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OPTIMIZATION OF BIOCATALYTIC 3-DEHYDROSHIKIMIC ACID PRODUCTION FROM D-GLUCOSE IN *ESCHERICHIA COLI*

By

Mark Raymond Mikola

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemical Engineering

1996

ABSTRACT

OPTIMIZATION OF BIOCATALYTIC 3-DEHYDROSHIKIMIC ACID PRODUCTION FROM D-GLUCOSE IN *ESCHERICHIA COLI*

By

Mark Raymond Mikola

Biocatalysis is an environmentally friendly alternative to petrochemical processing for production of small molecules. The biocatalytic process objective is efficient and high-yielding conversion of starting material to product. Success of the process depends on manipulation of metabolic fluxes through desired pathways by genetic manipulation and process engineering.

A strain of *Escherichia coli* was genetically engineered to overproduce dehydroshikimic acid (DHS). This intermediate of the common aromatic amino acid pathway is of commercial interest, because it can readily converted into a variety of valuable products. This *E. coli* strain features a feedback-insensitive DAHP synthase (*aroF*^{Tbr}) that increases carbon flux into the common pathway. A fed-batch fermentation process was developed using this strain resulting in a DHS titer of 40 g L^{-1} in 48 h. A novel method was developed to obtain the *aroF*^{Tbr} where ultraviolet light mutagenesis was followed by phenotype selection utilizing chemotaxis in a diffusion gradient chamber (DGC).

To my fiancée Anna, and my family for their love and support

ACKNOWLEDGMENTS

First of all I would like to express my gratitude to Dr. R. Mark Worden and Dr. John W. Frost for their encouragement and support. I hope the collaboration between the two research groups will successfully continue.

I wish to thankfully acknowledge Dr. Karen Draths and Kai Li, each for their contributions to the research. I want to also thank Mark Widman for his help with the DGC. I am very grateful to all the members of both Dr. Worden's and Dr. Frost's research group, for their advice, support, and friendship. I wish everyone the best in their endeavors.

Lastly, I wish to thank to thank my family and especially Anna. My parents have given me encouragement and support at the most needed times. I wish to thank Anna because of her help and great patience enabling me to concentrate on my graduate career.

TABLE OF CONTENTS

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

CHAPTER 1
INTRODUCTION 1
Biocatalyst 2
Common Pathway of Aromatic Amino Acid Biosynthesis 2
Common Pathway Metabolite Uses 5
Central Metabolism 6
Selection of DAHP Synthase Isozyme for Overexpression 7
Feedback-Insensitive AroF 8
Increasing DHS Production 9
Metabolic Control Analysis and Metabolic Engineering 11
Plasmid Maintenance 12
Fermentor 14
Research Objectives 15
LIST OF REFERENCES 16

CHAPTER 2
TYROSINE FEEDBACK INSENSITIVE DAHP SYNTHASE 21
Background 21
Molecular Biology Background 23
Development of the Strain for Mutagenesis 23
UV Mutagenesis 29
Development and Use of the Diffusion Gradient Chamber Method 30
DGC Chemotaxis Experiments 34
Generation and Isolation of Feedback-Insensitive Mutant 36
Confirmation of Feedback-Insensitivity 38
Isolation of the Feedback-Insensitive *aroF* 41
Sequencing of the Feedback-Insensitive *aroF* 42
Feedback Insensitive AroF Specific Activity 43
Discussion and Conclusion 53
LIST OF REFERENCES 56

CHAPTER 3
FERMENTOR PRODUCTION OF DHS 59
Fermentor Advantages 59
Biosynthetic Pathways for DHS Production 60
Optimization Strategy for DHS Production 63

Development of a Microbial Catalyst 64
Development of a New Host 64
B. Braun Fermentor 65
Optimized Fermentor Conditions 66
NMR Assays 71
DAHP Synthase Assays 72
Confirmation of Plasmid Maintenance 79
Fermentations 79
Scaled-Up Fermentations 88
Discussion and Conclusions 95
LIST OF REFERENCES 99

CHAPTER 4
EXPERIMENTAL 101
General Methods 101
Spectroscopic Measurements 101
Bacterial Strains 101
Storage of Bacterial Stains 101
Culture Medium 102
^{1}H NMR Analysis of Culture Supernatant 102
Restriction Enzyme Digest of DNA 103
Agarose Gel Electrophoresis 103
Preparation and Transformation of Competent Cells 103
Enzyme Assay 104
Chapter 2 Experimental 106
Insertion of a Synthetic Cassette into *serA* of AB3248 106
UV Mutagenesis 107
Diffusion Gradient Chamber (DGC) Setup 107
Recovery of Cells 110
Culture Conditions for
DAHP Synthase Assay of DGC Mutant 111
Isolation of the Feedback-Insensitive *aroF* gene 111
Isolation of the Feedback-Insensitive *aroF* gene 111
Generation of Truncated *aroF*^{fbr} 112
Replacement of the *aroF*^{fbr} Native Promoter
with the *tac* Promoter 112
Shake Flask Culture Conditions for
aroF^{fbr} Specific activity Evaluations 112
Chapter 3 Experimental 114
B. Braun Fermentor 114
Fermentor Medium 115
Fermentor Inoculum for Evaluation of
KL3/pKL4.32B and KL3/pKL4.33B 115
Optimized Fermentor Inoculum 116
Fermentor Conditions 116
Fermentor Setup 117
Fermentor Samples 119
LIST OF REFERENCES 120

LIST OF TABLES

Table 1 - PCR Primers 27
Table 2 - UV Radiation Survival 30
Table 3 - Fermentor Medium Components 67
Table 4 - Trace Mineral Supplements 68
Table 5 - Phase 2 D.O. PID Controller Parameters 70
Table 6 - Starter Culture for Evaluation of the Truncated/Nontruncated *aroF* 80
Table 7 - Summary of Fermentations 94

LIST OF FIGURES

Figure 1 - The Common Pathway 3
Figure 2 - Alternate Molecule Production From The Common Pathway 5
Figure 3 - Plasmid Map of pKAD76A 25
Figure 4 - Synthetic Cassette 26
Figure 5 - Plasmid Map of pKAD10.156A 27
Figure 6 - Plasmid Map of pKAD10.186A 28
Figure 7 - Diagram of a Diffusion Gradient Chamber 32
Figure 8a - Chemotaxis of AB2.24 36
Figure 8b - Image Analysis of Centerline 36
Figure 9a - DGC Prior to Mutagenesis (3 days) 37
Figure 9b - DGC 7 Days after Mutagenesis 37
Figure 9c - DGC 9 Days after Mutagenesis 37
Figure 10 - Mutant Strain Tyrosine Insensitivity Assay 39
Figure 11 - Percent Relative Activity 40
Figure 12 - Phenylalanine and Tryptophan Sensitivity Test 41
Figure 13 - Plasmid Map of Cloned Mutants 42
Figure 14 - Map of Feedback Insensitive *aroF* 43
Figure 15 - Cloned AroF Specific Activity 44
Figure 16 - Map of *aroF tyr* Boxes 45
Figure 17 - Plasmid Map of pCL2-13A-trunc 46
Figure 18 - Nontruncated and Truncated AroF Specific Activity 46
Figure 19 - Minimal Medium Nontruncated and Truncated AroF activity 47
Figure 20 - Plasmid Map of pKL4.32B and pKL4.33B 48
Figure 21 - Truncated/Nontruncated AroF Activity and DHS Production 49
Figure 22 - Plasmid Maps of pKL4.66A and pKL4.66B 50
Figure 23 - Specific Activity and DHS Production of pKL4.66A/B 51
Figure 24 - Plasmid Map of pKL4.79B 52
Figure 25 - AroF Specific Activity and DHS Production of pKL4.79B 52
Figure 26 - Biosynthetic Pathways for DHS Production 61
Figure 27 - Flux Distribution for Maximum DHS Yield 63
Figure 28 - Braun Fermentor 65
Figure 29 - ^{1}H NMR of 3-Dehydroshkimic Acid (DHS) 73
Figure 30 - ^{1}H NMR of Glucose 74
Figure 31 - ^{1}H NMR of Gallic Acid 75
Figure 32 - ^{1}H NMR of Acetic Acid 76
Figure 33 - ^{1}H NMR of Formic Acid 77
Figure 34 - Typical ^{1}H NMR for 36 hour Fermentation 78
Figure 35 - Fermentation Evaluating KL3/pKL4.32B and KL3/pKL4.33B 81
Figure 36 - Nonoptimized Fermentation 82
Figure 37 - KL3/pKL4.66A Fermentation 84
Figure 38 - Fermentations of KL3/pKL4.66A and KL3/pKL4.66B 85
Figure 39 - Fermentations of KL3/pKL4.33B 86
Figure 40 - Fermentation of KL3/pKL4.79B 1.0 g IPTG 89

Figure 41 - Fermentations of KL3/pKL4.79B, IPTG Titration of *aroF*.....90
Figure 42 - DHS Production as a Function of AroF Activity.....91
Figure 43 - 50 L Scale Fermentation.....93

LIST OF ABBREVIATIONS

Ap	ampicillin
Cm	chloramphenicol
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DHQ	3-dehydroquinate
DHS	3-dehydroshikimic acid
E4P	D-erythrose 4-phosphate
h	hour
IPTG	isopropyl β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
LB	Luria broth
m-FT	*m*-fluorotyrosine
min	minute
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
RBS	Ribosome Binding Site
Spc	spectinomycin
TSP	sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d_4

CHAPTER 1

INTRODUCTION

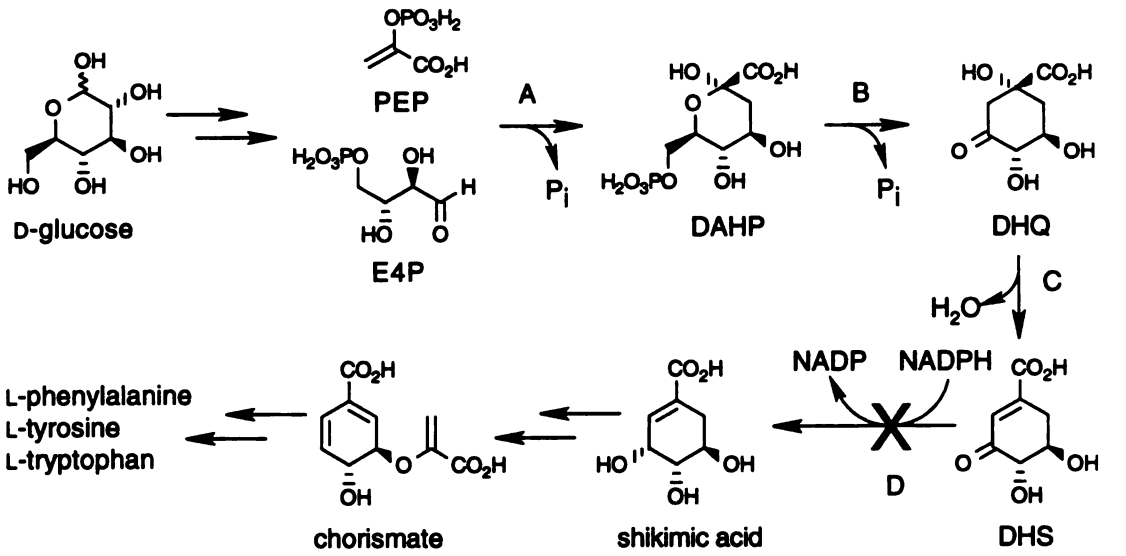
The development and use of microorganisms for biocatalytic production of small molecules is a growing technology as indicated by the increasing demand for bioreactors and projected biotechnology sales forecasts.[1] A factor in this increase is the use of biological production for aromatic compounds and their chemical precursors as an alternative to traditional synthetic routes based on petroleum feedstocks.[2] Biocatalysis utilizes benign, inexpensive, and renewable starting materials such as D-glucose. In comparison, traditional chemical synthesis utilizes petroleum-derived starting materials such as benzene and other hydrocarbons that are in many cases toxic. Chemical synthesis often has the disadvantage of producing a mixture of products and byproducts that require separation. Many of the environmentally harmful aspects of using petroleum feedstocks are a result of the chemistry required to add oxygen atoms to the starting materials and intermediates. Petroleum typically has a low ratio of oxygen to carbon, whereas plant-derived feedstocks, such as starch and cellulose, have a high oxygen to carbon ratio. Petroleum is a nonrenewable resource that has been plagued by inadvertent releases into the environment. Therefore, the advantages of biocatalytic production are the benefits of exploiting starting materials such as D-glucose and the initially high oxygen to carbon ratio of the feedstocks. However, efficient and high-yielding conversion of starting material to product requires optimization of the microbial catalyst by genetic modification and optimization of the fermentation conditions.

Biocatalyst

A plethora of engineered microorganism have been exploited for biocatalytic synthesis of aromatic amino acids. A partial listing of these microbes include *Corynebacterium glutamivum*,[3] *Brevibacterium lactofermentum*,[4] and *Escherichia coli*.[5] The organisms utilized in this study are *E. coli* K-12 derivatives. *E. coli* are facultative, gram-negative, rod shaped, prokaryotic bacteria of the family *Enterobacteriaceae*. There are many reasons for choosing *E. coli*. It is a well studied organism. The K-12 strain was given preferential treatment by the National Institutes of Health guidelines for work with recombinant organisms because the safety of K-12 strains were more actively investigated.[6] *E. coli* have high growth rates, rapidly metabolize substrates, require inexpensive medium components, are physically rugged, and survive in a wide variety of environmental conditions. Also, an advantage to using *E. coli* for the present study was the necessary phenotype to produce 3-dehydroshikimic acid (DHS, the desired fermentation product) had already been created.[7] Two derivatives of K-12, AB2834 and AB3248, were previously obtained by the Frost group from the Yale Genetic Stock Center. AB2834 had the desired phenotype for DHS production, and AB3248 had the phenotype necessary to be used as the host to study a key enzyme in the metabolic pathway.[8]

Common Pathway of Aromatic Amino Acid Biosynthesis

The elucidation in the early 1960s of the common aromatic amino acid pathway utilized bacterial auxotrophs of *E. coli* and *Kleblsiella areogenes*.[9] The pathway consists of seven enzymatically catalyzed reactions that convert D-erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to chorismic acid (Figure 1). The pathway is named for the fact that it is the source for the common precursor of aromatic amino acids and related secondary metabolites. This pathway is also present in other bacteria, plants, fungi, and molds.[10] PEP is produced through glycolysis, and E4P is produced from the non-oxidative branch of the pentose phosphate pathway.



(A) DAHP synthase (*aroF*, *aroG*, *aroH*); (B) DHQ synthase (*aroB*); (C) DHQ dehydratase (*aroD*); (D) shikimate dehydrogenase (*aroE*).

Figure 1 - The Common Pathway.

In *E. coli*, the first committed step in the common pathway is the condensation between E4P and PEP to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) and inorganic phosphate. This reaction is catalyzed by three DAHP synthase isozymes that are encoded by *aroF*, *aroG*, and *aroH*, whose products are sensitive to feedback inhibition[11] by L-tyrosine, L-phenylalanine, and L-tryptophan, respectively (typically, names of genes are italicized, and the first letter of protein names are capitalized). At the repressor-mediated transcriptional level, *aroF* and *aroG* are repressed by the TyrR protein and *aroH* is repressed by the TrpR protein.[12]

The tyrosine-sensitive isozyme of DAHP synthase (AroF) was used in the constructs for DHS production, and the choice for the use of this isozyme is detailed later in this chapter (p 7). It is an iron-containing dimer with subunit molecular weight of about 40,000.[13] When *E. coli* are grown in minimal salts medium supplemented with all the amino acids except the aromatic amino acids, AroF enzyme comprises the major isozyme.[14]

The reaction mechanism is ordered and sequential with PEP being the first substrate to bind. The K_m value (the substrate concentration at which the reaction rate is half its maximal value) for PEP has been reported to be 5.8 μM for the purified enzyme.[15] It is assumed that PEP-enzyme complex is the native form of the enzyme since the intracellular concentration of PEP never falls below 88 μM, and enzyme activity is lost and unrecoverable during purification if the enzyme buffer solution does not contain PEP.

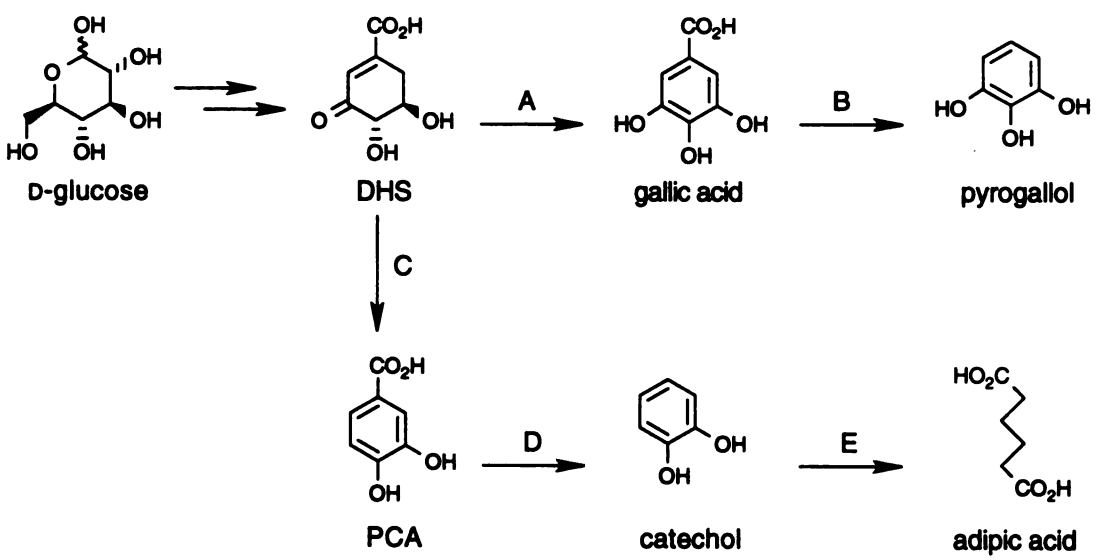
The second enzyme in this pathway, dehydroquinate synthase, is encoded by the *aroB* locus. Dehydroquinate (DHQ) and inorganic phosphate are formed from DAHP in this NAD-requiring reaction. AroB is a cobalt-requiring, monomeric protein with a molecular weight of 44,000.[16] Next DHQ dehydratase, encoded by *aroD*, catalyzes the dehydration of DHQ to form DHS. This enzyme does not require a metal as do the previous two enzymes. There are conflicting data concerning the molecular weight and structure of AroD. The native enzyme has been reported to be both a dimer and a tetramer with a molecular weights, respectively, of 40,000 to 60,000.[17] Both AroB and AroD are constitutively expressed and are not sensitive to feedback inhibition by any of the aromatic amino acids or chorsimate.[18] It is noteworthy that DHS contains the first double bond of what becomes the aromatic ring in phenylalanine, tyrosine, and tryptophan. The last enzyme of importance for DHS production in the common pathway is *aroE*-encoded shikimate dehydrogenase. This enzyme catalyzes the reduction of DHS to shikimic acid and requires NADPH. Shikimate dehydrogenase is a monomeric protein with a molecular weight of 32,000.[19] The host strain utilized in this study, AB2834, lacks shikimate dehydrogenase activity and therefore cannot further metabolize DHS.[20]

Since the strain is unable to further metabolize DHS, it is unable to produce chorismic acid, the last metabolite in the common pathway. Chorismic acid is the precursor for six end products. Three terminal pathways lead to the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The three other end products include folic acid,

ubiquinone, and enterochelin, which are involved in coenzyme biosynthesis, electron transport, and iron uptake, respectively.[21]

Common Pathway Metabolite Uses

The common pathway provides a metabolic route to not only aromatic amino acids and essential metabolites, but also to other pathway intermediates that are potentially valuable starting materials for chemical synthesis. Through metabolic engineering, key intermediates can be overexpressed, and their increased availability may further the use and the demand for these unique molecules as starting materials for chemical synthesis. Diversion of carbon flow from the common pathway to products not normally synthesized by *E. coli* is possible by the addition of foreign genes to a genetically modified host strain. Examples of novel syntheses using heterologous microbes include the production of protocatechuic acid,[22] catechol,[23] and adipic acid,[24] from glucose via the common pathway intermediate DHS (Figure 2). Also, DHS has several possible industrial uses for abiotic



(A) chemical oxidation; (B) enzymatic decarboxylation; (C) DHS dehydratase; (D) enzymatic decarboxylation; (E) (i) catechol 1,2-dioxygenase, (ii) chemical hydrogenation.

Figure 2 - Alternate Molecule Production from The Common Pathway.

synthesis of aromatic compounds such as gallic acid and pyrogallol.[25] Thus, research to increase DHS production in the fermentor can serve as the archetype for other common pathway metabolites products such as shikimic acid and other products that can be produced by the addition of foreign genes.

Central Metabolism

Glycolysis and the pentose phosphate pathway are two central routes of carbohydrate metabolism.[26] The pentose phosphate pathway connects glycolysis with several biosynthetic pathways. It consists of an oxidative and non-oxidative branch. The oxidative branch produces ribose 5-phosphate and carbon dioxide from glucose 6-phosphate, with concomitant production of NADPH. The non-oxidative branch coverts glycolytic intermediates such as fructose 6-phosphate and glyceraldehyde 3-phosphate into a variety of C-3 through C-7 monophosphate sugars, including E4P. Two enzymes of the non-oxidative branch, transketolase and transaldolase, work in concert to produce E4P. These two enzymes are essential to the formation of ribose 5-phosphate, sedoheptulose 7-phosphate and E4P, which are required, respectively, for the biosynthesis of nucleotides, lipopolysaccharide, and aromatic amino acids and vitamins.

The other substrate for DAHP synthase, PEP, is an intermediate in the glycolytic pathway. The production of PEP via glucose metabolism leads to a competition between the PEP-requiring biosynthetic steps and the PEP-dependent phosphorotransferase system (PTS) for glucose uptake.[27] PEP is the phosphate donor used by PTS in the uptake and phosphorylation of glucose. In the glycolytic pathway one mole of glucose produces two moles of PEP. During glucose transport by the PTS one mole of PEP is converted into one mole of pyruvate. Therefore, glucose transport results in only one mole of PEP per mole of glucose consumed being available for biosynthesis. Wild-type *E. coli* apparently do not recycle pyruvate back to PEP. Pyruvate formed from the PTS-mediated glucose uptake is further metabolized in the TCA cycle and used in the production of organic acids, carbon dioxide, or cell mass.

Selection of DAHP Synthase Isozyme for Overexpression

It was determined that the first step in the common pathway was limiting the carbon flow into the pathway and its overexpression was necessary to increase the pathway flux.[28] Since there are three isozymes of DAHP synthase present in wild-type *E. coli*, a selection of which isozyme to use had to be made. The most important criteria for this selection were optimal enzymatic activity and the effect enzymatic and transcriptional regulation.

The AroH isozyme was ruled out as the choice for overexpression. This isozyme normally accounts for only a small portion of the DAHP synthase activity under a variety of growth conditions.[29] In cells extracts, when fully derepressed, the activity of AroH is 10% of the AroG activity and 20% of the AroF activity.[30] It would be reasonable to assume the lower activity of AroH is indicative of a weak native promoter,[31] and significant overexpression of AroH is not possible.

There are many similarities between AroG and AroF. Both enzymes contain one mole of iron per mole of enzyme. There is approximately 50% identity in the nucleotide sequence of AroG and AroF.[32] Also *aroG* and *aroF* are transcriptionally repressed by the TyrR protein.[33] In addition, feedback insensitive mutants have been generated for both AroF[34] and AroG.[35]

AroG is a tetramer with a subunit molecular weight of 35,000. The AroF isozyme is a dimer with a subunit molecular weight of 40,000. During growth in minimal medium, without any amino acid supplementation, AroG comprises 80% or more of the total DAHP synthase activity. Similarly, when *E. coli* are grown in iron-starved conditions AroG comprises the majority of the DAHP synthase activity.[36] Although, when *E. coli* are grown on minimal salts medium supplemented with all amino acids except aromatic amino acids, AroF accounts for most of the DAHP synthase activity. The AroG isozyme is 50% inhibited by 13 μM phenylalanine, and AroF is 50% inhibited by 20 μM tyrosine. During the stationary phase of E. coli growth, AroF is significantly more sensitive to proteolysis relative to AroG.[37]

There are certain advantages to each of the isozymes. The isozyme, AroG or AroF, that comprises most of the DAHP synthase activity varies with growth conditions. Since iron is added to the medium, the higher specific activity of AroG during iron starvation is not significant. The higher specific activity of AroF under growth conditions where aromatic amino acid concentrations are limiting is relevant since these are the conditions anticipated for biocatalytic synthesis of DHS. Although, the main reason for use of a feedback-insensitive AroF was because it has been successfully used in high titer fermentations of L-phenylalanine and L-tryptophan.[38] Several different mutations in AroF confer insensitivity to feedback inhibition, and it was believed this would increase the likelihood that a feedback-insensitive AroF could be generated in this lab. The feedback-insensitive AroF was needed to increase the carbon flow into the pathway when *E. coli* is grown in medium supplemented with tyrosine.

Feedback-Insensitive AroF

Biocatalytic synthesis of various metabolites in high yields often requires alteration of the cell's natural regulatory mechanisms to increase carbon flow into selected biosynthetic pathways.[39] Alterations can be performed genetically by mutation of genes, changing the promoter, increasing the number of gene copies in the cell, or by overcoming transcriptional repression. Regulatory mechanisms governing carbon flow into the common pathway include both feedback inhibition of translated enzymes and transcriptional repression.[40]

Feedback inhibition involves reduction of activity of key biosynthetic enzymes by either pathway intermediates or products. Typically, the first enzyme in the common biosynthetic pathways and the first enzyme in branch pathways are subject to regulation by feedback inhibition. In the presence of excess inhibitory product, feedback inhibition reduces the affected enzyme's activity, which results in reduction of product biosynthesis. Feedback inhibition has been demonstrated to be the dominant regulatory mechanism controlling carbon flow into the pathway of aromatic amino acid biosynthesis in *E. coli*.[41]

Repression of transcription occurs when a repressor protein binds to a conserved DNA sequence upstream from the structural gene. In the presence of the repressor, transcription of the gene by RNA polymerase is blocked. Often the ability of a repressor molecule to bind to DNA is dictated by binding of an effector molecule to the repressor. Either biosynthetic end products or biosynthetic intermediates can be effector molecules. An example of such regulation occurs in the *aroF tyrA* transcriptional unit. The structural gene for *aroF* is preceded by three separate conserved sequences called *tyrR* boxes.[42] A repressor protein, TyrR, when activated by binding to the effector tyrosine, binds to the *tyrR* box such that transcription cannot occur. In the presence of tyrosine, transcriptional repression can lead to a 20-fold reduction in DAHP synthase activity relative to the activity in the absence of tyrosine.[43]

Since the key enzyme AroF is regulated by both feedback inhibition and transcriptional repression, increasing the in vivo activity of this isozyme of DAHP synthase was considered essential. The host strain used for producing DHS is an aromatic amino acid auxotroph that requires the addition of tyrosine, phenylalanine, and tryptophan, as well as the aromatic vitamins for growth in minimal medium. A feedback-insensitive AroF was thought to be needed to avoid feedback inhibition caused by in vivo, steady-state concentration of tyrosine. The intracellular tyrosine concentration of AB2834 may be high compared to the wild-type *E. coli* intracellular tyrosine concentration (since wild-type *E. coli* would be grown in medium that is not supplemented with tyrosine) if the rate of active transport of tyrosine into the cell is greater than the rate of tyrosine consumption by incorporation into proteins. In addition, the pseudo steady state concentration of tyrosine may be high even if the transport and initialization rates of tyrosine are the same. The nature of AroF inhibition is noncompetitive with respect to E4P and competitive with respect to PEP.[44] The enzyme is 50% inhibited by 20 μM tyrosine.[45]

Increasing DHS Production

The first step in the metabolic optimization of the common pathway was to increase DAHP synthase levels.[46] This was achieved by insertion of the locus that encoded for either *aroG* or *aroF* onto a multicopy plasmid. Plasmid-based expression of DAHP synthase increased the flux of carbon through the common pathway, resulting in a situation where subsequent enzymes became limiting.[47]

The next metabolic improvements were made using a strain that produced phenylalanine. In this system it was determined that 3-dehydroquinate (DHQ) synthase activity was insufficient to catalyze the conversion of DAHP into DHQ at a adequately rapid rate to avoid DAHP accumulation (termed rate-limiting). An amplification of *aroB*-encoded DHQ synthase expression as part of a synthetic cassette provided sufficient amplification to remove the rate-limiting character of DHQ synthase.[48] Based on these findings, the minimum requirement to have increased carbon flow through the common pathway to produce DHS is the overexpression both *aroF* and *aroB*.

Next, E4P in vivo availability was increased using the transketolase (*tktA*) locus inserted into a multicopy plasmid. The plasmid also contained the locus for *aroF*. The plasmid was transformed into a host that produced DAHP. The DAHP titer and yield increased two-fold.[49]

A theoretical analysis of the pathways involved indicated that the yield was then limited by PEP availability.[50] One option to increase PEP availability was to recycle pyruvate generated through the PTS back to PEP, by using the gene for phosphoenolpyruvate synthase (*pps*). This would increase the theoretical yield by a factor of two (from 43 mol % to 86 mol %), since two moles of PEP produced from one mole of glucose consumed would be available for biosynthesis. The gene was localized on a plasmid and transformed into a strain that accumulates DAHP. The result was an increase in the DAHP titer and an increase in the yield to near the new theoretical maximum.[51]

An attempt to increase in vivo PEP availability was examined by the localization of phosphoenolpyruvate carboxykinase (*pck*) on a plasmid. This enzyme converts oxaloacetate from the TCA cycle into PEP. Liao *et. al.* reported that the overexpression of *pck* adversely affected the growth.[52] The explanation given was *pck* overexpression altered the concentration of PEP-related metabolites which were previously unknown to be involved in global regulation. There was no mention of DAHP yield or titer, presumably because the growth was so poor that DAHP was not produced. This genetic modification illustrates that metabolic engineering techniques sometimes do not work because of unknown cellular processes.

Still another route to increase yield is to use xylose (a pentose) as a carbon source. Xylose does not utilize the PTS to phosphorylate the sugar.[53] Instead, its metabolism involves transport through the membrane, isomerization to xylulose, and ATP-dependent phosphorylation of xylulose. Most strains of *E. coli* grow on xylose, but a mutation is necessary for strain K-12 to grow on this carbohydrate.[54] A theoretical analysis of the pathways involved indicated that the yield should increase to 71 mol % since PEP is not consumed in the phosphorylation of xylose. This experiment was conducted using a strain that produced DAHP. The yield of DAHP from xylose was near the theoretical maximum and the DAHP titer was higher when compared to glucose as the carbon source.[55]

Most recently, the impact of amplified expression of transaldolase (*talB*) has been used to increase E4P in vivo availability. The *talB* locus was localized onto a multicopy plasmid that also contained *aroF* and *aroB*. The resulting plasmid was transformed into a host that produced DHS. The construct resulted in only slightly higher DHS titer compared to the control construct without *talB*.[56]

Metabolic Control Analysis and Metabolic Engineering

The methodology of metabolic control analysis has emerged since the early 1970s. It aims to characterize the sensitivity of metabolic responses to changes in enzymatic activities or parameters without the use of full mathematical models.[57] Metabolic control

analysis takes advantage of the relationship of characteristic parameters at steady-state. Valuable information can be gained by analysis with respect to identifying rate-limiting enzymes by using control analysis. Knowledge of which enzymes are rate-limiting is useful because it can identify enzymatic steps the exert the most control on the flux through the pathway. This methodology has been used and further developed by Liao *et. al.* for analysis the production of DAHP.[58] This analysis has shown, in the production of DAHP, that after increasing E4P availability, PEP becomes limiting. Therefore, PEP availability would be the next target for improving DAHP production, illustrating the usefulness of control analysis.

Metabolic engineering as stated by Stephanopoulos *et al* is, "not another form of classical manipulation of intermediary metabolism. It is, rather, the purposeful design of metabolic networks".[59] This methodology has been applied by Stephanopoulos to the production of the aspartate family of amino acids in *Corynebacterium lactofermentum*. The analysis identifies which branch points in the biosynthetic pathway for the amino acid production exert the most control. The objective of metabolic engineering is to identify rate-limiting enzymes for amplification that would most efficiently increase carbon flux through desired pathways. Flux control coefficients and elasticity coefficients are two tools used to implement the metabolic engineering objective. Flux control coefficients indicate how much an enzyme restricts the pathway as a whole. Elasticity coefficients indicate the sensitivity of single reaction to changes in metabolite concentrations. Similarly to the metabolic control analysis, metabolic engineering can provide the information necessary for rational design of a microbial catalyst. Both methodologies offer means for analysis of limiting steps in biosynthesis, and the results can be used to determine the next logical step for improvement.

Plasmid Maintenance

Significant plasmid loss during fermentation can result in low productivity and indicate the need for a better plasmid maintenance system. Many studies have investigated

plasmid loss and its cause. There are many environmental factors that influence plasmid maintenance, ranging from the level of dissolved oxygen to amino acid supplementation.[60] Plasmid maintenance has been a concern in the industrial use of recombinant microorganisms. Previous production of DHS in a fermentor indicated that after 48 h of fermentation, 50-90% of the cells were lacking plasmids. In actuality, the loss of plasmids by an individual host is a rare event.[61] However, cells lacking plasmids no longer have the metabolic burden caused by overproduction of the plasmid-encoded enzymes, and therefore have a higher growth rate and soon out grow the plasmid bearing cells. This was addressed by using a nutritional requirement to maintain the plasmid instead of antibiotic resistance. There have been several examples of nutritional requirements used for plasmid maintenance. One such method was employed by Porter *et al.*[62] The *ssb* gene, whose product is responsible for DNA replication, was deleted from the *E. coli* chromosome. The locus for *ssb* was placed onto a plasmid. Therefore, a cell lacking *ssb*-encoding plasmid would be unable to further replicate. Plasmid stability was achieved under nonselective culture conditions.

Another method for plasmid maintenance relies on the postsegregational killing of plasmid-free cells.[63] The plasmid carries for the *parB* locus which encodes two genes, Hok and Sok. The Hok gene (host killing) encodes for a small polypeptide that results in rapid death of the host. The Sok gene (suppression of killing) product inhibits the translation of the Hok mRNA. The Hok mRNA is much more stable than the Sok mRNA. In plasmid-carrying cells Sok RNA prevents the synthesis of the Hok protein and the cell remains viable. In a plasmid-free cell, the Sok RNA quickly decays thereby allowing translation of the Hok mRNA accessible for translation. The Hok protein is synthesized, and cell death occurs. Other methods rely on a similar strategy, wherein the plasmid free cells no longer have the ability to prevent cell death due to lack of a protein. The use of the *parB* locus may not be the best choice for plasmid maintenance since it involves more cloning and will increase the metabolic burden on the host cell.

The use of antibiotic resistance for plasmid maintenance in itself is problematic for many reasons. Antibiotics such as ampicillin are degraded by β-lactamase enzymes which are exported from the cell.[64] This enables cells lacking β-lactamase to grow because the enzyme conferring resistance has been transported into the culture broth. It is likely that during 48 h fermentations, all of the ampicillin initially present in the growth medium has been hydrolyzed.[65] Another problem with the use of some antibiotics is their influence on cellular metabolism. Chloramphenicol is one such example.[66] The mechanism of chloramphenicol deactivation entails acetylation of chloramphenicol using acetyl-CoA as the donor. Since the acetyl donor group is from acetyl-CoA this reaction may have the unwanted effect of depleting the acetyl-CoA pool. Lastly, it is undesirable to have an antibiotic resistant organism used for industrial processes due to possible release into the environment.

Fermentor

Significant work has been performed towards the optimization of *E. coli* fermentations to produce phenylalanine and tryptophan.[67] This previous work served as a useful starting point for optimizing DHS production.

At the laboratory scale, fermentors provide a significant advantage over shake flasks. The fermentor can control many variables on-line, such as temperature, pH, dissolved oxygen, and substrate addition. Through computer usage, it is also possible to have a variety of complex control algorithms calculated from on-line data. Another advantage is the greater aeration capacity provided by the air sparger and the impeller in the fermentor, which is necessary for aerobic growth of high density *E. coli* cultures.[68] Much higher cell densities and growth rates are typically achieved due to the increased aeration in a stirred-tank fermentor than in a shake flask, which results in a higher concentration of biocatalyst.

There are also many operating advantages in utilizing a fermentor. Dissolved oxygen can be controlled by the impeller speed and or air flow rate in the Braun fermentor.

A better degree of mixing is achieved by the impeller and baffles. In high density culture, foaming is a significant problem, but it can be controlled by a foam sensor and computer-controlled additions of antifoam. Alteration of medium temperature can be conveniently accomplished in a fermentor, which allows for temperature-dependent expression systems to be used. Samples can be removed under sterile conditions using the harvest pipe. On-line monitoring and electronic storage allows for data recording and retrieval of essential parameters.

Research Objectives

This research was a collaborative effort between Drs. John W. Frost and R. Mark Worden to develop a microbial catalysts and a fermentation process for industrial production of DHS. The first objective was to develop a feedback-insensitive AroF. The second chapter of this thesis will discuss the use of a novel technique to generate and select for a feedback-insensitive *aroF* mutant in a diffusion gradient chamber. The feedback-insensitive *aroF* obtained was then used in constructing strains relevant to industrial synthesis of hydroaromatic and aromatic compounds. Also investigated was the effect of removing part of the *tyrR* boxes upstream of the feedback-insensitive *aroF* in an effort to remove transcription regulation by repression.

The third chapter of this thesis and the second objective was the optimization of DHS production in a fermentor through both genetic and reaction engineering. The parameters optimized were DHS titer and yield. Optimization studies investigated the effects of several variables, including fermentor medium composition, starter culture medium, dissolved oxygen setpoint, pH setpoint, and glucose feeding strategies. Genetically modified constructs were evaluated for DHS production with the use of the feedback-insensitive *aroF*. The effect of DAHP synthase activity on DHS production was studied utilizing the *tac* promoter and repressor $lacI^q$ to manipulate the DAHP synthase activity by transcription control. The alterations of *aroF* orientation with respect to a promoter and the number of *aroF* copies on the plasmid were also investigated.

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CHAPTER 2

TYROSINE FEEDBACK-INSENSITIVE DAHP SYNTHASE

Background

Biocatalytic synthesis of various metabolites often requires alteration of a microbe's natural regulatory mechanism so that increased carbon can be directed into selected biosynthetic pathways.[1] Alterations can be performed genetically by mutation of genes, changing the promoter, increasing the number of gene copies in the cell, or by overcoming transcriptional repression. Regulatory mechanisms governing carbon flow in the common pathway include both feedback inhibition of enzymes and repressor-mediated transcriptional repression. [2]

In *Escherichia coli* the first committed step in the common aromatic biosynthetic pathway is the condensation between erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). This reaction is catalyzed by three DAHP synthase isozymes encoded by *aroF*, *aroG*, and *aroH*, which are feedback inhibited by L-tyrosine, L-phenylalanine, and L-tryptophan, respectively.[3] At the repressor-mediated transcriptional level, *aroF* and *aroG* are repressed by the TyrR protein, and *aroH* is repressed by the TrpR protein.[4] Feedback inhibition has been demonstrated to be the dominant regulatory mechanism in controlling carbon flow into the common pathway of aromatic amino acid biosynthesis in *E. coli*.[5]

Repressor-mediated transcriptional repression is a second mode of control for *aroF* expression at the gene level. In the case of *aroF*, the transcriptional regulation is a result of the *aroF tyrA* transcriptional unit. The structural gene for *aroF* is preceded by three

separate conserved sequences called *tyrR* boxes.[6] A repressor protein, TyrR, when activated by binding to the effector tyrosine, binds to the *tyrR* box such that transcription cannot occur. In the presence of tyrosine, transcriptional repression can lead to a 20-fold reduction in DAHP synthase activity relative to the activity in the absence of tyrosine.[7]

Since the key enzyme AroF is regulated by both feedback inhibition and transcriptional repression, increasing the in vivo activity of this isozyme of DAHP synthase was essential. The host used for producing dehydroshikimate (DHS) is an aromatic amino acid auxotroph that requires the addition of tyrosine, phenylalanine, and tryptophan, as well as aromatic vitamins for growth in minimal medium. The aromatic vitamins *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid are biosynthetic precursors to folic acid, enterochelin, and ubiquinone, respectively. A feedback-insensitive AroF was thought to be needed to avoid feedback inhibition caused by in vivo, steady-state concentration of tyrosine. Another motivation for creating the feedback-insensitive AroF is the intracellular tyrosine concentration could be higher than the extracellular concentration. The intracellular tyrosine concentration of AB2834 may be high compared to the wild-type *E. coli* intracellular tyrosine concentration (since wild-type *E. coli* would be grown in medium that is not supplemented with tyrosine) if the rate of active transport of tyrosine into the cell is greater than the rate of tyrosine consumption by incorporation into proteins. In addition, the pseudo steady state concentration of tyrosine may be high even if the transport and initialization rates of tyrosine are the same.

The tyrosine-sensitive form of DAHP synthase is an iron-containing dimer with subunit molecular weight of about 40,000.[8] Under minimal growth conditions that are supplemented with all amino acids except the aromatic amino acids, AroF comprises the major isozyme. The reaction mechanism is ordered and sequential with PEP being the first substrate to bind. The K_m value for PEP has been reported to be 5.8 μM for the purified enzyme.[9] It is assumed that PEP-enzyme complex is the native form of the enzyme since the intracellular concentration of PEP never falls below 88 μM, and enzyme activity is lost

and unrecoverable during purification if the enzyme buffer solution does not contain PEP. The enzyme is 50% inhibited by 20 μM tyrosine.[10] The enzyme is also inhibited by the nonmetabolized analog *m*-fluorotyrosine (m-FT).[11] The nature of the feedback inhibition is noncompetitive with respect to E4P and competitive with respect to PEP.

To obtain a feedback-insensitive AroF, a novel method was developed where ultaviolet light mutagenesis was followed by phenotype selection utilizing chemotaxis in the diffusion gradient chamber (DGC). Chemotaxis is the ability of an organism to move in response to a gradient of a chemical species.[12] A chemical species that attracts an organism is referred to as a chemoattractant. The separation and selection of the desired mutant was aided by the exploitation of chemotaxis.

Molecular Biology Background

In this chapter, several molecular biology techniques are described, and the following terminology is defined to help clarify these techniques. An open reading frame (OPR) contains a series of DNA base triplets coding for amino acids without any termination codons, and the sequence is potentially translatable into protein. The OPR does not contain the genetic sequence for a promoter. Polymerase chain reaction (PCR) was used to isolate the feedback-insensitive *aroF*. It is a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase, are used to amplify the number of copies of a target DNA sequence by $>10^6$ times[13]. The ribosome-binding site is the DNA sequence at which initiation of transcription occurs. It is a short sequence of bases that precedes the actual coding region.

Development of the Strain for Mutagenesis

The *E. coli* strain mutagenized contained only the AroF DAHP synthase isozyme. The activity of the other two DAHP synthase isozymes were not present. It was developed from host, AB3248, that lacks all three DAHP synthase isozyme activities,[14] and is also auxotrophic for the amino acids histidine (H), isoleucine (I), proline (P), arginine (R), and valine (V). Minimal salts medium must be supplemented with these amino acids in order

for AB3248 to grow. To obtain the correct phenotype, a copy of *aroF* was added either by insertion of a multicopy plasmid or insertion into the genome. Insertion into the genome was the method chosen for several reasons. This strategy eliminates the need to add an antibiotic for selective pressure to maintain a plasmid. One problem with using *aroF* on a multicopy plasmid stems from overexpression of the AroF protein. With use of plasmid-encoded *aroF*, this enzyme may constitute 10-15% of the total soluble protein of the organism.[15] The selection might not work well under these conditions since a significant amount of DAHP synthase activity may remain in the presence of m-FT. The genomic insertion would provide only one copy of *aroF*. This would reduce the possibility that the enzyme concentration may become too high relative to the inhibitor concentration. A further advantage of the genomic versus plasmid localization of *aroF* arises from the presence of only one copy of the mutated gene requiring isolation. Use of a plasmid-bearing *aroF* would complicate the isolation of the mutant gene since multiple copies of mutated and unmutated genes may be present. The insertion of *aroF* into the genome of AB3248 was conducted by Dr. Karen Draths.

Genomic insertion of genetic sequences into *E.* coli is possible by several methods. Successful insertions have been performed using transposons,[16] circular plasmids,[17] and linear DNA fragments[18] as a introduction vehicle. The disadvantage of many of these methods is the required use of special strains with specific mutations in order to achieve insertion. Site specific insertion independent of the genotype of the recipient strain was desired for our purpose.[19]

Usually plasmids are maintained as extrachromosomal, circularized DNA in *E. coli*, but occasionally recombination events occur such that plasmid DNA is integrated into the host cell's genome. Exploitation of this rare event provides a method for simple site-specific insertion of a gene flanked by sequences homologous to the desired insertion location in the genome. Plasmid-bearing cells are usually differentiated from cells without plasmid by selection due to antibiotic resistance conferred by the plasmid. The difficulty in

differentiation between the cells with integrated plasmid DNA and replicating plasmids is that both are resistant to the antibiotic. The use of a non-replicating plasmid allows for the exclusive selection of integrated plasmid DNA since cells will possess antibiotic resistance only if the plasmid resides in the genome.

A plasmid in which the replication mechanism can be turned on and off is desirable for genomic insertions. Under conditions where the replication of the plasmid is inactive, genomic insertions can be accomplished, whereas normal cloning and preparation of the plasmid can be performed with conditions conductive to active replication. Plasmids possessing a temperature-sensitive replicon [rep (*ts*)] are capable of being manipulated in this fashion. Plasmid pKAD76A[20] (Figure 3) contains such a temperature sensitive pSC101 replicon, a chloramphenicol acetyltransferase (*cm*) gene conferring resistance to chloramphenicol, and a copy of the *serA* locus, which has a suitable cloning site (*Eco*RI). The *serA* gene on the plasmid was necessary for homologous recombination into the genomic *serA*. The *serA* gene converts 3-phosphoglycerate into 3-phosphohydroxypyruvate for serine biosynthesis. The plasmid is able to replicate normally when the host cell is grown at 30 °C, but it is unable to replicate when the host cell is cultured at 44 °C. Thus genetic manipulation of the plasmids is carried out at 30 °C while cells that had the integration of the plasmid into the genome can be selected at 44 °C.

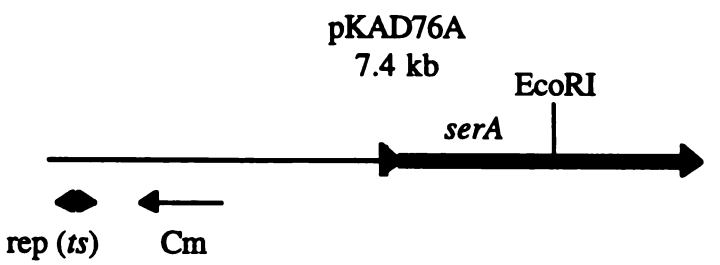


Figure 3 - Plasmid Map of pKAD76A.

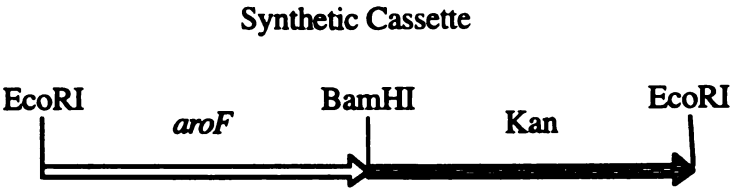


Figure 4 - Synthetic Cassette.

A synthetic cassette was developed to aid in the selection of the integration of the plasmid. An *aroF* gene fragment with *Eco*R1 and *Bam*HI ends was produced from the polymerase chain reaction (PCR), using primers JWF-22 and JWF-83 (Table 1), which have *Eco*RI and *Bam*HI ends, respectively. The *aroF* fragment was ligated into a linearized plasmid (pKAD62A, a kanamycin resistance-bearing plasmid) at a cloning site next to the kanamycin (*kan*) resistance gene. This product was then digested with *Eco*RI to produce a cassette of the *aroF* and kanamycin (*kan*) resistance genes (Figure 4). The fragment was then cloned into pSU18 (ampicillin and chloramphenicol resistance-bearing plasmid) creating the plasmid pKD10.156A (Figure 5). The correct ligation was confirmed by plasmid DNA digestion and plating onto minimal medium (M63) plates with kanamycin. The cells would not grow on the M63/Kan plates unless both the *kan* resistance and a functional *aroF* was contained on the plasmid. Next, the planned insertion of the cassette into the genomic copy of *serA* in strain AB3248 necessitated construction of the plasmid containing the synthetic cassette flanked by the *serA* locus.

The synthetic cassette was isolated from pKD10.156A as an *Eco*RI fragment and was cloned into the *Eco*RI site of *serA* in pKAD76A. The resulting 9.8 kb plasmid,

Table 1 - PCR Primers.

Primer Name	Sequence
	Eco RI
JWF-19	5' GGAATTCTTAAGCCACGCGAGCCGT 3'
	EcoR I
JWF-22	5' GGAATTCAAAGGGAGTGTAAATTTAC 3'
JWF-79	5' GCTTTTCCATTGAGCCTGCA 3'
JWF-80	5' AACGATCCCCATATGGATGG 3'
JWF-81	5' GCAGTCTGGCAACAGCAATT 3'
JWF-82	5' AAGATTATCGCCGTCAGCCT 3'
	Bam HI
JWF-83	5' GCGGATCCTCTTAAGCCACGCGAGCCGT 3'
	Eco RI
JWF-94	5' GGAATTCTGTACGAAATATGGATTGAA 3'
	Eco RI
JWF-97	5' GGAATTCTTAAGCCACGCCCGT 3'
	Eco RI
JWF-103	5' GCAATTCTATGCAAAAAGACGCGCTGA 3'

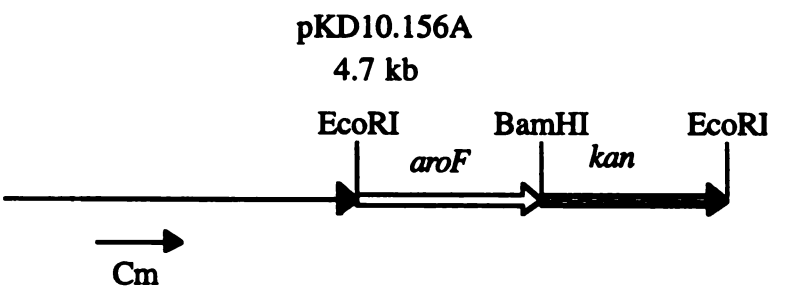


Figure 5 - Plasmid Map of pKD10.156A.

pKD10.186A (Figure 6), contained the synthetic cassette flanked by portions of the *serA* gene in a host vector containing a temperature-sensitive replicon. The plasmid was transformed into the host JC158, a serine auxotroph lacking *serA* activity. After transformation and plating, the colonies were then replicate plated onto M63/Cm plates with and without serine supplementation. The desired insertion inactivated the *serA* locus on the host cell's genome resulting in an inability of the cells to grow on minimal plates lacking serine supplementation.

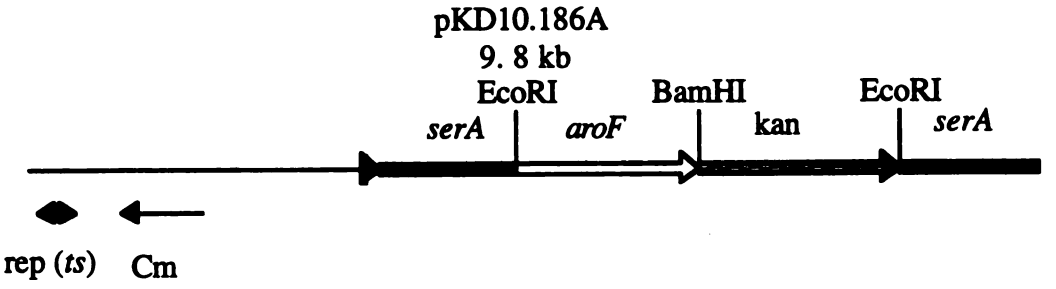


Figure 6 - Plasmid Map of pKD10.186A.

The synthetic cassette was inserted into the genome of AB3248 using homologous recombination into the genomic locus of *serA*. Competent AB3248 cells were transformed with pKD10.186A, and integration of the plasmid into the genome was selected for at 44 °C on LB plates containing chloramphenicol and kanamycin. The importance of the chloramphenicol resistance marker is two-fold. First, it confirms plasmid integration into the genome, and later chloramphenicol sensitivity was used to confirm that the plasmid had been excised from the genome. Only those colonies containing integrated plasmid DNA are able to grow in the presence of the antibiotics since the temperature-sensitive replicon in pKD10.186A does not allow replication at 44 °C. Eleven cointegrates were isolated in this manner after a series of transformations. Removal of the plasmid from the genome was

performed by growing cointegrates at 30 °C in LB medium without antibiotics. Two more cycles of growth were carried out at 30 °C by diluting cultures (1:20,000) into fresh LB medium without antibiotics. Growth of the cointegrate strain at a temperature permissive to plasmid replication (30 °C) creates an unstable environment for the integrated plasmid and a second spontaneous recombinational event occurs allowing the excision of the plasmid from the genome. The removal occurs such that the plasmid is either excised with its original synthetic cassette insert or with an intact *serA* sequence on the plasmid indicating that the cassette remained in the genome. Subsequent growth in liquid culture at 44 °C resulted in the loss of the excised plasmids from the progeny.

Colonies were finally selected for kanamycin resistance and chloramphenicol sensitivity at 44 °C to identify cells that retained the *kan* resistance and excised the *cm* plasmid marker from the genome. Fifteen such colonies originating from the same cointegrate exhibited the correct markers from which two were identified and characterized further. These two strains were designated AB2.23 and AB2.24. Plasmid DNA preparations confirmed that no plasmid DNA remained in the strains.

The strains were replicate plated on M63 plates with and without serine to verify that genomic insertion of the synthetic cassette disrupted the *serA* gene. The colonies were unable to grow without supplemented serine, signifying that site-specific insertion into *serA* had occurred.

UV Mutagenesis

The conditions for mutagenesis were adapted from the method by Miller.[21] The desired survival rate was 0.1 to 0.01 % to obtain efficient mutagenesis. Conditions giving the desired survival rate were determined by manipulating the distance of the culture from the UV source and the UV radiation exposure time. This was accomplished by growing AB2.24, followed by dilution and plating onto M63 plates lacking aromatic amino acid supplementation. The lack of aromatic amino acid supplementation was selected for cells that have a functional *aroF*. The plates were then exposed to the UV radiation (Sylvania

germicidal 8W lamp) for a duration of 0 (control), 5, 10, 15, 30, 60, 80, or 100 seconds immediately after plating. Two UV source distances were investigated: 18 and 24 inches. The optimal time, at a distance of 24 inches, was 10 seconds which resulted in a 0.6% survival rate (Table 2).

Table 2 - UV Radiation Survival.

UV Exposure Time (s)	Number of Colonies	Survial Rate (%)
0	169	100.0
5	75	44.4
10	1	0.6
15	3	1.8
30	2	1.2
60	1	0.6
80	0	0.0
100	0	0.0

In the DGC, the cells grew and migrated (by chemotaxis) through the agarose gel rather than only on the surface. As a confirmation that UV radiation would penetrate through the agarose gel, the UV absorbance of the gel was determined. The gel did not absorb significantly in the wavelength range of 200 to 800 nm.

Development and Use of the Diffusion Gradient Chamber (DGC) Method

Experiments using replicate plating to select for feedback-insensitive mutants after chemical mutagenesis resulted in false positives (growth in the presence of m-FT without a feedback-insensitive AroF). The selection was for growth on minimal plates in the

presence of m-FT. The false positives were believed to have resulted from enough overexpression of the feedback-sensitive AroF to allow for growth even in the presence of moderate levels of m-FT. The false positives greatly increased effort required to find the desired mutant.

One advantage of the DGC method is it allows for simultaneous selection and mutagenesis in the chamber, resulting in a significant reduction in effort to isolate the desired mutant. The DGC has other advantages over plate selection. The replicate plating assay compares growth of a single colony to growth of a single colony on a plate supplemented with m-FT. It is difficult to know in advance the optimal concentration of m-FT to use for selection using the plating technique. The growth of the sensitive AroF strains on the m-FT plates raised questions about the strength of the selection pressure provided by m-FT. The DGC allows for a range of m-FT tyrosine concentration to be applied simultaneously in the DGC. A second possible reason for the false positive phenomenon on plates is the fact that some cells uptake the m-FT leaving a zone lacking m-FT thereby allowing the m-FT-sensitive cells to grow. This would be avoided in the DGC since the m-FT gradient is continuously replenished throughout the experiment.

Another advantage is that differentiation between phenotypically (or possibly) genetically different mutants is possible in the DGC, based on their different sensitivities to m-FT. One mutation may allow for partial insensitivity to the inhibitor. Presumably the partially insensitive mutant would only be able to grow up to a certain distance towards the source of the m-FT. Beyond that point, the m-FT concentration would be to high for growth. A more desirable mutant that was completely insensitive to inhibition, however, could grow into the region of highest m-FT. The DGC may be able to select among many different mutations for the one with the highest m-FT insensitivity.

The DGC (Figure 7) is part of the diffusion gradient system commercially produced by Koh Development (Ann Arbor, Michigan). The chamber consists of an arena and the

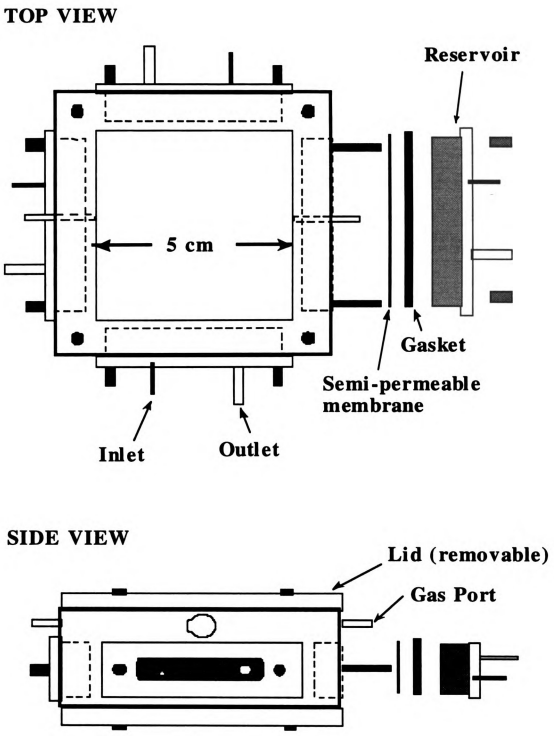


Figure 7 - Diagram of a Diffusion Gradient Chamber.

recesses that form the cavity of the four reservoirs. There is an opening between each recess and the arena. The reservoirs contain stainless steel inlet and outlet ports (1.0 and 2.5 mm diameters, respectively). The outlet port is higher than the inlet port to allow the reservoir to fill with liquid and release gas bubbles. The purpose of the larger outlet port diameter is to prevent back pressure of the outflowing liquid. The membrane allows for diffusion of small molecules from the reservoir into the gel, which is contained in the arena, and prevents organisms from moving from the gel into the reservoirs. The arena and the reservoirs are both machined from polycarbonate (PC). For reservoirs in use during the experiments, a 0.05 μM pore size PC filter membrane (Koh Development) and a gasket (on both sides of the membrane) were placed between each opening into the arena and the reservoir. The reservoirs not in use were sealed off from the arena with non-permeable silastic sheeting (Dow Corning Inc., Midland, Michigan). The reservoirs are secured to the DGC with thumb screws. The volume of each reservoir is 3 mL. The total volume of the arena, excluding the reservoirs, is 50 mL (5 cm x 5 cm x 2 cm). The lid and bottom plates are clear PC and are fastened by thumb screws. The entire system is sterilized by autoclaving prior to use.

The DGC was placed on a transilluminator box (TB) which can accommodate up to three DGCs. The TB contained two 30 cm fluorescent light fixtures (single 8W, cool white bulb). The inner walls of the TB were white and a piece of black felt was placed on the bottom and the sides of the TB to provide contrasts for the pictures. This design provided cool, diffuse, even illumination from beneath the DGC, which was essential for visualization of microbial growth patterns in the chamber. The light was turned off when not in use as to prevent heating of the DGC. The TB also had a bracket for mounting a camera above the DGC to record growth patterns photographically. The camera used was a PULNiX TM-7CN CCD-camera (Sunnyvale, CA). The pictures were taken using the programs Photofinish (Zsoft, Marietta, GA) and AutoCap. AutoCap written in the Worden

lab is the program responsible for recording the images of the DGC. Photofinish is a commercially available program to view and manipulate pictures.

To generate the gradient, solutes contained in Erlenmeyer flasks were continuously pumped through the reservoirs of the DGC. The flow rate of 2.5 mL h^{-1} was controlled with a dual channel peristaltic pump (LAB Bromma Microplex). An effluent chamber mounted on a stand next to the TB served three functions. Its height relative to the height of the DGC reservoir outlets regulated the back pressure in the chamber reservoirs. This was critical since excessive back pressure causes flooding of the gel due to bulk flow of liquid through the membrane. Insufficient back pressure causes shrinkage of the gel due to siphoning of the liquid. The effluent chamber also consolidated all of the reservoir outflows into one large waste flask as well as serving as a sterile break in the liquid flow.

DGC Chemotaxis Experiments

Chemotactic experiments in the DGC were carried out similarly to the experiments described in Emerson et al.[22] Because strain AB3248 had undergone several rounds of mutagenesis, we needed to prove that neither the chemotactic response nor motility were destroyed. A minimal salt medium (M63) was used for all DGC experiments. This medium was supplemented with 5 mM glycerol, and 40 mg L^{-1} of the amino acids histidine (H), isoleucine (I), proline (P), arginine (R), valine (V), and serine (S). Serine was supplemented in order to complement the serine auxotrophy that was created through the homologous recombination. The glycerol served as the carbon source for growth, but it is not a chemoattractant for *E. coli*.[23] The arena medium, which was stabilized with 0.15% agarose, was supplemented as described above and initially did not contain glucose. The low percentage of agarose provided enough strength for a stable gel matrix, but did not prevent movement of the cells through the gel. The sink reservoir (800 mL) contained supplemented M63 medium only. The source reservoir (800 mL) contained the supplemented M63 medium and was additionally supplemented with 5 mM glucose as a chemoattractant.[24] The sink and source reservoirs were on opposite sides of the DGC.

This arrangement created a 1-dimensional gradient that spanned 0 to 5 mM glucose from sink to source.

The first step was inoculation of the starter culture (5 mL of LB medium) with a single colony of AB2.24. The culture was then grown overnight at 37 °C with shaking in a water bath. Next, 1 mL of the starter culture was added to 100 mL of supplemented M63 medium containing 10 mM glycerol in a 250 mL Erlenmeyer flask. This culture was grown (37 °C and 250 rpm) to stationary phase (24 h, OD_{600} 3). Then four 1 mL aliquots were concentrated (4x) by microcentrifugation, combined and centrifuged again to give a final concentration of 16x. The center point of the DGC was inoculated with 15 µL of the 16x concentrated culture using a micropipette to disperse the cells evenly throughout the depth of the agarose as the pipette was withdrawn from the gel. The DGC and TB were set up in an approximately 30 °C warm room.

The flow of liquid through the reservoirs was started 6 h before inoculation to initiate the glucose gradient. Photographs were taken every 1 h for the 72 h length of the experiment. The photograph shown in Figure 8a (48 h) illustrates the chemotatic response of AB2.24 towards glucose. A clear bias of the *E. coli* growth towards the higher glucose concentration confirmed that AB2.24 is chemotatic towards glucose. Figure 8b is a computer image analysis of the relative light intensity (grey scale) of the Figure 8a centerline (vertical). In this figure, 0 mm is the glucose source reservoir (5 mM glucose) and the top of Figure 8a. The 50 mm position corresponds to the sink reservoir (0 mM glucose). Inoculation was at about position 23 mm. Significantly more cells moved towards the highest glucose concentration at 0 mm, as indicated by the light intensity. There was little chemotaxis towards the low concentration of glucose at the 50 mm position. This pattern is indicative of chemotaxis since the cell concentration is biased towards the glucose.

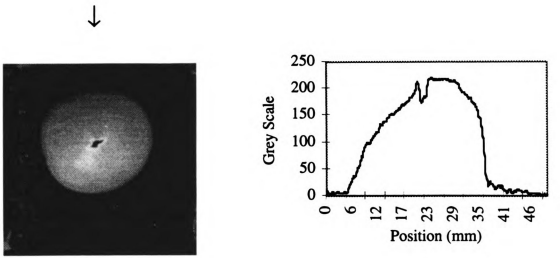


Figure 8 - a) Chemotaxis of AB2.24, Glucose Gradient Source at Top (↓); b) Image Analysis of Centerline.

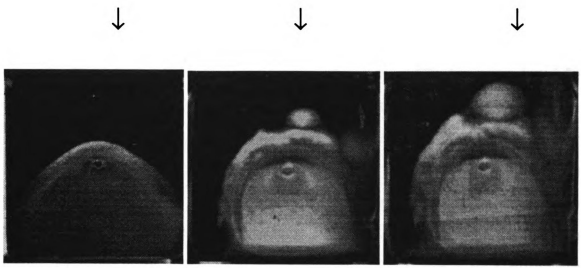
Generation and Isolation of Feedback-Insensitive Mutant

After the chemotatic response towards glucose was established, the DGC was used for mutagenesis and selection of a mutant containing feedback-insensitive AroF. The purpose of proving chemotaxis was to utilize chemotaxis to isolate feedback-insensitive mutants. Chemotaxis was used to draw the cells into higher glucose (and m-FT) concentrations, where only mutants with a feedback-insensitive AroF could survive.

The experimental setup was the same as for the previous DGC experiment, with one alteration. The glucose source reservoir now was also supplemented with 125 μM m-FT. This created an additional gradient, that spanned 0 to 125 μM m-FT from sink to source.

The cells were allowed to grow in the DGC for 3 days before mutagenesis was performed. This allowed for growth of a large initial pool of cells and establishment of the glucose and m-FT gradient. At the top of the picture (Figure 9a, 9b, and 9c) is the glucose source reservoir and therefore the highest glucose and m-FT concentrations in the DGC.

The m-FT clearly inhibited the cell growth prior to mutagenesis (Figure 9a). After the 3 days of initial growth the DGC lid was removed and the cells were exposed to UV radiation using the optimum conditions determined from the kill-curve experiments. The lid was quickly replaced, and, the cells were allowed to continue growth until the putative feedback-insensitive mutants had grown into the regions of high m-FT concentrations (Figures 9b and 9c). In Figure 9b, 7 days after mutagenesis, several "nodes" were observed growing into higher m-FT concentrations. The most predominant growth was from the central region and less distinctly is the growth on the left side. Also, there is a more diffuse growth on the right side growing into higher m-FT concentration. In Figure 10c, 9 days after mutagenesis (the conclusion of the experiment) the growths extended into still higher m-FT concentrations and had become more distinct. The difference in the contrasts of the pictures is an artifact of the camera.



a b c

Figure 9 - a) DGC Prior to Mutagenesis (3 days); b) DGC 7 Days after Mutagenesis; c) DGC 9 Days after Mutagenesis.

Confirmation of Feedback Insensitivity

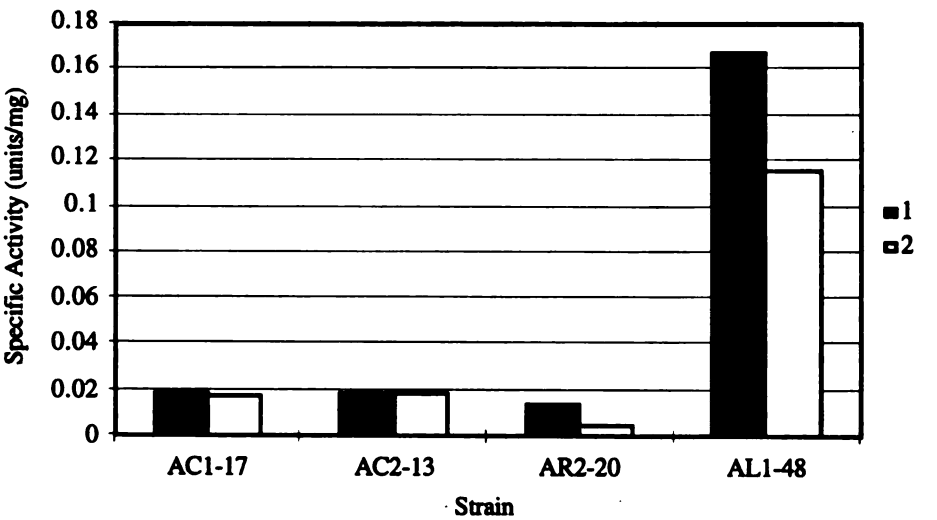
After the completion of the DGC experiment, cells samples were taken from the three regions showing growth in high m-FT concentrations by streaking onto LB/Kan plates and incubated at 37 °C for 10 h. Two methods of sampling were explored. In the first method a sterile wooden applicator was stuck into the gel and used to streak out colonies. In the second method, a micropipette was used to remove a plug of gel to streak the plates. Two plates were streaked, one by each method of sampling, for each of the three regions described above. This resulted in six plates of possible mutants.

After the growth on the plates, single colonies were selected and replicate plated onto M63/HIPVRS plates with 0, 30, and 150 μM m-FT and a LB/Kan master plate. The plating was performed in the listed order to assure that an adequate number of cells were introduced onto each plate. Since, if there were not a sufficient amount of cells on the applicator to plate onto all four plates, there would be no growth on the last plate (LB/Kan). Thus, the LB/kan plate was to assure that the lack of growth on any minimal plate was due to inhibition and was not a result of poor replicate plating. Colonies that grew well on either the 30 or 150 μM m-FT, as compared to the 0 μM m-FT plate, were tested for tyrosine insensitivity. Four colonies were tested for insensitivity and two were insensitive.

To assay for DAHP synthase specific activity colonies were taken from the LB/Kan master plates. The colony was removed with an applicator and first used to make a master plate of the colonies tested, then used to inoculate the starter culture, 50 mL LB/Kan in a 250 mL Erlenmeyer flask (37 °C and 250 rpm). After 10 h of growth, 5 mL of the starter culture was added to the growth flask consisting of 500 mL LB/Kan in a 2 L Erlenmeyer flask (37 °C, 250 rpm, 12 h). To harvest the culture for the enzyme assay, centrifugation of the culture (4000 rpm, 5 min 4 °C) was followed by resuspension of the cell pellet in the resuspension buffer. The cells were disrupted by two passes through a French Pressure cell (SML Aminco) at 11,000 psi. Cellular debris was removed from the lysate by centrifugation (20,000 rpm, 20 min, 4 °C). Protein was quantified using the Bradford

dye-binding procedure.[25] A standard curve depicting the absorbance at 595 nm versus protein concentration was prepared using bovine serum albumin. The protein assay solution (5x concentration) was purchased from Bio-Rad. The specific activity of *aroF* was quantified by the DAHP synthase/thiobarbituric acid (TBA) assay.[26] One unit of DAHP synthase activity was defined as 1 μmol of DAHP formed per minute.

To confirm insensitivity to tyrosine, the assay was performed first without tyrosine in the assay buffer to obtain the control specific activity. Next, the buffer was supplemented with tyrosine to a final concentration of 125 μM and the assay performed with another aliquot. From the test of four possible mutants, two were completely insensitive (AC1-17 and AC2-13), one was sensitive (AR2-20), and one was sensitive (AL1-48) but had much higher specific activity relative to the other three tested mutants (Figure 10). The two insensitive mutants were both from the central "node", while the



The assay buffer containing: (1) No tyrosine; (2) 125 μM tyrosine.

Figure 10 - Mutant Strain Tyrosine Insensitivity Assay.

sensitive strain (AR2-20) was from the diffuse growth on the right side. The mutant with higher activity was from the left "node" of the DGC. Mutant *aroF* (isolated from AC2-13) and wild-type *aroF* relative specific activities in the presence of tyrosine were compared. The mutant *aroF* was not inhibited by tyrosine concentrations as high as 330 μM (Figure 11), and may have even been somewhat stimulated by tyrosine, which was also reported for a feedback-insensitive AroF by Herrmann.[27]

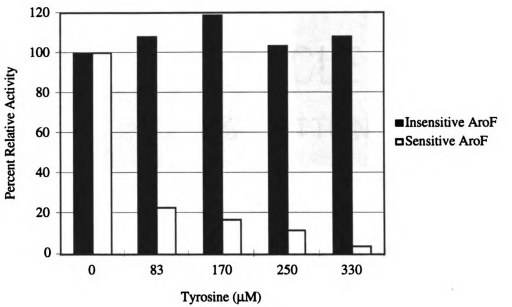
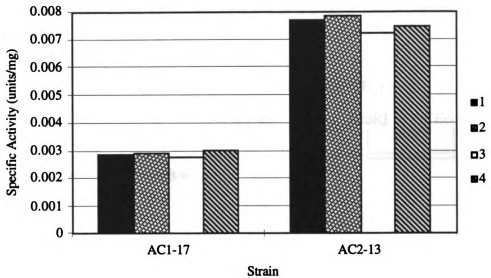


Figure 11 - Percent Relativity Activity.

Next, the strains were tested for inhibition by phenylalanine and tryptophan to ensure that the new mutation did not confer sensitivity to the other aromatic amino acids. The concentrations of phenylalanine and tryptophan used to detect inhibition were determined from the concentrations necessary to inhibit AroG and AroH, respectively. In cell free extract, AroG is 60% inhibited by 10 μM phenylalanine and AroH is 20% inhibited by 10 μM tryptophan.[28] A concentration of 100 μM of phenylalanine or tryptophan was used to ensure the detection of any sensitivity. The assay for sensitivity

was performed as before. A control assay was performed without added phenylalanine or tryptophan, and then the assay was performed in the presence of 100 μM phenylalanine or tryptophan. Neither AC1-17 nor AC2-13 was sensitive to phenylalanine or tryptophan (Figure 12).



The assay buffer containing: (1) No phenylalanine; (2) 100 μM phenylalanine; (3) No tryptophan; (4) 100 μM tryptophan.

Figure 12 - Phenylalanine and Tryptophan Sensitivity Test.

Isolation of the Feedback Insensitive *aroF*

The feedback-insensitive *aroF* (*aroF*^{fbr}) was then cloned from the genome. The cloning and isolation of *aroF*^{fbr} were conducted by Kai Li (Ph.D. student) and Dr. Karen Draths. The first step was the isolation of genomic DNA. The method used was modified from Silhavy.[29] Three genomic isolations were performed. One colony of AC1-17 and two colonies of AC2-13, designated AC2-13A and AC2-13B, were used. The second step was the amplification of *aroF*^{fbr} from the genomic DNA.

The PCR amplification unexpectedly had problems for unknown reasons. After several attempts, the *aroF*^{Tyr} gene was amplified from the genome using the primers JWF-19 and JWF-22 (Table 1) which have *Eco*RI ends. The PCR products were then ligated into the low copy number plasmid pCL1920 (spectinomycin (Spc) resistance-bearing plasmid) for sequencing. The three resulting plasmids, one from each genomic DNA isolation, were designated pCL1-17-1, pCL2-13A-1, and pCL2-13B-1 (Figure 13).

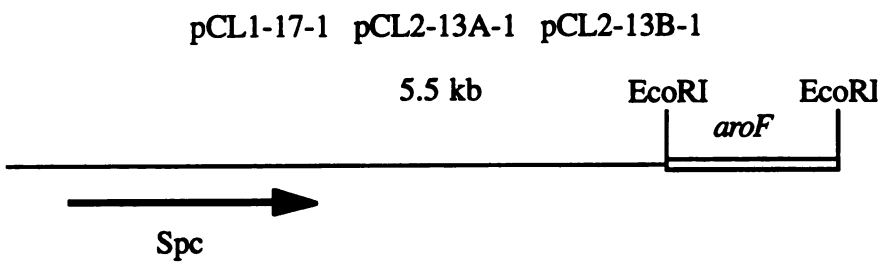


Figure 13 - Plasmid Map of Cloned Mutants.

Sequencing of the Feedback-Insensitive *aroF*

The polyethylene glycol precipitation method was used by Dr. Karen Draths to isolate the DNA fragment for sequencing.[30] The primer used for sequencing were JWF-22, JWF-79, JWF-80, JWF-81, and JWF-82 (Table 1). The primers and template were sent to the Molecular Sequencing Facility at Michigan State University for sequencing. The sequence of the *aroF*^{Tyr} was found to be different from the tyrosine sensitive *aroF* by a cytosine to a thymine base change. This base change causes a proline (residue 148) to leucine substitution conferring insensitivity. This is the same mutation that was obtained by Herrmann.[31] This base change introduced a *Bgl*II restriction site into the gene (Figure 14). Digestion of the three plasmids containing *aroF*^{Tyr} with both *Eco*RI and *Bgl*II confirmed the presence of the new restriction site.

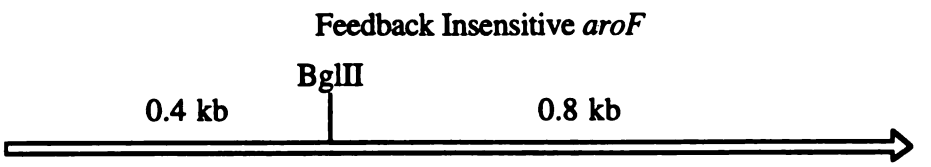
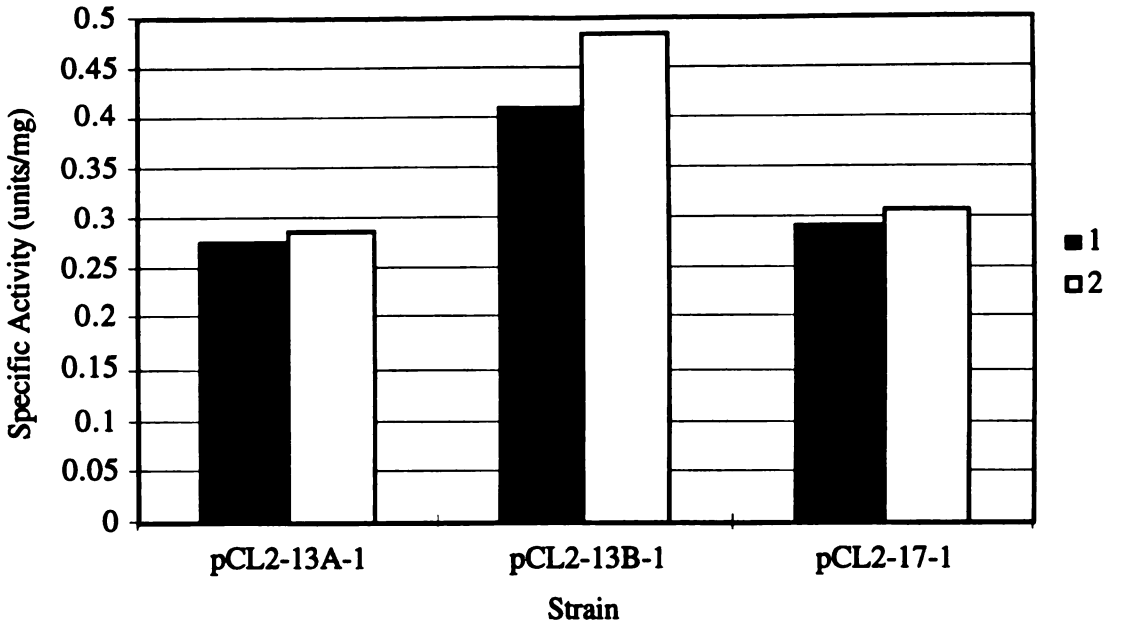


Figure 14 - Map of Feedback Insensitive *aroF*.

As stated by Herrmann,[32] this particular amino acid residue change is noteworthy. In tyrosine-sensitive AroF residue 148 is proline. The corresponding residue in AroG and AroH is methionine. Leucine and methionine are similar amino acids in respect to hydrophobicity and their effect on protein secondary structure. Residue 148 of AroF is in a region of little sequence homology to AroH. This region of AroH has been identified as a major part of the allosteric binding site. Changes of AroH amino acid residues flanking residue 148 have conferred insensitivity to tryptophan. The corresponding region in AroF appears to be involved in the allosteric binding site also. The variation of the sequence in this region between AroF and AroH are critical to the different allosteric binding pockets of the enzymes. It appears that AroH and AroF have similar domains that are critical to allosteric binding sites.

Feedback Insensitive AroF Specific Activity

The three plasmids containing feedback-insensitive *aroF* were each transformed into competent AB3248 in order to assay the specific activity of *aroF*^{fbr}. AB3248 does not possess any native DAHP synthase activity. The strains were grown as previously described for specific activity assays, except the medium contained spectinomycin (50 mg L^{-1}) for plasmid selection pressure. The activities are illustrated in Figure 15. The results show that PCR did not alter feedback insensitivity.



The assay buffer containing: (1) No tyrosine; (2) 125 μM tyrosine.

Figure 15 - Cloned AroF Specific Activity.

The next genetic manipulation was the removal of *tyrR* boxes in an attempt to eliminate or reduce transcriptional repression by tyrosine (Figure 16). A similar approach was used in the production of phenylalanine, whereby the entire native promoter was removed and replaced with a temperature-sensitive promoter.[33] For our purpose, all of *tyrR* Box 3 and half of *tyrR* Box 2 were eliminated by PCR amplification of *aroF*^{tyr} from pCL2-13A using the primers JWF-94 and JWF-22 (Table 1). The PCR product was cloned into pCL1920 generating the new plasmid pCL2-13A-trunc (Figure 17), which was transformed into competent AB3248.

The two constructs were assayed for DAHP synthase activity using the previously described conditions. The results are illustrated in Figure 18. Activity of the truncated *aroF*^{tyr} was lower. The lower activity of the truncated *aroF* was unexpected. It was

thought that the specific activity of the truncated *aroF* would either be the same or better then the nontruncated *aroF* because only the regulatory sequence of the gene was changed.

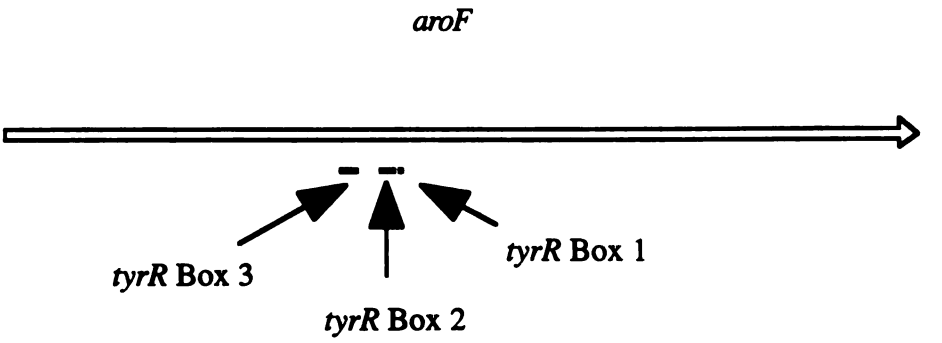


Figure 16 - Map of *aroF tyrR* Boxes.

The AroF specific activity was then assessed during shake flask experiments under conditions used to accumulate DHS. This was done to better simulate the actual growth conditions used to produce DHS. The experiment was performed for both AB3248/pCL2-13A (as a control) and AB3248/pCL2-13A-trunc. A single colony was used to inoculate 50 mL of LB in a 250 mL Erlenmeyer flask. After growth for 10 h (37 °C and 250 rpm), 5 mL aliquots of this starter culture were used to inoculate, in triplicate, 500 mL of supplemented M9 in a 2 L flask. The carbon source was 10 g L^{-1} (56 mM) glucose, and supplemented with was 40 mg L^{-1} phenylalanine, tyrosine, and tryptophan each, and 10 mg L^{-1} *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid. These accumulation cultures were grown for 36 h (37 °C and 250 rpm). At 12, 24, and 36 h, one culture of each construct was harvested for the enzyme assay. The results are summarized in Figure 19. Under these conditions the specific activity of the truncated *aroF*^{tr} is higher. Since the trend is the opposite to that shown in Figure 18, it appears that the AroF specific activity is dependent on the growth conditions. The truncated and nontruncated AroF

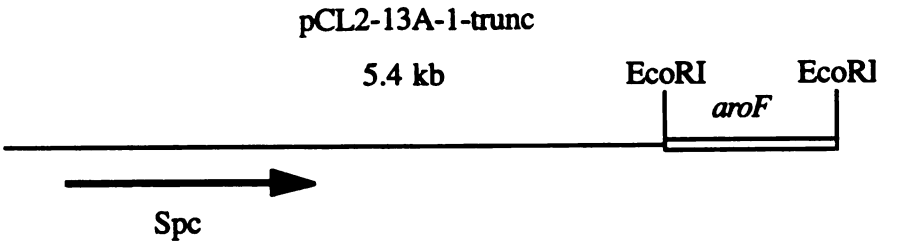
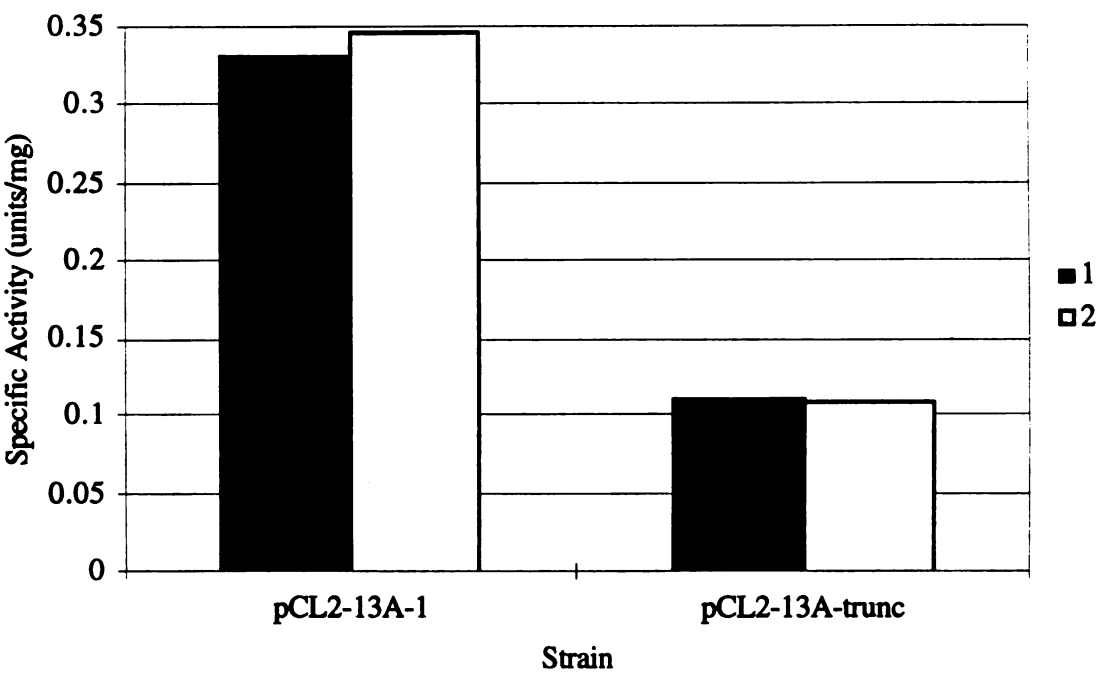


Figure 17 - Plasmid Map of pCL2-13A-trunc.



The assay buffer containing: (1) No tyrosine; (2) 125 μM tyrosine.

Figure 18 - Nontruncated and Truncated AroF Specific Activity.

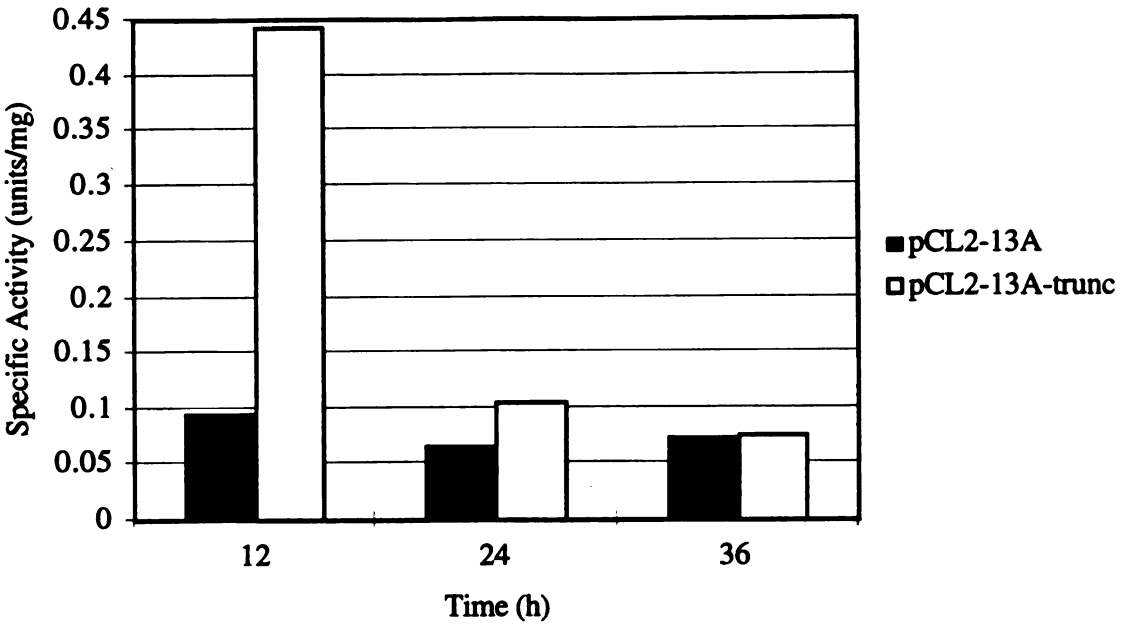


Figure 19 - Minimal Medium Nontruncated and Truncated AroF Activity.

specific activities both decreased with time. This trend has been reported before for the *aroF* isozyme of DAHP synthase[34] and it has been suggested[35] to be the result of selected proteolysis of the *aroF* isozyme during stationary phase.

The plasmid vector was subsequently changed, so that the *aroF*^{Tbr} could be used in a host/plasmid system and grown under conditions more similar to the fermentor conditions. This would also allow for monitoring of DHS production to investigate the relationship between AroF specific activity and DHS production. DHS was quantified by ^{1}H NMR analysis which is described in Chapter 3 (p 71). Both the *aroF*^{Tbr} and truncated *aroF*^{Tbr} were cloned into the plasmid vector pSU18. The truncated *aroF*^{Tbr} was only obtained in the opposite direction of the *lac* promoter that is on the pSU18 plasmid vector, and this new plasmid was designated pKL4.19B. The nontruncated *aroF*^{Tbr} was obtained in both orientations with respect to the *lac* promoter yielding plasmids pKL4.20A and pKL4.20B. Subsequently the *serA* locus was cloned into pKL4.19B and pKL4.20B creating

pKL4.33B and pKL4.32B, respectively (Figure 20). The *serA* was added to complement the lack of *serA* activity in the host. Kai Li used the same homologous recombination method described previously (p 23) to create a new host to use for DHS accumulation. The original strain was AB2834, and the new host was designated KL3. AB2834 and KL3 lack shikimate dehydrogenase (*aroE*) activity and therefore produce DHS. KL3 also carries an additional copy of *aroB* (encoded dehydroquinate synthase) inserted into *serA* locus of the host. This allowed for enough amplification of *aroB* to ameliorate the rate-limiting character of DHQ synthase. A nutritional requirement was used, by localization of *aroB* onto the plasmid, to maintain the plasmid instead of an antibiotic resistance. The method of growth was similar to the previous experiment. An important difference was that the starter culture medium was the same as the accumulation medium (supplemented M9). This required the starter culture to be grown for 24 h to reach the same optical density (OD) obtained when LB medium was used as the starter culture. The two resulting accumulation

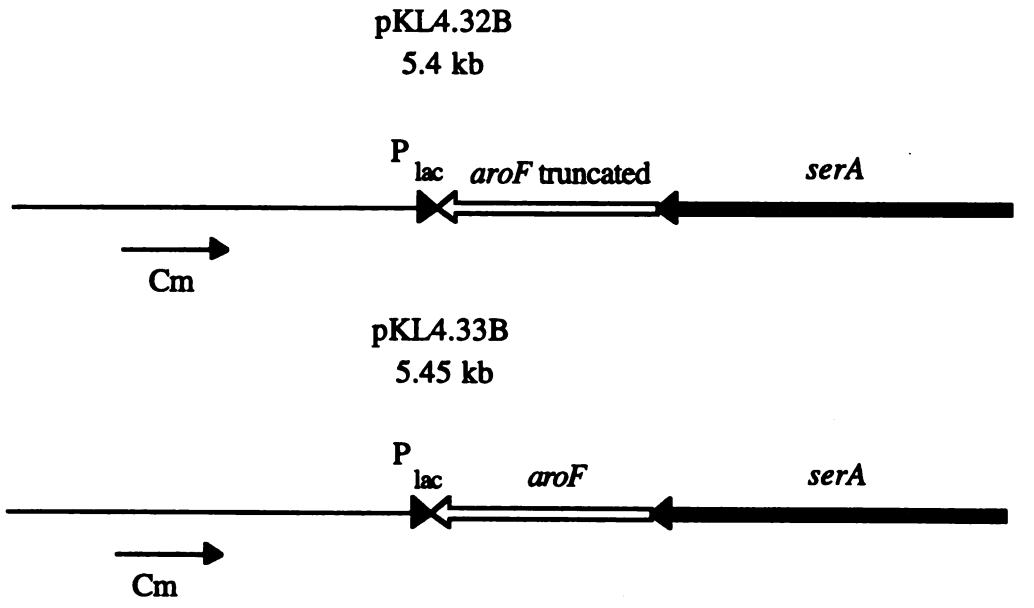


Figure 20 - Plasmid Map of pKL4.32B and pKL4.33B.

cultures were assayed for DHS production and AroF activity. The results are shown in Figure 21. The truncated *aroF*^{Tr} construct also was evaluated in the fermentor. These studies are summarized in Chapter 3 (p 79). At this point in the development of the microbial catalyst the truncated form of *aroF*^{Tr} was abandoned because of low specific activity and negligible DHS production in both shake flask and fermentor experiments.

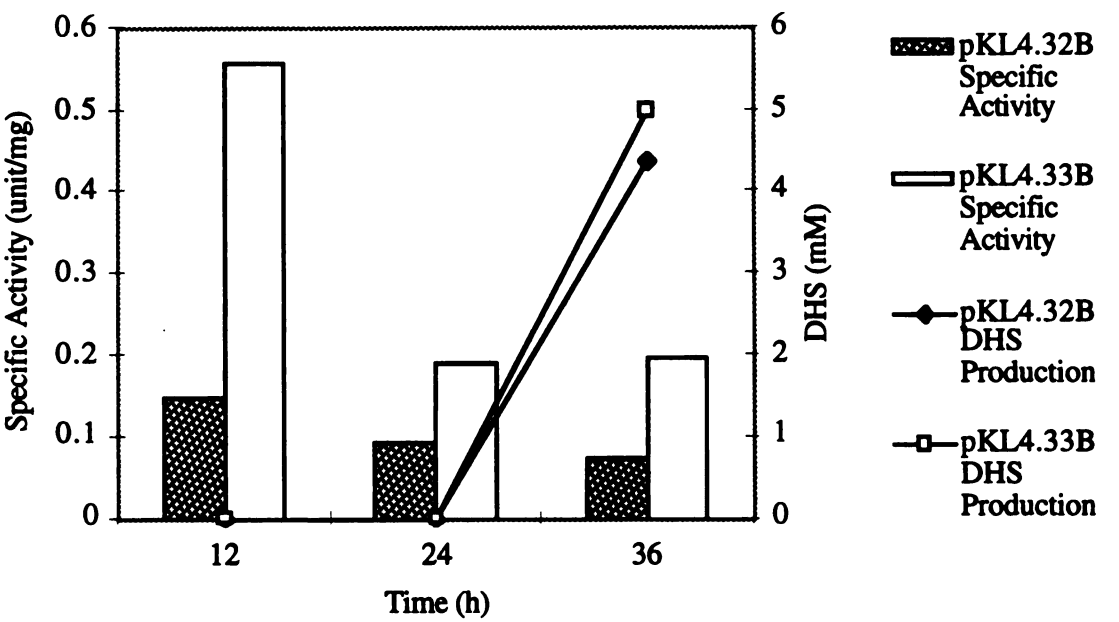


Figure 21 - Truncated/Nontruncated AroF Activity and DHS Production

To increase AroF activity, two more genetic approaches were tried. First, a second copy of *aroF*^{Tr} was inserted into pKL4.33B. The second *aroF*^{Tr} fragment had been PCR amplified with *xba*2 ends that were suitable for ligation into pKL4.33B. The two new plasmids were generated because of the two possible orientations of the insert and were designated pKL4.66A and pKL4.66B (Figure 22). This approach was used because Michael Farabaugh, of the Frost group, had previously seen a significant increase in AroF

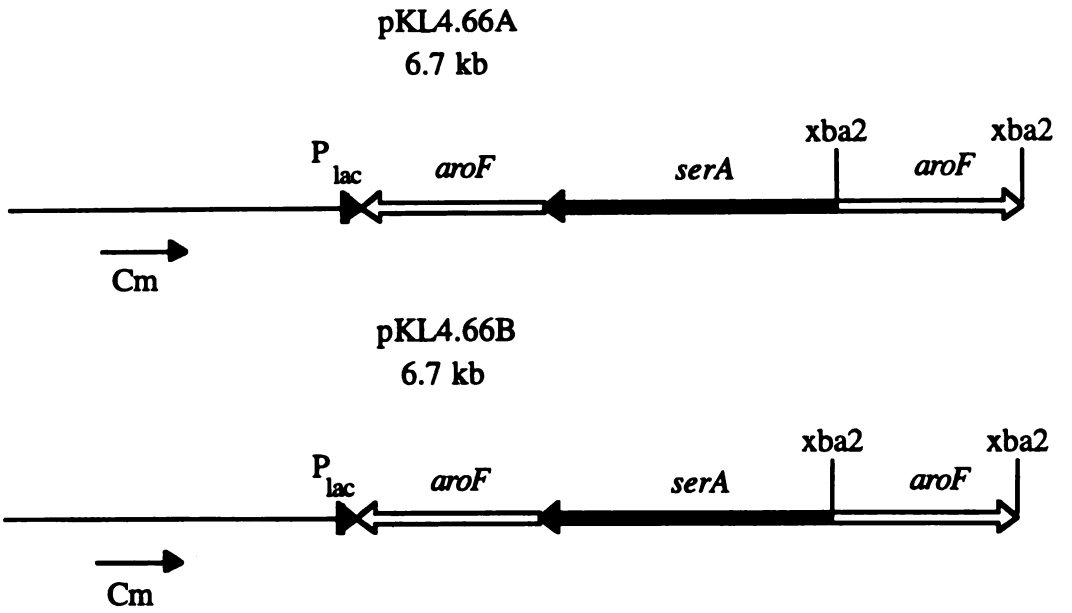


Figure 22 - Plasmid Maps of pKL4.66A and pKL4.66B.

activity when his constructs had two copies of *aroF* on a plasmid (unpublished data). The growth and enzyme assay conditions were the same as for the last experiment. The enzyme activities and DHS production for the two constructs are shown in Figure 23 (the supernatant was only analyzed at 24 and 36 h). The difference in DHS production indicates that there may be an effect due to the genes' orientations, although the effect might not be the same in the fermentor due to the differences in growth conditions.

The second approach was the complete removal of the native promoter and ribosome binding site (RBS). The inducible *tac* promoter[36] was used instead used to control *aroF* expression. The repressor protein, encoded by *lacI*^{q}, binds to the *tac* promoter region preventing transcription of the gene. The inducer isopropyl β-D-thiogalactopyranoside (IPTG) binds to the product of *lacI*^{q}, inactivating the repressor.

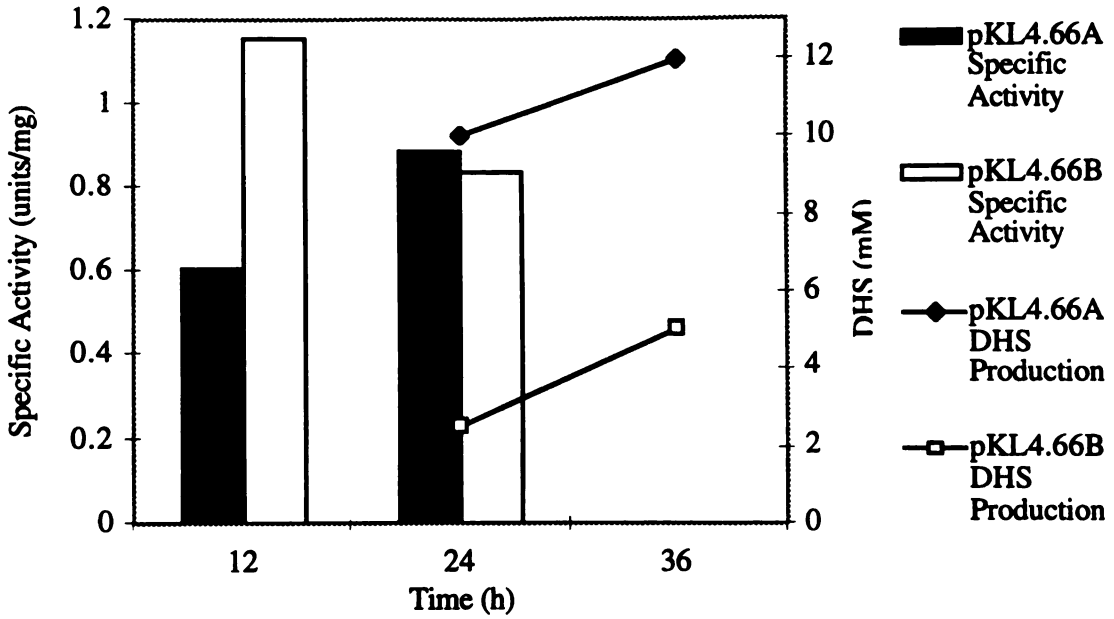


Figure 23 - Specific Activity and DHS Production of pKL4.66A/B.

Only the open reading frame of *aroF*^{fbr} was PCR amplified using primers JWF-103 and JWF-97 (Table 1). The *serA* locus and newly generated *aroF*^{fbr} fragment were cloned into pBR322, which contains the *tac* promoter, *tac* repressor *lacI*^{q}, and ampicillin resistance gene, generating pKL4.79B (Figure 24). The *aroF* fragment was cloned such that the *tac* promoter and RBS of pBR322 were in the correct location and orientation to express the gene. Three cultures were grown in triplicate using the same conditions and host (KL3) as previously used. Seven hours after inoculating the accumulation cultures, IPTG was added. To the first three flasks no IPTG was added, to the second set of three flasks IPTG was added to a final concentration of 0.05 mM, and to the remaining flasks IPTG was added to a final concentration of 0.05 mM. One flask of each of the three culture types (0, 0.05, and 0.5 mM IPTG) was harvested for the enzyme assay at 10, 21, and 44 h, and the supernatant was analyzed for DHS. The results are summarized in Figure 25. The induction of the expression of *aroF* appears to be time-dependent since the highest specific

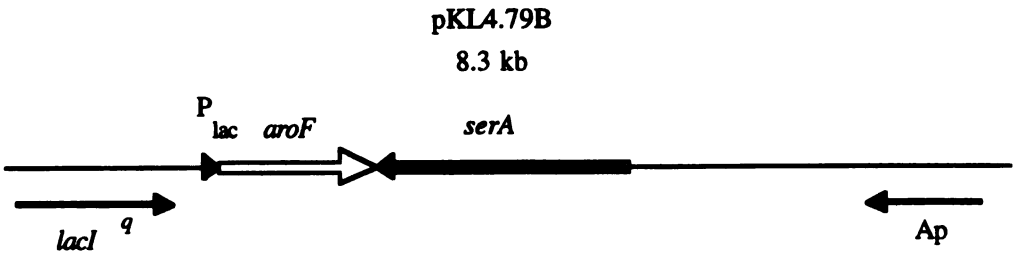


Figure 24 - Plasmid Map of pKL4.79B.

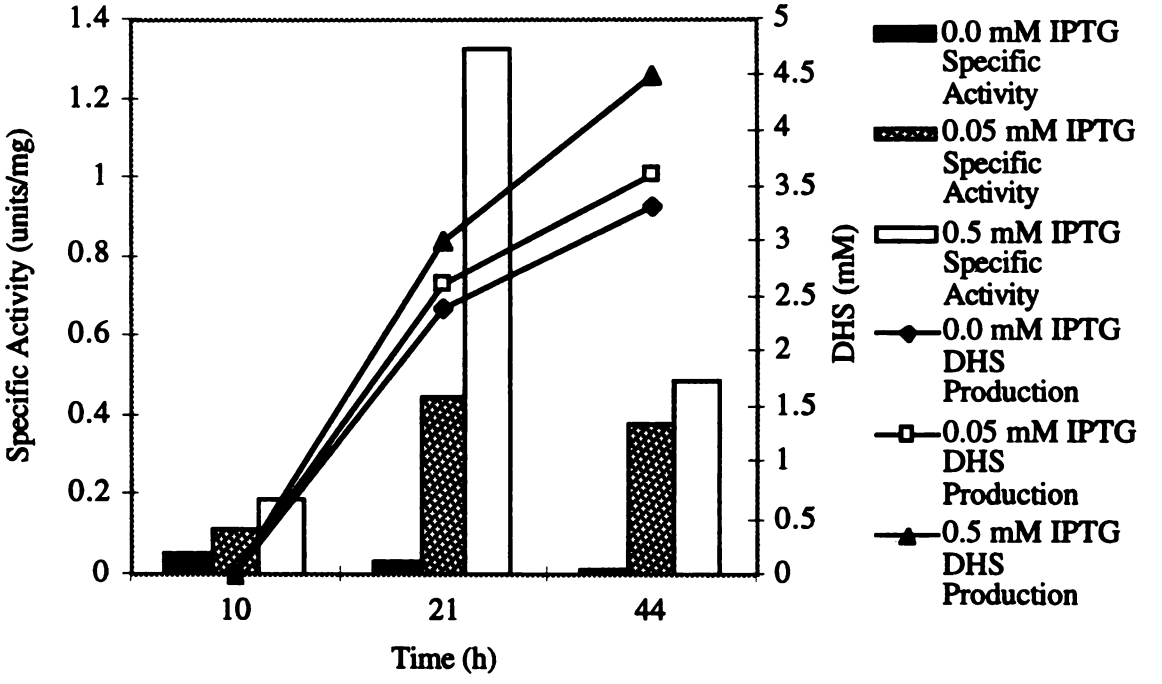


Figure 25 - AroF Specific Activity and DHS Production of pKL4.79B.

activity occurred 14 h after the addition of IPTG. The 0.05 mM IPTG culture had the highest specific activity. Again, the characteristic loss of specific activity was seen over the 48 h duration of the experiments. No DHS was produced in the 0 mM IPTG flasks, which suggests that the expression of *aroF* was tightly controlled. The 0.05 and 0.5 mM IPTG flasks produced about the same amount of DHS indicating that the difference in specific activity under these conditions had little effect on DHS production.

Discussion and Conclusion

The DGC experiment and related strain development resulted in a feedback-insensitive DAHP synthase (*aroF*^{fbr}). The mutation that conferred insensitivity was one base pair change that resulted in a proline (residue 148) to leucine substitution. The sequence of the *aroF*^{fbr} is different from the tyrosine-sensitive *aroF* by the a cytosine to a thymine base change. The single base change also introduces a *Bgl*II restriction site into the *aroF*^{fbr} gene which, upon digestion, yields 0.4 and 0.9 kb fragments. The characterization of *aroF*^{fbr} demonstrated that this isozyme was insensitive to feedback inhibition by tyrosine up to a concentration of 330 μM tyrosine. A slight stimulatory effect in the presence of tyrosine was observed for the *aroF*^{fbr}.

The novel, in-situ mutagenesis and selection approach demonstrated in the DGC is generic and can be applied to many other biological systems. The DGC method is a new approach to develop and isolate mutants. It is based on microbial chemotaxis and selection with respect to an inhibitory compound.

As stated by Herrmann, this particular amino acid residue change in *aroF*^{fbr} is noteworthy. In the tyrosine-sensitive AroF, residue 148 is proline, while the corresponding residue in AroG and AroH is methionine. Leucine and methionine are similar amino acids in respect to hydrophobicity and their effect on protein secondary structure. Residue 148 of AroF is in a region of little sequence homology to AroH. This region of AroH has been identified as an allosteric binding site. Changes of AroH amino acid residues flanking residue 148 have conferred insensitivity to tryptophan. The

corresponding region in AroF also appears to be involved in allosteric binding resulting in feedback inhibition.

Subsequent experiments evaluated the specific activity of *aroF*^{Tbr} under several different growth conditions. All growth conditions resulted in the decay of AroF activity over 48 h, and this has been previously reported for the native *aroF* isozyme of DAHP synthase.[37] This trend has been suggested to be the result of selected proteolysis of AroF during stationary phase.[38] The relative magnitude of the specific activity varied with the culture conditions, but the decay of activity was not avoided. To overcome this decay of activity, a *tac* promoter was used. At certain concentrations of the inducer IPTG, the AroF absolute specific activity was comparatively higher than previous experiments that did not use the *tac* promoter. Nevertheless, AroF specific activity still declined over time. It also did not translate into better DHS production.

The specific activity of *aroF*^{Tbr} varied greatly according to the growth conditions. This effect was very profound when comparing truncated and nontruncated *aroF*^{Tbr} specific activity. When grown in LB, the nontruncated *aroF*^{Tbr} had higher specific activity relative to the truncated *aroF*^{Tbr}. The lower activity of the truncated *aroF* was unexpected since it was thought that the specific activity of the truncated *aroF* would either be the same or better then the nontruncated *aroF* under these growth conditions. It was thought that removing the *tyrR* boxes of *aroF*^{Tbr} would not adversely affect expression since only the genetic sequence responsible for repression was removed. This relationship was reversed when the hosts were grown exclusively in minimal medium. The nontruncated *aroF*^{Tbr} specific activity decreased by more than an order of magnitude when grown entirely in minimal medium as compared to using LB as the starter culture medium. Another reversal of relative specific activities occurred after the host was changed from AB3248 to KL3 and the vector was changed from pCL1920 to pSU18. This change also necessitated insertion of a *serA* locus into the plasmid. Eventually, the truncated version was abandoned because

constructs that contained the truncated version did not produce DHS in the fermentor (Chapter 3, p 80), and had low activity in the shake flask.

DHS production was quantified to correlate the AroF activity with increased carbon flow directed into the common pathway of aromatic amino acid biosynthesis. Since the truncated *aroF* did not produce DHS, it appears that the promoter may have been affected when removing the *tyrR* boxes. The second *tyrR* box is near the ribosome binding site, and this may be responsible for the lack of DHS production.

The data obtained in shake flasks on the correlation between DHS production and DAHP synthase activity is of limited use. For consistent results DHS-synthesizing constructs need to be grown in a fermentor. These results did indicate that the level of DHS production varied with culture conditions. Since commercial fermentations are performed in stirred-tank fermentors, subsequent experiments were carried out in a 1 L, computer controlled stirred-tank fermentor. Chapter 3 describes studies of the medium components, fermentor conditions, and *aroF*^{fbr} activity of cultures grown in the fermentor. The studies provide insight into the relationship between DAHP synthase activity and DHS production under conditions which are more industrially relevant.

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CHAPTER 3

FERMENTOR PRODUCTION OF DHS

Fermentor Advantages

Significant work has been performed towards the optimization of fermentations to produce phenylalanine and tryptophan in *E. coli*.[1] This previous work would serve as a starting point for optimization of DHS production in the fermentor. At the laboratory scale, fermentors provide a significant advantage over shake flasks. The fermentor can control and manipulate many variables on-line, such as temperature which allows for temperature-dependent expression systems to be used, pH, dissolved oxygen, and addition of the carbon source. Through computer usage, it is also possible to have a variety of complex control algorithms calculated from on-line data. One significant advantage is the greater aeration provided by the air sparger and the impeller, which is necessary for aerobic growth of high density *E. coli* cultures.[2] Due to the increased aeration, much higher cell densities and growth rates are typically achieved in the fermentor than shake flasks.

There are also many operating advantages in utilizing a fermentor. Dissolved oxygen can be controlled by the impeller speed and or air flow rate in the Braun fermentor. A better degree of mixing is achieved by the impeller and baffles. In high density culture, foaming is a significant problem, but it can be controlled by a foam sensor and computer-controlled additions of antifoam. Samples can be removed under sterile conditions using the harvest pipe. On-line monitoring and electronic storage allows for data recording and retrieval of essential parameters.

The topic of this chapter is the optimization of DHS production in a fermentor through both genetic and reaction engineering. The first parameter optimized DHS titer, which resulted in the highest yield. The independent variables included the components and concentration of the starter culture and fermentor medium, dissolved oxygen, and pH. Genetically modified constructs were evaluated to obtain information relating the genetic changes of the microbial catalyst to DHS production. The feedback-insensitive *aroF* generated using the diffusion gradient chamber was used and tested in fermentations. Investigations included the relationship between DAHP synthase activity and DHS production using the *tac* promoter and repressor $lacI^q$ to manipulate the DAHP synthase activity by transcriptional control. DAHP synthase activity was also investigated by exploitation of the gene orientation with respect to a promoter and the number of gene copies on the plasmid.

Biosynthetic Pathways for DHS Production

Pathways required for biosynthetic production of DHS include, glycolysis, the pentose phosphate pathway, and the common pathway for aromatic amino acid biosynthesis (Figure 26). The TCA cycle is also involved because of its importance as a major sink for the pyruvate that is produced by the phosphorotransferase system (PTS). Glycolysis produces phosphoenolpyruvate (PEP) which is one of the two substrates required for DAHP (3-deoxy-D-*arabino*-heptulosonate 7-phosphate) synthase. The pentose phosphate pathway generates the second precursor erythrose 4-phosphate (E4P), which is the second substrate required by DAHP synthase. DAHP synthase is the first enzyme of the common pathway of aromatic amino acid biosynthesis.

The production of PEP via glucose metabolism leads to a competition between the PEP-requiring biosynthetic steps and the PEP-dependent PTS for glucose uptake (Chapter 1, p 6).[3] The utilization of the PTS is an important aspect in the metabolic model of DHS production.

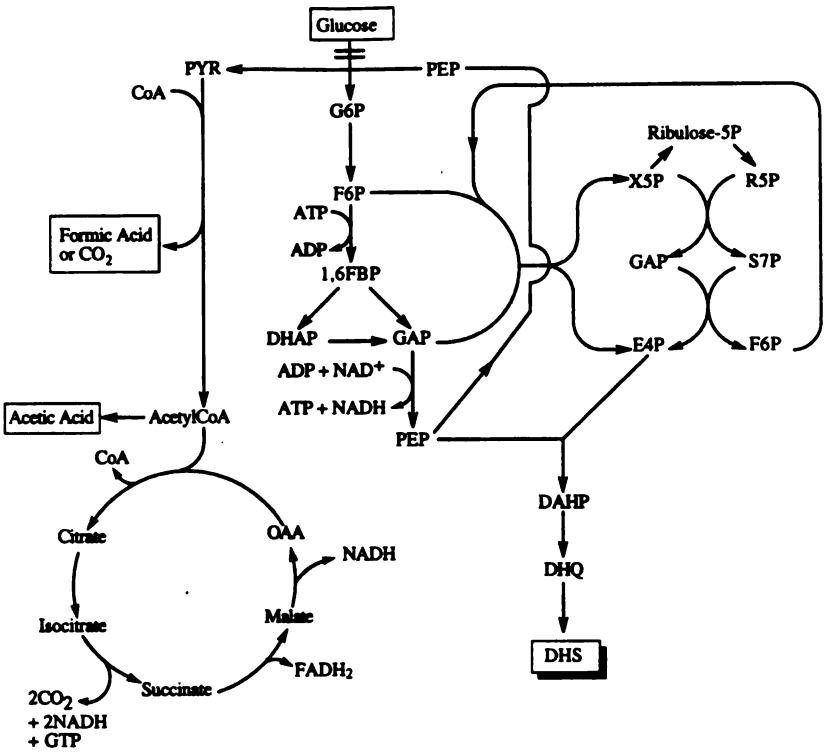


Figure 26 - Biosynthetic Pathways for DHS Production.

The pentose phosphate pathway connects glycolysis with several biosynthetic pathways. It consists of an oxidative and non-oxidative branch. Two enzymes of the non-oxidative branch, transketolase and transaldolase, work in concert to produce E4P.

The first step committed in the common pathway is the condensation between E4P and PEP to form DAHP. This reaction is catalyzed by three DAHP synthase isozymes that are encoded by *aroF*, *aroG*, and *aroH*, which are sensitive to feedback inhibition by L-tyrosine, L-phenylalanine, and L-tryptophan, respectively.[4] At the repressor-controlled level transcriptional level, *aroF* and *aroG* are repressed by the TyrR protein and *aroH* is repressed by the TrpR protein.[5] The second enzyme of the common pathway is DHQ synthase, which is encoded by the *aroB* locus. DHQ and inorganic phosphate are formed from DAHP in this NAD-requiring reaction catalyzed by AroB. The third enzyme of the pathway is DHQ dehydratase, encoded by *aroD*. It catalyzes the dehydration of DHQ

which results in DHS formation. Both AroB and AroD enzymes are constitutively expressed and are not sensitive to feedback inhibition mediated by any of the aromatic amino acids or intermediate metabolites of the common pathway.[6]

The last common pathway enzyme that is important to DHS production is shikimate dehydrogenase (*aroE*). This enzyme catalyzes the reduction of DHS to shikimic acid and requires NADPH. *E. coli* strain AB2834 and its derivative KL3 lack shikimate dehydrogenase activity and therefore cannot further metabolize DHS.[7]

During fermentation, there are often unwanted byproducts generated such as acetic and formic acid. Typically these result from either glucose overfeeding or anaerobic growth.[8] Acetic acid is inhibitory to growth. The presence of acetic acid will reduce the fermentation productivity, so avoidance of its accumulation is desirable. The production of either acid also constitutes an undesirable loss of carbon from the desired pathways. If the aeration of the culture is insufficient acetic acid and sometimes formic acid are formed. Formic acid production is indicative of severely anaerobic conditions, since the enzyme pyruvate-formate-lyase catalyzes the production of formic acid is irreversible and rapidly inactivated in the presence of oxygen.[9] Acetate can also be produced because of excessively high glucose concentration. If glucose is overfed, the TCA cycle and other biosynthetic pathways can no longer consume all the pyruvate that is being produced from glycolysis, and the extra pyruvate is converted into acetate and exported from the cell.[10] Knowledge of conditions promoting acetic acid and formic acid formation allows optimal fermentor conditions to be chosen to avoid their production.

In Figure 27 (only the pathways necessary for DHS production are shown) the numbers next to the reaction arrows represent the maximum theoretical flux distribution, as calculated by metabolic control analysis, for DHS production.[11] From these calculations, 24 moles of DHS are produced from 56 moles of glucose consumed. Therefore, the theoretical maximum molar yield of DHS from glucose is 43%.

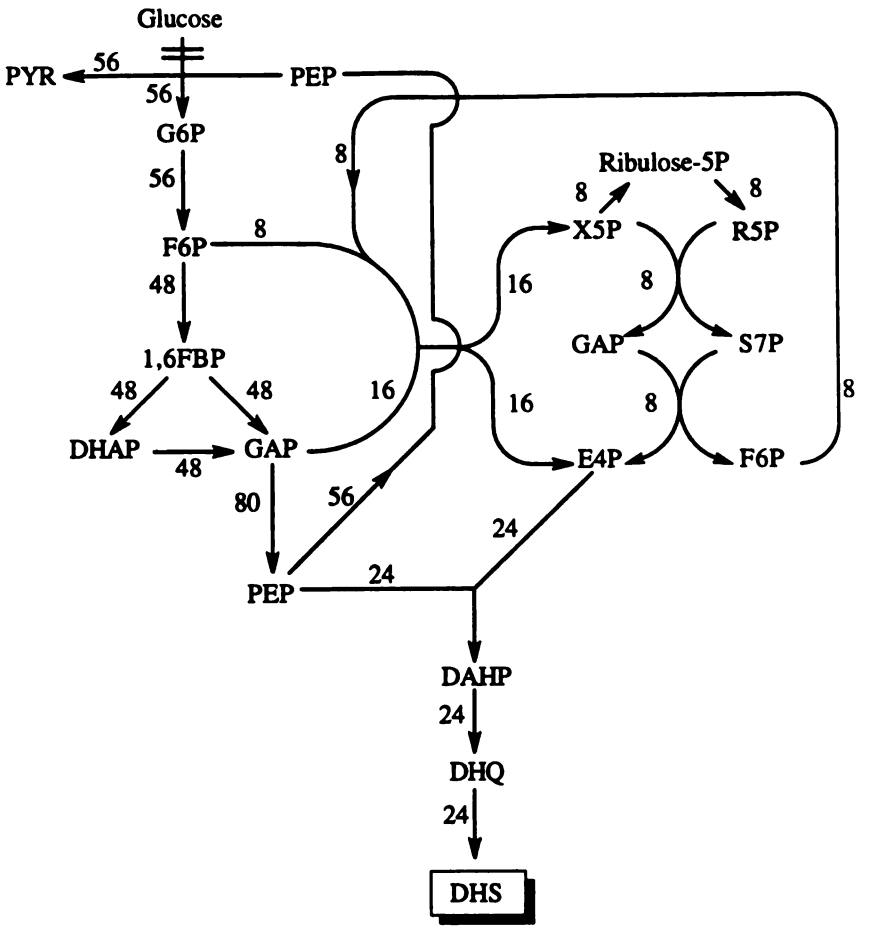


Figure 27 - Flux Distribution for Maximum DHS Yield.

Optimization Strategy for DHS Production

To exploit the advantages of biocatalysis for large scale production a strategy must be developed incorporating both industrial requirements and knowledge of the effect of environmental conditions on DHS production. The goal is the efficient and high-yielding conversion of D-glucose to DHS. It required genetic optimization of both microbial catalyst by genetic modification and the optimization of fermentation conditions.

To optimize the microbial catalyst, there are various important aspects for DHS production. An important strategy on the optimization is identification of the rate-limiting enzymes and enzymatic regulatory mechanisms. Both central and peripheral metabolism

must be understood in its relation to DHS production, such as the PTS.[12] Some of the genetic modifications must be evaluated in a fermentor since the physiology of *E. coli* varies greatly under differing growth conditions. Fermentor conditions and microbial constructs were simultaneously evaluated and optimized. The fermentor conditions investigated for optimization included, the starter culture medium, fermentor medium components, glucose feeding rate, amino acid supplementation, and aeration.

Development of a Microbial Catalyst

The necessary metabolic alterations necessary for DHS production were detailed in Chapter 1 (p 9). To review the key points of the first chapter, both *aroF* and *aroB* need to be overexpressed. The overexpression of *aroF* is accomplished by insertion of the *aroF* locus on a multicopy plasmid. The *aroB* locus was inserted into the genomic copy of *serA* in the strain AB2834, creating KL3. The insertion of *aroB* into the genome served an additional purpose besides overexpression, it was also used for plasmid maintenance, described below.

Development of a New Host

A nutritional requirement for plasmid maintenance was used in the development of a new host strain. The starting host strain was AB2834 which produces DHS. The methodology used for the generation of AB2.24 for mutagenesis was applied to the generation of the new host for DHS production. This time the homologous recombination into the genomic copy or *serA* was an *aroB* cassette. The inactivation of *serA* generated a new strain designated KL3. For plasmid maintenance, all plasmids transformed into KL3 had the *serA* locus. KL3 containing *serA*-encoding plasmids were cultured in minimal medium lacking supplementation. When KL3 was cultured in rich medium such as Luria Broth (LB), chloramphenicol or ampicillin was added to the medium for plasmid selection pressure.

B. Braun Fermentor

The fermentor utilized was a B. Braun Biotech Biostat® MD (Figure 28). The fermentor was equipped with a platinum thermister temperature probe, a polarizable dissolved oxygen probe, a pH probe, and an antifoam probe. It was equipped with three six-bladed disk impellers. The temperature was maintained at the setpoint by the circulation of either cooled or heated water through the jacket of the vessel. The vessel was sterilized at 121 °C for 25 min with the medium inside. The fermentor vessel was connected to a distributed control unit (DCU) for control. The DCU unit was interfaced to a Compaq personal computer running the Braun fermentor control software MFCS. The fermentor could be controlled by either the DCU or the MFCS software, but only when using the software would the data be recorded.

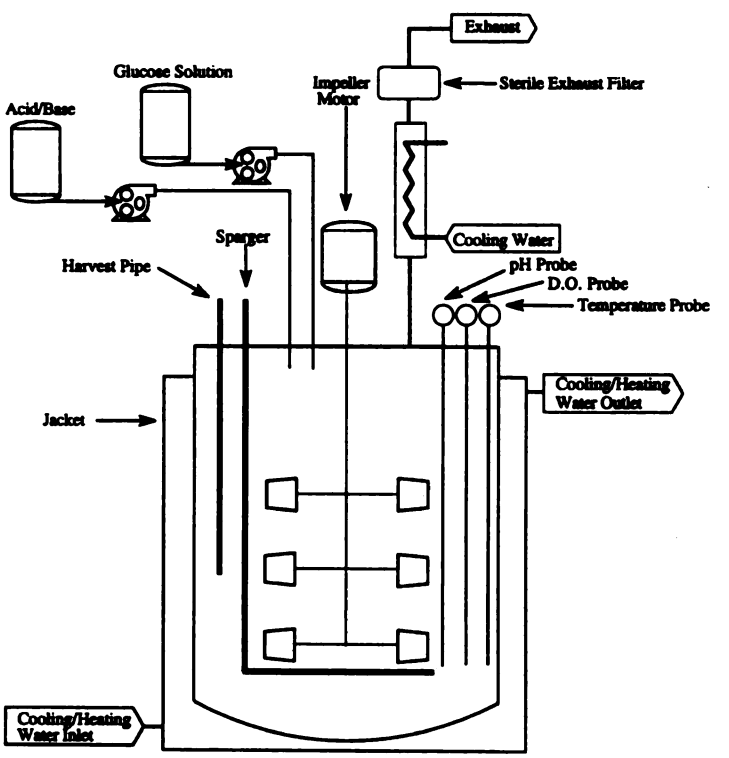


Figure 28 - Braun Fermentor.

The temperature, pH, and glucose feeding were controlled with a PID algorithm. Only the glucose feeding control constants were changed from the preset values from Braun for fermentations. The acid and base were added with peristaltic pumps that were part of the DCU assembly. The glucose feed was supplied by an external pump connected to the DCU.

Samples were removed from the vessel via the harvest pipe. There were 2 outlets for the exhaust. The main outlet was equipped with a condenser to minimize evaporative loss, and the second outlet was an emergency exhaust if the main exhaust became clogged. The exhausts were temporarily closed to create pressure inside the vessel, and the harvest pipe was opened to allow the sample to flow out. When the air flow into the vessel was high (greater than 1 L min^{-1}) the back pressure was enough to push the sample out the harvest pipe even without the exhausts closed off. The pH probe was calibrated before sterilization, but the oxygen probe was calibrated after sterilization. The D.O. probe was calibrated to 0 and 100% saturation with nitrogen and air, respectively.

Optimized Fermentor Conditions

The inoculum for the fermentor was 100 mL of LB supplemented with glucose to the final concentration of 20 g L^{-1}. The antibiotic chloramphenicol or ampicillin was added for plasmid maintenance. Since LB is a rich medium, the serine auxotrophy of KL3 would be ineffective as a plasmid maintenance strategy. The 100 mL inoculum was grown at 37 °C for approximately 14 h in a rotary shaker at 250 rpm.

To 850 mL of distilled and deionized water the optimized fermentor medium components listed in Table 3 were added (Fe(III) ammonium citrate concentration is not listed because the iron content varies from 28-32%). The pH was adjusted to 7 by the addition of KOH. The Fe(III) ammonium citrate was added to supply the Fe(III) for AroF. The AroF isozyme requires for activity one mole of iron per mole of enzyme. The citric acid was included to solubilize the Fe(III). The dibasic potassium phosphate and concentrated sulfuric acid were added as the phosphate and sulfur sources. The medium

was added to the fermentor vessel, which was then sterilized by autoclaving at 121 °C for 25 min. After autoclaving, the additional sterile trace minerals and nutrients, described below, were added.

Table 3 - Fermentor Medium Components.

Component	Grams	Concentration (mM)
K_2HPO_4	7.5	43
Citric Acid	2.0	10
Fe(III) Ammonium Citrate	0.30	
Concentrated H_2SO_4	1.2 (mL)	22

Since KL3 is unable to further metabolize DHS, it is unable to produce chorismic acid, the last metabolite in the common pathway. Chorismic acid is the precursor for six end products. Three terminal pathways lead to the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The three other end products are essential aromatics including folic acid, ubiquinone, and enterochelin, which are involved in coenzyme biosynthesis, electron transport, and iron uptake, respectively.[13] To overcome the aromatic amino acid auxotrophy of KL3, the growth medium was supplemented with phenylalanine (0.7 g L^{-1}), tyrosine (0.7 g L^{-1}), and tryptophan (0.35 g L^{-1}). Based upon the average *E. coli* requirements for aromatic amino acids,[14] about 25 g dry weight of cells L^{-1} should be produced given this supplementation. The concentration of tyrosine at 0.7 g L^{-1} is above its solubility limit. Therefore, the amino acids were added as a powder just before inoculation since it was not possible to make a concentrated stock solution. The essential metabolites were supplemented with biosynthetic precursors (aromatic vitamins). The aromatic

vitamins are *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid and supplements for the essential aromatic metabolites are, folic acid, ubiquinone, and enterochelin, respectively. The vitamins were each supplemented from a concentrated stock solution to a final concentration of 10 mg L^{-1}. A concentrated stock trace minerals solution was added; the constituents and their final concentrations are described in Table 4. The trace mineral and aromatic vitamin stock solution were sterile filtered through a 0.2 μm pore membrane.

Table 4 - Trace Mineral Supplements

Component	mg/L	Concentration (μM)
$(NH_4)_6Mo_7O_{24}(H_2O)_7$	3.7	3
H_3BO_3	24.7	400
$MnCl_2(H_2O)_4$	15.8	80
$ZnSO_4(H_2O)_7$	2.88	10
$CuSO_4(H_2O)_5$	2.49	10

A stock solution of glucose (18 g per 50 mL) was autoclaved separately. After autoclaving, the stock glucose solution was added to the fermentor medium. This addition adjusted the final concentration, including the 2 g of glucose which was added from the inoculum, to 20 g L^{-1}. For the constructs that had only one copy of *aroF* with the native promoter, the initial glucose concentration was 8 g L^{-1}.

The stock solution of $MgSO_4$ (1.0 M) was autoclaved separately. To the fermentor medium, 2 mL of the stock $MgSO_4$ solution was added (final concentration of 2.0 mM). Finally, the 100 mL inoculum was added to the fermentor to give a final volume of about 1

L. The pH was controlled with the addition of 2 N HCl and 28% NH_4OH. Sigma 204 antifoam was added manually when needed.

The choice for the dissolved oxygen (D.O.) setpoint was somewhat arbitrary. Konstantinov *et. al.* reported that phenylalanine production in *E. coli* was independent of the D.O. level in the range of 0-40%.[15] Their choice of a 20% D.O. setpoint was made out of convenience and simplicity. Also, at this D.O. level the growth rate is near its maximum.[16]

The fermentations were divided into two phases that had different control strategies. In the first phase, the D.O. was controlled by impeller speed and airflow rate. The experiment was begun with a D.O. value of about 100% saturation following the D.O. probe calibration. When then D.O. concentration fell to 20% the response was activated. The controller operated by increasing the impeller speed and then the airflow rate. The initial impeller speed was 50 rpm, and this value increased during the fermentation to a maximum vale of 900 rpm. After the maximum rpm was reached, the airflow rate was increased to maintain the 20% D.O. The minimum and maximum airflow rates were 0.06 and 3.0 L min^{-1}, respectively. The first phase lasted for 10 to 18 h depending on the construct used.

After the glucose in the first phase was consumed, the second phase of fermentation was started. At this time, the impeller was usually at the maximum rpm (900), and there was a rapid increase in the airflow rate to maintain the D.O. The airflow rate would then reach 3.0 L min^{-1} for a short time before rapidly decreasing which corresponded to depletion of the glucose. At this point, the use of the impeller rate and airflow rate to control D.O. concentration were stopped. Two different methods were used to start the second phase. For both methods the impeller rate was set to 900 rpm. Preferably the maximum airflow rate (3.0 L min^{-1}) had been reached and had not begun declining, allowing the flow rate to be set to this value. For some of the first fermentations using this method, the maximum airflow rate point was missed, and it had begun to decline. So, the

airflow rate was set to the current value at the time of the phase change, usually about 1.0 L min^{-1}. Then the flow rate was increased in increments of about 0.25 - 0.5 L min^{-1} to reach a final flow rate of 2.5 - 3.0 L min^{-1} within about 12 - 15 h after the start of phase two.

The D.O. concentration was controlled in the second phase by the glucose-feeding rate. The glucose feedstock had a concentration of either 400 or 600 g L^{-1}. Glucose was fed when the D.O. rose above the 20% setpoint, indicating glucose limitation by decreased respiratory activity. The PID controller parameters (Table 5) were found empirically. A dynamic simulation of the process was used to obtain a to obtain a first approximation of the controller parameters. Then based on experience with the fermentation, the parameters were significantly modified.

Table 5 - Phase 2 D.O. PID Controller Parameters.

Control Parameter	Value
τ_D	0
τ_I	999.9 s
X_P	950.0%

The equation for the output of the controller is given by Equation 1.[17] The controller response as a function of time is $c(t)$. The process gain (K_c) is defined as ratio of the change in output (D.O. level in our case) to the change of input (glucose feeding), Equation 2. Proportional control creates a controller response that is proportional to the error. The error as a function of time ($\varepsilon(t)$) is defined as the difference between the output (D.O.) and the setpoint (20%), Equation 2. The integral time constant (τ_I) defines the time needed by the controller to repeat the initial proportional control response to a

continuing deviation error. The derivative time constant (τ_D) and the derivative term anticipates what the error will be in the immediate future. The proportional band (X_P) is inversely related to the process gain, K_c (Equation 3). It characterizes the range over which the error must change in order to drive the controller's response over its full range. The larger the proportional band, the lower the sensitivity of the controller's response to the deviation error will be. This means that the larger the magnitude of the proportional band, the slower glucose feed rate is for a given deviation from the D.O. setpoint. The

$$c(t) = K_c\varepsilon(t) + \frac{K_c}{\tau_I}\int_0^t \varepsilon(t)dt + K_c\tau_D\frac{d\varepsilon}{dt} \qquad (1)$$

$$K_c = \frac{\Delta Output}{\Delta Input} \qquad (2)$$

$$X_p = \frac{100}{K_c} \qquad (3)$$

derivative control feature was turned off (τ_D= 0). The integral control parameter τ_I was set to the maximum value allowed by the computer field width, 999.9s (Equation 1). The control action due to integral control is inversely proportional to the parameter value; therefore the integral control action was set to a minimum. These control values typically worked well as demonstrated by little to no accumulation of glucose. The setpoint for the culture temperature was 37 °C. The pH was maintained at 7.0 ± 0.05 pH units.

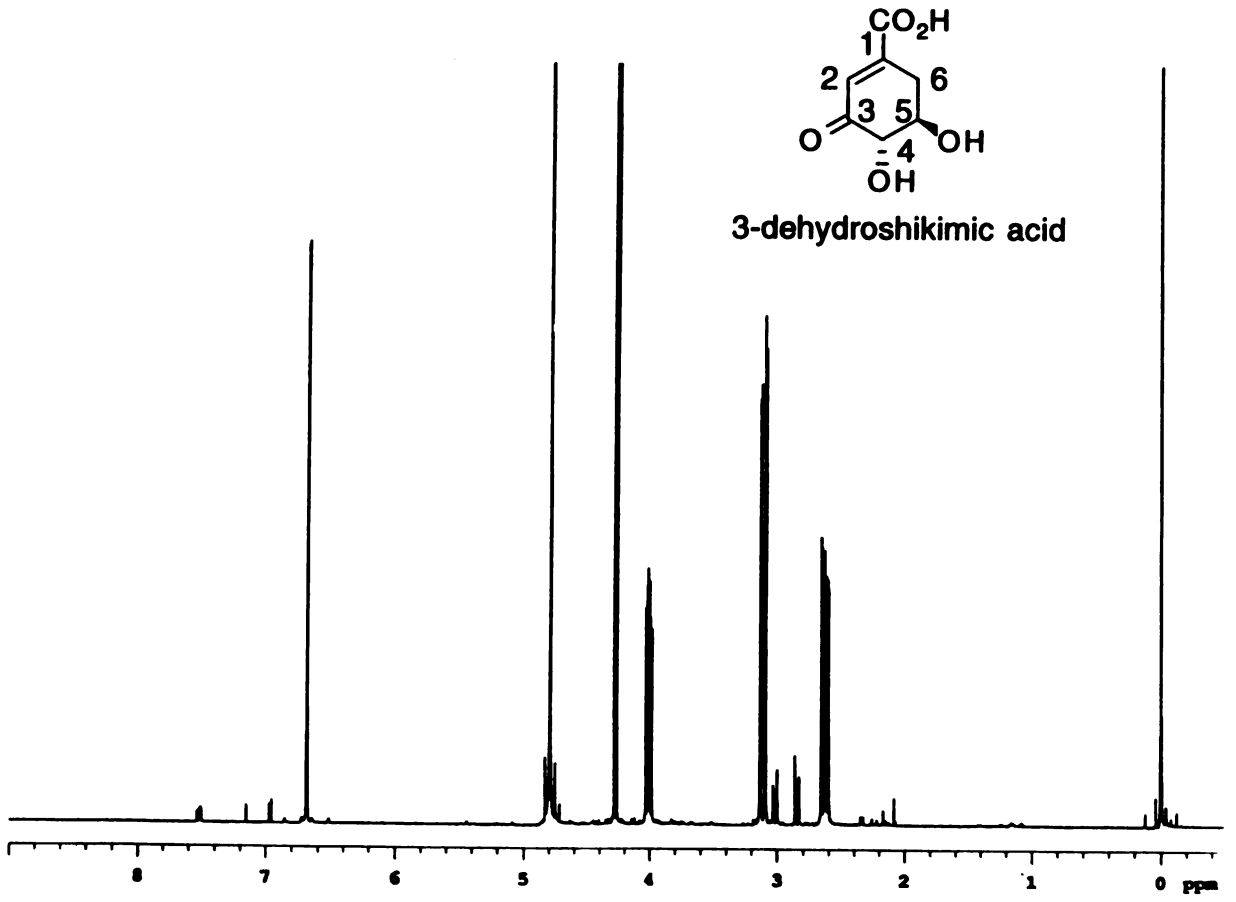
NMR Assays

DHS, glucose, gallic acid, acetic acid and formic acid were assayed by ^{1}H NMR (nuclear magnetic resonance) that were recorded on a Varian Gemini-300 spectrometer at

300 MHz. An aliquot (5-6 mL) of the culture was withdrawn, and cells were removed by centrifugation. A portion (0.25-3.0 mL) of the culture supernatant was concentrated to dryness under reduced pressure, concentrated to dryness two additional times from D_2O (1 mL), and then dissolved in D_2O (1 mL) containing a known concentration of TSP (δ 0.00). Concentrations of metabolites in the supernatant were determined by comparison of integrals corresponding to each metabolite with the integral corresponding to TSP in the ^1H NMR spectra. DHS resonances are at δ 6.4, 4.3, 4.0, 3.1, and 2.7 (citric acid also has overlapping resonances at δ 2.7). The resonances used for quantification are either δ 3.1 (doublet of doublets) or δ 4.2 (doublet), and each corresponds to a single proton (Figure 29). The resonance at δ 3.1 is preferred because the proton that corresponds to δ 4.2 is slightly exchangeable with deuterium. The resonance at δ 4.2 is used for quantification only when a significant amount of glucose is present which begins to overlap with the DHS peak at δ 3.2. Glucose is quantified by summing of the integrals of the resonances at δ 4.6 and 5.2, which corresponds to the total concentration of α and β enantiomers in solution (Figure 30). Gallic acid is quantified by the singlet resonance at δ 7.1 (Figure 31). This resonance corresponds to the protons of the aromatic ring. Acetic acid is quantified by the δ 2.1 singlet corresponding to 3 protons (Figure 32). Lastly, if formic acid is present, it is quantified by the singlet resonance δ 8.5 corresponding to one proton (Figure 33). A typical optimized fermentation spectrum at 36 h is shown in Figure 34. Glucose can also be assayed using the Glucose Trinder Assay marketed by Sigma (St. Louis, Missouri). The supernatant was typically quantified every 6 h after the start of the fermentation.

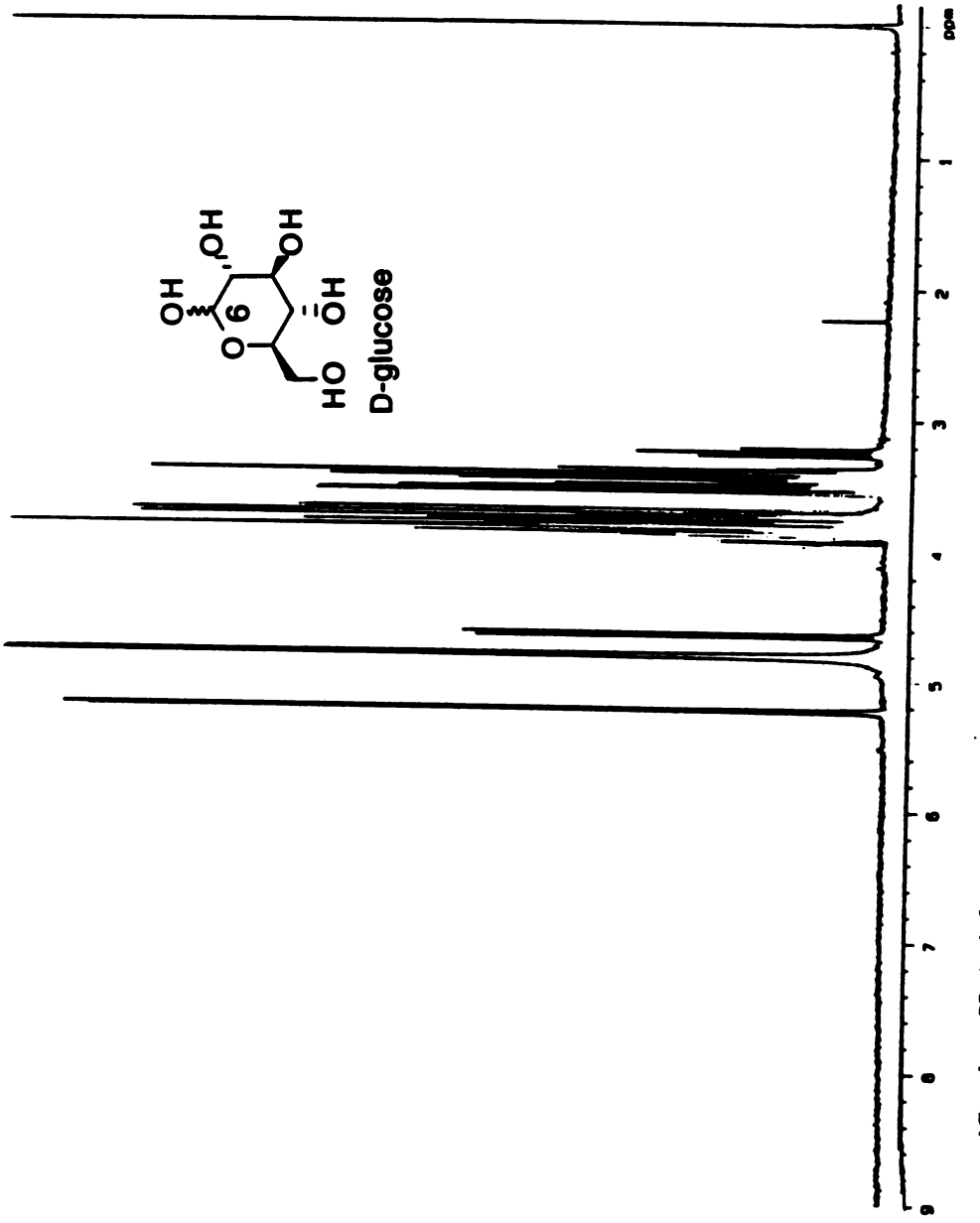
DAHP Synthase Assays

DAHP synthase activity was measured according to the procedure described by Schoner.[18] Fermentor samples for the assay were taken at 12, 24, 36, and 48 h. The volume of culture taken was 40 mL at the 12 h time point and 25 mL was taken for each point after that. The lysate was diluted with the appropriate buffer for the assay. The assay



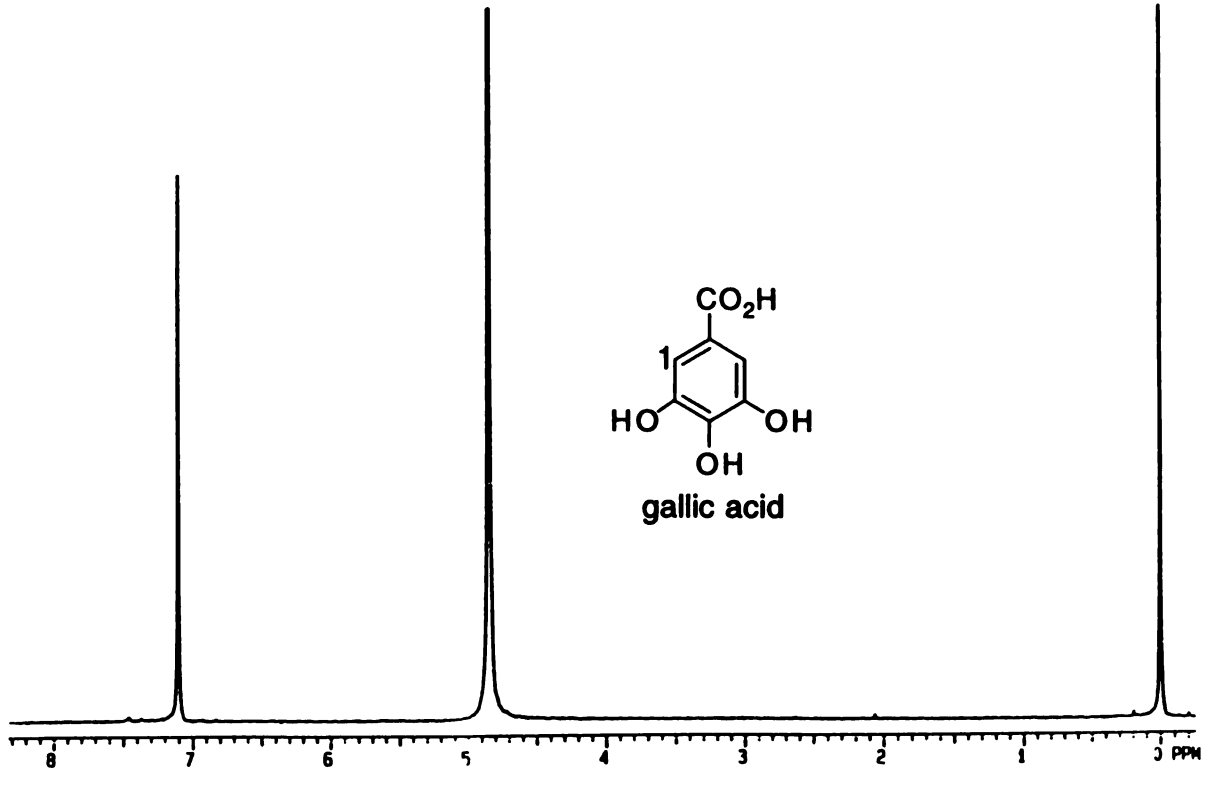
Resonances for quantification: H_{6e} (δ 3.07, dd, 1H) and H_4 (δ 4.28, d, 1H).

Figure 29 - ^{1}H NMR of 3-Dehydroshikimic Acid (DHS).



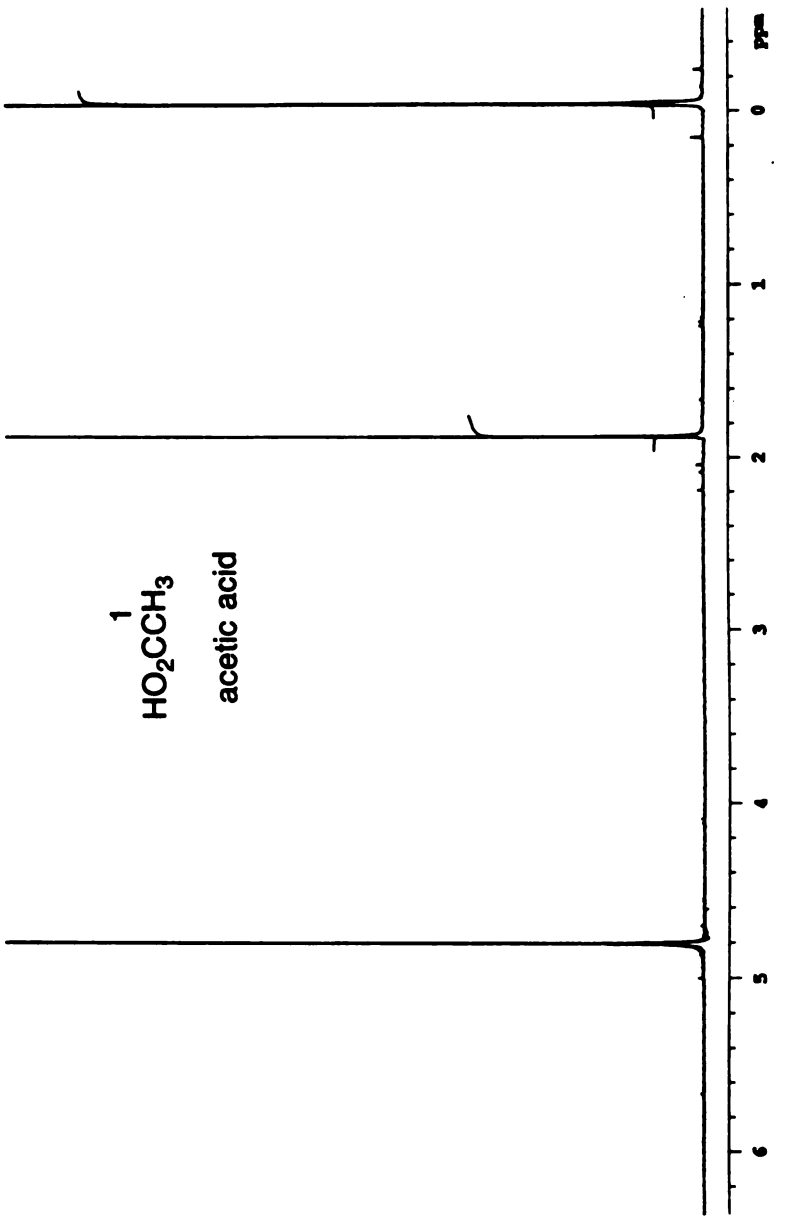
Resonances for quantification: H_1 (δ 4.6 α enantiomer and 5.2 β enantiomer, dd 1H).

Figure 30 - ^1H NMR of Glucose.



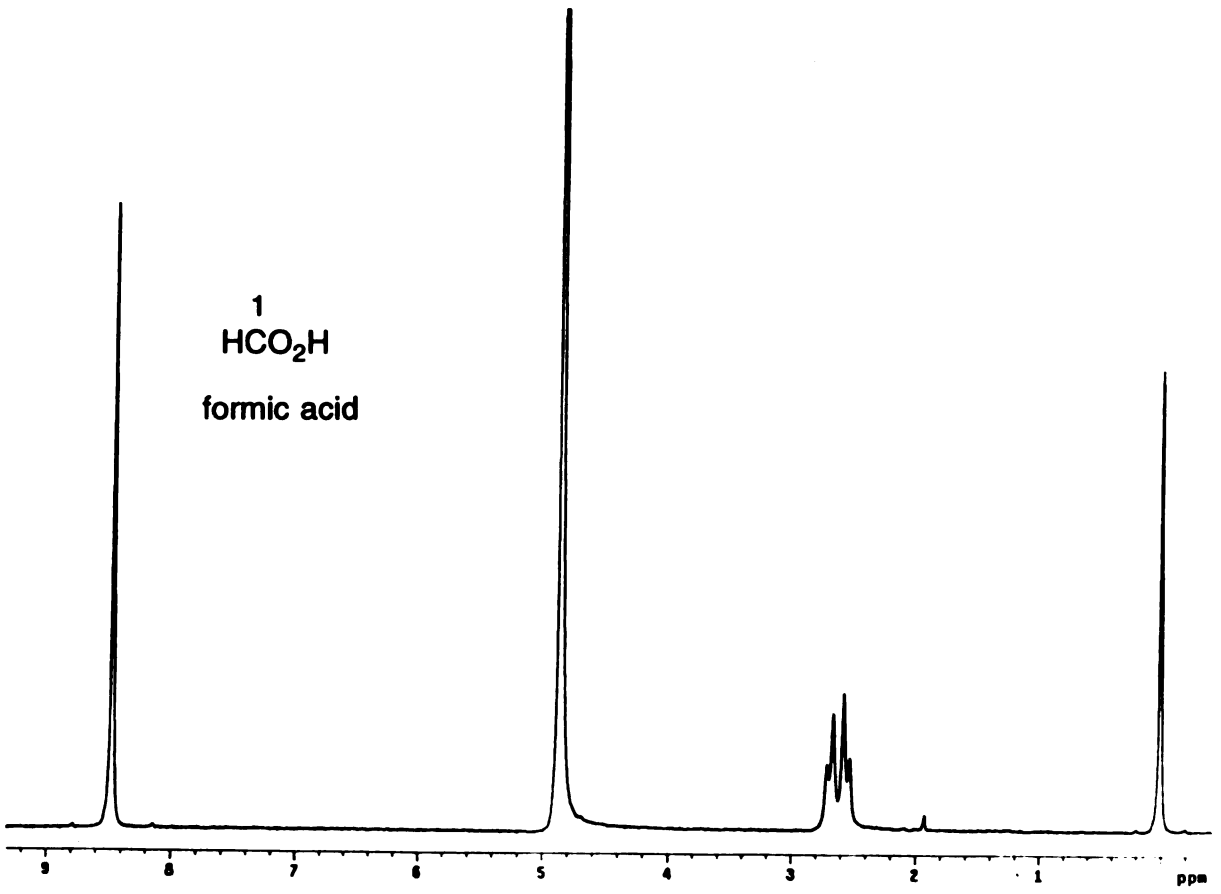
Resonance for quantification: H_1 (δ 7.1, s, 2H).

Figure 31 - ^{1}H NMR of Gallic Acid.



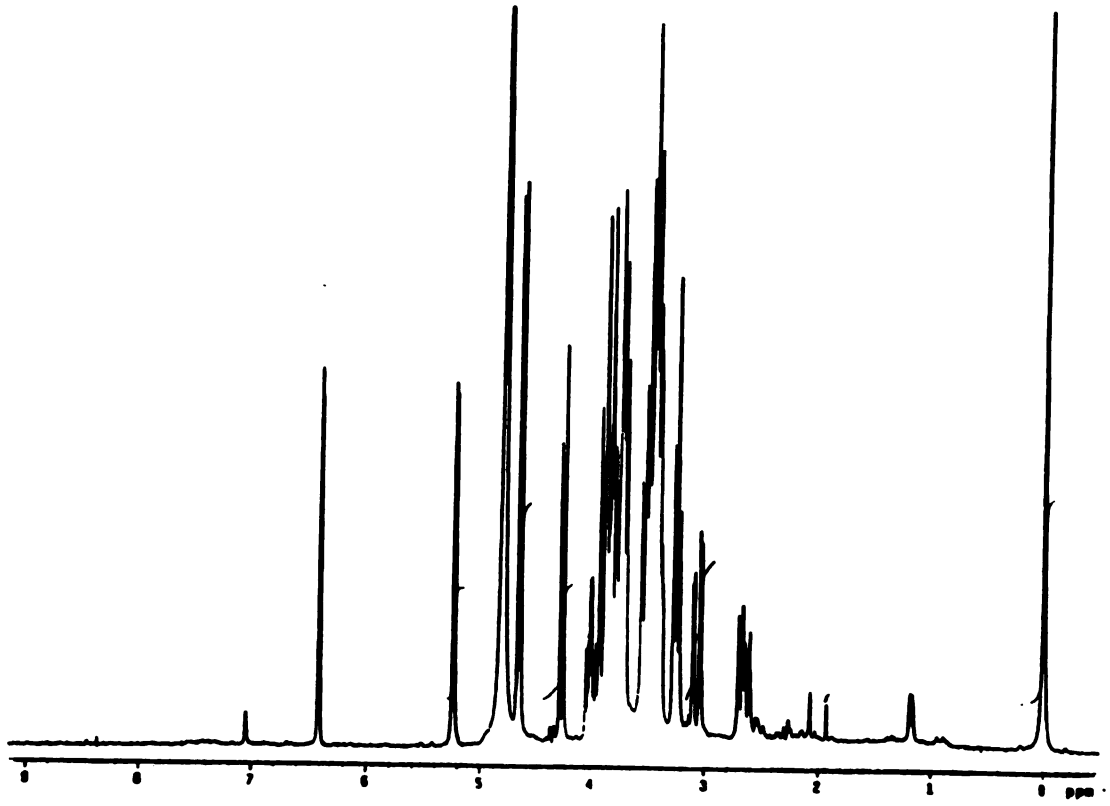
Resonance for quantification H_1 (δ 2.1, s, 3H).

Figure 32 - ^{1}H NMR of Acetic Acid.



Resonance for quantification H_1 (δ 8.5, s, 1H), in fermentor medium.

Figure 33 - ^{1}H NMR of Formic Acid.



DHS, Gallic Acid, and Acetate are present.

Figure 34 - Typical ^1H NMR for 36 hour Fermentation.

time points were taken every 15 or 30 s for a total of 6 data points. The concentration of DAHP produced was determined by thiobarbituric acid visualization of the periodate cleavage products as described by Gollub.[19] The absorbance of the cleavage products was measured at 549 nm for quantification.

Confirmation of Plasmid Maintenance

The effectiveness of the nutritional basis for plasmid maintenance was examined. After each fermentation, an aliquot of the culture broth was diluted and plated. The plates were LB, LB/Cm or LB/Amp depending on the plasmid host vector, and M9/glucose. The growth on LB indicated how many viable cells were present in the culture, while the LB/antibiotic plate indicated the number of cells that maintained the plasmid as verified by antibiotic resistance. The ratio of the number of colonies on the LB/antibiotic plate and LB plate gave an estimate of plasmid retention. The M9/glucose plate was used to confirm that the strain was still an aromatic amino acid auxotrophy and had not reverted to prototrophy. There should not be any growth on this plate because it lacked the proper supplementation to complement the aromatic amino acid auxotrophy.

A second confirmation of the plasmid stability was also used. The plasmid DNA was isolated and digested with proper endonucleases. The digested DNA was then separated by size using electrophoresis on a 0.7% agarose gel. The bands were stained with ethidium bromide to visualize the DNA fragments with a UV lamp. This confirmed not only the presence of the plasmid, but also the genetic composition of the plasmid.

Fermentations

The constructs used to compare the nontruncated and truncated *aroF*^{fbr}, KL3/pKL4.33B and KL3/pKL4.32B (Chapter 2, p 48) were further investigated in the fermentor. The objective of the two fermentations was to determine whether the truncated *aroF*^{fbr} gave higher titers or yields than the nontruncated *aroF*^{fbr}. The conditions were slightly different from the optimized conditions listed above since the strain development and optimization of the fermentor conditions occurred simultaneously. The composition of

the starter culture is listed Table 6. The starter culture volume was 80 mL, but the other growth conditions were the same as for the optimized fermentations. The D.O. was maintained at 5% saturation. This D.O. setpoint was used at this time because some previous fermentations produced higher titers at lower D.O. This trend was not observed for subsequent fermentations. The fermentor medium composition was the same as before although the initial concentration of glucose in the medium was 5 g L^{-1}. After inoculation (6 h) glucose was fed at approximately 1 g h^{-1} for 42 h. The D.O. control was that of phase one of the optimized conditions. The impeller speed and airflow rate were manipulated to maintain the D.O. at 5%.

Table 6 - Starter Culture for Evaluation of Truncated/Nontruncated *aroF*.

Component	g/L	Concentration (mM)
K_2HPO_4	4.8	28
KH_2PO_4	12.2	90
Yeast Extract	2.50	
$(NH_4)_2SO_4$	2.5	20
Glucose	1.2	6.6
$MgSO_4$	0.5	4.1

The first fermentation was performed for KL3/pKL4.32B (truncated *aroF*^{tr}). No DHS accumulated and, only acetic acid formation was observed. Fifty mL aliquots of the fermentor culture were taken at 24 h and 48 h for the DAHP synthase specific activity assays. The growth rate and DAHP synthase specific activity were low compared to the next fermentation (Figure 35). The experiment was repeated for KL3/pKL4.33B

(nontruncated *aroF*^{fbr}). There was a small amount of DHS (1 g L^{-1}) produced in this fermentation, and the DAHP synthase specific activity was higher at 24 h than the DAHP synthase specific activity at 24 h for the truncated *aroF*^{fbr}. Based on the lack of any DHS accumulation by KL3 containing the truncated *aroF*^{fbr} plasmid, this construct was abandoned.

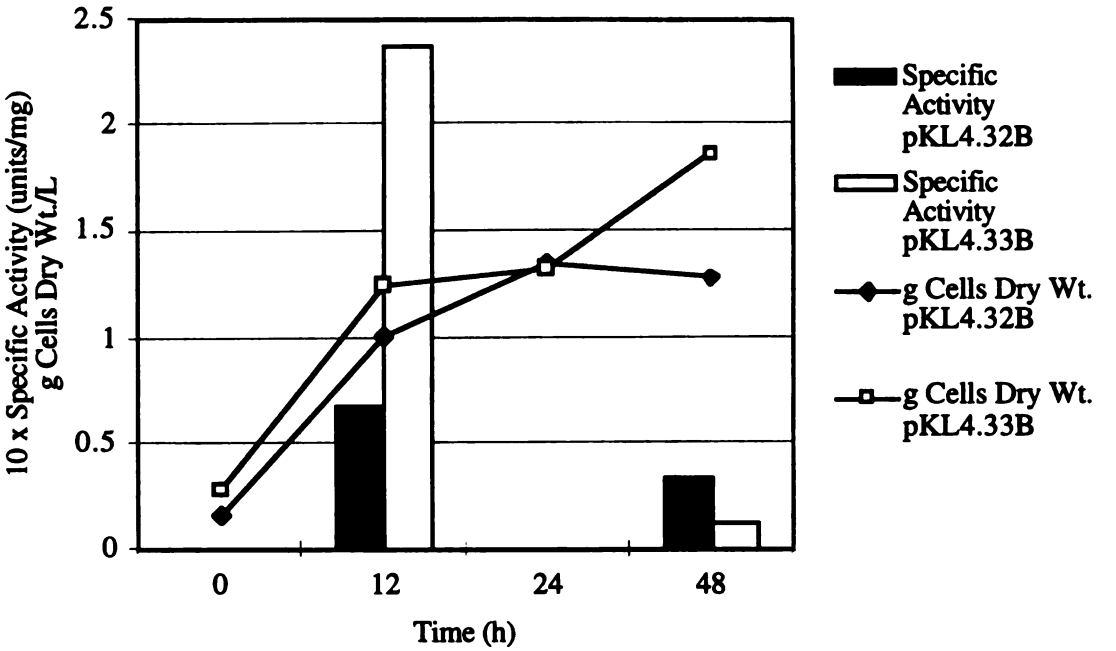


Figure 35 - Fermentation Evaluating KL3/pKL4.32B and KL3/pKL4.33B.

For a point of reference, a fermentation performed using unoptimized conditions is illustrated in Figure 36. The construct used for this fermentation was KL3/pKL4.66A which is a double *aroF*^{fbr}-bearing plasmid (Chapter 2, p 50) The starter culture was grown in 100 mL LB/Cm for 14 h (37 °C and 250 rpm). After 14 h the cells were harvested (4 °C and 3000 x *g*, for 5 min) and resuspended in 100 mL of the fermentor medium. Amino acids used as supplements included phenylalanine (1.0 g L^{-1}), tyrosine (1.0 g L^{-1}), and tryptophan (0.5 g L^{-1}). The initial glucose concentration was 8 g L^{-1}. The dissolved

oxygen was maintained at 5% saturation. The fermentation control was carried out in two phases as for the optimum conditions with the exception that the maximum impeller speed was 400 rpm. The cell growth was better than most previous fermentations, but the cell concentration was still about one third of the optimized fermentation's cell concentration. The DHS production was an order of magnitude less then for the optimized case. The molar yield of DHS was 15%. The acetic production was kept to a minimum. Typically,

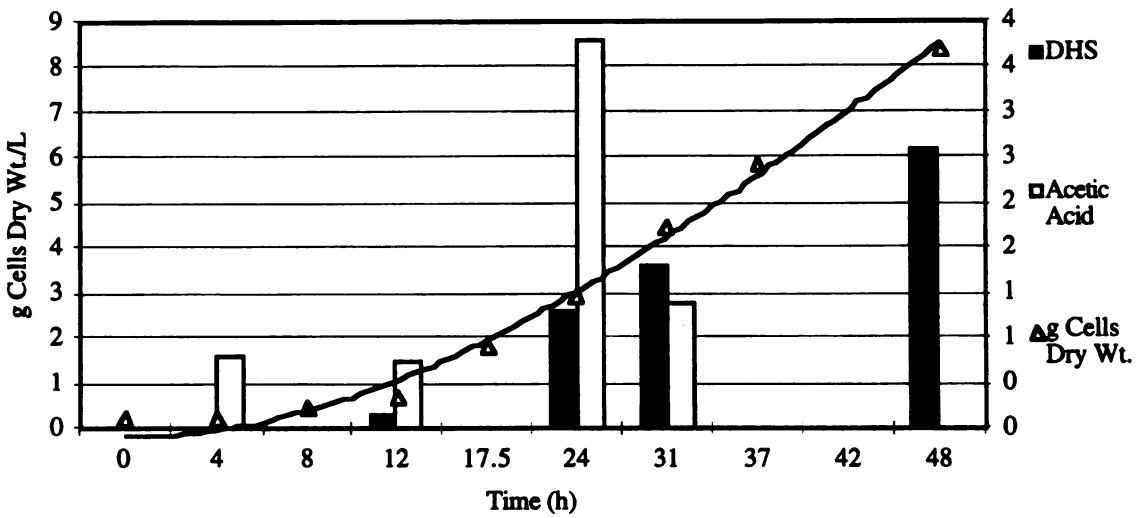


Figure 36 - Nonoptimized Fermentation.

the acetic acid was consumed during phase two due to the strict control of glucose feeding as is shown for this fermentation. This fermentation was included to demonstrate the differences between optimized and non-optimized fermentations. A key point learned was at 5% D.O. the cell concentration was much lower than the maximum cell concentration as calculated by the aromatic amino acid supplementation (25 g dry wt. cells L^{-1}).

All subsequent fermentations cited were conducted using the optimized conditions. The two double *aroF* constructs (pKL4.66A and pKL4.66B, Chapter 2 p 50) were tested

in the fermentor. Two effects were investigated in these fermentations. The effect of adding a second *aroF* gene to the construct was explored to increase the DAHP synthase specific activity. Secondly, since more than one orientation was obtained when ligating the second *aroF*, any effect due to the different orientation between the two construct was explored. The first fermentation (Fermentation 960037) using the optimized conditions was with KL3/pKL4.66A (double *aroF* construct, Figure 22, p 50). The fermentation was run for 60 h to explore the DHS production over a longer time period (Figure 37). During this fermentation, the airflow rate was incrementally increased in phase two. The DHS titer was 19 g L^{-1} and overall molar yield from glucose was 24%.

The next three fermentation described are discussed as a group. One fermentation was with KL3/pKL4.66A and the other two were run with KL3/pKL4.66B (Figure 22, p 50), designated KL3/pKL4.66B(1) and KL3/pKL4.66B(2) (Figure 38). Fermentation 960037 was not included in the comparison because some of the operation was different from the other fermentations (i.e. the airflow rate at the beginning of phase two was not 3.0 L min^{-1}), and it ran for much longer. The DHS titers were 40, 37, and 34 g L^{-1}, and the molar yields were 21%, 20%, and 17% for pKL4.66A, pKL4.66B(1), and pKL4.66B(2), respectively. Unlike the shake-flask experiments, the DHS production was nearly the same for the two constructs, and there was not a difference between the two constructs with respect to specific activity in the fermentor.

The single *aroF* construct, KL3/pKL4.33B, was grown twice in the fermentor (Figure 39). The DHS titers were 21 and 25 g L^{-1}. The molar yields were 18% and 10%. A fermentation was attempted using 20 g L^{-1} glucose, but after 24 h only acetic acid was produced. The conditions had to be slightly modified from the previous fermentations. The initial concentration of glucose in the fermentor was 8 g L^{-1} instead of 20 g L^{-1}. For the 20 g L^{-1} glucose run, the growth rate and glucose consumption were slower initially.

Since AroF and the PTS compete for PEP, the PEP availability may be the cause for the this run's lack of DHS production. It has been assumed that the PEP-enzyme

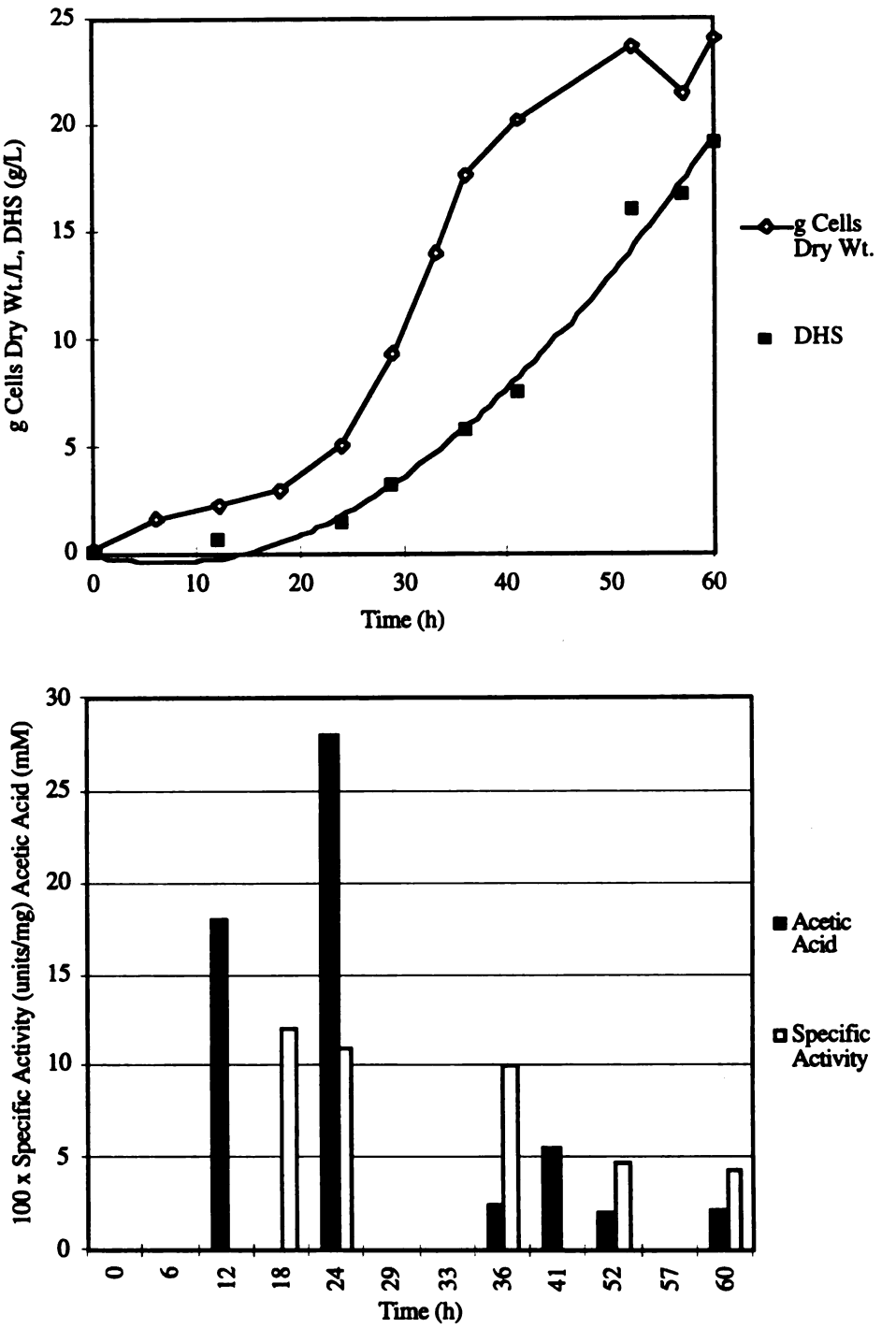


Figure 37 - KL3/pKL4.66A Fermentation.

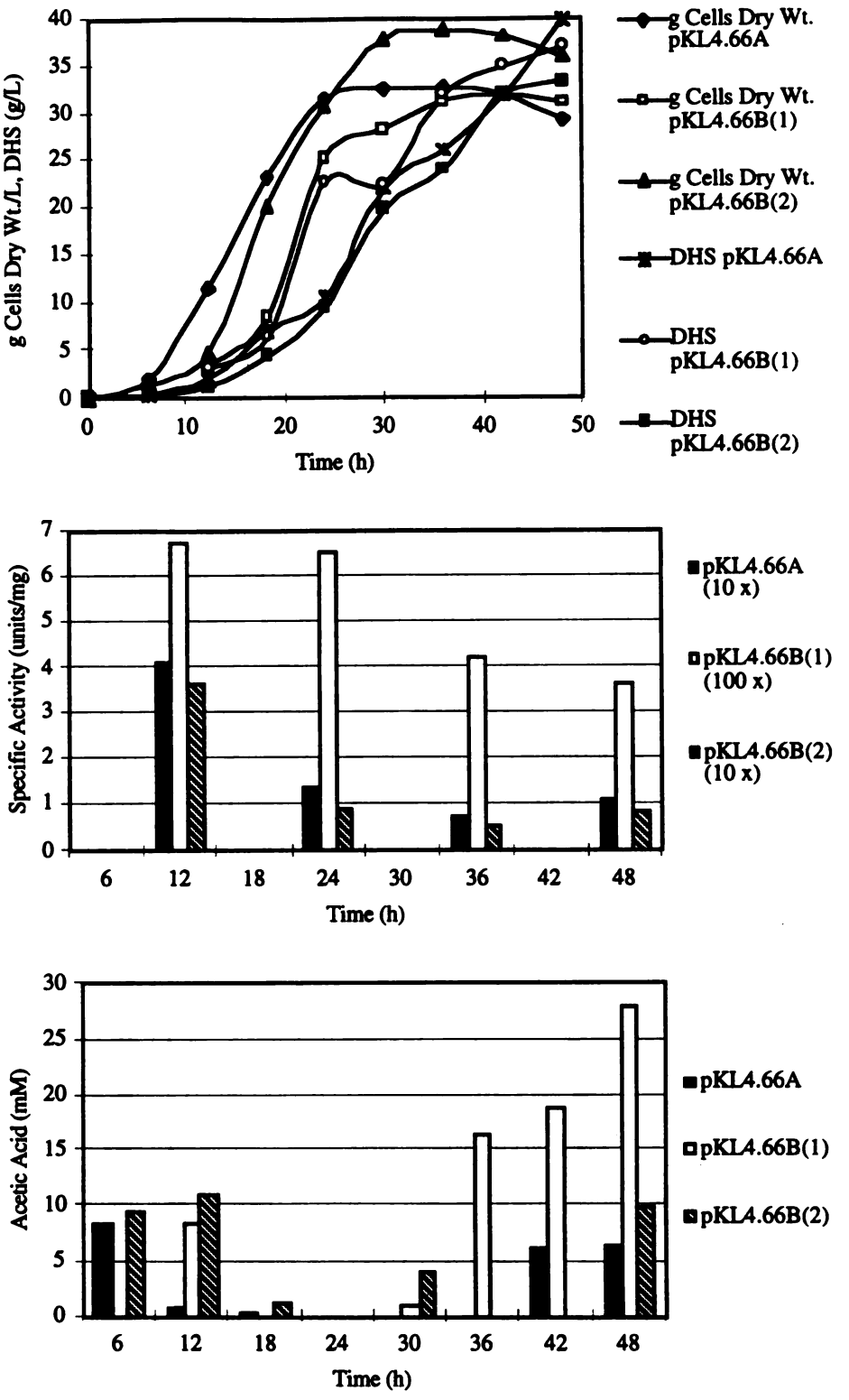


Figure 38 - Fermentations of KL3/pKL4.66A and pKL4.66B.

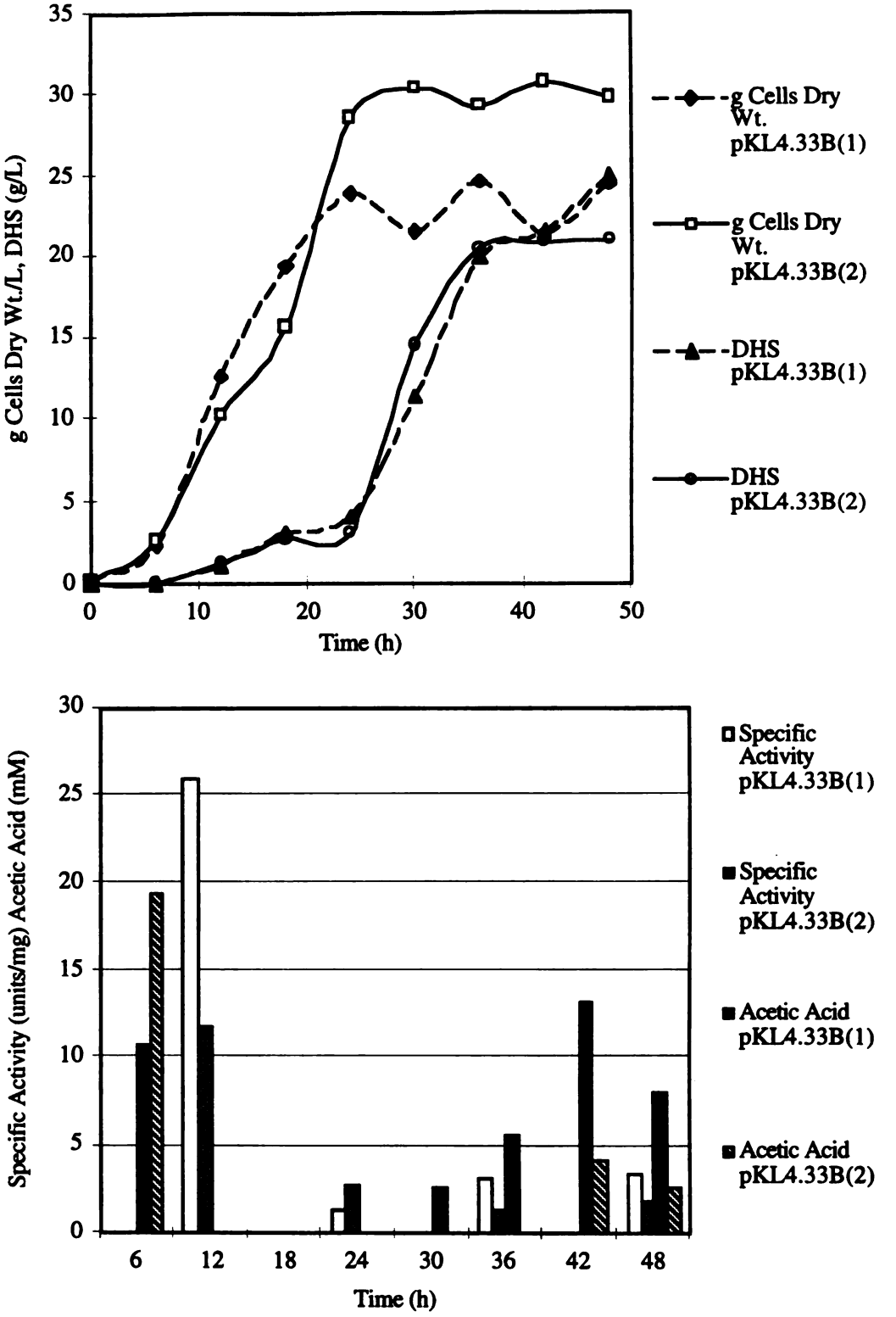


Figure 39 - Fermentations of KL3/pKL4.33B.

complex is the native form of *aroF* since the K_m for PEP is 5.8 μM, and the intracellular PEP concentration never falls below 88 μM.[20] With the double *aroF* construct, more PEP is channeled into the biosynthesis of DHS initially because there is more of the enzyme to bind the PEP, resulting in a reduction of glucose uptake. The construct with a single copy of *aroF* would siphon less PEP into the synthesis of DHS, thereby increasing the PEP availability for the PTS. Since initially more glucose is transported into the cell because of high PEP availability, the natural regulatory mechanism of catabolite repression would be significant. A kinetic rate expression for the PTS was derived by Liao *et. al.*,[21] and it predicts the rate of glucose transport is affected by the PEP/pyruvate ratio. Increases in the ratio raise the apparent maximum reaction velocity of the PTS (V_{max}) and reduce the apparent K_m for glucose. The kinetic rate expression demonstrates that there is a link between the PEP availability and glucose transport. The two single *aroF* fermentations had lower DHS titers and lower DAHP synthase activities as compared to the double *aroF* constructs. The DHS titers of the single *aroF* construct decreased almost 40% compared to the double *aroF* constructs. It appears that the double *aroF* under these conditions is a better construct.

A strain used the *tac* promoter to manipulate *aroF* expression was then tested. The gene that encoded for the *tac* repressor *lacI*^{q} on a plasmid allowed for manipulation of *aroF* expression into which a copy of *aroF* was cloned. Expression could be controlled by addition of the inducer isopropyl β-D-thiogalactopyranoside (IPTG). This compound binds to the repressor and changes its conformation so that the repressor can no longer bind to the ribosome binding site to stop transcription. The plasmid pKL4.79B was described in Chapter 2 (Figure 24 ,p 52). The first experiment was to add IPTG after the initial lag period, about 4 h after inoculation. The cell concentration was at least an order of magnitude greater in the fermentor than in shake flasks, so it was not clear how to correctly scale the IPTG. Addition of 1 g of IPTG was arbitrarily chosen for a first fermentation. The results are summarized in Figure 40. The initial specific activity was high but dropped

to about the activity of the double aroF constructs. DHS titer, 17 g L^{-1}, was lower than either the double or single *aroF* constructs. The molar yield was 29%.

For the next five fermentations, IPTG was added throughout the fermentation to attempt to maintain a stable level of DAHP synthase activity over the 48 h fermentation. The subsequent schedule for adding the IPTG was one addition at the time of the phase change and then one addition every 6 h. The last addition was after the start of stationary phase. The IPTG was typically added at about 8, 12, 18, 24, 30, 36, and 42 h after inoculation. The amount of IPTG added to the culture, was either 0, 0.32, 1.6, 8.0, or 40 mg per addition. For each fermentation the same amount of IPTG was added for each addition (i.e. one fermentor run had seven additions of 0.32 mg IPTG). The total amount added was 0, 2.24, 11.2, 56, or 280 mg IPTG. While this method produced a range of DAHP synthase specific activities, a constant specific activity throughout the fermentation was never achieved (Figure 41). The DHS molar yields were 30%, 42%, 52%, 30%, and 26%, respectively. The most striking result from this set of fermentations was that the highest specific activity did not produce the highest DHS titer. Apparently, there is an optimal specific activity for DHS production that does not correspond to the maximum specific activity (Figure 42). The highest DHS titer and best yield were for the 1.6 mg IPTG addition fermentation. Also, unlike the shake flask experiment with this construct, expression of *aroF* under these conditions cannot be completely stopped in the absence of IPTG. This finding further supports the idea that the physiology of the organism varies greatly with growth conditions.

Scaled-Up Fermentations

The fermentation conditions was scaled up to a 50 liter working volume fermentor at Biosys Inc. (Columbia, Maryland). This fermentor was also a B. Braun Biostat®. The physical design of the 50 L fermentor was very similar to the 1L fermentor. The same probes (temperature, pH, and D.O.) and impeller design were used, and the acid and base

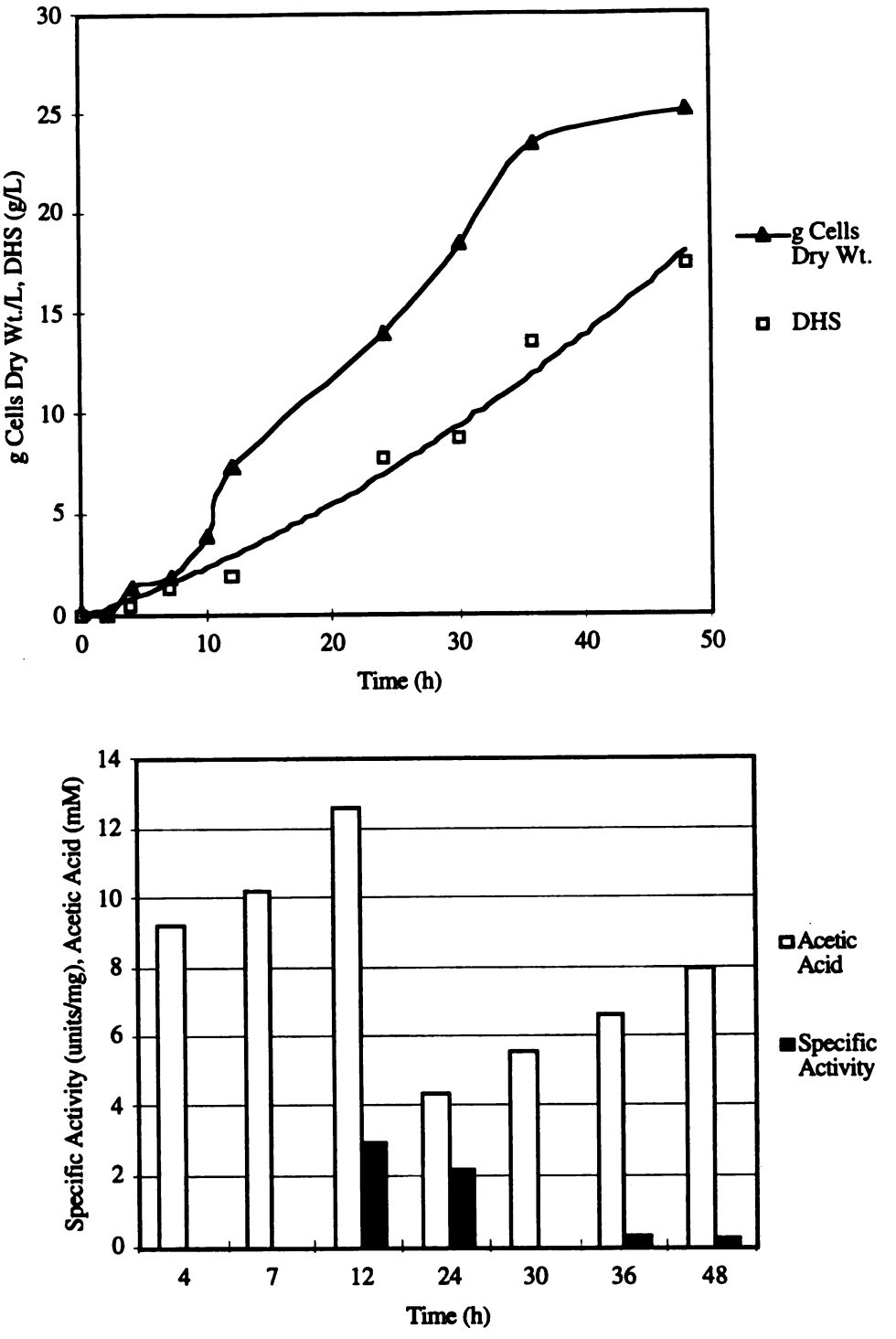


Figure 40 - Fermentation of KL3/pKL4.79B 1.0 g IPTG.

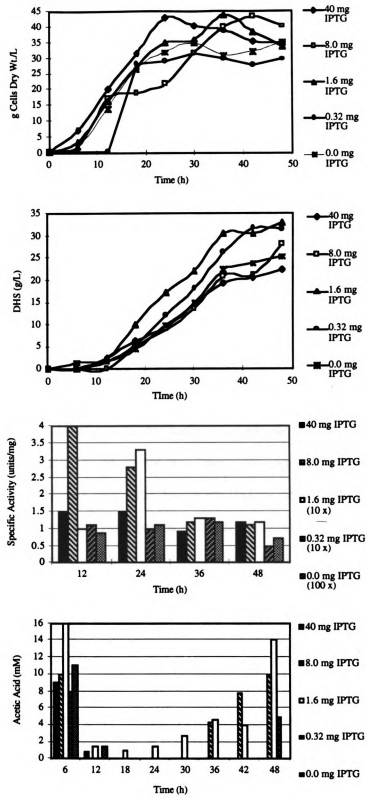


Figure 41 - Fermentations of KL3/pKL4.79B, IPTG Titration of *aroF*.

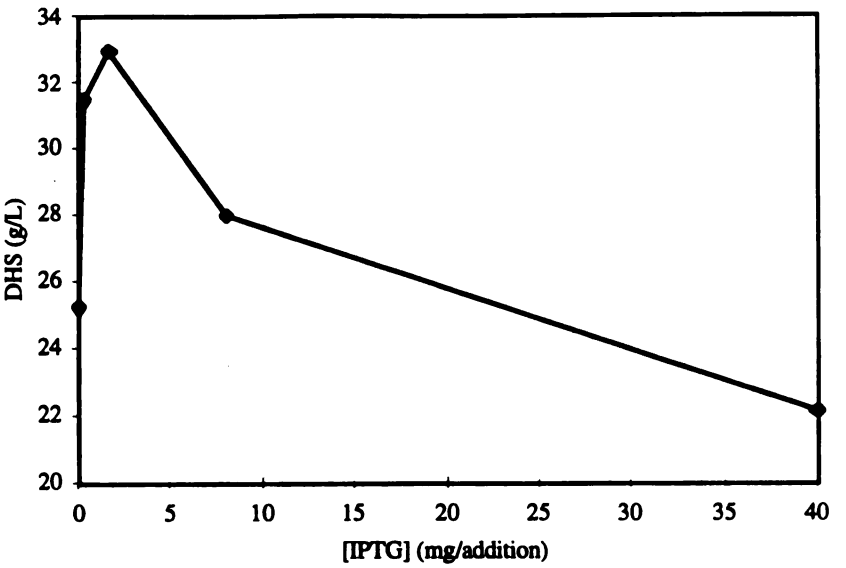


Figure 42 - DHS Production as a Function of AroF Activity.

were added through peristaltic pumps that were a part of the fermentor unit. Again, the glucose was fed by an external pump but a second D.O. probe was used to control the glucose feeding in phase two since the first D.O. probe could not be connected to a controller for glucose feeding. This fermentor did not have the capability to control D.O. using first the impeller and then the airflow rate. Instead, the air flow rate was set to 40 L min^{-1} throughout the fermentation, and the impeller speed was used to control D.O. The medium composition was the same as for the 1 L fermentations. After about 8 h, the culture stopped growing and the dissolved oxygen quickly rose. Upon addition of NH_4OH, the D.O. quickly dropped indicating an increased respiratory demand. The difference between this fermentation and the previous 1 L fermentations was at the 1L scale the pH dropped upon inoculation, and NH_4OH was added to increase the pH to near neutrality. It had been noted that he pH did not drop after inoculation of the 50 L culture and therefore no NH_4OH was added. These results suggest that nitrogen was limiting

growth. The first fermentation produced a significant amount of acetate (146 mM) and only about 6 g L^{-1} of DHS.

For the second fermentation, the pH of the medium was adjusted with NH_4OH instead of KOH to supply more nitrogen to the culture at the beginning of the fermentation. This fermentation accumulated 43 g L^{-1} of DHS (Figure 43), and the molar yield was 39%. The acetic acid production was very different from the 1 L scale. Acetic acid was produced throughout the fermentation, whereas the 1 L fermentations typically only produced acetic acid at beginning and end. The acetic acid production throughout the fermentation may be because that the glucose feeding control parameters were arbitrarily set, and may have cause over feeding of the culture or oxygen-liming conditions. A summary of all fermentation in this chapter is given in Table 7.

Discussion and Conclusions

The double *aroF* construct produced the highest DHS titer (40 g L^{-1}) obtained to date. None of the fermentations using the nutritional requirement for plasmid maintenance showed significant plasmid loss or instability. Through the manipulation of *aroF* activity, by addition of IPTG, it was indicated that an intermediate level of DAHP synthase activity gave the highest DHS titer. The fermentations have reached 25% DHS yield from glucose. There was a general correlation between DHS titer cell concentrations (Figures 37, 38, 39, 40, 41, and 43). This correlation has also been reported for tryptophan production.[22]

Using the data from the highest DHS titer fermentation (KL3/pKL4.66A) the yield coefficients, productivity, and average oxygen masss transfer rate were calculated. The coefficient for the production of DHS from glucose was 0.16 g DHS per gram glucose ($Y_{p/s}$ = 0.16), and the yield coefficient of DHS based on oxygen was 0.053 ($Y_{p/O}$ = 0.053). The productivity was 0.83 g DHS L^{-1} h^{-1}. An electron and carbon balance were also calculated for this fermentation to provide insight into the loss of energy and carbon. The electron balance showed that 66% of the electrons available from the glucose were not accounted for

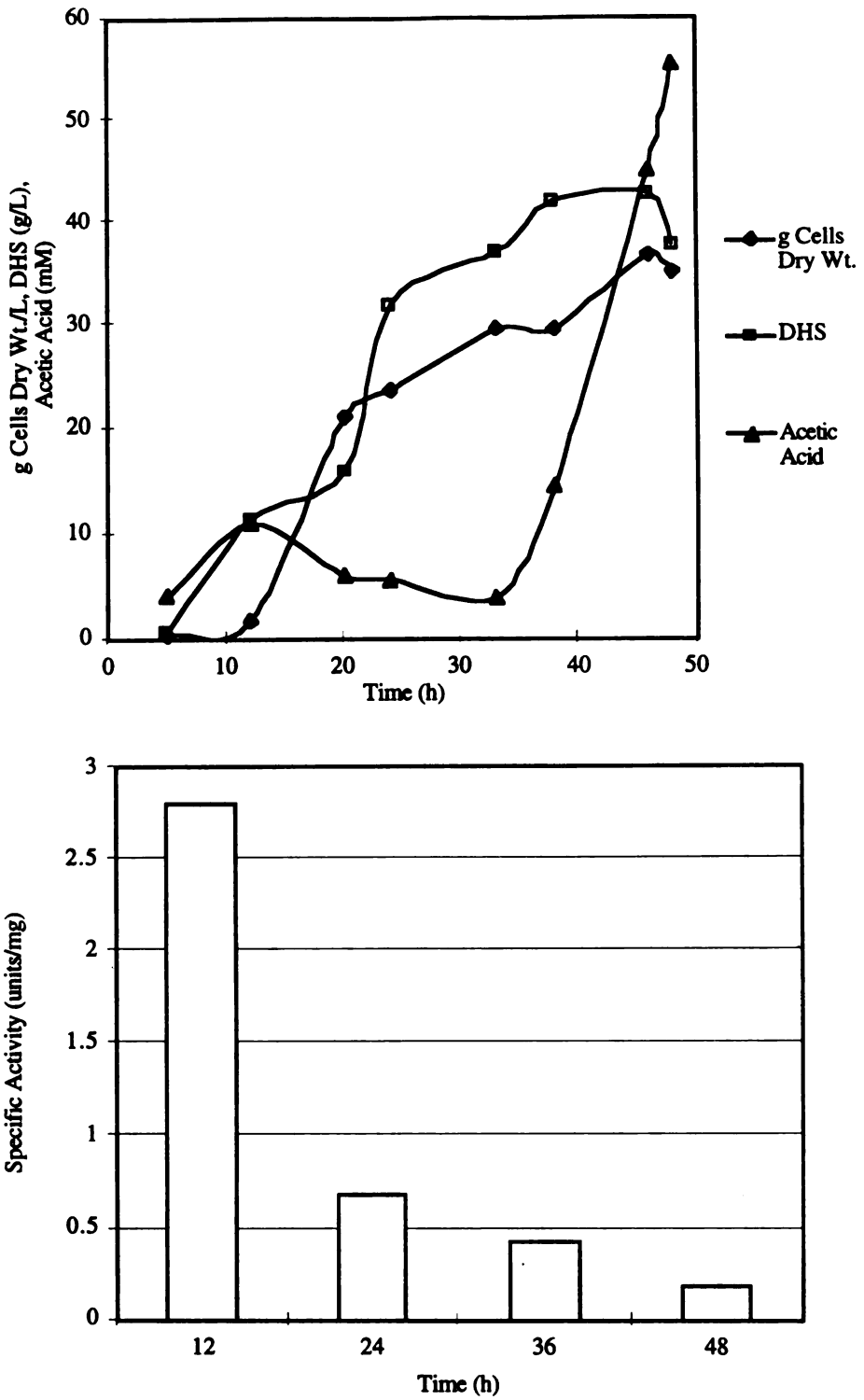


Figure 43 - 50 L Scale Fermentation.

Constructs	Relevant Characteristics	DHS Titer (g/L)	Molar Yield (%)	Comments
KL3/pKL4.32B	truncated *aroF* ^{fbr}	0	0	comparision with nontruncated *aroF* ^{fbr}
KL3/pKL4.33B	nontruncated *aroF* ^{fbr}	1	4.4	comparision with truncated *aroF* ^{fbr}
KL3/pKL4.33B	single *aroF* ^{fbr}	21	18	optimized conitions
KL3/pKL4.33B	single *aroF* ^{fbr}	25	10	optimized conitions
KL3/pKL4.66A	double *aroF* ^{fbr}	3.1	15	unoptimized conditions
KL3/pKL4.66A	double *aroF* ^{fbr}	19	24	optimized conitions
KL3/pKL4.66A	double *aroF* ^{fbr}	40	22	optimized conitions
KL3/pKL4.66B	double *aroF* ^{fbr}	37	20	optimized conitions
KL3/pKL4.66B	double *aroF* ^{fbr}	34	17	optimized conitions
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	17	15	1.0g IPTG (added once)
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	22	11	40 mg IPTG
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	28	13	8.0 mg IPTG
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	33	22	1.6 mg IPTG
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	32	18	0.32 mg IPTG
KL3/pKL4.79B	*tac* promoter *aroF* ^{fbr}	25	13	0.0 mg IPTG

in the products of cell mass and DHS, and from the carbon balance calculation, 131 g glucose (56%) was not accounted for in the products. From the carbon balance it was also calculated that 3.7 moles of carbon dioxide (CO_2) was produced per mole of glucose consumed. The theoretical minimum production of CO_2 is 3 moles CO^2 per mole of glucose, indicating the carbon balance does not violate the theoretical yield. The time averaged oxygen mass transfer rate (at 20% D.O.) was calculated to be 514 h^{-1} (K_la = 514 h^{-1}), which provides a minimum oxygen transfer rate that needs to be maintained during phase 2 of the fermentation.

Due to the large number of variables involved many of the parameters were not fully investigated. The concentrations of the medium components have not been optimized. Although DHS production medium components are all necessary, such as the Fe(III) ammonium citrate, the exact concentrations may not be critical.

The fermentation scaled well to 50 L. However, this fermentation demonstrates that several issues still need to be addressed. The air flow rate used the 1 L scale of 3 L min^{-1} was very high. This airflow rate corresponds to 3 volumes of air per volume of culture per minute (3 vvm). The air flow rate used for the 50 L run was only 40 L min^{-1}, or about 1 vvm. The new air flow rate did not alter the DHS titer. The choice for the D.O. setpoint was somewhat arbitrary. The choice was based upon a phenylalanine production study by Konstantinov *et. al.*,[23] in which phenylalanine production was found to be independent of the D.O. level in the range of 0-40%.[24] Therefore their criteria for the 20% D.O. setpoint was convenience and simplicity. Research has indicated this D.O. level is not always practical. To maintain 20% D.O. at the end of a fermentation, the airflow rate was 3 L min^{-1}, causing liquid to be carried out of fermentor and thereby clogging the hydrophobic exhaust-air filters. Also, this flow rate (3 vvm) is too high for industrial scale use. It will be important in the future to investigate the DHS production at lower D.O. values.

The glucose-feeding strategy for phase two was chosen for several reasons. Previous fermentation experience indicated that at high glucose concentrations very little, if any, DHS is produced. Also the feeding strategy needs prevent the glucose addition rate from exceeding the respiratory capacity of the cells for a given rate of oxygen transfer into the medium. The method chosen was to keep the glucose concentration low enough to be rate-limiting and to feed glucose when the D.O. rose above the setpoint value. This approach prevented accumulation of glucose to levels that would lead to metabolic overflow[25] or surpass the respiratory capacity and result in acetate production.

The inoculum size also deserves more investigation. The 10% (100 mL to a final volume of 1 L) value used in this study is larger than is typically used in industrial fermentations (2-5% inoculum). A smaller inoculum would greatly affect the costs associated with the inoculum, since a series of fermentors are used to grow the starter cultures for large-scale fermentations.

Significant plasmid loss using the nutritional requirement for maintenance was never detected by either the plating on antibiotic-containing medium relative to controls lacking antibiotics or by restriction enzyme analysis. This results demonstrates that the nutritional plasmid-maintenance strategy worked very well.

The use of a nutritional requirement for plasmid maintenance that would not be supplemented by a rich medium, such as LB, would be desirable for industrial-scale DHS production. In an industrial fermentation the aromatic amino acids would most likely be supplemented by a complex medium component (such as yeast extract). The complex medium component would also supplement serine, rendering the plasmid selection pressure based on *serA* complementation with the host KL3 ineffective. Currently, the aromatic amino acids are added as powders. The cost of pure aromatic amino acids is high compared to a complex medium component such as yeast extract. In addition, there has been the concern about the nonsterile powder additions of the aromatic amino acids. At the bench, scale this has not been a problem, but in an industrial setting, there would be cause

for concern. There have been other nutritional requirements, besides amino acids, used for plasmid maintenance.[26] One such method was employed by Porter et al.[27] The *ssb* gene whose product is responsible from DNA replication was deleted for the *E. coli* chromosome. The *ssb* locus was then placed onto a plasmid. Therefore any plasmid lacking cell would be unable to further replicate and produce more plasmid-lacking cells. Plasmid stability was achieved under nonselective culture conditions. An approach similar to this maybe more appropriate for the future development of the microbial catalyst. It is unlikely that undefined supplementation would complement the deficiency caused by the *ssb* chromosomal deletion.

The glucose feeding rate had a profound effect on DHS production. When the glucose concentration was high in the culture medium, acetic acid was formed. In the second fermentation at the 50 L scale, a significant amount of DHS was produced despite the high concentration of acetic acid present. There was no accumulation of glucose in the NMR spectra from this fermentation, so the acetic acid formed was probably a result of oxygen limitation. Formic acid was also produced in this fermentation which is only formed in anaerobic conditions. It appears that the fermentation was anaerobic for a period of time. However, there may be another cause to the low DHS production in fermentations with high glucose concentration other than the production of inhibitory acetic acid. The effect of the PTS on DHS production was discussed earlier. At very low glucose concentrations a second high affinity glucose transport system is utilized by *E. coli*.[28] The significance of this system is that glucose is phosphorylated by ATP and not by PEP. This transport system utilizes the galactose transport system and involves complex changes in the cell membrane. The optimum medium glucose concentration for the high affinity transport system is about 1 μM.[29] This level is well below the NMR detection limits of about 100 μM. At micromolar glucose concentrations, the *E. coli* growth rate was comparable to growth rates at millimolar glucose concentrations. Also the ability to uptake glucose through the high affinity system increased for at least 50 generations for cultures

grown in a chemostat. So, the *E. coli* can also mutationally adapt to the low glucose concentration environment. It is possible that the previous fermentations primarily have been using the high-affinity transport system. Use of the high-affinity glucose uptake system may be the ideal solution to the problem of in vivo PEP limitations. The cell uses its own regulatory mechanism to make more PEP available, and this method would circumvent problems associated with manipulation of the cell's central metabolism. In addition, micromolar levels of glucose induce the transport systems of other sugars opening the possibility to utilize multiple substrates during the fermentation.

The result that the optimum DAHP synthase activity for DHS production is not the maximum specific activity raises the question of which enzyme becomes rate-limiting. As stated in Chapter 1 (p 11), the hypothesis of metabolic engineering is identification and amplification of rate-limiting enzymes to most efficiently increase carbon throughput in a desired pathway. A next logical step might be to increase the in vivo availability of either PEP or E4P. Some possible methods to increase the availability of PEP and E4P have been tested in shake flask experiments and were discussed in Chapter 1 (p 9). A word of caution is necessary because increasing the PEP and E4P have unseen results. In Chapter 1 (p 10) overexpression of an enzyme whose product was PEP was found to adversely alter the central metabolism of the cell. Also, PEP is involved in more than one key step (both the PTS and DAHP synthase reactions) for the production of DHS, and therefore any changes involving PEP production may have unknown consequences.

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Chapter 4

EXPERIMENTAL

General Methods

Spectroscopic Measurements

^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer at 300 MHz. Chemical shifts were reported in parts per million (ppm) downfield from sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 (TSP δ 0.00) with D_2O as solvent. TSP was purchased from Lancaster. UV and visible measurements were recorded on a Perkin-Elmer Lambda 3b UV-vis spectrophotometer connected to a R100A chart recorder. Additional UV spectra were measured on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with HP 89532A UV-Visible Operating Software.

Bacterial Strains

E. coli AB2834 [*tsx*-352 *supE42* λ^- *aroE353 malA352* (λ^-)] and AB3248 [F^- *aroF363 aroG365 aroH367 proA2 argE3 ilv-7 his-4 lac gal-2 tsx-358 thi* (λ)] were obtained from the *E. coli* Genetic Stock Center at Yale University.[1] *E. coli* KL3 (*serA*::*aroB*) derived from AB2834 and AB2.24 derived from AB3248 (*serA*::*aroB/kan*) were generated for this study.

Storage of Bacterial Strains

All bacterial strains were stored at -78 °C in glycerol. Glycerol samples were prepared by adding 0.75 mL of an overnight culture to a sterile vial containing 0.25 mL of

sterile 80% (v/v) glycerol. The solution was mixed, left at room temperature for 2 h, and then stored at -78 °C.

Culture Medium

All solutions were prepared in distilled, deionized water. LB medium[2] contained (per liter) tryptone (10g), yeast extract (5g), and NaCl (10g). M9 medium[2] contained (per liter) Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), NaCl (0.5 g), $MgSO_4$ (0.12 g), and D-glucose (10 g). M63 medium[2] contained (per liter) KH_2PO_4 (13.6g), $(NH_4)_2SO_4$ (2g), $FeSO_4(H_2O)_7$ (0.5 mg), $MgSO_4$ (0.12 g), and either glycerol (0.46 g) or the combination of glycerol (1.1 g) and D-glucose (0.90 g). The M63 medium was adjusted to 7.0 pH with KOH before autoclaving. Isopropyl β-D-thiogalactopyranoside (IPTG) was added in varying concentration to the culture medium for strains possessing plasmids derived from pSU18 and pSU19. Ampicillin (50 mg L^{-1}), chloramphenicol (20 mg L^{-1}), kanamycin (50 mg L^{-1}), and spectinomycin (50 mg L^{-1}) were added to appropriate cultures. Stock solutions of antibiotics were prepared in water with the exception of chloramphenicol (95% ethanol). Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then combined. Antibiotics and IPTG were sterilized through a 0.2 μm membranes prior to addition to the culture medium. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to LB and 1.5% (w/v) agarose (Sigma, type II: medium EEO) to M9 or M63 medium.

^{1}H NMR Analysis of Culture Supernatant

A 5-6 mL aliquot of the fermentor culture or a 10 mL aliquot of shake flask culture was removed at a specific time point, and cells were removed by centrifugation (3000, 5 min). A portion (0.25-3.0 mL) of the culture supernatant was concentrated to dryness under reduced pressure, concentrated to dryness two additional times from D_2O, and then dissolved in D_2O (99.9 atom %D) containing a known concentration of TSP (δ 0.00).

Concentrations of metabolites in the supernatant were determined by comparison of integrals corresponding to each metabolite with the integral corresponding to TSP in the ^1H NMR spectrum.

Restriction Enzyme Digest of DNA

Restriction enzyme digests were performed using buffer solutions supplied by BRL or New England Biolabs. A typical digest contained approximately 1 μg of DNA in 8 μL TE (10 mM TrisHCl 1 mM EDTA, pH 8.0), 1 μL of restriction enzyme buffer (10X concentration), and 1 μL of restriction enzyme (10 units). The reaction was incubated at 37 °C for 1 to 2 h. The digest was quenched by addition of 1.1 μL of Endostop solution (10x concentration) followed by agarose gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gels were run in TAE buffer containing 40 mM Tris-acetate and 2 mM EDTA (pH 8.0) The concentration of the gel was 0.7% agarose (w/v) in TAE buffer. Ethidium bromide (0.5 μg mL^{-1}) was included in the agarose to visualize the DNA fragment with a UV lamp. The size of the DNA was determined by using two sets of DNA standards: λ DNA digested with *Hind*III or λ DNA digested with *Eco*RI and *Hind*III.

Preparation and Transformation of Competent Cells

Competent cells were prepared using a procedure modified from Sambrook et al.[3] An aliquot (1 mL) from an overnight culture (5 mL) was used to inoculate 100 mL of LB (500 mL Erlenmeyer flask) containing the appropriate antibiotics. The cells were cultured in a gyratory shaker (37 °C, 250 rpm) until they reached the mid-log phase growth (judged from the absorbance at 600 nm reaching 0.6). The culture was transferred to a large centrifuge bottle that had been previously sterilized with 10% (v/v) bleach solution and rinsed with sterile water. The cells were collected by centrifugation (3000 x *g*, 5 min, 4

°C) and the culture medium discarded. At this point all further manipulations were performed on ice. The cell pellet was washed with 100 mL of cold 0.9% (w/v) NaCl and then resuspended in 50 mL of cold 100 mM $CaCl_2$. The suspension was placed on ice for at least 30 min and then centrifuged (3000 x *g*, 5 min, 4 °C). The cell pellet was resuspended in 4 mL of cold 100 mM $CaCl_2$ containing 15% glycerol (v/v). Aliquots (0.25 mL) were dispensed into sterile 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen. The competent cells were stored at -78 °C with no significant decrease in transformation efficiency over a period of several months.

Frozen competent cells were thawed on ice for 5 min before transformation. A small aliquot (1 to 10 µL) of plasmid DNA was added to an aliquot of thawed competent cells (0.10 mL). The solution was gently mixed and placed on ice for 30 min. The cells were then heat shocked at 42 °C for 2 min and placed on ice (2 min). LB (1 mL, no antibiotics) was added to the cells, and the sample was incubated at 37 °C (no agitation) for 1 h. Cells were collected in a microcentrifuge (30 s), resuspended in a small volume of LB (0.1 mL), and then spread onto LB plates that contained the appropriate antibiotics. A sample of competent cells with no plasmid DNA added was also carried through the transformation procedure as a control. These cells were used to check the viability of the competent cells and to verify the absence of growth on selective medium. Strains were retransformed every 4 weeks to ensure homogeneity, since transformed colonies on plates older than 4 weeks can give variable results.

Enzyme Assay

Centrifugation of the appropriate culture (3000 x *g*, 5 min, 4 °C) was followed by resuspension of the cell pellet in the resuspension buffer. The resupension buffer was comprised of 50 mM potassium phosphate (pH 6.5) that contained 10 mM PEP and 0.05 mM $CoCl_2$. The volume of resuspension buffer (mL) was twice the wet weight (g) of the cells. The cells were disrupted by two passes through a French Pressure cell (SML

Aminco) at 11000 psi. Cellular debris was removed from the lysate by centrifugation (48,000 x *g*, 20 min, 4 °C). Protein was quantified using the Bradford dye-binding procedure.[4] A standard curve was prepared using Bovine serum albumin. The protein assay solution (5x concentration) was purchased from Bio-Rad.

DAHP Synthase activity was measured according to the procedure described by Schoner.[5] The cells were disrupted as previously described. The lysate was diluted in potassium phosphate (50 mM), PEP (0.5 mM) and 1,3-propanediol (250 mM), pH 7.0. The lysate dilution buffer, when assaying for aromatic amino acid sensitivity, also contained either 250 μM tyrosine, 200 μM phenylalanine, or 200 μM tryptophan. A dilute solution of E4P was concentrated to 12 mM by rotary evaporation and neutralized with 10 N KOH. A solution was the prepared that contained E4P (6 mM), PEP (12 mM), ovalbumin (1 mg mL^{-1}), and potassium phosphate (25 mM), pH 7.0. An aliquot (0.5 mL) of the diluted lysate was combined with 1 mL of the E4P/PEP solution after 5 min of incubation at 37 °C separately. After these solutions were mixed (time = 0), the reaction was incubated at 37 °C for 5 min. Aliquots (0.15 mL) were removed, during the 5 min, at time intervals and quenched by addition to 0.1 mL of 10% trichloroacetic acid (w/v). Precipitated protein was removed by microcentrifugation (2 min), and DAHP in each sample was quantified by the thiobarbituric acid assay.

The concentration of DAHP was determined by the thiobarbituric acid visualization of the periodate cleavage products.[6] An aliquot (0.1 mL) of solution was incubated at 37 °C for 5 min. The periodate oxidation was quenched by adding 0.5 mL of a solution containing 0.8 M Na_2SO_4 in 0.1 M H_2SO_4 (pH 7.0). The mixture was incubated at 100 °C for 15 min and the pink chromophore was extracted into 4 mL of distilled cyclohexanone. The organic layer was separated by centrifugation (2000 x *g*, 15 min, room temperature) The absorbance of the organic layer was measured at 549 nm (ε = 680000 L mol^{-1} cm^{-1}). One unit of DAHP synthase activity was defined as 1 μmol of DAHP formed per min.

Chapter 2 Experimental

Insertion of a Synthetic Cassette into *serA* of AB3248

Homologous recombination of the synthetic cassette at *serA* was performed with modified literature procedures.[7] Competent AB3248 cells were transformed with the temperature sensitive plasmid containing the cassette for insertion. Cells were heat shocked at 42 °C (2 min) followed by the addition of LB (1 mL) without antibiotics, and the cells were incubated at 44 °C for 1 h. Transformation mixtures were plated onto LB plates containing chloramphenicol and kanamycin and incubated at 44 °C until colonies appeared (24 h). Colonies with the ability to grow in the presence of chloramphenicol and kanamycin at 44 °C contained plasmid DNA inserted into the genome. After several transformations, 11 colonies were isolated.

Cointegrates were inoculated into test tubes containing LB without antibiotics (5 mL) and grown at 30 °C with agitation for 12 h to allow excision of plasmid DNA from the genome. Cultures were diluted (1:20,000) into fresh LB and incubated at 30 °C with agitation for 12 h. One additional cycle of growth at 30 °C was carried out to ensure the cells had excised plasmid DNA from the genome. Cultures were diluted (1:20,000) into fresh LB and incubated at 44 °C for 12 h to allow plasmid loss. Serial dilutions of each culture were spread on LB plates containing kanamycin and incubated overnight at 44 °C. Cells with the ability to grow on kanamycin at 44 °C still retained the kanamycin marker in the genome.

Single colonies were replicate plated on LB plates containing chloramphenicol and LB plates containing kanamycin and were scored for colonies that were kanamycin-resistant and chloramphenicol sensitive at 44 °C. Kanamycin resistance signifies retention of the kanamycin marker in the genome, whereas chloramphenicol-sensitivity signifies the loss of plasmid DNA from the genome. Digestion of the cells and isolation of plasmid DNA yielded no plasmid DNA, verifying the loss of free replicated plasmids for AB2.23

and AB2.24. To verify the cassette insertion inactivated *serA*, AB2.23 and AB2.24 were replicated plated on minimal plates with and without serine. The strains were unable to grow without serine supplementation.

UV Mutagenesis

The conditions for mutagenesis were determined through a modified method of Miller.[8] A single colony of AB2.24 was used to inoculate a 5 mL culture (LB, 50 mg L^{-1}). The culture was grown overnight (37 °C, 12 h, agitation) followed by dilution with M63 salts and spreading onto M63 medium plates supplemented with HIPRVS. The lack of aromatic amino acid supplementation selects for cells that still have a functional *aroF*. The plates were exposed immediately after plating to the UV radiation for a duration of 0 (control), 5, 10, 15, 30, 60, 80, or 100 s at a distance of 18 or 24 inches from the UV source. The UV Lamp (Sylvania Germicidal 5 W) was warmed up 0.5 h prior to the mutagensis. The plates were incubated at 37 °C for 24 h, and the viable colonies counted. The optimal time, at a distance of 24 inches, was 10 s. As a confirmation that UV radiation would penetrate through the agarose gel, the absorbance of the gel was examined. The DGC medium was prepared as describe below and an aliquot was added to a UV cuvette. After solidification, the UV absorbance was scanned through the wavelength range of 200 to 800 nm.

Diffusion Gradient Chamber (DGC) Setup

The DGC is part of the diffusion gradient system commercially produced by Koh Development (Ann Arbor, Michigan). For reservoirs in use during the experiments, a 0.05 μm pore size PC filter membrane (Koh Development) and a gasket (on both sides of the membrane) were placed between each opening into the arena and the reservoir. The reservoirs not in use were sealed off from the arena with non-permeable silastic sheeting (Dow Corning Inc., Midland, Michigan). The reservoirs are secured to the DGC with thumb screws. The lid and bottom plates are fastened by thumb screws.

The DGC was placed on a transilluminator box (TB) which can accommodate up to three DGCs. The TB contained two 30 cm fluorescent light fixtures (single 8W, cool white bulb). The insides of the TB were white and a piece of black felt was placed on the bottom and the sides of the TB to provide contrasts for the pictures. The light was turned off when not in use to prevent heating of the DGC. The TB also had a bracket for mounting a camera above the DGC to record growth patterns photographically. The camera used was a PULNiX TM-7CN CCD-camera. The pictures were taken using the programs Photofinish and AutoCap.

To generate the gradient in the DGC arena, solutes contained in Erlenmeyer flasks were continuously fed through the reservoirs of the DGC via tubing. The flow rate of 2.5 mL h^{-1} was controlled with a dual channel peristaltic pump (LAB Bromma Microplex). An effluent chamber was mounted on a stand next to the TB served three functions. The effluent chamber essential because it permitted control of back pressure in the chamber reservoirs by varying the inlet height into the effluent chamber relative to the height of the DGC reservoir outlets. This was critical since excessive back pressure may cause flooding of the gel due to bulk flow of liquid through the membrane, or insufficient back pressure may cause shrinkage of the gel due to siphoning of the liquid. The effluent chamber also consolidated all of the reservoir outflows into one large waste flask as well as serving as a sterile break in the liquid flow.

A minimal salt medium (M63) was used for the DGC experiments, and all solutions were prepared with distilled, deionized water. The glycerol stock solution (92 g L^{-1}), glucose stock solution (181 g L^{-1}), and $MgSO_4$ stock solution (121 g L^{-1}) were made to a 1 M concentration and autoclaved separately. The amino acid stock solution contained 20 mg mL^{-1} of histidine (H), isoleucine (I), proline (P), arginine (R), valine (V), and serine (S) each. The stock amino acid solution was sterilized through a 0.2 μm membrane prior to use. The m-FT tyrosine was added as a powder to the medium. To a liter of M63 medium 5 mL of glycerol stock solution (5 mM glycerol), 1 mL of $MgSO_4$ stock solution (1 mM

$MgSO_4$), and 2 mL of the stock amino acid solution (40 mg L^{-1} of each amino acid) was added (supplemented M63).

Chemotactic experiments in the DGC were carried out similarly to the experiments described by Emerson *et al.*[9] The arena medium (supplemented M63) was stabilized with 0.15% agarose and initially contained neither glucose nor *m*-fluorotyrosine (m-FT). The sink reservoir (800 mL) contained supplemented M63 medium only. The source reservoir (800 mL) contained the supplemented M63 medium and was additionally supplemented to the final concentration of either 5 mM glucose or 5 mM glucose plus 125 μM m-FT. Sink and source reservoirs were placed opposing each other. This arrangement created a 1 dimensional gradient that spanned 0 to 5 mM glucose, and 0 to 125 μM m-FT if present, from sink to source reservoir.

The DGC, effluent chamber, and empty flasks were autoclaved as one unit. The source and sink flasks containing medium were autoclaved separately, since the pressure of the liquid and the vapor in the flask during autoclaving would disturb the reservoirs gasket seals. After sterilization, the DGC was placed in a sterile laminar flow hood, the empty flasks were exchanged for the full flasks, the supplements were added. Since the m-FT was added as a powder, it was stirred overnight to dissolve after addition to the medium. While still in the laminar flow hood, the arena is filled with 40 mL of the sterile molten 0.15% agarose stabilized medium cooled to approximately 50 °C. This creates a layer (1.6 cm thick) that covers the openings between the arena and each reservoir.

The starter culture (5 mL LB, 50 mg L^{-1} kanamycin) was inoculated with a single colony of AB2.24 and was grown overnight (37 °C with shaking in a water bath). A portion of the starter culture (1 mL) was added to 100 mL of supplemented M63 medium containing 10 mM glycerol and 5 mM glucose in a 250 mL Erlenmeyer flask. This culture was grown (37 °C, 250 rpm) to stationary phase (24 h). Four 1 mL aliquots were concentrated (4x) by microcentrifugation, combined, centrifuged and combined again to give a final concentration of 16x. The center point was inoculated with 15 μL of the 16x

concentrated culture using a micropipette to disperse the cells evenly throughout the depth of the agarose as the pipette was withdrawn from the gel. The DGC and TB were set up in an approximately 30 °C warm room.

The flow of liquid through the reservoirs was started 6 h prior to the inoculation to begin the establishment of the gradients. For the experiment to prove chemotaxis of AB2.24, the photographs were taken every 1 h for the 72 h length of the experiment. For the mutagenesis experiment, the DGC lid was removed 3 days after inoculation, and the cells were exposed to UV radiation for 10 s at a distance of 24 inches from the source, and the lid was quickly replaced. The cells in the DGC were allowed to continue growth until the mutants had separated from the regions of inhibited growth (15 d). The mutagenesis experiment pictures were taken as just prior to mutagenesis and at several time points to document the cells growth patterns.

Recovery of Desired Cells

The cells were recovered by streaking onto LB/kan plates and grown at 37 °C for 10 h. Two methods of sampling from the 3 desired locations of the gel were used. The first method of sampling was a sterile applicator stuck into the gel and used to streak out colonies. The alternative method used a micropipette for removing a plug of gel for streaking. Two plates were streaked out for each region of interest, one by each method of sampling. This resulted in 6 plates of possible mutants.

After the growth on the plates, single colonies were selected and replicate plated onto M63/HIPRVS plates with 0, 30, and 150 μM m-FT and a LB/kan master plate. The plating was performed in the listed order to assure that an adequate number of cells were introduced onto each plate. Too few plated cells would be reflected by an absence of growth on the last LB/kan plate. The LB/kan plate was a check that the lack of growth on the minimal plates was due to inhibition and was not a result of poor replicate plating.

Colonies that grew well on either the 30 or 150 μM m-FT, as compared to the 0 μM m-FT plate, were tested for tyrosine insensitivity. Four colonies were tested for insensitivity.

Culture Conditions for DAHP Synthase Assay of DGC Mutants

The appropriate colonies for the assays were taken from the LB/kan master plates. Each colony was removed with an applicator, used to make a master plate of the colonies tested, and then used to inoculate the starter culture (50 mL LB, 50 mg L^{-1} kanamycin in a 250 mL Erlenmeyer flask). After 10 h of growth (37 °C, 250 rpm), 5 mL of the starter culture was added to the growth flask (500 mL LB, 50 mg L^{-1} kanamycin, in a 2 L Erlenmeyer flask) and incubated for 10 h (37 °C, 250 rpm). The cells were harvested and the DAHP synthase/TBA assay was performed as described in the general methods section.

Isolation of the Feedback-Insensitive *aroF* gene

The cloning and isolation of the feedback-insensitive *aroF* (*aroF*^{fbr}) was conducted by Kai Li and Dr. Karen Draths. The first step was the isolation of genomic DNA. The method used was from Silhavy.[10] Three genomic isolations were performed. One colony of AC1-17 and two colonies of AC2-13, designated AC2-13A and AC2-13B, were used. The second step was the amplification of *aroF*^{fbr} from the genomic DNA.

The *aroF*^{fbr} gene was amplified from the genome using the primers JWF-19 and JWF-22 (Table 1, Chapter 2) which have *Eco*RI ends. The PCR products were then ligated into the low copy number plasmid pCL1920 for sequencing. The three resulting plasmids were designated pCL1-17-1, pCL2-13A-1, and pCL2-13B-1.

Sequencing of Feedback-Insensitive *aroF* gene

The polyethylene glycol precipitation method of isolation of the DNA fragment for sequencing was used. The experiment was performed by Dr. Karen Draths.[11] The primer used for sequencing were JWF-22, JWF-79, JWF-80, JWF-81, and JWF-82 (Table 1,

Chapter 2). The primers and template were sent to the Molecular Sequencing Facility at Michigan State University for sequencing. The mutation introduced a *Bgl*II restriction site into the gene, as indicated by the sequencing information. Digests of the *aroF*^{Tbr} with *Bgl*II confirmed the presence of the new restriction site.

Generation of Truncated *aroF*^{Tbr}

The truncated *aroF*^{Tbr} gene was created by eliminating *tyrR* box 3 and half of *tyrR* box 2. This was accomplished by PCR amplification of *aroF*^{Tbr} from pCL2-13A using the primers JWF-94 and JWF-22 (Table 1, Chapter 2). The PCR product was ligated into pCL1920 generating the new plasmid pCL2-13A-trunc.

Replacement of the *aroF*^{Tbr} Native Promoter with the *tac* Promoter

The *aroF*^{Tbr} open reading frame was PCR amplified with JWF-103 and JWF-97 (Table 1, Chapter 2). The *serA* locus and the new generated *aroF*^{Tbr} fragment were cloned into pBR322, generating the plasmid pKL4.79B, which contains the *tac* promoter. The *aroF*^{Tbr} fragment was cloned such that the tac promoter and RBS of pBR322 was in the correct location and orientation to express the gene.

Shake Flask Culture Conditions for *aroF*^{Tbr} Specific Activity Evaluations

Three plasmids, pCL1-17-1, pCL2-13A-1, and pCL2-13B-1 were transformed into competent AB3248 in order to assay the specific activity of *aroF*^{Tbr}. A single colony of AB3248/pCL1-17-1, AB3248pCL2-13A-1, and AB3248pCL2-13B-1 each was used to inoculate a starter culture and (50 mL LB, 50 mg L^{-1} Spectinomycin, in a 250 mL Erlenmeyer flask) grown for about 10 h (37 °C, 250 rpm). After 10 h, 5 mL of each starter culture was used to inoculate the growth flask (500 mL LB, 50 mg L^{-1} spectinomycin, in 2 L Erlenmeyer flask) and grown for 10 h (37 °C, 250 rpm). The exact experimental conditions were repeated for AB3248/pCL2-13A (as a control) and

AB3248/pCL2-13A-trunc. The assay for specific activities was performed as described on the general methods section of this chapter.

Both AB3248/pCL2-13A (as a control) and AB3248/pCL2-13A-trunc were grown using culture conditions for DHS accumulation. A single colony of each strain was used to inoculate a starter culture (50 mL LB, 50 mg L^{-1} spectinomycin, in a 250 mL Erlenmeyer flask) and grown for 10 h (37°C, 250 rpm). At 10 h 5 mL of the starter culture was used to inoculate, in triplicate, growth flasks for each construct (500 mL M9, 40 mg L^{-1} aromatic amino acids, 10 mg L^{-1} vitamins, and 50 mg L^{-1} spectinomycin, in a 250 mL Erlenmeyer flask). The growth culture was grown for 36 h (37 °C, 250 rpm). One culture of each construct was harvested for the enzyme assay at 12, 24, and 36 h.

A single colony of the constructs KL3/pKL4.32B (truncated *aroF*^{fbr}) and KL3/pKL4.33B (nontruncated *aroF*^{fbr}) was used to inoculate starter cultures (50 mL M9, 40 mg L^{-1} aromatic amino acids, 10 mg L^{-1} vitamins, and 20 mg L^{-1} chloramphenicol, in a 250 mL Erlenmeyer flask) which were grown for 24 h (37 °C and 250 rpm). After 10 h, 5 mL of the starter culture was used to inoculate triplicate growth flasks (500 mL M9, 40 mg L^{-1} aromatic amino acids, 10 mg L^{-1} vitamins, and 50 mg L^{-1} chloramphenicol, in a 250 mL Erlenmeyer flask) for each construct. The growth culture was grown for 36 h (37 °C, 250 rpm). After 12, 24, and 36 h, one culture of each construct was harvested for the enzyme assay and quantification of DHS.

For the double *aroF*^{fbr} constructs (KL3/pKL4.66A and KL3/pKL4.66B), the growth conditions were exactly the same as for the previous experiment. The cultures were harvested at 12, 24, and 36 h for the enzyme assay, but the supernatant was only analyzed for DHS at 24 and 36 h.

For KL3/pKL4.79B three cultures were grown, in triplicate, (9 cultures total) using the just previously described conditions, except 7 h after the inoculum was added to the growth cultures, IPTG was added. To the first three flasks no IPTG was added, to the second set of three flasks IPTG was added to a final concentration of 0.05 mM, and to the

remaining flasks IPTG was added to a final concentration of 0.5 mM. One flask of each of the three culture types (0, 0.05, and 0.5 mM IPTG) was harvested for the enzyme assay at 10, 21, and 44 h, and the supernatants were analyzed for DHS.

Chapter 3 Experimental

B. Braun Fermentor

The fermentor utilized was a B. Braun Biotech Biostat® MD. The fermentor was equipped with a platinum thermister temperature probe, a polarizable dissolved oxygen probe, a pH probe, and an antifoam probe. It was equipped with three six-bladed disk impellers. The temperature was maintained at the setpoint by the circulation of either cooled or heated water through the jacket of the vessel. The fermentor vessel was interfaced to a DCU unit for control. The DCU unit was connected to a Compaq personal computer running the Braun fermentor control software MFCS. The fermentor could be controlled be either the DCU or the MFCS software, but usually the fermentor was controlled by the MFCS software because only it could record and store the data.

The temperature, pH, and glucose feeding were controlled with a PID controller. Only the glucose feeding control constants were changed for fermentations. The acid and base were added with peristaltic pumps that were part of the DCU unit. The glucose feed was supplied by an external pump connected to the DCU.

Samples were removed from the culture via the harvest pipe. There were 2 outlets for the air exhaust. The main outlet was equipped with a condenser to minimize evaporative loss of the culture and the second outlet was an emergency exhaust if the main exhaust became clogged. The exhausts were temporarily closed to create pressure inside the vessel, and the harvest pipe was opened to allow the sample to flow out. When the air flow into the vessel was high (greater than 1 L min^{-1}) the back pressure created by aeration was enough to push the sample out the harvest pipe without the exhausts closed off.

Fermentor Medium

The optimized fermentation medium contained (per liter) K_2HPO_4 (7.5 g), Fe(III) ammonium citrate (0.3 g), citric acid (2.0 g), concentrated H_2SO_4 (1.22 mL), and $MgSO_4$ (0.24 g). The medium was prepared by adding the salts (except $MgSO_4$) to 850 mL of distilled and deionized water. The reason for using only 850 mL was to have a 1 L final volume after all supplements and inoculum were added. The pH was adjusted to 7 by the addition of KOH, except the 50 L fermentation was adjusted with NH_4OH. The $MgSO_4$ was autoclaved separately from the fermentor medium.

Aromatic amino acids were supplemented as a dry powder by the addition of phenylalanine (0.7 g L^{-1}), tyrosine(0.7 g L^{-1}), and tryptophan (0.7 g L^{-1}). The aromatic vitamins *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid, were prepared as a stock solution (10 mg mL^{-1}). The stock trace mineral solution contained (per liter) $(NH_4)_6Mo_7O_{24}(H_2O)_7$ (3.7 g), H_3BO_3 (24.7 g), $MnCl_2(H_2O)_4$ (15.8 g), $ZnSO_4(H_2O)_7$ (2.88 g), and $CuSo_4(H_2O)_5$ (2.49 g). The trace mineral and aromatic vitamin stock solution were sterilized by filtration through a 0.2 μm pore membrane prior to addition

A stock solution of glucose was prepared and autoclaved separately for each fermentation. Either 18 or 7.2 g of glucose was added to 50 mL of water and autoclaved. This was to add to the fermentor to adjust the final concentration of glucose in the fermentor at the start of the experiment to either 20 or 8 g L^{-1}, including the glucose that was added from the inoculum.

Fermentor Inoculum for Evaluation of KL3/pKL4.32B and KL3/pKL4.33B

The starter culture for the evaluation was (per liter) K_2HPO_4 (4.8 g), KH_2PO_4 (12.2 g), yeast extract (2.5 g), $(NH_4)_2SO_4$ (2.5 g), $MgSO_4$ (0.5 g), and glucose (1.2 g). A single colony was used to inoculate the starter culture (80 mL medium, 50 mg L^{-1} chloramphenicol, in a 250 mL Erlenmeyer flask). The start culture was grown for 12 h (37

°C , 250 rpm) and harvested (3000 x *g*, 5 min, 4 °C). The cell pellet was resupsended in the optimized fermentor medium and added to the fermentor vessel. The only other difference in culture conditions from the optimized conditions was the D.O. setpoint at 5% instead of 20% during the fermentation.

Optimized Fermentor Inoculum

A single colony was used to inoculate the starter culture (100 mL LB in a 500 mL Erlenmeyer flask) supplemented with glucose to the final concentration of either 8 g L^{-1} or 20 g L^{-1}. The antibiotic chloramphenicol (20 mg L^{-1}) or ampicillin (50 mg L^{-1}) was added for selection pressure. The inoculum was grown at 37 °C for approximately 14 h at 250 rpm.

Fermentor Conditions

The fermentation conditions were divided into two phases. In the first phase, the D.O. was controlled by impeller speed and airflow rate. Dissolved oxygen started at approximately 100% saturation. The liquid phase dissolved oxygen concentration was maintained at 20% air saturation by first the impeller speed, and then the airflow rate. The impeller speed was set to a minimum value of 50 rpm, and a maximum value of 900 rpm. After the controller reached the maximum rpm, the airflow rate was increased to maintain the 20% dissolved oxygen. The airflow rate minimum and maximum was 0.06 and 3.0 L min^{-1}, respectively. The first phase lasted for 10 to 18 h depending on the construct.

After the glucose in the first phase was consumed, the second phase of fermentation was started. At this time, the impeller was usually at the maximum rpm (900), and there was a rapid increase in the airflow rate to maintain the D.O. The airflow rate would then reach 3.0 L min^{-1} for a short time before rapidly decreasing which corresponded to consumption of the glucose. At the point of the phase change, the control of the impeller and airflow rate was stopped. Two different methods, were used to start the second phase.

For both methods the impeller was set to 900 rpm. Preferably, the point of maximum airflow rate (3.0 L min^{-1}) was reached and had not begun declining, allowing the flow rate to be set to this value. For some of the first fermentations the second method was used. When the point of maximum airflow rate was missed and had begun to decline, the airflow rate was set to the current value at the time of the phase change (usually about 1.0 L min^{-1}). Then the flow rate was increased in increments of about 0.25 - 0.5 L min^{-1} to reach a final flow rate of 2.5 - 3.0 L min^{-1} within about 12 - 15 h after the start of phase two.

The D.O. was controlled, in the second phase, by glucose feeding. Glucose was fed with either a 400 or 600 g L^{-1} solution. The glucose was fed on demand based upon the increase of the D.O. from the 20% saturation set point. The PID controller parameters were found empirically. The parameter were set to 0.0 (off) for the derivative control (τ_D), 999.9 s (minimum control action) for the integral control (τ_I), and 950.0% for the proportional band (X_P). A simulation of the process was performed to obtain a first approximation of the controller parameters, but when this was done, very different conditions were employed for the fermentation (i.e. glucose feed rate and final cell density). As a result, the parameters were significantly modified. The setpoint for the culture temperature was 37 °C. The pH was maintained at 7.0 ± 0.05 pH units. The pH was controlled with the addition of 2 N HCl and 28% NH_4OH. Sigma 204 antifoam was added manually when needed.

Fermentor Setup

The fermentor vessel was prepared for autoclaving by first adding medium (850 mL) to the fermentor vessel. Sparger, impeller motor connection, and all probe connections were protected during autoclaving by covering in aluminum foil. The harvest pipe and the gas exhausts were closed off by clamps. One unused port was left loose when autoclaved. This was to prevent pressure build-up in the fermentor during the depressurize cycle of autoclaving and was critical. An additional 1 L of the

fermentor medium was autoclaved to replace any volume loss in the fermentor due to autoclaving.

The fermentor was autoclaved for 25 min at 121 °C, at least 12 h before the start of the fermentation. The fermentor vessel was allowed to cool in the autoclave for about 20 min after the end of the autoclaving cycle. This was to prevent the medium from boiling out of the fermentor vessel.

The D.O. probe was connected to the DCU after autoclaving. The D.O. must be polarized before use and was connected about 12 h before the start of the fermentation. All other connections (probes, water, and airline) were made about 1 h before the start of the fermentation. The pH probe was calibrated with buffer solution of pH 4.01 and 7.0 before autoclaving. The D.O. probe was calibrated about 1 h before the start of the fermentation. Before D. O. probe calibration, the fermentor vessel was warmed to the operating temperature of 37 °C and the impeller speed was set to 100 rpm. The probe was calibrated with N_2, for the 0% dissolved oxygen, and then was calibrated with air, for the 100% dissolved oxygen. The flow rate for both calibrations was 3 L min^{-1}.

Just prior to the start of the fermentation, the supplementation was added. To the medium, 2 mL of stock MgSO4, 1 mL of stock vitamin solution, the 50 mL glucose solution, 1 mL of trace mineral solution, and the aromatic amino acids were added through an open port. Lastly the inoculum was added and the data collection by the MFCS software was begun.

Fermentor Samples

Culture broth samples were withdrawn from the vessel every 6 - 8 h. When only the DHS concentration was to be determined, a 5 mL aliquot was taken. The cells were removed by microcentrifugation, and the supernatant was analyzed for DHS. For DHS concentration and enzyme assay sample, 50 mL was withdrawn for the first

sample (12 h) and subsequent sample were 25 mL (24, 36, and 48 h). The cells were pelleted as described in the enzyme assay section in this chapter, and the supernatant was analyzed for DHS. For all samples the first 5 mL of sample was discarded due to the dead volume present in the harvest pipe.

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