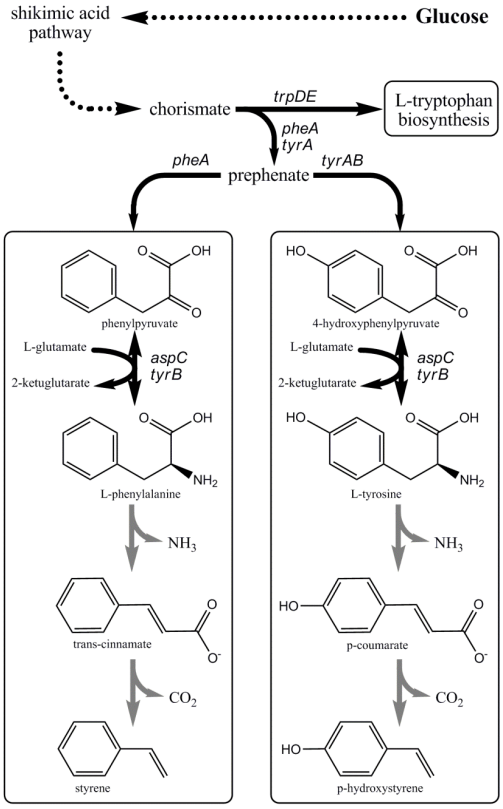


Figure 1: *Novel pathways engineered for the production of p-hydroxystyrene and styrene from renewable glucose.*

The first styrenic biomonomer to be synthesized from renewable resources was pHS (Qi et al., 2007). Its innate optical properties make pHS useful for producing photoresist polymers used in semiconductor manufacturing, for example (Pawlowski et al., 1991). As seen in Figure 4, the engineered pHS biosynthesis pathway stemmed from L-tyrosine as its immediate endogenous precursor. Only two enzymatic steps were then required to convert L-tyrosine to pHS. First, the bi-functional phenylalanine/tyrosine ammonia lyase (PAL/TAL) of the yeast *Rhodotorula glutinis* catalyzed p-coumaric acid formation. Then, p-coumaric acid was subsequently decarboxylated by phenylacrylate decarboxylase of either *Bacillus subtilis* (encoded by *pdc*) or *Lactobacillus plantarum* (encoded by *padC*). Initial *E. coli* strains expressing the pHS pathway achieved final titers as high as only 400 mg/L. Accumulation beyond this mark was severely limited as a result of pHS toxicity imposed on the *E. coli* host (Qi et al., 2007). To overcome toxicity limitations, the well-known aromatic-tolerant *Pseudomonas putida* S12 was next investigated as a pHS production platform. Utilizing two strains derived from *P. putida* S12 to overproduce precursor L-tyrosine as hosts, the pHS pathway was introduced. Due to the innate ability of *P. putida* S12 to degrade numerous aromatic solvents, multiple competing pathway genes were also targeted for deletion. These included both *smo*, which encodes styrene monooxygenase, and *fcs*, the first gene in the p-coumaric acid degradation pathway. All told, engineered *P. putida* S12 strains were able to reach and tolerate pHS titers as high as 545 mg/L, nearly a 50% improvement.

More recently, our own group engineered a novel enzymatic pathway to enable *E. coli* to produce styrene directly from glucose (McKenna and Nielsen, 2011). The styrene biosynthetic pathway is analogous to the pHS pathway (Figure 1), except in that it stems from L-phenylalanine as its endogenous precursor and as such requires different pathway enzyme 'parts'. In this case, L-phenylalanine ammonia lyase (PAL) first converts L-phenylalanine to trans-cinnamic acid which is then decarboxylated to styrene. Upon screening numerous enzyme candidates, it was ultimately found that PAL2 from *Arabidopsis thaliana* and FDC1 from *Saccharomyces cerevisiae* were most effective for these steps, respectively. By over-expressing PAL2 and FDC1 in a L-phenylalanine over-producing *E. coli* host, styrene titers as high as 264 mg/L were achieved. As with pHS, styrene titers too approached *E. coli*'s toxicity limit (~300 mg/L) and must similarly be overcome if economical styrene biomonomer production is to be achieved.

More examples in recent years of metabolic and pathway engineering's influence on biomonomer production includes the enantiomerically pure hydroxyacids. Hydroxyacids can serve as biomonomers to derive renewable polyesters. Hydroxyacids of prominent interest to bioplastics applications include terminal hydroxyacids such as 3-hydroxypropionate (3HP) as well as the *β*-hydroxyacids 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). Each of these hydroxyacid monomers have been produced microbially from renewable resources via pathway engineering.

3HP was named one of the twelve 'Top Value Added Chemicals from Biomass' (U.S. Department of Energy, 2004) by the Department of Energy. In addition to serving as polyester monomer, 3HP can also be used to produce several other specialty and commodity chemicals through additional chemocatalytic processing (Rathnasingh et al., 2009). While several distinct enzymatic routes have been proposed for microbial 3HP production (Jiang et al., 2009), two have demonstrated the greatest promise to date. The first was a two-step pathway consisting of: 1) an adenosylcobalamin dependent glycerol dehydratase to catalyze the dehydration of glycerol to 3-hydroxypropionaldehyde (3HPA), and 2) an aldehyde dehydrogenase to oxidize 3HPA to 3HP (Suthers and Cameron, 2000). Using this pathway, Rathnasingh and co-workers engineered *E. coli* to produce 38.7 g/L 3HP in a fed-batch bioreactor with glycerol as substrate. In their strain, glycerol dehydratase (DhaB) from *Klebsiella pneumoniae* DSM 2026 and the α-ketoglutaric semialdehyde dehydrogenase (KGSADH) from *Azospirillum brasilense* were selected (Rathnasingh et al., 2009). A key shortcoming of this approach, however, was the uneconomical requirement for exogenous coenzyme-B12 supplementation. To address this limitation, *E. coli* was later engineered to produce 3HP from glucose in a coenzyme-B12-independent manner.

Microbial production of *(R)*-3HB by recombinant *E. coli* was first reported by Gao *et al.*. Their engineered pathway was composed of *phaA* and *phaB* from *Ralstonia eutropha* (a natural PHB producer), encoding *β*-ketothiolase and an (*R*)-

specific 3-hydroxybutyryl-CoA (*R*-3HB-CoA) dehydrogenase, respectively, to convert endogenous acetyl-CoA to 3-HB-CoA through acetoacetyl-CoA (Figure 5). The *ptb-buk* operon from *Clostridium acetobutylicum* was lastly co-expressed to convert *R*-3-HB-CoA to *R*-3HB (via *R*-3-hydroxybutyryl phosphate), and shake flask titers exceeding 2 g/L were demonstrated as a result (Gao et al., 2002). Tseng *et al.* later built upon these works, adding the ability to produce both (*R*)- and (*S*)- 3HB isomers with high stereoselectivity and increasing product titers (Tseng et al., 2009). Stereoselective control was achieved by utilizing two different enantioselective 3HB-CoA dehydrogenases: (*R*)-selective PhaB from *R. eutropha* and (*S*)-selective Hbd from *Clostridium acetobutylicum*. (*R*)-3HB biomonomer, which is natural excreted from *E. coli*, can be used to make biodegradable PHB with well-controlled properties. (*S*)-3HB biomonomer, meanwhile, can be used to produce novel PHBs with new features and properties.

Some useful diol monomers provide examples of short-chain monomers suitable for bioplastics applications that have been produced microbially via both natural and engineered pathways. For example, the four-carbon diol 2,3-butanediol (2,3-BDO) is a natural fermentation product of many microbes, including many species of *Klebsiella*, *Bacillus*, and lactic acid bacteria (Voloch et al., 1985). Microbial production of 2,3-BDO by both natural and engineered strains will not be discussed here, however, as it has been extensively reviewed over the years (Garg and Jain, 1995;Celinska and Grajek, 2009;Ji et al., 2010;Nielsen et al., 2010;Ji et al., 2011). More recent efforts have focused on

engineering microbes to produce the three-carbon diol 1,3-propanediol (1,3-PDO) and the four-carbon diol 1,4-butanediol (1,4-BDO). Both 1,3-PDO and 1,4-BDO are non-natural metabolites. Like 2,3-BDO, 1,4-BDO can be similarly converted to butadiene. More importantly, however, both 1,3-PDO and 1,4-BDO are directly useful as biomonomers for the polyesters poly(propylene terephthalate) (PPT) and poly(butylene terephthalate) (PBT).

While microbial production of 1,3-PDO has been recently and comprehensively reviewed (Nakamura and Whited, 2003;Sauer et al., 2008;Saxena et al., 2009;Celinska, 2010), the engineering of *E. coli* to produce up to 18 g/L 1,4-BDO from glucose (as well as other substrates) was only recently reported for the first time (Yim et al., 2011). Bioproduction of 1,4-BDO was accomplished through the development of two novel pathways, the design of which was facilitated using the SimPheny Biopathway Predictor; a design algorithm that considers chemical structure to predict possible routes from central metabolite precursors to end-product targets (Smolke, 2009). It is also noted that both 1,4-BDO pathways include the common intermediate 4-hydroxybutyrate (4HB), a hydroxyacid. As described, hydroxyacids are excellent precursors for polyester production. Thus, by further leveraging this technology an additional, non-natural hydroxyacid building block could also be added to the list of potential polyester biomonomers.

1.3.1 ***Microbial Production of Polyamide Building Blocks***

Polyamides (i.e., nylons) are a class of plastics that balance mechanical strength and durability with chemical resistance, and which find extensive use as textiles and mechanical parts. Polyamides are formed as homopolymers of terminal amino acids or as co-polymers via condensation of diamines with diacids, as illustrated in figure 2 below:

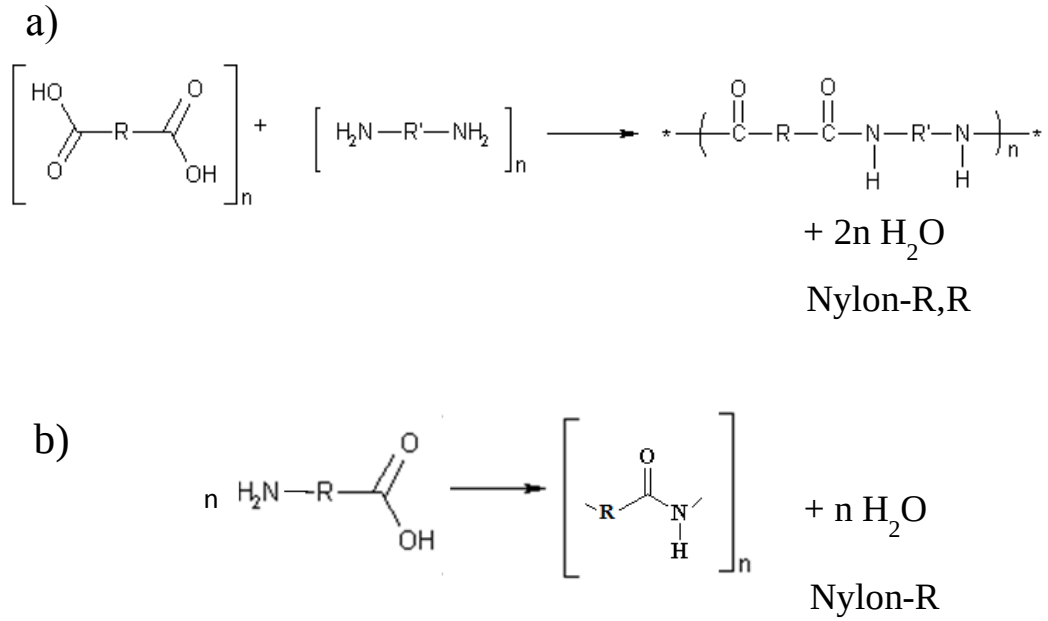


Figure 2: *General Polymerization Reactions a) Dicarboxylic acid molecules undergo polymerizing condensation reactions with diamine molecules. b) Identical monomers with an amino group and a carboxylic acid group at each terminus undergo a polymerizing condensation reaction.*

The most common commercial polyamides are nylon-6 (a homopolymer of 6-aminohexanoic acid) and nylon-6,6 (a copolymer of adipic acid and 1,6-hexanediamine) which account for more than 85-90% of the global market, which

will approach 6.6 million tons by 2015 (Nexant Chem Systems, 2009). As carbon chain length (typically 2 to 12) between amide bonds strongly influences material properties, a large diversity of polymers can be generated from a limited monomer pool by a combinatorial approach. Figure 3 highlights the diversity of polyamides that can be produced from a small number of biomonomers.

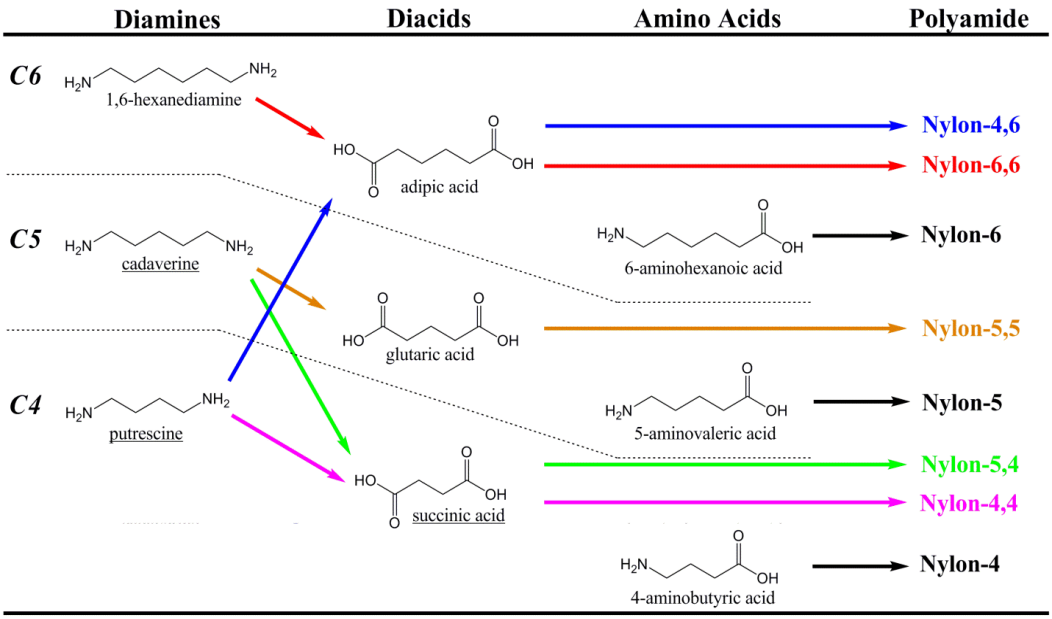


Figure 3: *Examples of polyamides produced from combinations of C4-C6 diamines, diacids, and amino acids. To date, molecules with names underlined have been produced microbially from renewable resources with the aid of metabolic engineering.*

Putrescine is the 4-carbon diamine counterpart to succinic acid. DSM Engineering Plastics currently uses biologically-derived putrescine (derived from

castor oil) to produce nylon-4,6 and nylon-4,10, which are marketed as Stanyl™ and EcoPaXX™, respectively. Although putrescine occurs as an amino acid biodegradation product, no natural over-producers have been discovered or isolated. In *E. coli*, for example, putrescine can generate at low levels by two different pathways that stems from either L-arginine (through agmatine) or L-ornithine (from L-glutamate) by SpeAB or SpeC and SpeF, respectively (Tabor and Tabor, 1985;Igarashi and Kashiwagi, 2000). Several researchers have also taken to rationally engineering putrescine over-producing microbes. For example, Qian *et al.* reported the development of a putrescine over-producing *E. coli* strain by increasing metabolite flux through L-ornithine (Qian et al., 2009a). This was achieved by overcoming allosteric and transcriptional regulation of the L-ornithine pathway at multiple levels through promoter replacements and genetic deletions. The transcriptional regulation of the L-ornithine biosynthesis pathway by L-arginine at the promoters P*argCBH*, P*argD*, and P*argE* was alleviated by replacement of these promoters with the strong P*trc* promoter. Additionally, P*speC* and P*sepF-potE* (*potE* codes for the putrescine export protein) were replaced with P*trc*, relieving regulation of transcription caused by putrescine on P*speC*, and increasing the concentration of *potE*, which increases putrescine export from the cell. Furthermore, the *argI* gene coding L-ornithine carbamoyltransferase was deleted, significantly reducing flux to L-arginine, and any allosteric or transcriptional regulation L-arginine caused. The result was an *E.*

coli strain capable of producing 24.2 g/L putrescine in fed-batch cultures in glucose media (Qian et al., 2009b).

Corynebacterium glutamicum has also been engineered to over-produce putrescine by both the L-ornithine and L-arginine pathways, reaching up to 6 g/L with a plasmid based *speC*, L-ornithine decarboxylase from *E. coli* (Schneider and Wendisch, 2010). In comparison, putrescine yields achieved by *C. glutamicum* via the L-arginine pathway were 40 times lower with *adiA* (L-arginine decarboxylase) and *speB* (agmatinase) overexpressed, likely due to the production and allosteric inhibition of urea on *adiA*. (Schneider and Wendisch, 2010)

Just as putrescine, the 5-carbon diamine cadaverine also naturally occurs as an amino acid degradation product, though only at low levels in natural organisms. In this case it is the decarboxylation of L-lysine, and not L-ornithine, that leads to cadaverine. Polyamides derived from cadaverine possess such desirable properties as high melting points and low water absorption (Kind and Wittmann, 2011). In addition, nylon-5,4, nylon-5,5 and nylon-5,10 (co-polymers of cadaverine with succinic acid, glutaric, and sebacic acid, respectively) have been proposed as bio-based alternatives to conventional, petroleum-derived polyamides of similar properties (Kind and Wittmann, 2011;Qian et al., 2011a).

Mimitsuka *et al.* were first to engineer a microbe for cadaverine production from glucose by inserting the acid inducible lysine decarboxylase (encoded by *cadA*) of *E. coli* into the genome of a L-lysine over-producing strain

of *C. glutamicum*, as seen in Figure 4 (Mimitsuka et al., 2007). *E. coli* and *C. glutamicum* have since both been extensively engineered for its over-production from renewable substrates (Mimitsuka et al., 2007;Tateno et al., 2009;Kind et al., 2010a;Qian et al., 2011a) at titers up to 9.6 g/L in *E. coli* and over 10 g/L in *C. glutamicum* In both systems, the over-expression of lysine decarboxylase (*cadA* and *ldcC* of *E. coli*) in a L-lysine over-producing host were essential strategies. *cadA* was expressed in *E. coli* by Qian et. al, along with deletion of the following cadaverine degredation pathway enzymes: spermacetyl transferase, spermidine synthase, cadaverine oxidase, and putrescine aminotransferase coded by *speG*, *speE*, *ygjG*, and *puuA*, respectively (Qian et al., 2011b). Additional increases in cadaverine formation were achieved by the native promoter replacement of *dapA* (dihydrodipicolinate synthase) with Ptrc. Final titers by fed batch cultivation reached 9.61g/L of cadaverine.

In Mimitsuka et al.'s strain of *C. glutamicum*, the *cadA* gene was introduced in place of the *hom* gene on the chromosome, making a cadaverine producer, but also an L-homoserine auxotroph, which is less than ideal. Kind et al. showed that it is the *ldcC* gene that is best for overexpression because of its physiological pH optimum (Kind et al., 2010b). Codon-optimization of *ldcC*, and the addition of 1 mg/L pyridoxal (the cofactor of ldcC) helped further improve the yield of cadaverine in this strain to 0.17 g/g on glucose. Kind et al. further investigated the byproduct formation of the *C. glutamicum* cadaverine producing strains, and noted up to 20% of cadaverine being formed as N-acetylcadaverine

(Kind, 2010). Genome-wide transcript analysis then identified a permease (named cg2893), as illustrated in Figure 3, whose deletion reduced cadaverine production by 90%. Additionally, genome based overexpression of cg2893 increased cadaverine yield by 20% and reduced N-acetylcadaverine formation by 75% (Kind et al., 2011). This genome based overexpression was accomplished by replacing the natural promoter of cg1893 with the strong promoter of superoxide dismutase, Psod. The improved results come from the fact that export escapes cadaverine from allosteric inhibition of L-lysine decarboxylase and degradation into N-acetylcadaverine. Also, lysine excretion as a by-product was completely eliminated by the deletion of LysE, the lysine export protein in *C. glutamicum*, which is also illustrated in Figure 4. This deletion effectively increases the available intracellular lysine concentration subjected to decarboxylation. In a final tuning of the strain, the N-acetyltransferase was identified. Out of the 17 possible identified N-acetyltransferases, the deletion of NCgl1469 completely eliminated the N-acetylcadaverine formation (Kind, 2010).

Nylon homopolymers are produced from terminal amino acid monomers, the 4-carbon of which is 4- or γ-aminobutyrate (GABA). GABA is naturally produced by many organisms by decarboxylation of L-glutamate via glutamate decarboxylase. Whereas a microbe that produces GABA directly from glucose has yet to be reported, GABA production has been investigated in strains expressing heterologous glutamate decarboxylase in media supplied with L-glutamate.

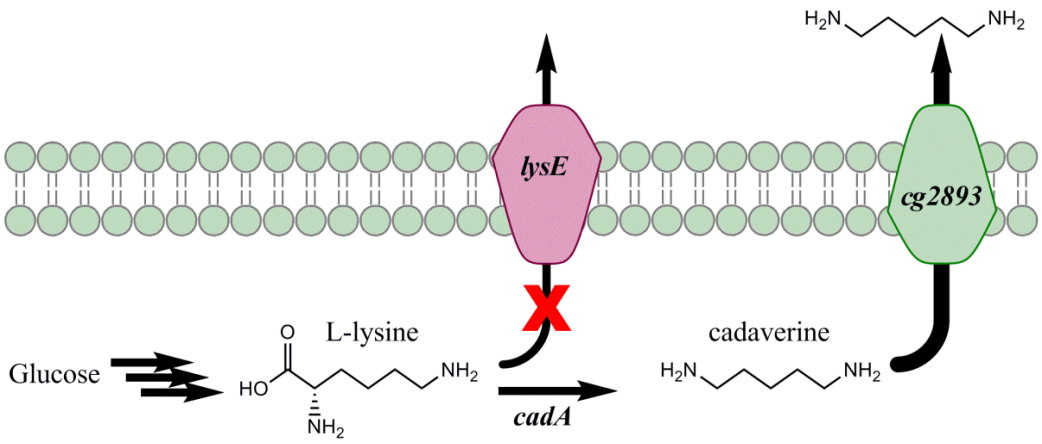


Figure 4: *Engineering cadaverine production in Corynebacterium glutamicum by i) over-expressing L-lysine decarboxylase (cadA), ii) disrupting L-lysine the exporter (lysE), and iii) over-expressing an identified cadaverine exporter (cg2893).*

For example, up to 5.5 g/L GABA was obtained from 10 g/L of monosodium glutamate in *E. coli*, wherein the glutamate decarboxylase encoded by *gadB*, and the GABA/Glutamate antiporter of *gadC* were expressed via plasmids (Le Vo et al., 2012). This antiporter functionally excretes formed GABA simultaneously with the import of L-glutamate molecules. Additionally, the GABA aminotransferase *gabT* was deleted from the genome to prevent GABA from being degenerated and funneled into the tricarboxylic acid cycle. In another example, the glutamate decarboxylase from *Lactobacillus planatarum* ATCC 14917 was isolated and expressed in *Lactobacillus sakei B2-16* with the use of the *Lactobacilli-E. coli* shuttle-expression vector pTRKH2 (Kook et al.,

2010). This yielded 27.4 g/L GABA in a media supplemented with 70 g/L of L-glutamate. More work needs to be done in coupling the glutamate decarboxylase expression and activity demonstrated with a strain that has been proven to overproduce glutamate.

As seen in earlier in Figure 3, bio-production of several other useful polyamide monomers has yet to be realized. There is a particular gap in the renewable production of homopolymer building blocks. GABA is the closest demonstration, but has yet to be demonstrated on glucose. Since this problem is currently being worked on, other impactful monomers that do not have a current proposed or demonstrated metabolic engineering strategy including the 5-carbon polyamide monomers δ-aminovaleric acid and glutaric acid. Rational engineering of microorganisms as cellular factories to produce these polyamide building blocks from renewable resources would push the biopolyamide field and biopolymer field in general, forward by filling these crucial gaps.

1.4 ***Pathways to δ-Aminovalerate and Glutaric Acid Production***

Like cadaverine, the 5-carbon monomers δ-aminovalerate and diacid glutaric acid are known to be natural L-lysine degradation products. Numerous *Pseudomonas* sp., for example, are known to degrade L-lysine through δ-aminovaleric acid and on to glutaric acid via the AMV pathway, before it is then further degraded to central pathway metabolites. (Revelles et al., 2007) Thus, if select parts of the AMV pathway can be functionally reconstructed in an L-lysine

over-producing host, renewable routes to bio-based nylon-5 and nylon-5,5 could be possible. The microbial production and subsequent polymerization of δ-aminovalerate would yield Nylon 5. While nylon-5 has not achieved commercial realization, it is known to possess properties close to nylon-4,6 and could serve as a suitable substitute (Bermudez et al., 2000). Glutarate can be polymerized with both putrescine and cadaverine, producing the completely sustainable nylon-4,5 and nylon-5,5, respectively. In particular nylon-5,5 is an odd-odd polymer with additional piezoelectric and ferroelectric properties useful in sensors and electronics (Navarro et al., 1996).

As is illustrated in Figure 5 below, there are many documented biochemical pathways that can convert renewable feedstocks into AMV and on to glutarate. Since the chemistry from AMV to glutarate is more straightforward, the options are either DavT or GabT, and DavD or GabD. These activities and production kinetics of the enzyme homologues from AMV to glutarate will be compared. For AMV production from L-lysine there are a number of options. DavB and DavA were deemed to be the most direct, and also are completely characterized in terms of gene sequences and enzyme chemistry (Revelles et al., 2007). Thus the DavBA pathway was the first pathway attempted for cloning.

1.5 ***Research Objectives and Structure of Thesis***

To address the grand challenges of sustainability that faces our society, I envisioned applying the metabolic engineering toolbox to rationally engineer *E. coli* to produce both δ- aminovalerate and glutarate from glucose. To accomplish this feat, three foreseeable challenges first had to be met. First, metabolite flux through the endogenous lysine had to be enhanced to provide adequate supply of the pathway precursor from glucose. Second, appropriate enzymes constituting the production pathway had to be identified, introduced into *E. coli*, and characterized. Third, production of the compounds of interest from glucose had to be demonstrated by expressing the pathway genes in a lysine over-producing *E. coli* host. The last step then involved debottlenecking of the pathway and fine-tuning of its enzymes in the final production strain. The work contained within this document constitutes the first demonstration of the bioproduction of δ-aminovalerate and glutarate from glucose, and effectively expanding the repertoire of polyamide monomers that can be produced microbially from renewable resources. The last step then involved debottlenecking of the pathway and fine-tuning of its enzymes in the final production strain. The work contained within this document constitutes the first demonstration of the bioproduction of δ-aminovalerate and glutarate from glucose, and effectively expanding the repertoire of polyamide monomers that can be produced microbially from renewable resources.

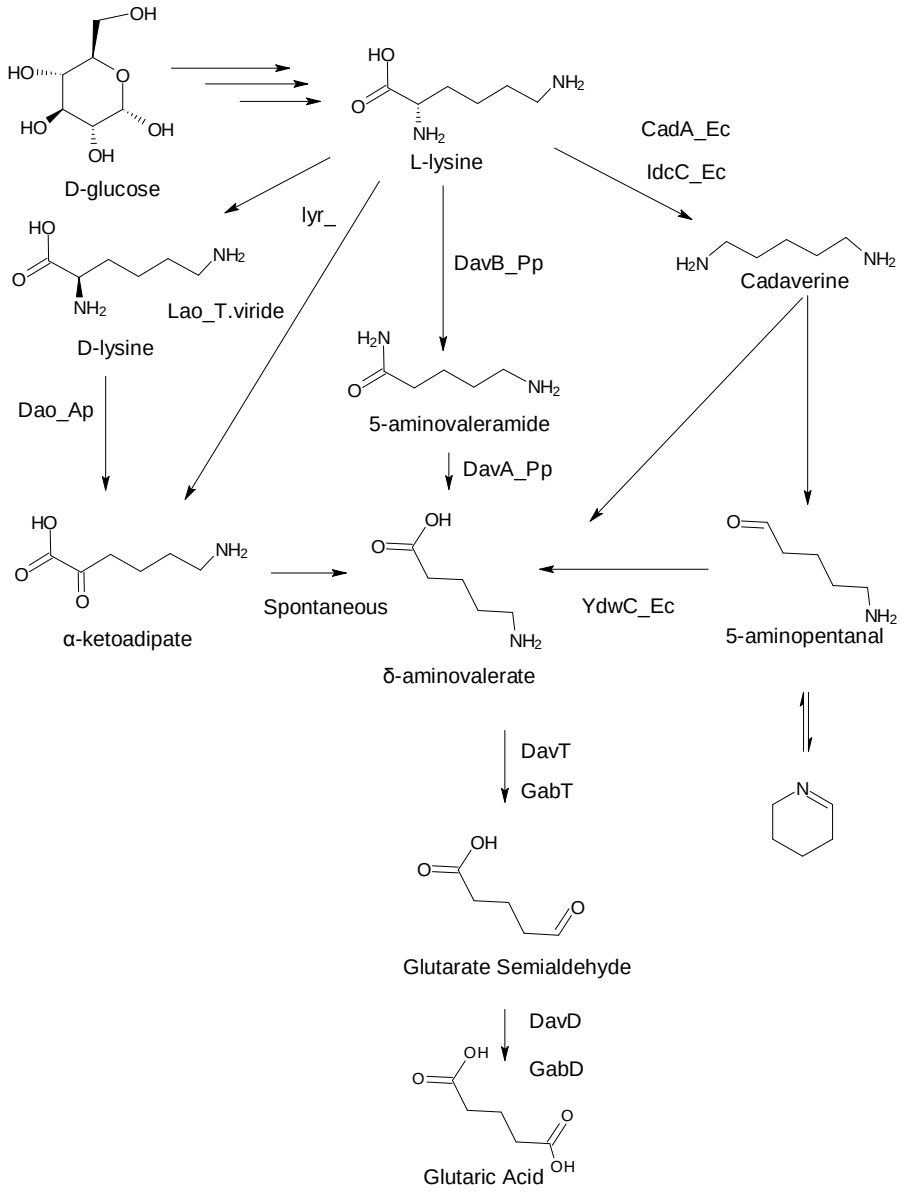


Figure 5: *Multiple known biochemical pathways from L-lysine to δ-aminovalerate and glutarate, and the previously engineered pathway to cadaverine.*

This thesis consists of four chapters. Chapter 2 is a presentation of the analytical and experimental methods that were central to this research. Chapter 3 presents the relevant results of this research, as well as critical analysis and discussion. Chapter 4 provides a summary of the work, along with conclusions and several recommendations for future work. These chapters are followed by an appendix and a list of all references.

Chapter 2

MATERIALS AND METHODS

2.1 ***Introduction***

As discussed in Chapter 1, guiding *E. coli's* metabolism through the introduction of exogenous biochemistry could potentially yield a polyamide building block production host. This chapter outlines the material and chemicals, enzymes, strains, plasmids, and cloning strategies used. This includes how various mutations were introduced through splicing overlap extension. The various pathway characterization methods and cellular culturing strategies in production hosts are also detailed. At times, it is necessary to provide some background as to why various experimental methods were employed.

2.2 ***Chemicals and Enzymes***

Various organic acids were used as standards for analytical detection and as cofactors. Glutaric Acid (≥99%) in its free acid form and α-Ketoglutaric acid sodium salt (≥98%) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The amino acids used for analytical standards and production kinetic experiments L-lysine (≥97%) and δ-aminovalerate (≥97%) were also obtained from Sigma-Aldrich. The diamine cadaverine (MP Biomedical; Santa Ana, CA; ≥97%), D-(+)-glucose (Amresco; Solon, OR; ≥99.5%) were used for analytics and culturing, respectively. Glycerol (≥99%), and Yeast Extract were obtained from Sigma-

Aldrich. The inorganics used as media components and buffers include $(NH_4)_2SO_4$ (Sigma-Aldrich, ≥99%), $MgSO_4$ (EMD Chemicals Inc.; Darmstadt, Germany; ≥99.5%), KH_2PO_4 (Amresco, ≥98%), $CaCO_3$ (Sigma-Aldrich, ≥99%). The buffers used for resting cell assays, and nucleotide and protein gels were Phosphate Buffered Saline (PBS; Cellgro; Manassas, VA) buffer, Tris-Acetate-EDTA (TAE; S-Prime; Gaithersburg, MD) buffer, and Tris/Glycine/SDS (Bio-Rad; Hercules, CA) buffer.

All DNA polymerases, ligases, restriction enzymes and buffers were purchased from New England Biolabs (Ipswich, MA). For routine DNA amplification *taq* DNA polymerase was used and for DNA cloning Phusion DNA polymerase was utilized. For all ligations T4 DNA ligase was utilized. The various restriction enzymes used for DNA digestion were: *Bam*HI, *Bgl*II, *Eco*RI, *Nco*I, *Nhe*I, *Bsp*HI, and *Nde*I. All of the enzyme specific buffers were purchased from the same companies as the respective enzymes.

2.3 ***Plasmid Construction and Cloning***

2.3.1 ***Strains and DNA Plasmids, Primers***

E. coli strains used in this study are listed in Table 1. *E. coli* strains NEB10β and *5-α F'I*^{q} were used for general gene cloning. *E. coli* BW25113, a K-12 strain, was used as a parent strain to develop lysine overproduction strain because of its tractability. For the construction of plasmids and strains, cells were grown in Luria-Bertani (LB) broth or on LB plates (1.5wt% agar) containing

appropriate antibiotics at the following concentrations: 50 mg L^{-1} Streptomycin (Sr), 50 mg L^{-1} Chloramphenicol (Cm), 100 mg L^{-1} Ampicillin (Amp), and 50 mg L^{-1} Kanamycin (Kan).

Plasmids used in this study are also listed in Table 1. Polymerase chain reaction (PCR) primers (obtained from IDT; Coralville, IA) for plasmid construction and genetic engineering are listed in Table 2. For the overexpression of *davB* and *davA*, the *davBA* operon of *P. putida* KT2440 was amplified using Phusion polymerase and F_DavBA_EcoRI and R_DavBA_HindIII (Table 2). The PCR product was digested completely with *Eco*RI and *Hind*III. This fragment was ligated with *Eco*RI-*Hind*III digested plasmid pSTV28. For the overexpression of *davT* and *davD*, the *davDT* operon of *P. putida* KT2440 was amplified using Phusion polymerase and F_DavDT_EcoRI and R_DavDT_BglII. The PCR product was digested completely with *Eco*RI and *Bgl*II. The fragment was ligated with *Eco*RI-*Bam*HI digested plasmid pTRC99a. *Bgl*II and *Bam*HI are isoschizomers making their digested overlap regions palindromic and compatible for ligation. These constructed plasmids were named pSTVDavBA and pTRCDavDT, and allow for the gene expression under the strong, inducible *trc* promoters.

To create a lysine overproducer, *dapA* from *Corynebacterium glutamicum* 13032 was PCR amplified using F_dapA_NcoI and R_dapA_EcoRI. *dapA* is a naturally feedback resistant version of Aspartate kinase III. This amplicon was purified and digested with *Nco*I and *Eco*RI. This fragment was ligated with *Nco*I-

*Eco*RI digested plasmid pCDFduet-1. *LysC* was made feedback resistant by introducing a mutation at the 1055 bp of the *E. coli* MG1655 l*ysC,* aspartate kinase III. This was accomplished using splicing overlap extension. This is a well characterized method of introducing single point mutations in genes through properly designed primers. A PCR with F_lysC_NdeI and R_lysCmutation amplified an ~1150 bp segment of the gene. The primers (Table 2) F_lysCmtuation and R_lysC_BglII were used to introduce the mutation into an ~450 bp segment. These two DNA amplicons were used as templates in another PCR which amplifies a mutated (feedback resistant) version of *lysC*. *LysC*^{fbr} was digested completely with *Nde*I and *Bgl*II. *Nde*I-*Bgl*II digested pCDFdapA^{fbr} was ligated with digested *lysC*^{fbr}.

pCDFduet-1 was was chosen because pSTV99a and pTRC28 confer chloromphenocol and ampicillin resistance, respectively. pCDFduet-1 confers streptomycin resists, thus was a natural choice for screening experiments that is compatible in the presence of pSTV99a and/or pTRC28, or their derivative plasmids (i.e. pSTVDavBA). To confirm that these genes had been properly cloned into pCDFduet-1, PCR and digestion screening was employed. Additionally, to confirm that the appropriate mutation was introduced into the *lysC* gene via splicing overlap extension, the pCDFDapA^{fbr} and pCDFdapA^{fbr}lysC^{fbr} were both sequenced at the Biodesign Institute at Arizona State University via their DNA sequencing process.

2.3.2 ***DNA Purification***

PCR amplified DNA fragments were purified using DNA Clean and Concentrator Kits(Zymo Research, Irvine, CA). Plasmid DNA was miniprepped and purified using Zyppy plasmid miniprep kits (Zymo Research). Cell harboring the plasmid of interest were cultured in 5mL LB with appropriate antibiotic overnight. The following day the kit was used to yield purified plasmid DNA in water for transformations, PCR, and digestions. Genomic DNA purification kits from Zymo Research Co. for all genomic template DNA extractions used for polymerase chain reactions and cloning was purified according to the genomic DNA purification kits. Gel DNA extraction kits from Zymo Research Co. were used on DNA samples that were to be used for ligation reactions were viewed and concentrated on nucleotide agarose gels. Agarose gel was cut out and the contained DNA was purified according to the Gel DNA purification kit.

Table 1: *Bacterial strains and DNA plasmids used in this study*

Strains/Plasmids	Description[a]
***E. coli* NEB10β**	*araD139*Δ(*ara-leu*)7697 *fhuA lacX74 galK* (ϕ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^R) Δ(*mrr-hsdRMS-mcrBC*)
E. coli 5-α F'I^q	F´ *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)*M15 *zzf::Tn10* (Tet^R) / *fhuA2*Δ*(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)*M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*
E. coli BW25113	F' λ$^-$ Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), lambda$^-$, *rph-1*, Δ*(rhaD-rhaB)568, hsdR514*
E. coli ΔcadA	*BW25113ΔcadA (l-lysine decarboxylase)*
E. coli ΔldcC	*BW25113ΔldcC (l-lysine decarboxylase)*
E. coli MG1655	F$^-$ λ$^-$ *ilvG*$^-$ *rfb*$^-$ 50 *rph*-1
P. putida KT2440	originates from the toluene degrading bacterium P. putida mt-2
C. glutamicum 13032	Gram-positive bacterium of the class Actinobacteria
pTRC99a	Expression vector, Amp^r, *trc* promoter, pBR322 Ori, 4178 bp
pSTV28	Expression vector, Cm^r, *trc* promoter, pACYC184 Ori, 2999 bp
pCDFDuet-1	Duet expression vector, Sr^r, *T7* promoter, CDF Ori, 3781 bp
pSTVDavBA	*P. putida KT2440 davBA* operon in *Eco*RI-*Hind*III site of pSTV28
pTRCDavDT	*P. putida KT2440 davDT* operon in *Eco*RI-*Bam*HI site of pTRC99a
pTRCGabDT	*E. coli MG1655 gabDT* operon in *Eco*RI-*Bam*HI site of pTRC99a
pCDFlysC^{fbr}dapA^{fbr}	*E. coli MG1655 lysC* gene in NdeI-BglII site and *C. glutamicum 13032 dapA* gene in NcoI-EcoRI site of pCDFduet-1 with a single base pair mutation at the 1055bp (cytosine to thymine)

*2.3.3**Chemically Competent Cell Transformations***

Chemically competent cells were prepared by first preparing a seed culture grown in 5mL LB overnight. From this seed, 500µL was then used to inoculate 50mL LB in a 250mL shake flask, which was then grownat 37 ^{o}C and shaken at 200rpm Until reaching an OD_{600} of ~0.4. Cells were then collected by centrifuging at 1600xg for 5 minutes and at 4 ^{o}C before then being washed with 10 mL of 60mM $CaCl_2$. This wash step was repeated three times before the cells were then incubated on ice for 30 min. Cells were then again centrifuged before being resuspended in 2 mL 60mM $CaCl_2$. This final solution was divided into 100 µL aliquots and stored in the -80^{o}C. Transformations of chemically competent cells were performed by first allowing frozen cells thaw on ice for 15 minutes. Then, 1µL of purified plasmid or 10µL of ligation reaction was added to cells and the mixture was allowed to incubate for another 15 minutes on ice. The mixture was then heat shocked at 42°C for thirty seconds. Immediately after this heat shock step, 500µL Super Optimal Broth (SOC; Teknova; Hollister, CA) was added to the mixture, and plated on LB with appropriate antibiotics. 500µL Super Optimal Broth (SOC; Teknova; Hollister, CA) was added to the mixture and the sample was incubated in a 37°C while shaking at 200 rpm for one hour before being plated on LB with appropriate antibiotics and incubating overnight at 37^{o}C.

Table 2: *DNA primers used in this study for PCR and Splicing Overlap Extension*

Primer	Sequence (5'—3')[a]
F_DavBA_EcoRI	TATGAATTCCTAAGGGGAATATACATGAACAAGAAGAACCGCCACC
R_DavBA_HindIII	TTAAAGCTTTCAGCCTTTACG
F_DavDT_EcoRI	TATGAATTCCTAAGGGGAATATACATGCAGCTCAAAGACGCTCA
R_DavDT_BglII	TATAGATCTTCAGGCGATTC
F_DapA_NcoI	CCCGGGCCATGGCTACAGGTTTAACAGCTAAGACCGGAGTAGA
R_DapA_EcoRI	GATATCGAATTCTCATTATAGAACTCCAGCTTTTTTC
F_LysC_NdeI	GATATCCATATGTCTGAAATTGTTGTCTCCAAATTTGGCGG
R_LysC_BglII	GCGATTAGATCTTTACTCAAACAAATTACTATGCAGTTTTTG
F_LysC_Mutation	GTGGCATTAATCCTTGATACC
R_LysC_Mutation	GGTATCAAGGGTTAATGCCAC

a) Restriction sites are underline

2.3.4 ***DNA Ligations and Digestions***

Ligations were performed according to the following recipe: 1µL T4 DNA ligase was mixed with 1µL T4 DNA ligase buffer, 4µL digested plasmid DNA, and 4µL digested DNA fragment making a 10µL ligation reaction. These 10µL ligation reaction mixtures were allowed to react for 16 hours or overnight at 4^{o}C

before transformation. T4 DNA ligase buffer was purchased from New England Biolabs (Ipswich, MA)

Digestions were accomplished according to the following recipe: 32 µL Plasmid or PCR amplified DNA was mixed with 4µL Restriction Enzyme(s) and 4µL Appropriate Restriction Enzyme Buffer making a 40µL ligation reaction. 40µL restriction digestions were allowed to react for 3 hours at 37°C, and digestion products were recovered by DNA gel purification. All restriction enzyme buffers were purchased from New England Biolabs

2.4 ***Pathway Expression and Characterization***

Various methods were employed to characterize the pathway. First protein gels and Bradford assays were employed to discern the expression of the proteins and to get a feel for the amount of total protein that is being produced. Next, whole cell assays were done in order to get a feel for the functionality and kinetics of the various enzymes when fed substrates of the AMV pathway.

2.4.1 ***Protein and Nucleotide Gel Electrophoresis***

Protein gels were run to determine that the appropriately cloned genes were successfully being translated. Appropriately cloned plasmids were transformed into a production strain of *E. coli* BW25113, which have the DE3 prophage necessary to translate the RNA polymerase for the T7 promoter of the duet vectors (i.e. pCDFduet-1). Cells were given the DE3 prophage by following

the standard procedure of the Novagen λDE3 lysogenization kit (Darmstadt, Germany). These production strains were then cultured in 5mL LB with appropriate antibiotics. 500µL seeds were inoculated into 50mL LB with appropriate antibiotics and cultured at 37°C. After 3 hours 12.5µL 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to growing cultures. Cultures were allowed to grow for 12 hours after addition of IPTG. Cells were then lysed, centrifuged, and protein lysates taken as the supernatant. 15 µL of the lysates were then stained with 15 µL a mixture of 118.75 µL lammeli buffer (Bio-Rad) and 6.25 µL 2-mercaptoethanol (Sigma-Aldrich, ≥98%). The lysates were then denatured at 100°C for 5 minutes. Premade 4-12% gradient polyacrylamide gels stored in NaN_3, purchased from Bio-Rad were then placed vertically into the gel apparatus and stained samples injected into the wells. Protein gels were run at 200V for 35minutes against a Precision Plus Protein Unstained Standard (Bio-Rad). Protein gels were then washed with water, stained with 50mL Coomassie Brilliant Blue G-250 Solution (G-Biosciences, St. Louis, MO) for one hour and left to shake overnight in water.

Nucleotide gels were prepared dissolving a ratio of 0.2g Agarose per 25mL 1x TAE buffer and heating. The liquefied gel was poured into a gel tray and allowed to cool with a well comb. Samples were stained with Safe-View DNA white dye (abm Inc.; Richmond, BC) and ran against a New England Biolabs 1.0 kB DNA ladder prepared as 20µL Safe-View white dye, 20µL 1.0 kb DNA ladder, and 160µL 1x TAE. Gels were viewed on a UV block.

2.4.2 ***Bradford Assays***

For Bradford assays the protein lysates were prepared identically to that of a protein gel. A calibration curve was made using 0, 0.2, 0.4, 0.6, 0.8, and 1 g/L Bovine Serum Albumin (BSA) protein standards stained with Quick Start Bradford 1x Dye Reagent (Bio-Rad), vortexed, and allowed to stain for 10 minutes. Optical Density (OD) at 595nm of these standard solutions was taken and made into a calibration curve. Lysates were then stained with Bio-Rad dye. The OD_{595} was measured, and the total protein concentration was back calculated based on the BSA calibration.

2.4.3 ***Whole Cell Kinetic Assays***

Appropriately cloned plasmids (i.e. pSTVDavBA, pTRCDavDt, pCDFDapA^{fbr}, and/ or pCDFDapA^{fbr}LysC^{fbr}) were transformed into a production strain of *E. coli* BW25113, which has the DE3 prophage. These production strains were then grown overnight in 5mL LB with appropriate antibiotics (5µL). 500µL seeds of these overnight cultures inoculated 50mL LB with appropriate antibiotics and grown at 37˚C. After 3 hours 12.5µL 1M IPTG was added to growing cultures. Cultures were allowed to grow for an additional 12 hours. 50mL of cell culture was then spun down to a pellet at 1700g and washed twice with 10mL PBS. The pellet was finally resuspended in 30mL PBS and split into three 10mL samples in 15mL vials to run experiments in triplicate. Appropriate sterilized chemicals were fed to the resting whole cells for kinetic assays at that point at

various concentrations. Samples were taken for analytical quantification as 500µL samples, centrifuged at 2000g, and the supernatant removed from the pellet. This supernatant was either immediately analyzed by high pressure liquid chromatography or frozen at -20˚C for future analysis.

2.5 ***Culture Media***

Three types of culture media were used for this research: LB, SOC, and a production minimal media (M10). LB (company, state) media was used for seeds and routine growth of all *E. coli* strains with a composition of (per L): 10 g Tryptone, 5 g Yeast Extract, 10 g NaCl. SOC was used for cultivation of cells during chemically competent transformations. SOC has a composition of (per L): 20 g Tryptone, 5 g Yeast Extract, 0.5 g NaCl, 0.186 g KCl, 0.952 g $MgCl_2$, and 3.603 g Glucose. M10 was used for production experiments. The recipe for M10 is as follows (per L): 25 g Glucose, 16 g $(NH_4)_2SO_4$, 1 g $MgSO_4$, 1 g KH_2PO_4, 2 g Yeast Extract, 30 g $CaCO_3$. When culturing with M10 media, prior to inoculation, seed cultures were always grown overnight in 5 mL LB. 500µL samples were then washed twice with 500µL 0.9% NaCl, and added into 50 mL M10 media in a 250mL shake flask. Frozen stocks of all bacteria strains were prepared by growing up a seed culture in 5 mL LB over night at 37˚C and combining in a 1:1 (v/v) manner with a 30% (w/v) glycerol solution in tubes. These tubes were stored for up to 1 year in a -80˚C freezer. Seed cultures prepared from frozen stock were

scraped from frozen stock with a pipette tip, and introduced to 5mL LB shaking at 37 °C, 200rpm, and with appropriate antibiotics added.

2.5.1 **Toxicity Experimental Setup**

If the toxicity were too high it may be ineffective to attempt to produce these compounds with E. *coli* as a host. Here we define the toxicity limit as the point at which the rate of cellular growth is zero, and any further compound addition results in a negative cellular growth rate. (i.e. cell death). Seed cultures of BW25113 (a tractable *E. coli* strain for production of protein) The following day 900 µL of these seed cultures were centrifuged, and the pellets suspended in 0.9% NaCl (filter sterilized). This wash step was repeated twice, a final resuspension was given, and used to inoculate 50 mL M10 media. Once the OD_{600} reached 0.5, varying amounts of δ-aminovalerate and glutarate in water were added. At this point the experiment was underway and growth over time was monitored at OD_{600}.

2.5.2 ***Assays for Lysine, AMV, and Glutarate Production***

In order to test various strains for engineered lysine, AMV, and/or glutarate production it is critical to use a minimal media with a limited amount of phosphate. Imaizumi et al. suggested the M10 media in their publications with *E. coli* WC196 and WC196R (mutagenized and evolved L-lysine overproducers). To detect various culture metabolites, seeds were cultured overnight in 5 mL LB with

appropriate antibiotics. These seeds were then washed with 0.9% NaCl. These washed seeds inoculated into 50 mL M10 media, after 3 h 12.5 µL 1M IPTG was added to the culture. Samples of the culture were taken periodically and the OD_{600} read. Then the cells were centrifuged to a pellet, and the supernatant was taken for analysis including quantification of media components with time. These experiments were repeated in triplicate as 3x50 mL cultures in 250 mL shake flasks.

2.6 ***Analytical Methods***

High pressure liquid chromatography (HPLC; 1100 series, Agilent, Santa Clara, CA) was used to examine aqueous concentrations of various L-lysine, AMV, cadaverine, glutarate, 2-oxoglutarate, and glucose. Samples thought to contain amino acids and diamines were first derivatized in-needle with o-phthalaldehyde (OPA) (OPA Reagent Solution Complete, Sigma-Aldrich, St. Louis, MO) as described below, before being separated on a ThermoFischer Hypersil Gold aQ (Anthem, AZ), 150x 4.6mm column with 5µm diameter particles. A constant column temperature of 40^{o}C was used. A 50/50 mixture of filtered and degassed deionized water and methanol (≥99.9%, HPLC grade, Sigma-Aldrich, St. Louis, MO) was used as the mobile phase at a constant flow rate of 0.77 mL/min. Analytes were detected using a Diode Array Detector (DAD) set at a wavelength of 338nm. Analyte concentrations were determined with the use of external standards for calibration.

OPA derivatization was required for the detection of each of lysine, cadaverine, and δ-aminovalerate . The thioisoindole reaction products absorb light maximally at 338nm (Qian et al. 2009b). Derivatization was accomplished by programmed mixing of cold OPA reagent with samples in a 3:1 (v/v) proportion, typically in a ratio of 6 µL analyte to 2µL OPA mixture, inside the auto-injector needle for one minute before injection.

Organic acids (glutaric acid and 2-oxoglutarate) and glucose were separated using a Bio-Rad Aminex HPX-87H column, 300x7.8mm, with 9µm diameter particles (Hercules CA), operated at a constant temperature of 50°C and using a mobile phase of 0.005 M H_2SO_4. A Refractive Index Detector (RID) was used for analyte detection. Analyte concentrations were determined with the use of external standards for calibration. Injection volumes of 10 µL and detection time frames of 15 minutes were enough to detect all relevant culture components, and elute all components past the RID.

Cell growth was measured by means of changes in optical density (OD) as measured as the absorbance of 600nm light relative to a standard. A Beckman Coulter DU800 spectrophotometer was used (Brea, CA) The spectrophotometer was blanked using uninoculated media. Occasionally, for analysis of the OD_{600} of cultures grown in LB specifically, a Buglabs (Concord, CA) handheld OD Scanner was calibrated and used.

Chapter 3

ENGINEERING *E. COLI* FOR THE PRODUCTION OF δ-AMINOVALERATE AND GLUTARATE FROM GLUCOSE

3.1 ***Introduction***

While the AMV pathway of *P. putida* KT2440 is naturally used for L-lysine degradation, its piecewise and heterologous reconstruction could also provide a route to the renewable production of polyamide biomonomers δ-aminovalerate and glutarate. Chapter 3 outlines and characterizes the 'bottom up' engineering of *E. coli* to produce both δ-aminovalerate and glutarate through the rational engineering of a L-lysine over-producing host strain and functional reconstruction and characterization of the AMV pathway, followed by their ultimate integration to demonstrate their production directly from renewable glucose. In addition, metabolite toxicity analyses were performed to understand the impacts of producing these chemicals on *E. coli* and to set targets for maximum achievable titers.

3.2 ***Approach***

Figure 6 illustrates the general approach followed in this study to engineer *E. coli* for δ-aminovalerate and glutarate production from glucose. As shown in Figure 6, the complete AMV pathway is composed of four enzymes, DavBADT, which, as noted by Revelles et al. are organized into two operons (Revelles et al., 2007). The genes *davBA* form one operon while *davDT* form the other. As seen in

Table 3, it was predicted that the AMV pathway enzymes should function well in *E. coli* due to the abundant of available cofactors that are conserved between the strains.

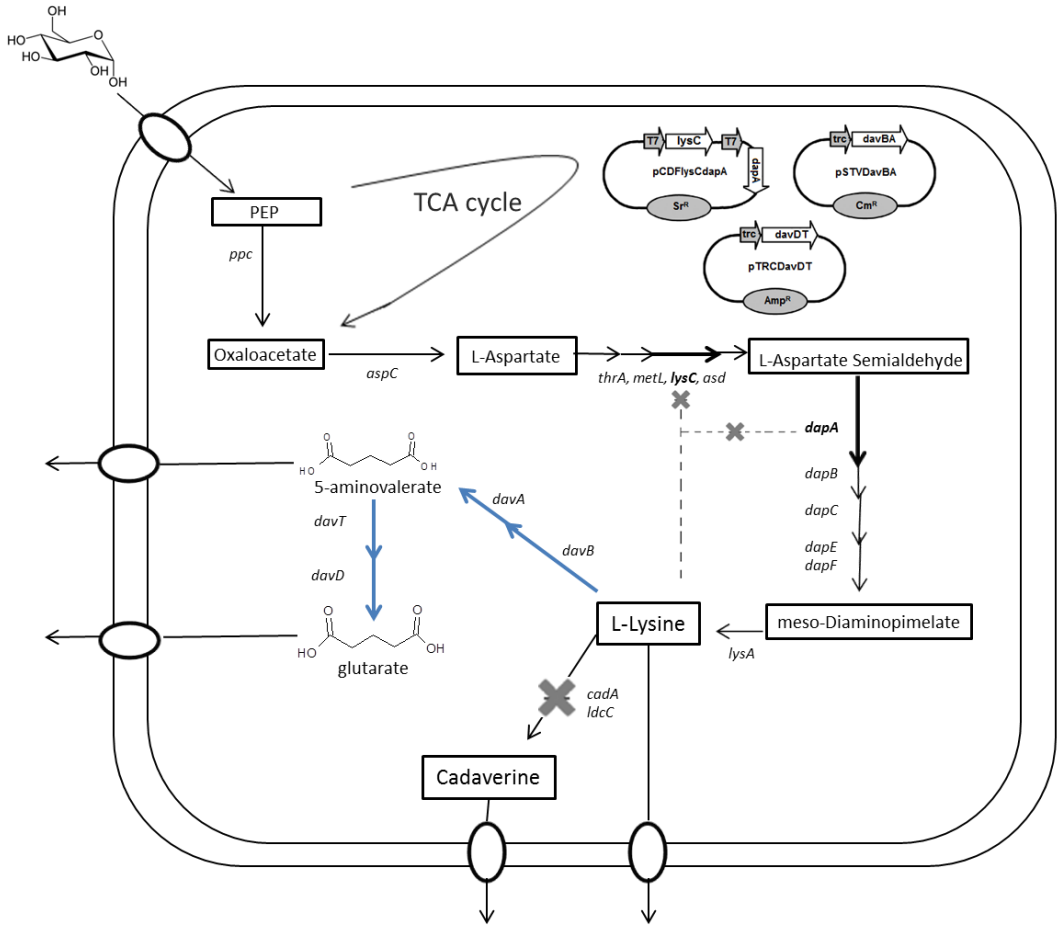


Figure 6: *The general approach followed in this study to engineer E. coli for δ-aminovalerate and glutarate production from glucose. The lysine biosynthesis pathway was allosterically deregulated, the competing cadaverine biosynthesis pathway was deleted, and the AMV pathway was introduced.*

DavT, specifically, is a transaminase that requires 2-KG, for example. After examining the metabolism of *E. coli* in depth, it was hypothesized that 2-KG limitations may not be a concern since a net of one molecule of 2-KG is produced for every L-lysine molecule that is produced. Together davBA convert L-lysine to δ-aminovalerate, one of the desired products. Then, davDT is responsible for converting for converting δ-aminovalerate to glutarate, the second desired product, for converting δ-aminovalerate to glutarate, the second desired product.

A modular approach to constructing pathways for each product was adopted to improve efficiency. This involved cloning and expressing the AMV pathway genes as part of their native operons. Therefore, as depicted in Figure 7, *davBA* was cloned from *P. putida* KT2440 into pSTV28, while *davDT* was cloned from *P. putida* KT2440 into pTRC99a, resulting in the construction of plasmids pSTVDavBA and pTRCDavDT. While plasmid X in theory would provide the genes required for δ-aminovalerate production, when paired with the plasmid pCDFLysC^{fbr}DapA^{fbr}, the three plasmids would complete the entire pathway to glutarate.

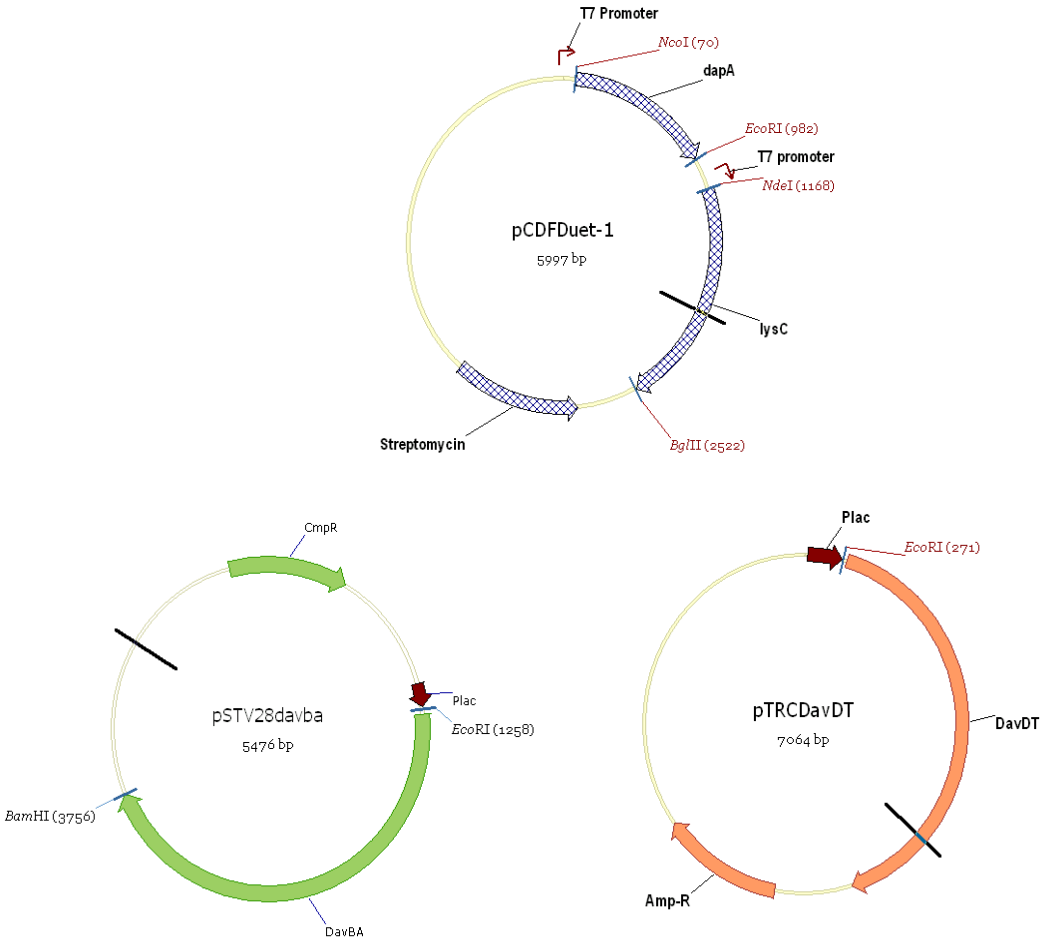


Figure 7: *Plasmid constructs for lysine pathway deregulation and introduction of the AMV pathway. DavBA and davDT were cloned as operons into the depicted restriction sites. dapA^{fbr} and lysC^{fbr} were cloned into the first and second cloning sites of the pCDFduet-1.*

3.3 ***Results and Discussions***

3.3.1 ***Expression of DavBADT in E. coli***

Whole cells (BW25113) transformed with the various pathway plasmid constructs pSTVDavBA and/or pTRCDavDT were grown up to be used for protein analysis. Figure 8 below shows the protein lysate contents. As outlined in table 3, the predicted size of the *davB* protein is 61kD. This is consistent with the circled protein band in lane 3. However, davA was not easily seen on this gel. The absence of davA is unexplained, and was not found in repeat assays. However, since digestion and PCR colony screens of the plasmid based operon appeared successful it was decided to test for enzyme functionality. As explained in detail later, wild-type BW25113, when supplemented with L-lysine, does not produce any δ-aminovalerate; However, BW25113 transformed with pSTVDavBA does. This demonstrates that davA is expressed and functional.

Table 3: *Characteristics of the key AMV pathway enzymes*

Enzyme	**DavB**	**DavA**	**DavT**	**DavD**
Chemistry	Monooxygenase	Amidohydrolase	Transaminase	Dehydrogenase
Cofactors	O_2	H_2O	2-ketoglutarate	$NADP^+$
Byproducts	CO_2, H_2O	NH_3	L-glutamate	NADPH, 2 H^+
Molecular Weight	61kD	67kD	44.82kD	51.55kD
K_m	620μM	2000μM	2000μM	63μM

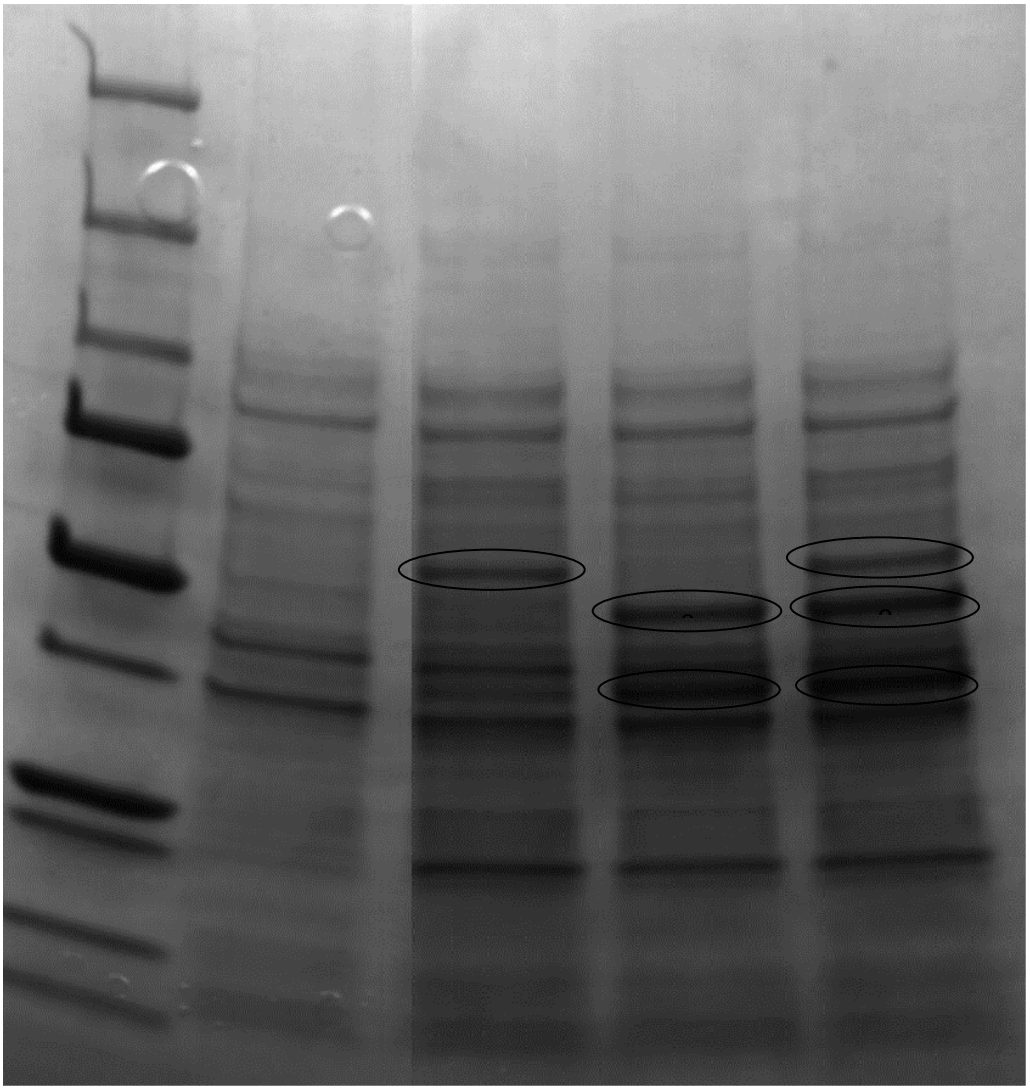


Figure 8: *Protein gel showing the successful expression of* davB *and* davDT *in* BW25113. *The lanes contain protein from the following sources (Left to Right): Precision Plus Protein DNA ladder, BW25113 pSTV28 pTRC99a (control), BW25113 pSTVDavBA, BW25113 pTRCDavDT, BW25113 pSTVDavBA pTRCDavDT (entire AMV pathway).*

Additionally, the protein gel depicts, in accordance with the experimental (when available) or predicted protein molecular weights, the successful expression of the proteins davD, and davT. In lane four, two bands are circled that correspond to davD and davT, and the molecular weights listed for them in table 3. The last lane (all the way to the right) contained both cloned plasmids (BW25113 pSTVDavBA pTRCDavDT) and the same three bands. Based on the protein gel alone it was apparent that three of the four exogenous pathway proteins were being translated. Small samples of protein lysates were also taken for Bradford total protein assays. Based on BSA protein calibrations, total protein concentrations of BW25113 transformed with pSTVDavBA and pTRCDavDT were 1.05 and 0.89 mg/mL of total protein. Cells transformed with both pSTVDavBA and pTRCDavDT had 1.29 mg/mL total protein based on the assay.

3.3.2 **Assaying Function and Kinetics of DavBADT in *E. coli***

Kinetic assays were next performed using either resting cells or cell lysates to determine if the pathway enzymes were functional, and to provide a measure of their activity in *E. coli*. Collective function of DavBA was first studied by feeding lysine directly to resting whole cells of *E. coli BW25113* in PBS, and monitoring its consumption together with the corresponding production of δ-aminovalerate (as determined by HPLC-DAD) as a function of time. The dynamic results are present in Figure 9, which also compares the impact of different initial concentrations of lysine. It can be seen that nearly all of the lysine

added is efficiently converted to δ-aminovalerate in under 20 h and the amount produced is limited only by the initial amount of L-lysine added at the start of the assay. This demonstrates that DavBA are functional in *E. coli*, despite the inability to specifically detect DavA in protein gels. Using the data from Figure 9, yields of L-lysine-to-δ-aminovalerate through DavBA have been determined and are compared in Table 4 as a function of initial L-lysine concentration.

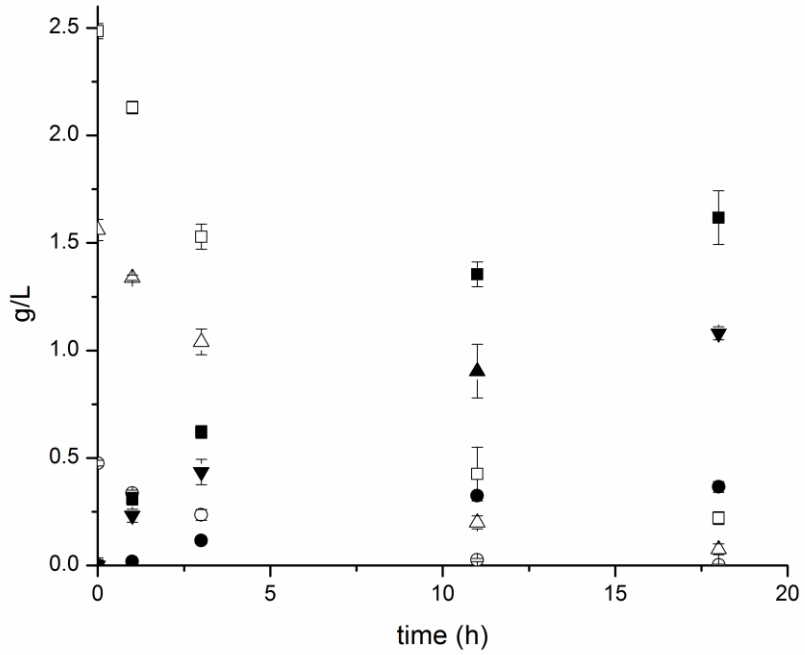


Figure 9: *Lysine consumption kinetics and the resultant production of δ-aminovalerate as a function of time in resting whole cell assays of E. coli BW25113 pSTVDavBA. Data shown are the averages of three independent replicates with the standard deviations calculated. Concentrations are: open symbols, lysine; filled symbols, AMV; Square, triangle, and circle data correspond to the experiments fed 2.5, 1.5, 0.5 g/L Lysine, respectively.*

Table 4: *AMV production yields from E. coli BW25113 pSTVDavBA resting whole cell kinetic data*

Starting Lysine Concentration [g/L]	Yield of $[AMV]_{prod.}$ /$[Lys]_{cons.}$ [mmol/mmol]
0.46	0.948
1.61	0.951
2.45	0.891

With DavBA found to be functional and capable of providing reasonable rates of δ-aminovalerate production from lysine, the first half of the pathway had been successful kinetically. Next, the second half of the pathway, namely DavDT, required similar testing and validation. To do so, resting cell experiments were again performed, though this time on strain *E. coli* BW25113 pTRCDavDT and by adding δ-aminovalerate as a substrate. Initially it was found that, despite the observance of DavD and DavT in cell extracts, little to no activity was detectable, as seen in Figure 10. We hypothesized that this unfavorable result could have arisen due to several potential limiting factors, including, non-functional enzymes (DavDT), poor import of δ-aminovalerate into the cells, poor export of glutarate out of the cells, or co-factor limitations. A series of follow-up studies were

performed to explore these prospects in an effort to achieve functional expression of enzymes constituting the second half of the glutarate pathway.

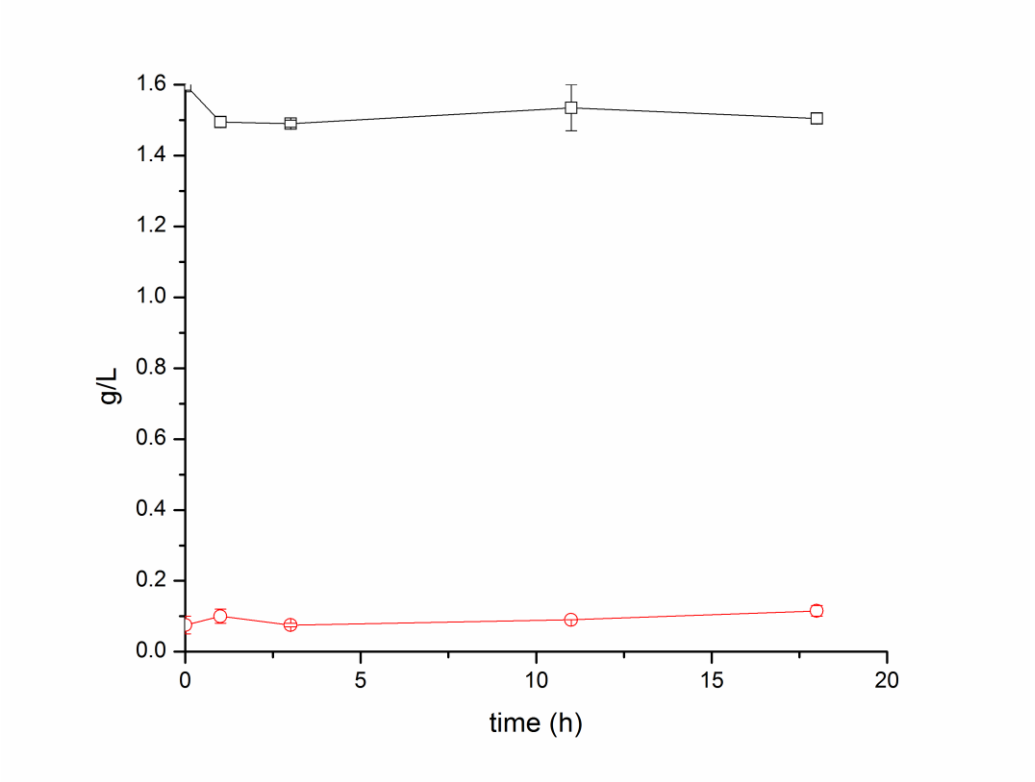


Figure 10: *AMV consumption and the resultant production of glutarate as a function of time in resting cell assays of E. coli BW25113 pTRCDavDT. Square data are AMV concentrations and circles are glutarate concentrations.*

3.3.3 ***Troubleshooting the Second Half (DavDT) of the AMV Pathway***

To test if poor import of δ-aminovalerate was limiting, resting cell assays with were performed on *E. coli BW25113* pSTVDavBA pTRCDavDT, which

expresses both DavBA and DavDT. As was shown in Figure 11, δ-aminovalerate is endogenously produced in this strain, and thus should also be available to DavDT in the cell. However, it was also found that, although lysine was again efficiently converted to δ-aminovalerate, a bottleneck then occurred in the pathway as a result of low DavDT activity and almost no glutarate production was detected.

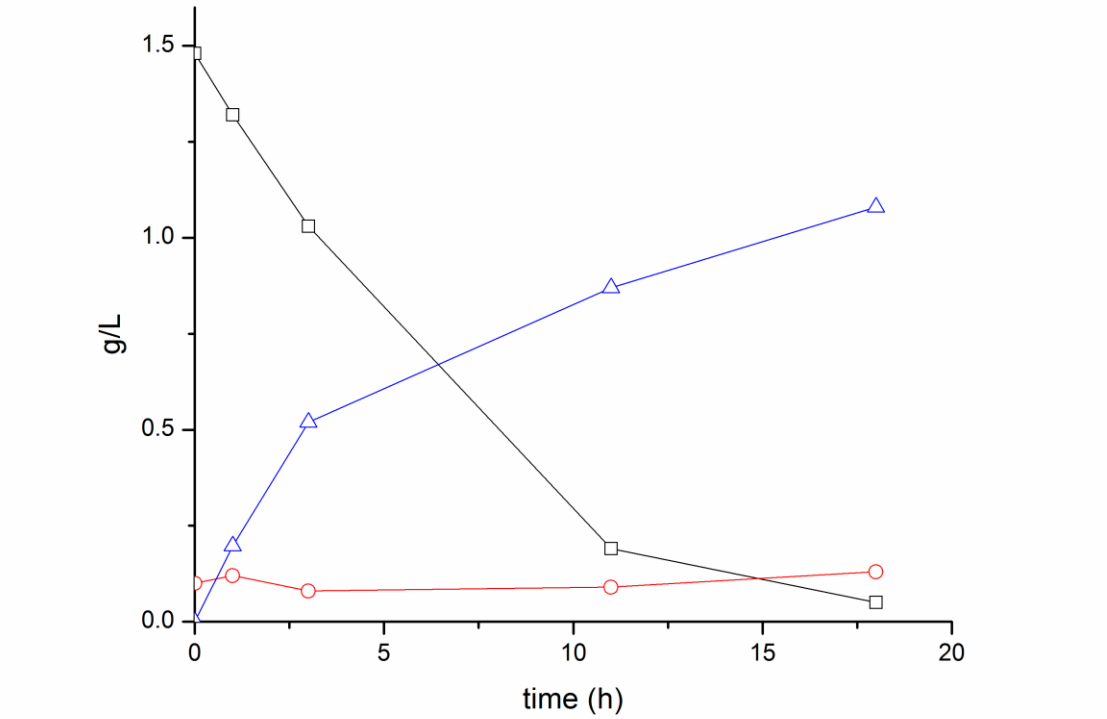


Figure 11: *Lysine consumption and the resultant production of δ-aminovalerate and glutarate as a function of time in resting cell assays of BW25113 pSTVDavBA pTRCDavDT (entire pathway): Squares (lysine),*

Next the possibility of cofactor limitations was tested because, as seen in Table 3, the transaminase reaction catalyzed by DavD is known to also require 2-ketoglutarate (2-KG). Cofactor limitations are common in resting cell assays because, although cells are provided with one of the required substrates (in this case δ-aminovalerate), because cells are not growing they are also unable to synthesize the required cofactor. To test this possibility, resting cell experiments with strain *BW25113* pTRCDavDT expressing DavDT were repeated, but in this case 2-KG was also provided to the cells. In this case, a fixed concentration of δ-aminovalerate (1.5g/L) was provided together with varying initial concentrations of 2-KG (0.85, 1.71, or 3.41 g/L, corresponding to 0.5, 1.0, or 2.0-times the stoichiometric requirement). As seen in Figures 12, 13, and 14, the addition of 2-KG to the medium promptly led to glutarate formation, which was excreted from the cells to accumulate in the medium.

While 2-KG supplementation resulted in alleviation of the bottleneck at DavT, the effect was clearly concentration dependent. When 2-KG is added at a limiting concentration, conversion of δ-aminovalerate to glutrate promptly stops once all 2-KG is consumed. Likewise, when 2-KG is added at excessive concentrations, conversion of δ-aminovalerate to glutarate goes to completion and leaves residual 2-KG as unreacted in the culture.

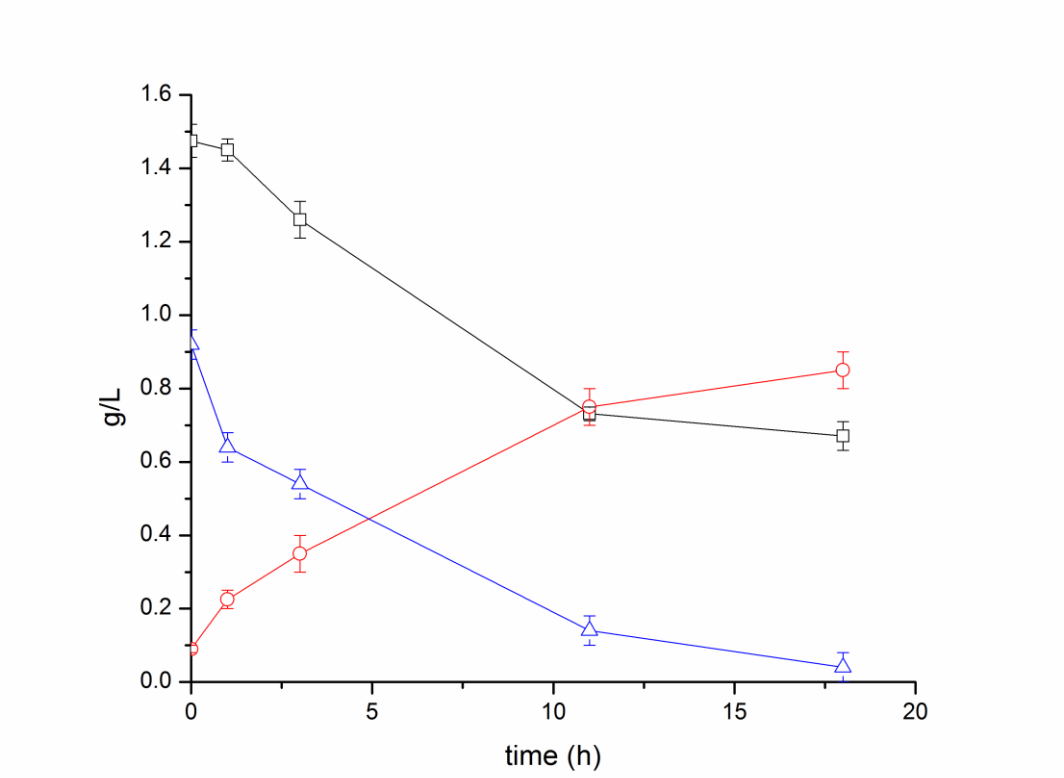


Figure 12: *AMV consumption and the resultant production of glutarate as a function of time in resting whole cell assays of BW25113 pTRCDavDT supplemented with 0.5x stoichiometric (limiting) 2-KG: Squares represent AMV concentration, triangles represent 2-KG concentration, and circles*

At last, the function of the entire pathway (from lysine to gutarate) was tested in resting cells of *E. coli* BW25113 pSTVDavBA pTRCDavDT expressing DavDABT and provided with both lysine and 2-KG. Varying the amount of 2-KG were added (relative to its stoichiometric requirement, as based on the initial amount of lysine added) to again demonstrate its essential importance and how it can limit resting cell assays. Glutarate was efficiently produced at yields of .94 mmol $mmol^{-1}$ on lysine, as shown in Figure 15. Another reason to test the entire

pathway construction was to discern bottlenecks at the step of *davT*, or any other step for that matter, and if bottlenecking was happening at *DavT* at low cellular concentrations of 2-KG. However, no significant bottlenecking, as would be indicated by the accumulation of an intermediate was observed.

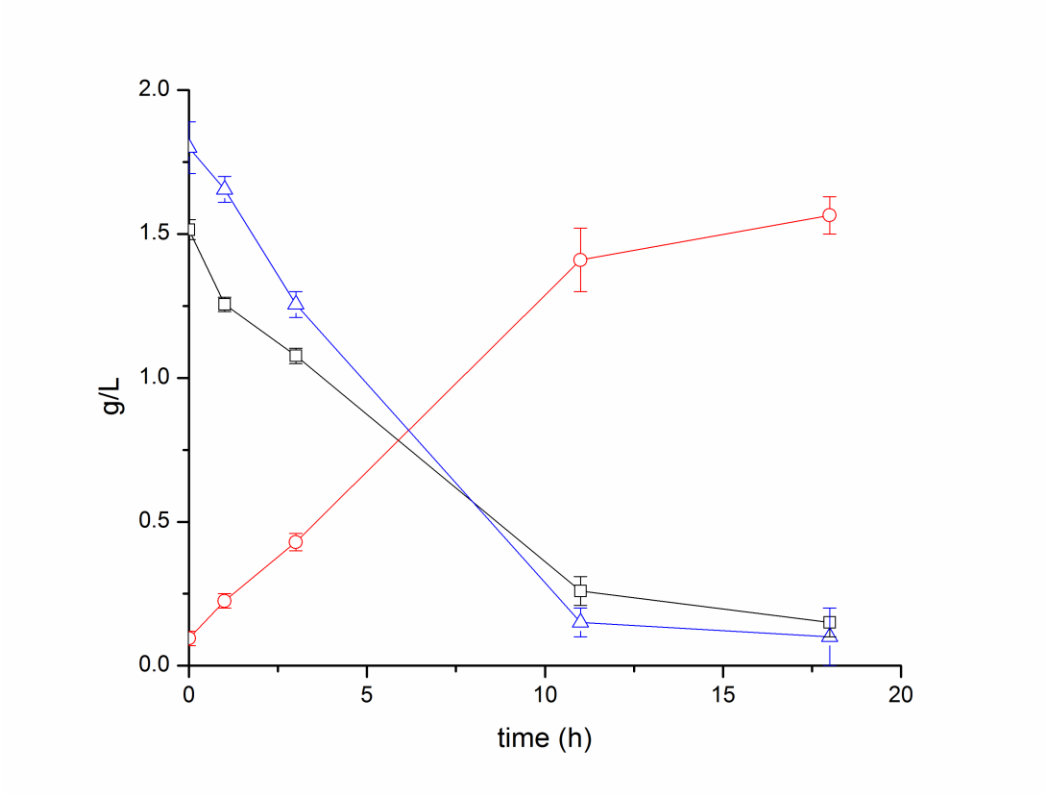


Figure 13: *AMV consumption and the resultant production of glutarate as a function of time in resting whole cell assays of BW25113 pTRCDavDT supplemented with 1.0x stoichiometric (equimolar) 2-KG: Squares represent AMV concentration, triangles represent 2-KG*

It is possible however, that some degree of bottlenecking is taking place and that this experiment did not detect it. More specifically, δ-aminovaleramide and glutarate semialdehyde were not measured analytically due to the fact that these chemicals are not commercially available. It can be argued, though, that this bottle necking cannot be significant because the rates of AMV formation and glutarate formation compared to L-lysine consumption and AMV consumption, respectively, are comparable.

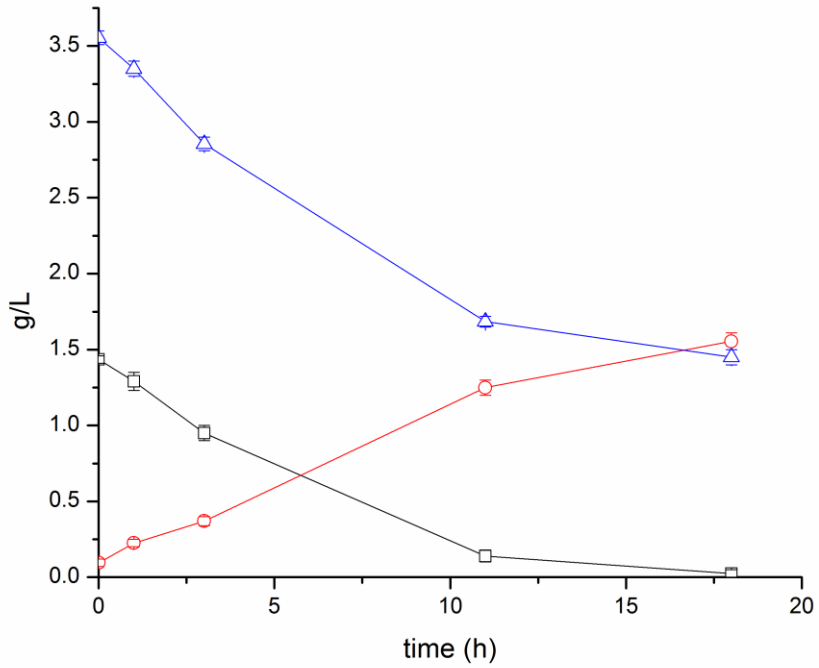


Figure 14: *AMV consumption and the resultant production of glutarate as a function of time in resting whole cell assays of BW25113 pTRCDavDT supplemented with 2.0x stoichiometric (excessive) 2-KG: Squares represent AMV concentration, triangles represent 2-KG concentration, and circles represent glutarate formation.*

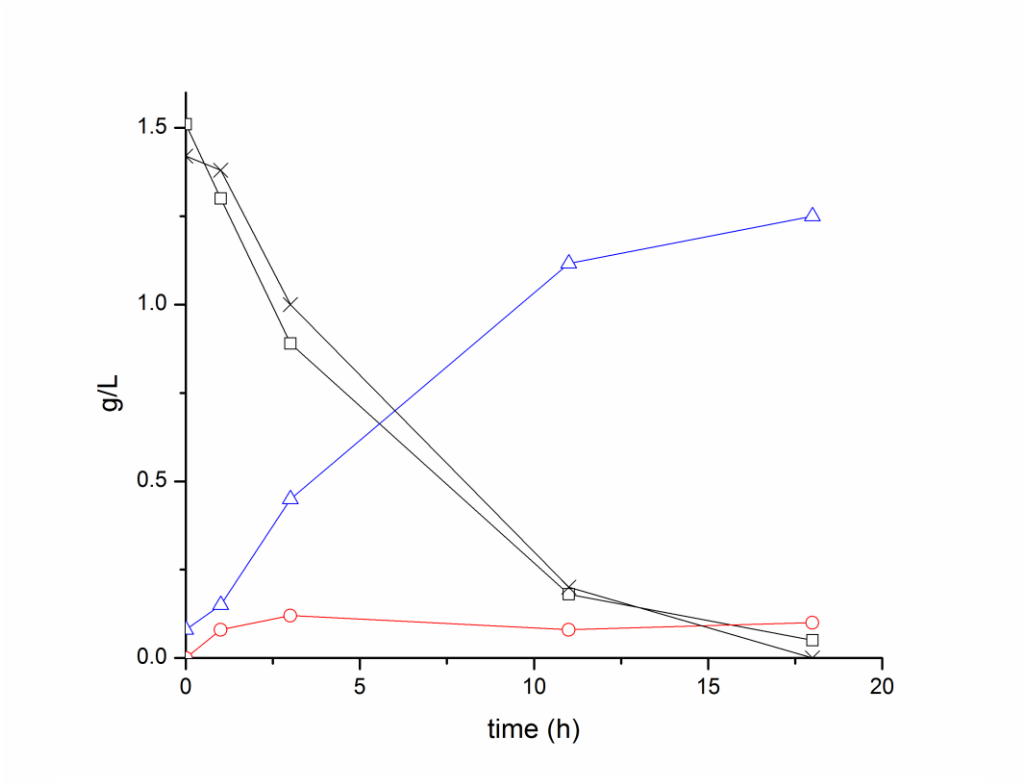


Figure 15: *Lysine consumption and the resultant production of δ-aminovalerate and glutarate as a function of time in resting cell assays of BW25113 pSTVDavBA pTRCDavDT (entire pathway), when supplemented with 1.0x stoichiometric (equimolar) 2-KG: Squares (lysine), crosses (2-KG), triangles (glutarate), and circles (AMV)*

3.3.4 ***Testing the Toxicity of δ-Aminovalerate and Glutarate against E. coli***

With a functional pathway elucidated, the next objective was to integrate the pathway together with endogenous lysine over-production to explore the production of δ-aminovalerate and glutarate from glucose. However, before

doing so it is useful to first assess potential inhibitory effects that these products may illicit againt the *E. coli* host. This knowledge will help to set a baseline for maximal product titers that may be expected in cultures of our engineered strains.

Production rates of δ-aminovalerate and glutarate are essentially a function of cellular growth rate. If product accumulation disrupts cellular processes in one way or another then production rates will likely drop. If the toxicity were too high it may be ineffective to attempt to produce these compounds with *E. coli* as a host. As mentioned in Chapter 2, we define the toxicity limit as the point at which the rate of cellular growth is zero, and any further compound addition results in cell death. This represents the maximum achievable product titer.

Toxicity assays conducted by the direct addition of either δ-aminovalerate or glutarate to growing culture of BW25113 are shown in figures 16 and 17. From these tests it is apparent that *E. coli* can survive and grow in the presence of high concentrations (at least 4g/L) of both δ-aminvalerate or glutarte. Growth rates were always at least 85% of that of the control strains (BW25113 grown in M10 without δ-aminvalerate or glutarate). The high tolerance of *E. coli* to these chemicals is ideal as it suggests that reaching titers as high as these may one day be possible, and that product toxicity will be of little concern at this point in the strain construction.

These results coupled with those of the production kinetic studies give strong support for the potential of this technology to directly convert renewable

glucose to δ-aminovalerate and glutarate. The next requirement is to engineer a host strain capable of over-producing lysine, the precursor to the AMV pathway, from glucose.

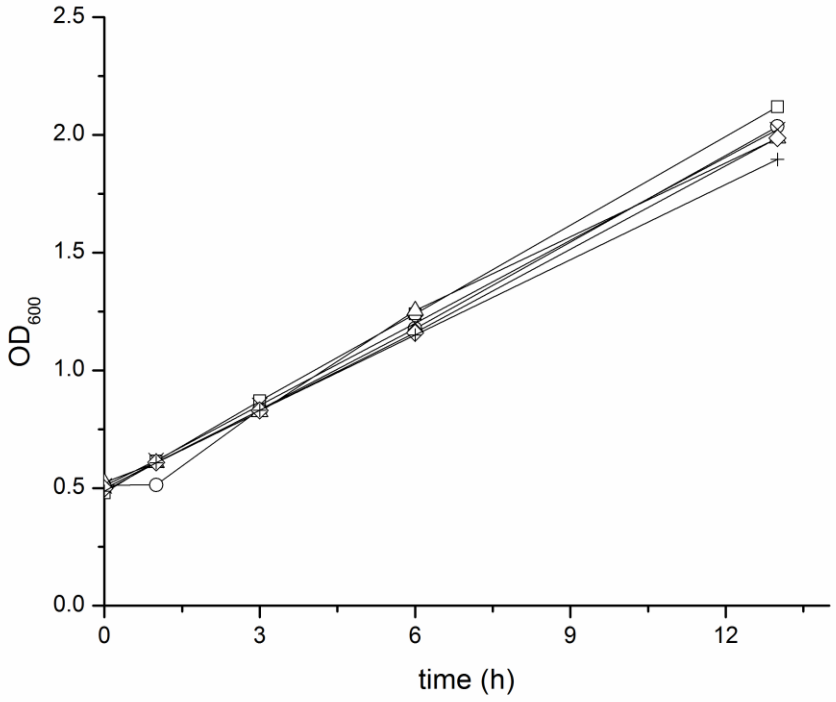


Figure 16: *Toxicity of AMV on BW25113. Glutarate concentrations: 0 g/L (squares), 0.5 g/L (crosses, x), 1.0 g/L (circles), 2.0 g/L (diamonds), 3 g/L*

3.4 ***Engineering a L-lysine Over-producing E. coli Strain as a Host Platform***

After discerning that the compounds of interest are not toxic to the cells, even at a high concentrations, and that both pathways can be functionally expressed in *E. coli* without major bottlenecks, the next step was to engineer *E. coli* to produce large quantities of L-lysine. Lysine is the pathway precursor, from glucose.

Resting cells could be fed lysine as a system for producing these compounds; however, at this point supplementation with L-lysine is not preferable because of costs, even though lysine production is an evolving green technology. A strain that can grow on glucose as a single, renewable carbon source would enable δ-aminovalerate and glutarate production from glucose.

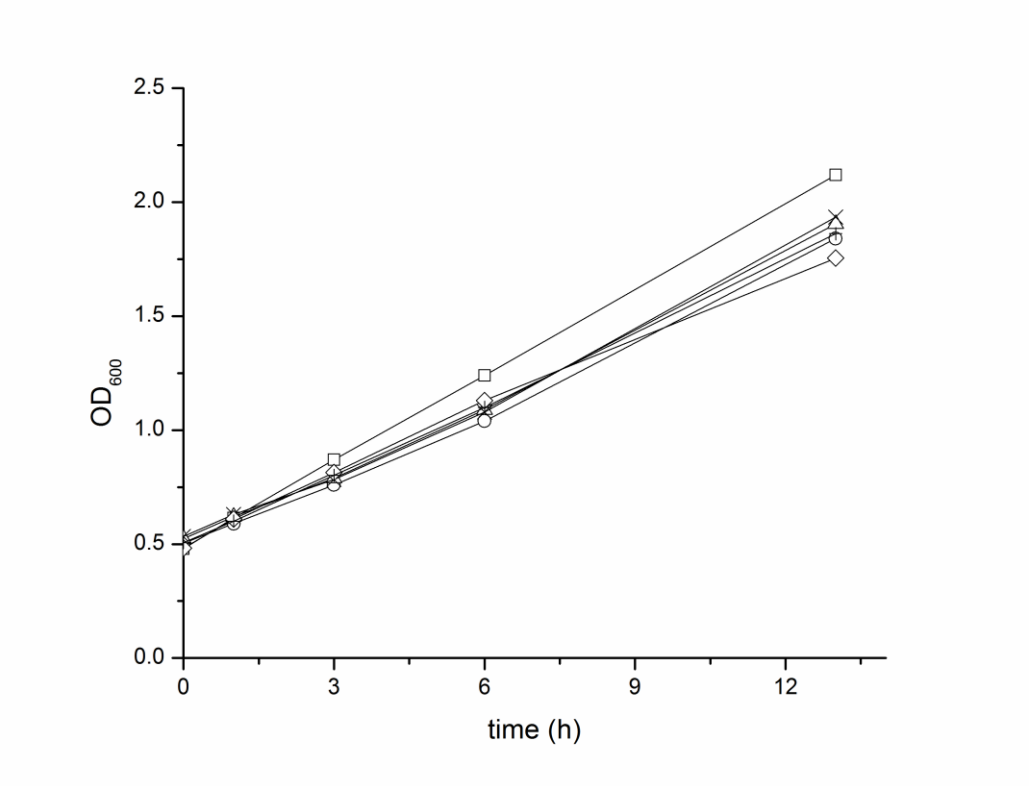


Figure 17: *Toxicity of glutarate on BW25113. Glutarate concentrations: 0 g/L (squares),0.5 g/L (crosses, x), 1.0 g/L (triangles), 2.0 g/L (pluses, +), 3 g/L (circles), 4 g/L (diamonds)*

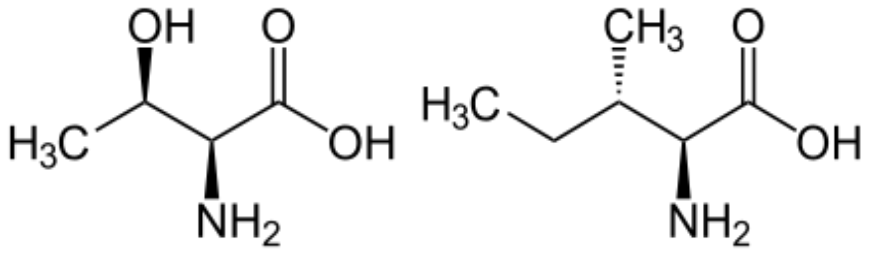
To develop a lysine overproducing *E. coli* strain with the goal of achieving maximal lysine titers and yields was the goal. To engineer a novel lysine over-

producing *E. coli* strain based on BW25113, as achieved by deregulating its natural lysine biosynthesis pathway. Lysine biosynthesis is naturally regulated by the feedback inhibition of two key enzymes in its production pathway. Figure 6 details the lysine biosynthesis pathway with the feedback inhibition illustrated. These enzymes are DapA and LysC, a dihydrodipicolinate synthase and aspartate kinase, respectively. Feedback inhibition is a natural mechanism evolved to prevent precious cellular resources from being used inefficiently. However, feedback resistant variants of both enzymes have been evolved over the years and also been sequenced. It was discovered that *dapA* in *Corynebacterium glutamicum* 13032 is naturally a feedback resistant dihydrodipicolinate synthase. Additionally, using S-aminoethyl-L-cysteine as an L-lysine analog, researchers have developed feedback resistant aspartate kinase III. Exposure to this analog puts stress on cells, and in order to survive, eventually will evolve to make feedback resistant enzymes.

Characterization and sequencing of *lysC* in one of these evolved strains showed a substitution at the 1055th nucleotide from a cytosine to a thymine, causing a mutation at the 352nd amino acid from a threonine to an isoleucine. Using splicing overlap extension, as described in Chapter 2, this single base pair mutation (Cytosine to Thymine) was introduced into *lysC.*

As schematically depicted in figure 18, this mutation changed a polar hydroxyl bearing carbon side chain to a strongly hydrophobic alkyl side chain. As lysine is extremely hydrophilic, this mutation precludes from L-lysine binding to

the active site of aspartate kinase III, thereby relieving its inhibitory effect. The sequence of the single point mutant was confirmed at the Biodesign Institute at Arizona State University using a DNA sequencing process. To illustrate, the DNA, and corresponding amino acid, sequencing results are provided in Table 5. Running a Bradford assay with *BW25113* pCDFDapA^{fbr} and *BW25113* pCDFDapA^{fbr}LysC^{fbr}, yielded 1.05 and 1.28 mg mL^{-1} of total protein, respectively.



Threonine Isoleucine

Figure 18: *Amino acid mutation in LysC was from a threonine at the 352 amino acid to an isoleucine.*

Table 5: *Sequencing Results of the DNA plasmid pCDFLysC^{fbr}DapA^{fbr} at the region of the mutation*

Native *lysC* Nucleotide Sequence:	…G AGC GTG GCA TTA ACC CTT GAT ACC ACC GG …
Mutated *lysC* Sequence :	… G AGC GTG GCA TTA ATC CTT GAT ACC ACC GG …
Native lys*C* Amino Acid Sequence:	… L I T T S E V S V A L T L D T T G S T S …
Mutated lysC Amino Acid Sequence	… L I T T S E V S V A L I L D T T G S T S …

3.4.1 ***Experiments of L-lysine Over-production in E. coli***

Co-expression of *dapA*^{fbr} and *lysC*^{fbr} in *E. coli BW25113* pCDFLysC^{fbr}DapA^{fbr} was next evaluated for its ability to enable lysine over-production. This strain was grown in M10 media and induced by the addition of 12.5 µL 1 M IPTG after 3 h of growth. First the effects of the individual gene *dapA*^{fbr} was compared head to head with *BW25113* pCDFLysC^{fbr}DapA^{fbr}.

Table 6: *Lysine and cadaverine production in BW25113 pCDFDapA*^{fbr} *and BW25113 pCDFLysC*^{fbr}*DapA*^{fbr}*. Tabulated data calculated as averages of*

	BW25113 pCDFDapA^{fbr}		BW25113 pCDFLysC^{fbr}DapA^{fbr}	
Time	L-lysine [g/L]	Cadaverine [g/L]	L-lysine [g/L]	Cadaverine [g/L]
0	0	0	0	0
5.25	0.136	0.05	0.155	0.05
18.25	0.784	0.15	0.716	0.358
48	0.154	0.82	0.27	0.64

Since it was apparent in these experiments (Table 6) that the addition of both *dapA*^{fbr} and *lysC*^{fbr} was better than just *dapA*^{fbr} for lysine production, for all further experiments only this plasmid (pCDFLysC^{fbr}DapA^{fbr}) was used. It is intuitive that the addition of both feedback resistant genes would lead to the best lysine producer. The regulation of the lysine biosynthesis pathway occurs allosterically at both locations, and if only a single feedback resistant enzyme is introduced then feedback inhibition can still occur. Since the lysine production was markedly depressed after the 18 hour mark in these producers, it was reasoned that the elimination of the cadaverine biochemical pathway, which diverts flux away from the AMV pathway, could further increase titers. Cadaverine is a natural lysine degradation product of *E. coli*. In *E. coli* it is known that there are two different L-lysine decarboxylase enzymes, *cadA* and *ldcC*. The former is an acid inducible enzyme with a pH optimum of 5, whereas *ldcC* is constitutively expressed by a weak promoter.

With regards to maximizing AMV and glutarate production, cadaverine coproduction is problematic as it competes for lysine, the required pathway precursor. Therefore, we hypothesized that the deletion of this competing pathway could further increase lysine accumulation, and therefore its availability to our pathways. BW25113 with single deletions of both *ldcC* and *cadA* were purchased from the Keio collection (Mori et al. 2006). BW25113 Δ*ldcC* and BW25113 Δ*cadA* were subsequently evaluated for lysine production upon their

transformation with pCDFLysC^{fbr}DapA^{fbr}, the results of which are compared in Figure 19.

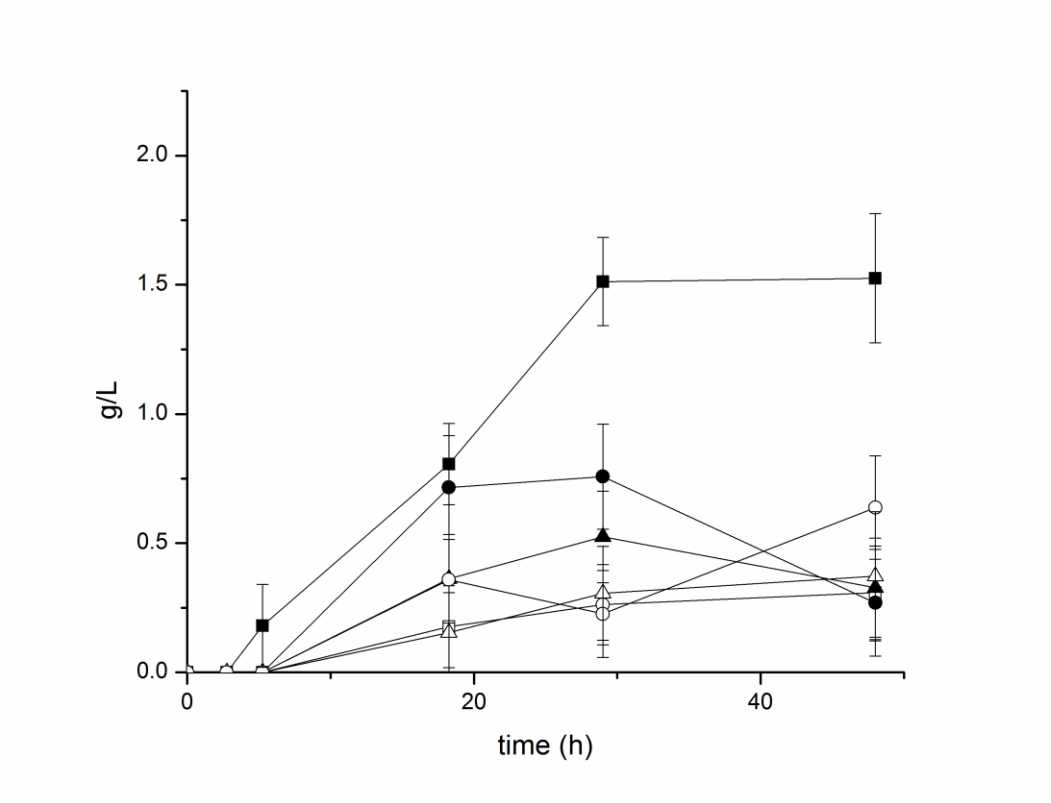


Figure 19: *Lysine and cadaverine production in BW25113ΔcadA and BW25113ΔldcC (l-lysine decarboxylase knockout strains): Squares (BW25113ΔcadA), circles (BW25113ΔldcC), triangles (BW25113 wild-type); Filled symbols are lysine data points and open symbols are cadaverine data*

From Figure 19 it is apparent that after about 18 h mark BW25113 Δ*cadA* becomes the superior lysine over-producer. However, cadaverine production was still a problem, albeit less than the wild-type, in both single gene knockout strains. It was reasoned that BW25113 Δ*ldcC* performed less ideally than BW25113

Δ*cadA* because after a certain amount of culturing time, the pH of the media dropped to a level in which *cadA* transcription increased to a significant level. The pH of the cultures at 48h was approximately 5 (the optimal pH of cadA).

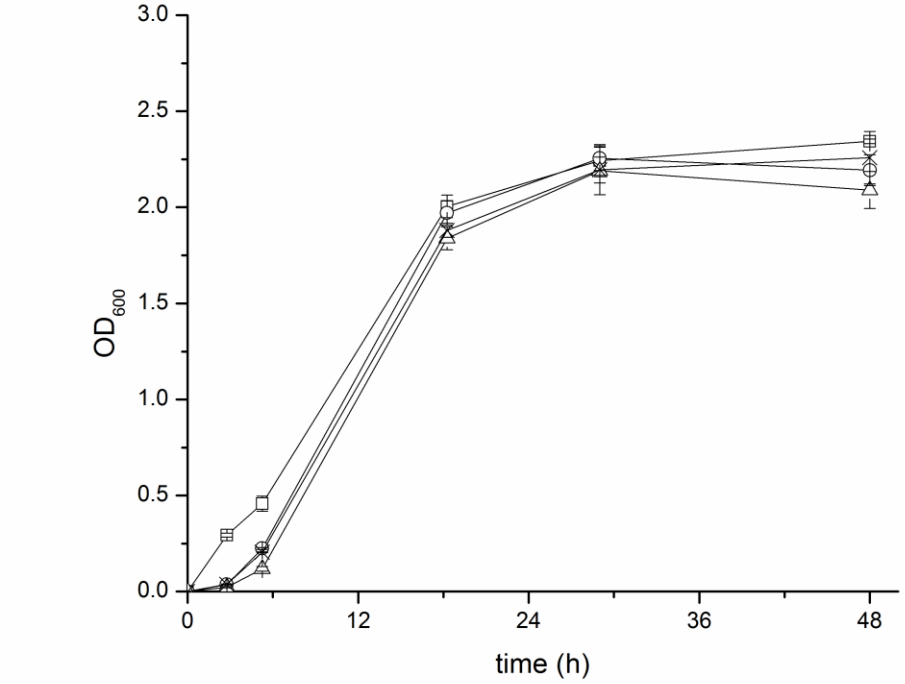


Figure 20: *Growth characteristics of pCDFLysC^{fbr}DapA^{fbr} bearing strains and single mutants: Squares (BW25113 wild-type), crosses (BW25113 pCDFLysC^{fbr}DapA^{fbr}), circles (BW25113ΔcadA*

Also, BW25113 Δ*cadA* still produced cadaverine because *ldcC* is weakly constitutive, and is always being expressed. This hints to the fact that a double mutant, BW25113Δ*cadA*Δ*ldcC*, may actually be the best option, as long as complete elimination of the cadaverine biochemical pathway does not affect

cellular function as a whole. However, it can be argued that in a fermenter of any appreciable size (≥ 1L) there will be control of pH, agitation speed (dissolved oxygen), and glucose levels. With pH control, the BW25113 Δ*ldcC* will be essentially the same phenotypically as BW25113 Δ*cadA*Δ*ldcC*, because the acid-inducible, acid optimal cadA will not be translated.

With the addition of the AMV pathway, it is possible that most lysine will not be available for the L-lysine decarboxylase enzymes. For these reasons, AMV and glutarate production experiments (Figures 21 and 23) were done in BW25113Δ*ldcC* while a double mutant (BW25113 Δ*cadA*Δ*ldcC*) was developed.

Growth experiments showed that BW25113 Δ*cadA* and BW25113 Δ*ldcC* had no effect on the growth rate or final OD_{600} (compared to wild type) of cell cultures during production transformed with pCDFLysC^{fbr}DapA^{fbr}, as seen in Figure 20. In light of these considerations, subsequent studies focused on the engineering of a lysine over-producing strain emphasized the use of strains derived of BW25113 Δ*cadA* and BW25113 Δ*ldcC* and which were transformed with pCDFLysC^{fbr}DapA^{fbr}.

3.5 ***AMV and Glutarate Production Experiments on Glucose***

With all of the pieces in place, a functional pathway and a developed lysine producing host strain, it was appropriate to test for the production of AMV and then glutarate on M10 glucose media. Experiments were begun with the same

procedures as before but with the strains *E. coli* BW25113 Δ*ldcC* pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA and *E. coli* BW25113 Δ*ldcC* pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA pTRCDavDT. This strain is what is depicted in figure 6. Seed cultures were grown up and 50mL M10 innoculated and induced. Experiments were repeated in triplicate.

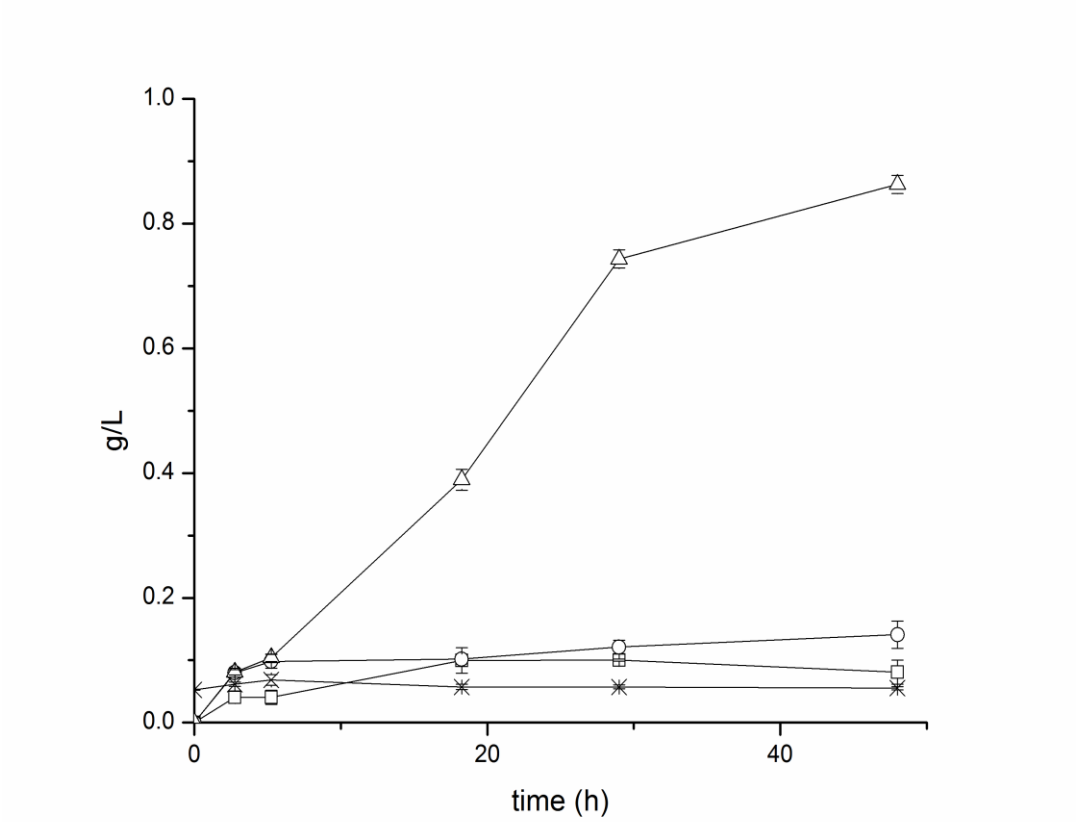


Figure 21: *AMV production curve of E. coli BW25113ΔldcC pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA grown in M10 media. Triangles represent AMV concentration, circles represent cadaverine concentration, squares represent lysine concentration, and crosses represent glutarate*

Figure 21 above, depicts the metabolite concentrations of the constructed AMV producing strain. After 48 hours of growth over 0.86 g/L AMV accumulated in the culture with minimal accumulation of byproducts cadaverine, lysine, or glutarate (a byproduct when AMV production is desired). As depicted in the AMV produced versus glucose consumed plot (Figure 25) the yield of AMV remained fairly steady at 69.5 mmol/mol with a specific growth rate of 0.243 h^{-1} (calculated from figure 22) Additionally, it can be seen from Figure 22 that the media was not completely exhausted at 48 hours, and may have had potential for a higher cell density and AMV titer.

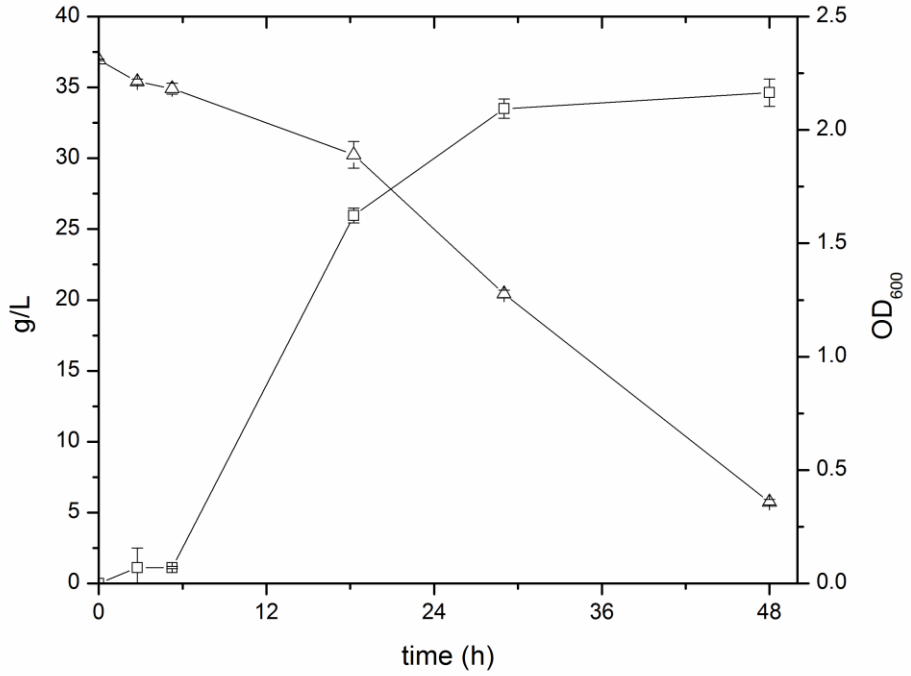


Figure 22: *Glucose consumption and growth curve of E. coli BW25113ΔldcC pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA grown in M10 media. Triangles represent glucose and squares represent OD_{600} measurements.*

Next the entire pathway was constructed to check for a glutarate producing phenotype. As depicted in figure 23 glutarate was also accumulated to an appreciable titer of 0.82 g/L in 48 h at a fairly constant yield of 68.4 mmol/mol. As was the case in figure 22, figure 24 shows the media was nearly exhausted and a longer culture could potentially reach higher glutarate titer and cell density.

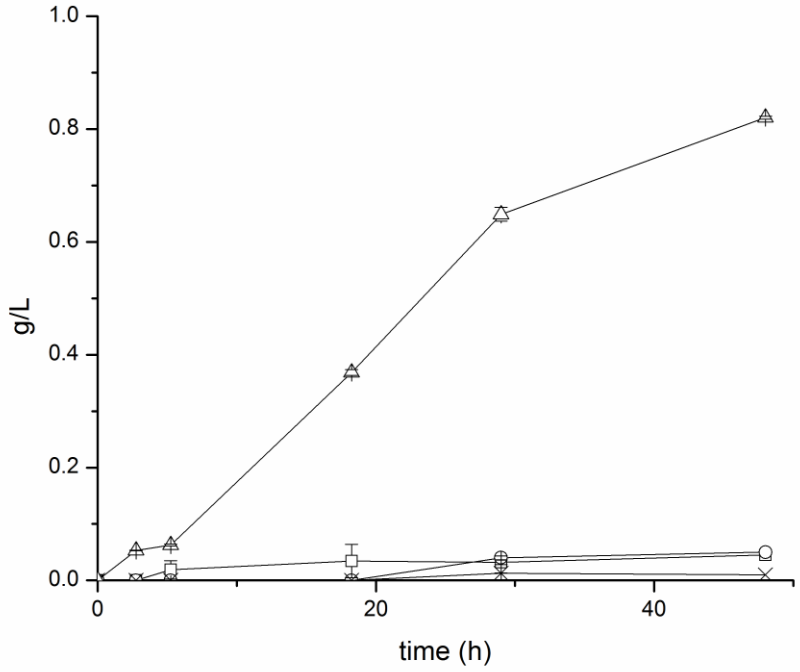


Figure 23: *Glutarate production curve of E. coli BW25113*ΔldcC *pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA pTRCDavDT (entire AMV pathway) grown in M10 media. Triangles represent glutarate concentration, circles represent cadaverine concentration, squares represent lysine concentration, and crosses represent AMV concentration.*

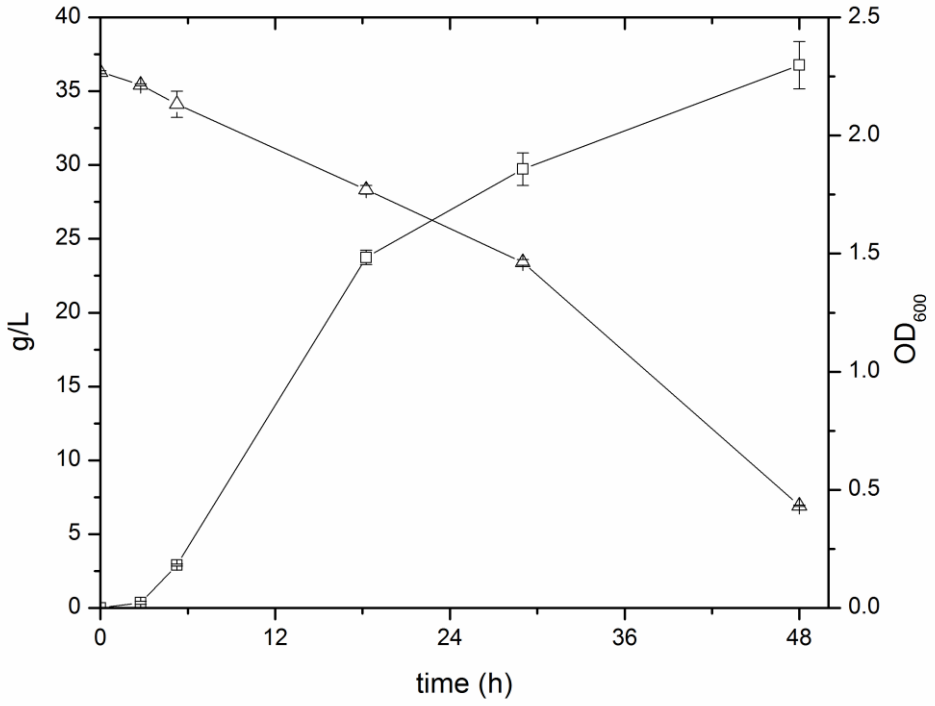


Figure 24: *Glucose consumption and growth curve of E. coli BW25113ΔldcC pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA pTRCDavDT grown in M10 media. Triangles represent glucose and squares represent OD_{600}*

This demonstrates the first microbial overproduction of both AMV and glutarate to titers of 0.86 and 0.82 g/L, respectively at 48 hours. Side product formation was present, but minimal in the production strains with the functional AMV pathway diverting flux from lysine. These results are promising, and support further research toward the development of AMV and glutarate production using metabolic engineering and microbe based biotechnology.

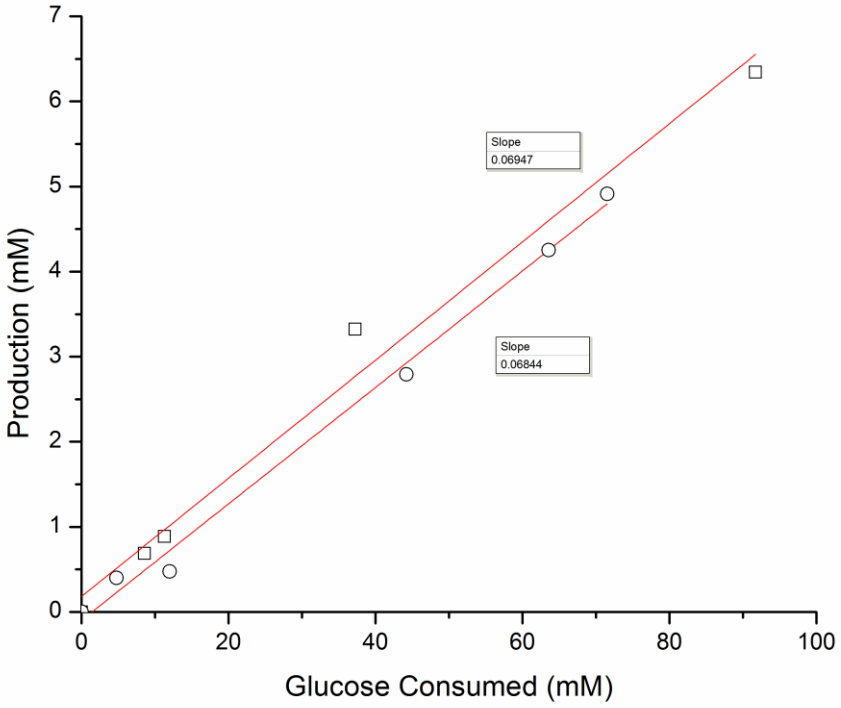


Figure 25: *AMV and Glutarate produced as a function of glucose consumed in E. coli BW25113ΔldcC pCDFLysC^{fbr}DapA^{fbr} pSTVDavBA pTRCDavDT grown in M10 media. Squares represent*

Chapter 4

CONCLUSIONS AND FUTURE WORK

4.1 ***Conclusions***

Volatile oil prices, environmental concerns, and finite oil reserves are strong motivating factors for the development of a bio-based, sustainable society. With few exceptions, most conventional polymers are derived from these non-renewable and polluting petroleum resources. Sustainable methods that meet our polymer material demands must be discovered, and polyamide whose molecular building blocks are ubiquitously petrochemicals, are not the exception. In the year 2010 alone there was a global market demand of 3.5 million tons of various polyamides. The functionality of these polyamide monomers is similar to many in vivo metabolites (i.e. γ-aminobutyrate, cadaverine, succinate) making a sustainable biosynthetic production route to these monomers feasible. However, to date there have been no useful homopolyamide, or homopolyamide precursors produced microbially on glucose. Additionally, there have been no other dicarboxylic acids produced in a sustainable manner, aside from the natural metabolite succinate, thus limiting the potential types of sustainable polyamides that can be produced renewably. The demonstration herein of the sustainable production of glutarate and δ-aminovalerate helps push this field forward by filling these gaps.

First, a lysine degradation pathway found in *Pseudomonas sp.* was reconstructed in *E. coli BW25113* which passes through δ-aminovaleric acid and glutaric acid. The respective pathway enzymes were cloned into expression vectors and tested for enzyme production and functionality. Because of its tractability, *E. coli* was developed into a lysine over-producer through deregulation of the native lysine superpathway through over-expression of feedback resistant $lysC^{fbr}$ and $dapA^{fbr}$, aspartate kinase and dihydrodipicolinate synthase, respectively. The AMV pathway was then constructed with genes from *E. coli*, *C. glutamicum*, and *P. putida* and imparted to the best lysine over-producing strains to test for novel δ-aminovaleric acid and/or glutaric acid production. Final titers of 0.86 and 0.82 g/L, respectively in forty-eight hours were achieved with yields of 69.5 mmol AMV mol^{-1} glucose and 68.4 mmol Glutarate mol^{-1} glucose.

4.2 ***Future Work***

This project signals the proof of concept for the production of AMV and glutarate from glucose. There are many aspects of the process that must logically next be addressed to move forward with this project. Creation of a double mutant of both Δ*cadA*Δ*ldcC* would seemingly be ideal and could possibly increase flux toward the reconstructed AMV pathway and reduce cadaverine formation completely. This could be done following Datsenko and Wanner's well-known gene knockout procedure (Wanner et al., 2000).

Flux to lysine is another important aspect of the project. The major limiting factor is the amount of glucose that is eventually converted to lysine. Once the lysine is present the vast majority is funneled through the AMV pathway. Further increasing flux to lysine through upstream modifications would be desirable. There are currently *Corynebacterium* strains that produce millions of tons of lysine per year, and modeling some of these characteristics in *E. coli* could lead to increasing results. Or alternatively, now that this concept has been demonstrated in the tractable *E. coli*, reconstructing the pathway inside of a suitable *Corynebacterium* strain, with compatible expression vectors, could potentially lead to an outstanding AMV and Glutarate producing strain.

Another future point of work will undoubtedly be the scale-up process. One major limitation of these experiments was that each production experiment was limited to batch shake flasks. Batch shake flasks are less than ideal because of the conditions of the experiments are poorly controlled. A scale-up to a laboratory sized fermenter with on-line temperature, dissolved oxygen, pH, and glucose concentration control would more than likely lead to titers much higher than those demonstrated herein, even without altering the production strains any further.

REFERENCES

Asada, Y., Miyake, M., Miyake, J., Kurane, R., and Tokiwa, Y. (1999). Photosynthetic accumulation of poly-(hydroxybutyrate) by cyanobacteria - the metabolism and potential for CO_2 recycling. *Int J Biol Macromol* 25**,** 37-42.

Atsumi, S., Hanai, T., and Liao, J.C. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451**,** 86-89.

Atsumi, S., and Liao, J.C. (2008). Metabolic engineering for advanced biofuels production from Escherichia coli. *Curr Opin Biotechnol* 19**,** 414-419.

Bechthold, I., Bretz, K., Kabasci, S., Kopitzky, R., and Springer, A. (2008). Succinic Acid: A New Platform Chemical for Biobased Polymers from Renewable Resources. *Chemical Engineering & Technology* 31**,** 647-654.

Bermudez, M., Leon, S., Aleman, C., and Munoz-Guerra, S. (2000). Comparison of lamellar crystal structure and morphology of nylon 46 and nylon 5. *Polymer* 41**,** 8961-8973.

Braunegg, G., Lefebvre, G., and Genser, K.F. (1998). Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. *J Biotechnol* 65**,** 127-161.

Campbell, C.D., and Vederas, J.C. (2010). Biosynthesis of lovastatin and related metabolites formed by fungal iterative PKS enzymes. *Biopolymers* 93**,** 755-763.

Carothers, J.M., Goler, J.A., and Keasling, J.D. (2009). Chemical synthesis using synthetic biology. *Current Opinion in Biotechnology* 20**,** 498-503.

Celinska, E. (2010). Debottlenecking the 1,3-propanediol pathway by metabolic engineering. *Biotechnol Adv* 28**,** 519-530.

Celinska, E., and Grajek, W. (2009). Biotechnological production of 2,3-butanediol-Current state and prospects. *Biotechnology Advances* 27**,** 715-725.

Chen, G.Q. (2009). A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem Soc Rev* 38**,** 2434-2446.

Freedonia Group (2009). , *World Bioplastics to 2013.*

Gao, H.J., Wu, Q., and Chen, G.Q. (2002). Enhanced production of D-(-)-3-hydroxybutyric acid by recombinant Escherichia coli. *FEMS Microbiol Lett* 213**,** 59-65.

Garg, S.K., and Jain, A. (1995). Fermentative Production of 2,3-Butanediol - a Review. *Bioresource Technology* 51**,** 103-109.

Global Industry Analysts (2012). Plastics: A Global Outlook.

Haas, T., Jaeger, B., Weber, R., Mitchell, S.F., and King, C.F. (2005). New diol processes: 1,3-propanediol and 1,4-butanediol. *Applied Catalysis A: General* 280**,** 83-88.

Hu, Y.C., Zhan, N.N., Dou, C., Huang, H., Han, Y.W., Yu, D.H., and Hu, Y. (2010). Selective dehydration of bio-ethanol to ethylene catalyzed by lanthanum-phosphorous-modified HZSM-5: Influence of the fusel. *Biotechnology Journal* 5**,** 1186-1191.

Igarashi, K., and Kashiwagi, K. (2000). Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* 271**,** 559-564.

Ji, X.J., Huang, H., and Ouyang, P.K. (2011). Microbial 2,3-butanediol production: A state-of-the-art review. *Biotechnology Advances* 29**,** 351-364.

Ji, X.J., Nie, Z.K., Li, Z.Y., Gao, Z., and Huang, H. (2010). Biotechnological Production of 2,3-Butanediol. *Progress in Chemistry* 22**,** 2450-2461.

Jiang, X., Meng, X., and Xian, M. (2009). Biosynthetic pathways for 3-hydroxypropionic acid production. *Appl Microbiol Biotechnol* 82**,** 995-1003.

Keshavarz, T., and Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. *Current Opinion in Microbiology* 13**,** 321-326.

Kind, S., Jeong, W.K., Schroder, H., and Wittmann, C. (2010a). Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane. *Metab Eng* 12**,** 341-351.

Kind, S., Jeong, W.K., Schröder, H., and Wittmann, C. (2010b). Systems-wide metabolic pathway engineering in Corynebacterium glutamicum for bio-based production of diaminopentane. *Metabolic Engineering* 12**,** 341-351.

Kind, S., Kreye, S., and Wittmann, C. (2011). Metabolic engineering of cellular transport for overproduction of the platform chemical 1,5-diaminopentane in Corynebacterium glutamicum. *Metabolic Engineering* 13**,** 617-627.

Kind, S., and Wittmann, C. (2011). Bio-based production of the platform chemical 1,5-diaminopentane. *Appl Microbiol Biotechnol* 91**,** 1287-1296.

Kind, S.W., Christoph (2010). Identification and Elimination of the Competing N-Acetyldiaminopentane Pathway for Improved Production of Diaminopentane by Corynebacterium glutamicum. *Applied and Environmental Microbiology* 76**,** 5175-5180.

Kosakai, Y., Soo Park, Y., and Okabe, M. (1997). Enhancement of L+-lactic acid production using mycelial flocs of Rhizopus oryzae. *Biotechnol Bioeng* 55**,** 461-470.

Le Vo, T., Kim, T., and Hong, S. (2012). Effects of glutamate decarboxylase and gamma-aminobutyric acid (GABA) transporter on the bioconversion of GABA in engineered <i>Escherichia coli</i&gt. *Bioprocess and Biosystems Engineering* 35**,** 645-650.

Lee, S.Y., Choi, J.I., and Wong, H.H. (1999). Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. *International Journal of Biological Macromolecules* 25**,** 31-36.

Leonard, E., Runguphan, W., O' Connor, S., and Prather, K.J. (2009). Opportunities in metabolic engineering to facilitate scalable alkaloid production. *Nat Chem Biol* 5**,** 292-300.

Lunt, J. (1998). Large-scale production, properties and commercial applications of polylactic acid polymers. *Polymer Degradation and Stability* 59**,** 145-152.

Martens, J.H., Barg, H., Warren, M.J., and Jahn, D. (2002). Microbial production of vitamin B-12. *Applied Microbiology and Biotechnology* 58**,** 275-285.

Martin, C.H., Nielsen, D.R., Solomon, K.V., and Prather, K.L.J. (2009). Synthetic Metabolism: Engineering Biology at the Protein and Pathway Scales. *Chemistry & Biology* 16**,** 277-286.

Mazumdar, S., Clomburg, J.M., and Gonzalez, R. (2010). Escherichia coli strains engineered for homofermentative production of D-lactic acid from glycerol. *Appl Environ Microbiol* 76**,** 4327-4336.

Mckenna, R., and Nielsen, D.R. (2011). Styrene biosynthesis from glucose by engineered E. coli. *Metab Eng*.

Mendez-Perez, D., Begemann, M.B., and Pfleger, B.F. (2011). Modular synthase-encoding gene involved in alpha-olefin biosynthesis in Synechococcus sp. strain PCC 7002. *Appl Environ Microbiol* 77**,** 4264-4267.

Mimitsuka, T., Sawai, H., Hatsu, M., and Yamada, K. (2007). Metabolic engineering of Corynebacterium glutamicum for cadaverine fermentation. *Biosci Biotechnol Biochem* 71**,** 2130-2135.

Nakamura, C.E., and Whited, G.M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Curr Opin Biotechnol* 14**,** 454-459.

Navarro, E., Aleman, C., Subirana, J.A., and Puiggali, J. (1996). On the crystal structure of nylon 55. *Macromolecules* 29**,** 5406-5415.

Nexant Chem Systems (2009). New Process Evaluation and Research Program: Nylon 6 and Nylon 6,6.

Nielsen, D.R., Yoon, S.H., Yuan, C.J., and Prather, K.L. (2010). Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in E. coli. *Biotechnol J* 5**,** 274-284.

Niu, W., Draths, K.M., and Frost, J.W. (2002). Benzene-Free Synthesis of Adipic Acid. *Biotechnology Progress* 18**,** 201-211.

Pawlowski, G., Dammel, R., Eckes, C., Lindley, C.R., Meier, W., Przybilla, K.J., Roschert, H., and Spiess, W. (1991). Substituted Polyhydroxystyrenes as Matrix Resins for Chemically Amplified Deep Uv Resist Materials. *Microelectronic Engineering* 13**,** 29-32.

Peralta-Yahya, P.P., and Keasling, J.D. Advanced biofuel production in microbes. *Biotechnol J* 5**,** 147-162.

Phillips, A.L. (2008). Bioplastics boom. *American Scientist* 96**,** 109.

Plasticseurope Market Research Group (2009). , The compelling facts about plastics.

Qi, W.W., Vannelli, T., Breinig, S., Ben-Bassat, A., Gatenby, A.A., Haynie, S.L., and Sariaslani, F.S. (2007). Functional expression of prokaryotic and

eukaryotic genes in Escherichia coli for conversion of glucose to p-hydroxystyrene. *Metab Eng* 9**,** 268-276.

Qian, Z.-G., Xia, X.-X., and Lee, S.Y. (2009a). Metabolic engineering of Escherichia coli for the production of putrescine: A four carbon diamine. *Biotechnology and Bioengineering* 104**,** 651-662.

Qian, Z.G., Xia, X.X., and Lee, S.Y. (2011a). Metabolic engineering of Escherichia coli for the production of cadaverine: a five carbon diamine. *Biotechnol Bioeng* 108**,** 93-103.

Rathnasingh, C., Raj, S.M., Jo, J.E., and Park, S. (2009). Development and evaluation of efficient recombinant Escherichia coli strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnol Bioeng* 104**,** 729-739.

Rathnasingh, C., Raj, S.M., Lee, Y., Catherine, C., Ashok, S., and Park, S. (2012). Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant Escherichia coli strains. *J Biotechnol* 157**,** 633-640.

Reddy, G., Altaf, M., Naveena, B.J., Venkateshwar, M., and Kumar, E.V. (2008). Amylolytic bacterial lactic acid fermentation - a review. *Biotechnol Adv* 26**,** 22-34.

Reinecke, F., and Steinbuchel, A. (2009). Ralstonia eutropha strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *Journal of Molecular Microbiology and Biotechnology* 16**,** 91-108.

Revelles, O., Wittich, R.-M., and Ramos, J.L. (2007). Identification of the Initial Steps in d-Lysine Catabolism in Pseudomonas putida. *Journal of Bacteriology* 189**,** 2787-2792.

Ro, D.K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C., Withers, S.T., Shiba, Y., Sarpong, R., and Keasling, J.D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440**,** 940-943.

Rojas-Rosas, O., Villafana-Rojas, J., Lopez-Dellamary, F.A., Nungaray-Arellano, J., and Gonzalez-Reynoso, O. (2007). Production and characterization of polyhydroxyalkanoates in Pseudomonas aeruginosa ATCC 9027 from glucose, an unrelated carbon source. *Can J Microbiol* 53**,** 840-851.

Rude, M.A., Baron, T.S., Brubaker, S., Alibhai, M., Del Cardayre, S.B., and Schirmer, A. (2011). Terminal olefin (1-alkene) biosynthesis by a novel p450 fatty acid decarboxylase from Jeotgalicoccus species. *Appl Environ Microbiol* 77**,** 1718-1727.

Sauer, M., Marx, H., and Mattanovich, D. (2008). Microbial production of 1,3-propanediol. *Recent Pat Biotechnol* 2**,** 191-197.

Saxena, R.K., Anand, P., Saran, S., and Isar, J. (2009). Microbial production of 1,3-propanediol: Recent developments and emerging opportunities. *Biotechnol Adv* 27**,** 895-913.

Schirmer, A., Rude, M.A., Li, X., Popova, E., and Del Cardayre, S.B. (2010). Microbial biosynthesis of alkanes. *Science* 329**,** 559-562.

Schneider, J., and Wendisch, V. (2010). Putrescine production by engineered <i>Corynebacterium glutamicum</i&gt. *Applied Microbiology and Biotechnology* 88**,** 859-868.

Schubert, P., Steinbuchel, A., and Schlegel, H.G. (1988). Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. *J Bacteriol* 170**,** 5837-5847.

Singh, M., Patel, S.K.S., and Kalia, V.C. (2009). *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. *Microbial Cell Factories* 8.

Smolke, C.D. (2009). "The metabolic pathway engineering handbook fundamentals". (Boca Raton: CRC Press/Taylor & Francis).

Sodergard, A., and Stolt, M. (2002). Properties of lactic acid based polymers and their correlation with composition. *Progress in Polymer Science* 27**,** 1123-1163.

Song, H., and Lee, S.Y. (2006). Production of succinic acid by bacterial fermentation. *Enzyme and Microbial Technology* 39**,** 352-361.

Sri (2010). *Styrene.* Access Intelligence LLC Inc.

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., Mcclure, A., Del Cardayre, S.B., and Keasling, J.D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463**,** 559-562.

Straathof, A.J.J., Sie, S., Franco, T.T., and Van Der Wielen, L.A.M. (2005). Feasibility of acrylic acid production by fermentation. *Applied Microbiology and Biotechnology* 67**,** 727-734.

Suthers, P.F., and Cameron, D.C. (2000). *Production of 3-hydroxypropionic acid in recombinant organisms*. US Patent 6852517.

Tabor, C.W., and Tabor, H. (1985). Polyamines in microorganisms. *Microbiol Rev* 49**,** 81-99.

Takahara, I., Saito, M., Inaba, M., and Murata, K. (2005). Dehydration of ethanol into ethylene over solid acid catalysts. *Catalysis Letters* 105**,** 249-252.

Tateno, T., Okada, Y., Tsuchidate, T., Tanaka, T., Fukuda, H., and Kondo, A. (2009). Direct production of cadaverine from soluble starch using Corynebacterium glutamicum coexpressing alpha-amylase and lysine decarboxylase. *Appl Microbiol Biotechnol* 82**,** 115-121.

Thakker, C., Martinez, I., San, K.Y., and Bennett, G.N. (2012). Succinate production in Escherichia coli. *Biotechnol J* 7**,** 213-224.

Thompson, R.C., Swan, S.H., Moore, C.J., and Vom Saal, F.S. (2009). Our plastic age. *Philosophical Transactions of the Royal Society B-Biological Sciences* 364**,** 1973-1976.

Thorball, V. (1967). Styrofoam - an Extruded Polystyrene Foam for Low Temperature Insulation. *Modern Refrigeration and Air Conditioning* 70**,** 51-&.

Tseng, H.C., Harwell, C.L., Martin, C.H., and Prather, K.L. (2010). Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered E. coli. *Microb Cell Fact* 9**,** 96.

Tseng, H.C., Martin, C.H., Nielsen, D.R., and Prather, K.L. (2009). Metabolic engineering of Escherichia coli for enhanced production of (R)- and (S)-3-hydroxybutyrate. *Appl Environ Microbiol* 75**,** 3137-3145.

Tsuruta, H., Paddon, C.J., Eng, D., Lenihan, J.R., Horning, T., Anthony, L.C., Regentin, R., Keasling, J.D., Renninger, N.S., and Newman, J.D. (2009). High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in Escherichia coli. *PLoS One* 4**,** e4489.

U.S. Department of Energy (2002). , *Steam System Opportunity Assessment for the Pulp and Paper, Chemical Manufacturing, and Petroleum Refining Industries.*

U.S. Department of Energy (2004). , *Top Value Added Chemicals From Biomass*

U.S. Energy Information Administration (2011). www.eia.gov.

Voloch, M., Jjansen, N.B., Ladisch, M., Tsao, G.T., Narayan, R., and Rodwell, V.W. (1985). "2,3-Butanediol," in *Comprehensive Biotechnology: The Principles, Applications and Regulations of Biotechnology in Industry,*

Agriculture and Medicine, ed. M. Moo-Young. 1st Ed. ed (New York: Pergamon Press).

Wee, Y.J., Kim, J.N., and Ryu, H.W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technology and Biotechnology* 44**,** 163-175.

Whited, G., Feher, F., Benko, D., Cervin, M., Chotani, G., Mcauliffe, J., Laduca, R., Ben-Shoshan, E., and Sanford, K. (2010). *Industrial Biotechnology* 6**,** 152-163.

Wierckx, N.J., Ballerstedt, H., De Bont, J.A., and Wery, J. (2005). Engineering of solvent-tolerant Pseudomonas putida S12 for bioproduction of phenol from glucose. *Appl Environ Microbiol* 71**,** 8221-8227.

Wu, C., Koylinski, T., and Bozik, J. (1981). Preparation of styrene from ethylbenzene *US Patent 4,255,599.*

Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick, J.D., Osterhout, R.E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H.B., Andrae, S., Yang, T.H., Lee, S.Y., Burk, M.J., and Van Dien, S. (2011). Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nature Chemical Biology* 7**,** 445-452.

Zhou, S., Causey, T.B., Hasona, A., Shanmugam, K.T., and Ingram, L.O. (2003). Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered Escherichia coli W3110. *Appl Environ Microbiol* 69**,** 399-407.

Zhou, Y., Dominguez, J.M., Cao, N., Du, J., and Tsao, G.T. (1999). Optimization of L-lactic acid production from glucose by Rhizopus oryzae ATCC 52311. *Appl Biochem Biotechnol* 77-79**,** 401-407.