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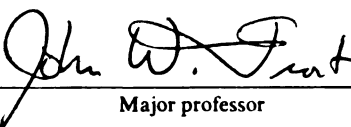
MICROBIAL SYNTHESSES OF VALUE-ADDED
CHEMICALS FROM D-GLUCOSE

presented by

Kai Li

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Chemistry


Major professor

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MICROBIAL SYNTHESSES OF VALUE-ADDED CHEMICALS FROM D-GLUCOSE

By

Kai Li

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Chemistry

1999

ABSTRACT

MICROBIAL SYNTHESIS OF VALUE-ADDED CHEMICALS FROM D-GLUCOSE

By

Kai Li

Microbial syntheses of value-added chemicals from renewable resources are becoming important alternatives for current petroleum-based chemical syntheses in industry. Environmental considerations and the non-renewable character of petroleum necessitate the development of microbial biocatalysis through a combination of metabolic engineering and fermentation technologies. The primary tasks are to develop novel environmentally-benign routes to target molecules, to increase the titer and yield of the synthesized product, and to reduce production costs through alternative feedstocks. In this thesis, the synthesis of vanillin from D-glucose,¹ the fed-batch fermentor synthesis of 3-dehydroshikimic acid (DHS) using recombinant *Escherichia coli*,² and the selection of optimal carbon sources^{3,4} illustrate these three challenging aspects of microbial biocatalysis.

Vanillin is the major flavor component of vanilla extract. Synthesis of vanillin from glucose has been achieved using a microbe-catalyzed conversion of glucose into vanillic acid followed by an enzyme-catalyzed reduction of the vanillic acid to afford vanillin. A genetically engineered *E. coli* synthesized 5.0 g/L of vanillic acid from glucose under fed-batch fermentor conditions. Aryl-aldehyde dehydrogenase purified from *Neurospora crassa* was used to reduce vanillic acid to vanillin in 66% isolated yield.

DHS is a hydroaromatic intermediate in the common pathway of aromatic amino acid biosynthesis. In addition to being a potent antioxidant, it is the most advanced intermediate shared by aromatic amino acid biosynthesis and biocatalytic synthesis of adipic acid, catechol, vanillin, and gallic acid. Microbial synthesis of DHS, like other intermediates in the common pathway, has previously been examined only under shake

flask conditions. A series of *E. coli* biocatalysts have been constructed to evaluate the synthesis of DHS under fed-batch fermentor conditions. The limiting factors for achieving high DHS yields and titers were identified and alleviated. DHS titers of 69 g/L were synthesized in 30% yield.

Carbon source selection is one of the most important considerations confronting microbe-catalyzed chemical synthesis. The phosphotransferase uptake system imposes an upper limit on the yields and concentrations of chemicals which can be synthesized when microbes such as *E. coli* employ glucose as a carbon source. The concentration and yield of microbially synthesized DHS has been examined as a function of using D-xylose, L-arabinose, D-glucose, or a mixture consisting of a 3/3/2 molar ratio of glucose/xylose/arabinose as carbon sources under fed-batch fermentor conditions. The sugar mixture is a direct simulation of corn fiber hydrolysate. In addition, butane-derived succinic acid has been evaluated as a glucose adjunct for the synthesis of DHS to determine how glucose might be improved upon as a source of carbon. Catabolite repression of the uptake of both pentoses and succinic acid in the presence of glucose were successfully circumvented under fed-batch fermentor conditions. Our discovery of higher-titer, higher-yielding syntheses of DHS using these alternative feedstocks carries significant ramifications relevant to the employment of corn fiber and the recruitment of butane feedstocks in microbial synthesis of value-added chemicals.

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1. Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1998**, *120*, 10545.
2. Li, K.; Mikola, M. R.; Draths, K. M.; Worden, R. M.; Frost, J. W. *Biotechnol. Bioeng.* **1999**, *64*, 61.
3. Li, K.; Frost, J. W. *Biotechnol. Prog.* **1999**, In press.
4. Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, In press.

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**To my parents and my wife
For their love and support**

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LIST OF ABBREVIATIONS

ADC	4-amino-4-deoxychorismic acid
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ap	ampicillin
bp	base pair
CA	chorismic acid
cAMP	cyclic adenosine monophosphate
cDNA	complimentary DNA
FADH ₂	flavin adenine dinucleotide, reduced form
CIAP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
COMT	catechol- <i>O</i> -methyltransferase
DAH	3-deoxy-D- <i>arabino</i> -heptulosonic acid
DAHP	3-deoxy-D- <i>arabino</i> -heptulosonic acid 7-phosphate
DCU	digital control unit
DEAE	diethylaminoethyl
2, 3-DHB	2,3-dihydroxybenzoic acid
DHQ	3-dehydroquinic acid
DHS	3-dehydroshikimic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
DO	dissolved oxygen
DTT	dithiothreitol
E4P	D-erythrose 4-phosphate

EPSP	5-enolpyruvoylshikimate 3-phosphate
FBR	feedback resistant
FBS	feedback sensitive
GA	gallic acid
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl β -D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
K _m	Michaelis constant
M	molar
min	minute
mL	milliliter
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance spectroscopy
OD	optical density
ORF	open reading frame
PABA	<i>p</i> -aminobenzoic acid
PCA	protocatechuic acid
PEG	polyethylene glycol
PEP	phosphoenolpyruvic acid
PHB	<i>p</i> -hydroxybenzoic acid

PID	proportional-integral-derivative
Pck	PEP carboxykinase
PCR	polymerase chain reaction
Phe	L-phenylalanine
PMSF	phenylmethanesulfonyl fluoride
Ppc	PEP carboxylase
Pps	PEP synthase
PTS	phosphotransferase system
QA	quinic acid
RBS	ribosome binding site
rpm	rotations per minute
SA	shikimic acid
SAM	<i>S</i> -adenosylmethionine
SDS	sodium dodecyl sulfate
S3P	shikimate 3-phosphate
Spec	spectinomycin
TBA	thiobarbituric acid
Tc	tetracycline
TCA	tricarboxylic acid
Trp	L-tryptophan
TSP	sodium 3-(trimethylsilyl)propionate-2,2,3,3- <i>d</i> ₄
Tyr	L-tyrosine
UDP	uridine diphosphate
UV	ultraviolet
V _{max}	maximal velocity

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CHAPTER 1

INTRODUCTION

Chemistry is moving into an era in which renewable resources and starting materials such as D-glucose, D-xylose, and L-arabinose will likely be prominent features of industrial chemical manufacture. Current chemical manufacture typically relies on abiotic, chemical catalysts and on starting materials derived from petroleum. As a nonrenewable natural resource, petroleum has several negative environmental and geopolitical problems associated with its use. For example, aromatics are currently derived predominantly from the benzene, toluene, xylene (BTX) fraction of petroleum refining. With annual production levels at 5.4×10^9 kg in the United States, benzene is the most important component of the BTX fraction.¹ Benzene is a potent carcinogen,² and it is also included by the Environmental Protection Agency on the list of chemicals covered by the Chemical Manufacturing Rule that requires drastic reductions in emissions of hazardous organic air pollutants.³

Beyond the health costs associated with benzene, the costs of deriving this starting material from nonrenewable fossil fuel feedstocks are important to consider. Oil spills and land reclamation along with geopolitical complications substantially add to the true cost of aromatic manufacture from fossil fuel-derived BTX. By contrast, plant-derived starch, cellulose, and hemicellulose are abundant, renewable sources of glucose, xylose, and arabinose. The comparatively low temperatures, near-atmospheric pressures, and use of water as reaction solvent, which characterize microbial biocatalysis, are environmentally benign. In addition, there is a net consumption of carbon dioxide from the environment when glucose, xylose, and arabinose are employed as starting materials for chemical synthesis. Add to this the avoidance of toxic intermediates, reagents, and byproducts, and the ability to synthesize a chemical by microbial biocatalysis from renewable feedstocks and

nontoxic starting materials presents itself as an appealing alternative to traditional chemical manufacture.⁴

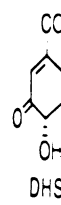
In Chapter 2 of this thesis, a novel route to synthesize vanillin biocatalytically is described, which serves as a good example for the comparison of biocatalysis with traditional chemical manufacture. The main portion of the route was developed through metabolic engineering of the common pathway of aromatic amino acid biosynthesis (the shikimate pathway) in *Escherichia coli*. Chapter 3 and 4 of this thesis focus on increasing the carbon flow directed into the shikimate pathway to improve titers and yields of biosynthesized chemicals. 3-Dehydroshikimic acid (DHS) was used as a model compound because it is the most advanced intermediate shared by both biosynthesis of aromatic amino acids and biocatalytic syntheses of a variety of value-added chemicals.

The Shikimate Pathway

The shikimate pathway, also referred to as the common pathway of aromatic amino acid biosynthesis, has been the subject of considerable study due to its role in the transformation of simple carbohydrate precursors into aromatics in plants, bacteria, fungi, and molds.⁵ It consists of seven enzyme-catalyzed reactions converting phosphoenolpyruvic acid (PEP) and erythrose 4-phosphate (E4P) into chorismic acid (Figure 1). Three terminal pathways then lead from chorismic acid to L-tryptophan, L-tyrosine, and L-phenylalanine. In addition, biosynthetic pathways leading to ubiquinone, folic acid and enterochelin also branch away from the common pathway at chorismic acid (Figure 1).⁵ Folic acid-derived coenzymes are frequently involved in the biosynthetic transfer of one carbon fragments; ubiquinones are involved in electron transport; and enterochelin is an iron chelator responsible for iron uptake in numerous microorganisms.

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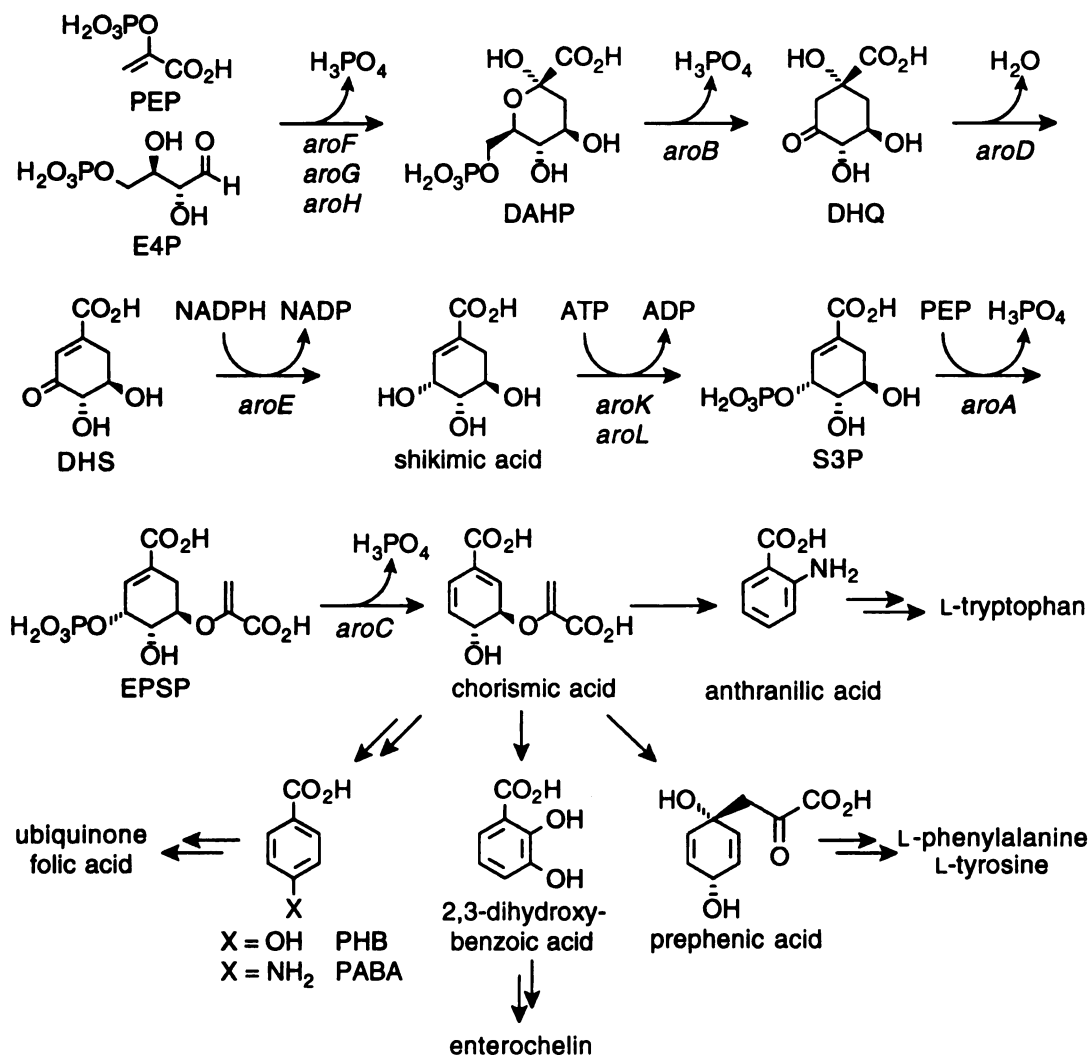


Figure 1. The common pathway of aromatic amino acid biosynthesis. Genetic loci are as follows: *aroF aroG aroH*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroL aroK*, shikimate kinase; *aroA*, EPSP synthase; *aroC*, chorismate synthase.

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Individual pathway enzymes have received attention due to the novel mechanisms employed during turnover of substrate into product and as targets for inhibition. The existence of the shikimate pathway in plants and bacteria but not in humans provides an appealing mode of action for enzyme-targeted herbicides and antibiotics.

The substrates and products of the seven enzymatic reactions that convert PEP and E4P into chorismic acid (Figure 1) were identified by the early 1960s from studies of bacterial auxotrophs of *Escherichia coli* and *Klebsiella aerogenes*. The first committed step of aromatic amino acid biosynthesis involves the condensation of PEP and E4P to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) and is catalyzed by DAHP synthase.⁵ Three isozymes of DAHP synthase exist in *E. coli*, each of which is sensitive to feedback inhibition by one of the three aromatic amino acids. The genes *aroF*, *aroG* and *aroH* encode for tyrosine-sensitive, phenylalanine-sensitive, and tryptophan-sensitive isozymes of DAHP synthase, respectively. DAHP is converted into 3-dehydroquinic acid (DHQ) by DHQ synthase which is encoded by *aroB*⁶ in a complex reaction where active site NAD is used as a catalytic active site residue.⁶ A *syn* elimination of water from DHQ affords 3-dehydroshikimic acid (DHS).⁷ This reaction is catalyzed by DHQ dehydratase, which is encoded by *aroD*. Reduction of DHS to shikimic acid in the presence of NADPH is catalyzed by *aroE*-encoded shikimate dehydrogenase.⁸ Shikimic acid is further converted to shikimate 3-phosphate by phosphoryl group transfer from ATP. This reaction is catalyzed by two isozymes of shikimate kinase encoded by the loci *aroL*⁹ and *aroK*.¹⁰ 5-Enolpyruvoylshikimate 3-phosphate (EPSP) synthase, encoded by *aroA*,¹¹ catalyzes the reversible condensation of shikimic 3-phosphate and PEP. Product EPSP is formed along with inorganic phosphate. The last enzyme of the common pathway is chorismate synthase.¹² Encoded by *aroC*, it catalyzes the elimination of inorganic phosphate from EPSP to afford chorismic acid.

Microbial Syntheses of Value-Added Chemicals Utilizing the Shikimate Pathway

Aromatic amino acids such as phenylalanine and tryptophan figure prominently among the chemicals being microbially manufactured from glucose.¹³ A variety of other chemicals are then synthesized from these aromatic amino acids. For example, phenylalanine can be enzymatically or chemically converted to aspartame (Figure 2),¹⁴ which has the largest sales volume of all food additives.¹⁵ Introduction of naphthalene dioxygenase into a tryptophan-synthesizing microbe that also expresses tryptophanase results in biocatalytic synthesis of indigo (Figure 2),¹⁶ the vat dye that gives blue jeans their faded-blue coloration. Indigo is the highest volume dye produced worldwide. Tyrosine can be converted into melanin (Figure 2),¹⁷ the mammalian pigmentation that is responsible for hair coloring as well as protection from solar irradiation.

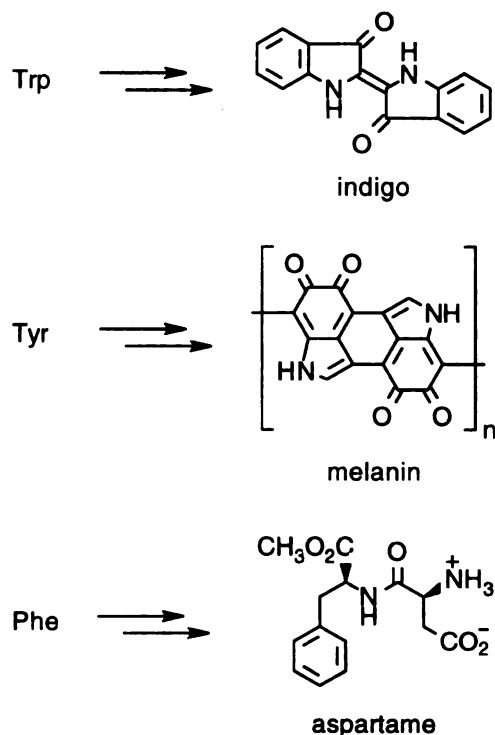


Figure 2. Chemicals synthesized from tryptophan, tyrosine, and phenylalanine.

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Some common pathway intermediates are important value-added chemicals. For example, shikimic acid is a valuable chiral synthon used in the synthesis of neuraminidase inhibitors effective in the treatment of influenza infection.¹⁸ Because the current isolation of shikimic acid from the fruit of *Illicium* plants¹⁹ precludes its use in kilogram-level synthesis, an *E. coli* biocatalyst has been constructed which synthesizes 50 g/L of shikimic acid from glucose under fed-batch fermentor conditions. This shikimate-synthesizing biocatalyst resulted from disruption of the genomic *aroL* and *aroK* loci in *E. coli* and overexpression of feedback-insensitive, *aroF^{FBR}*-encoded DAHP synthase to channel more carbon into the common pathway.²⁰

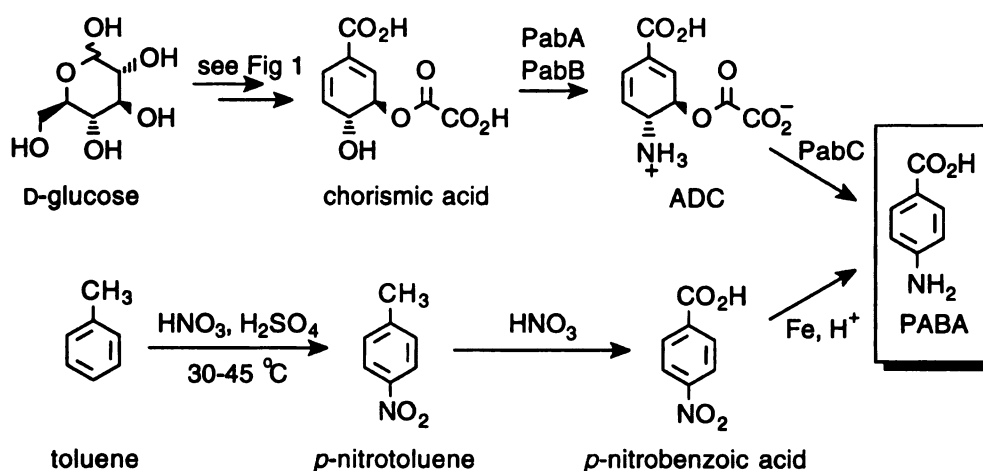


Figure 3. Comparison of microbial and chemical synthesis of *p*-aminobenzoic acid (PABA).

Intermediates in the common pathway of aromatic amino acid biosynthesis can also be transformed into valuable chemicals either through chemical conversion or bioconversion. For instance, *p*-aminobenzoic acid (PABA) is biosynthesized by initial conversion of chorismic acid to 4-amino-4-deoxychorismic acid (ADC) followed by elimination of pyruvic acid (Figure 3). ADC synthase consists of two subunits encoded by the *pabA* and *pabB* loci. ADC lyase is encoded by *pabC*. Each of the *E. coli* genes associated with the conversion of chorismic acid into PABA has been cloned and

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sequenced.²¹ Although its biological role is as an intermediate in the biosynthesis of folic acid, PABA's chemical use is as an ingredient in UV-blocking formulations, and it can be esterified to form the local anesthetic known as benzocaine.²²

PABA is currently industrially synthesized from toluene (Figure 3). The first step entails reaction of toluene with a 20/60 (v/v) of nitric acid/sulfuric acid at 30-45 °C.²² *p*-Nitrotoluene is then separated from the other nitrotoluene isomers by sequential distillation and crystallization. Oxidation of the methyl group of *p*-nitrotoluene with nitric or chromic acids is followed by reduction to afford PABA. Almost every step of this manufacturing route has to contend with a health/safety hazard. These range from strong acid solutions to manipulation of toxic, flammable toluene and highly toxic *p*-nitrotoluene.²³

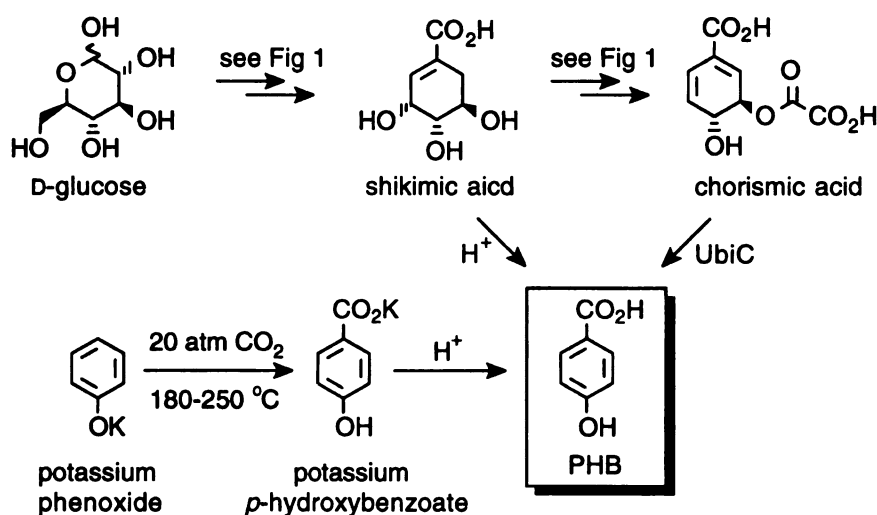


Figure 4. Comparison of *p*-hydroxybenzoic acid (PHB) synthesis from glucose versus potassium phenoxide.

Chorismic acid can also be converted directly into *p*-hydroxybenzoic acid in a reaction catalyzed by *ubiC*-encoded²⁴ chorismate-pyruvate lyase (Figure 4). An *E. coli* biocatalyst has been constructed consisting of a genome-localized *aroAaroLaroBaroCkan*^R cassette and plasmid-localized *aroF^{FBR}*, *tktA*, and *ubiC*. The genes encoding other chorismate-utilizing enzymes are disrupted to prevent biocatalytic conversion of chorismic

acid into anthranilic acid and prephenic acid (Figure 1). The biocatalyst can synthesize 12 g/L of *p*-hydroxybenzoic acid from glucose under fed-batch fermentor conditions.²⁵ *p*-Hydroxybenzoic acid can also be synthesized by means of chemical dehydration of shikimic acid. This reaction is catalyzed by 1 M sulfuric acid in refluxing acetic acid (Figure 4).²⁵ *p*-Hydroxybenzoic acid is a component of liquid crystal polymers such as Xydar,²⁶ which have attracted considerable attention because of their use in high-performance applications. Esters of *p*-hydroxybenzoic acid are also widely used as food preservatives.²⁷

p-Hydroxybenzoic acid is currently manufactured by Kolbe-Schmitt reaction of dried potassium phenoxide with 20 atm dry carbon dioxide at 180-250 °C (Figure 4).²⁷ Product *p*-hydroxybenzoate potassium salt is converted to its free acid upon addition of mineral acid. Besides the required temperatures and pressures, *p*-hydroxybenzoic acid manufacture has to contend with handling of phenol which is listed as a highly toxic, corrosive chemical.²³

Quinic acid is another useful molecule that can be microbially synthesized through the utilization of the shikimate pathway (Figure 5).²⁸ Quinic acid is a widely used chiral starting material in multi-step chemical synthesis.²⁹ It is currently obtained by an expensive isolation from plant sources. An *E. coli* biocatalyst has been constructed via mutational inactivation of *aroD*-encoded DHQ dehydratase, overexpression of *aroF^{FBR}*-encoded feedback-insensitive DAHP synthase and overexpression of *aroE*-encoded shikimate dehydrogenase. The biocatalyst can synthesize up to 80 g/L of quinic acid.³⁰ Quinic acid can be converted into either benzoquinone or hydroquinone through simple chemical oxidation (Figure 5).²⁸ Hydroquinone's selective reduction of photoactivated silver ion is the basis for this organic's widespread use in photography. Benzoquinone is an important chemical precursor in the manufacture of various chemicals.³¹

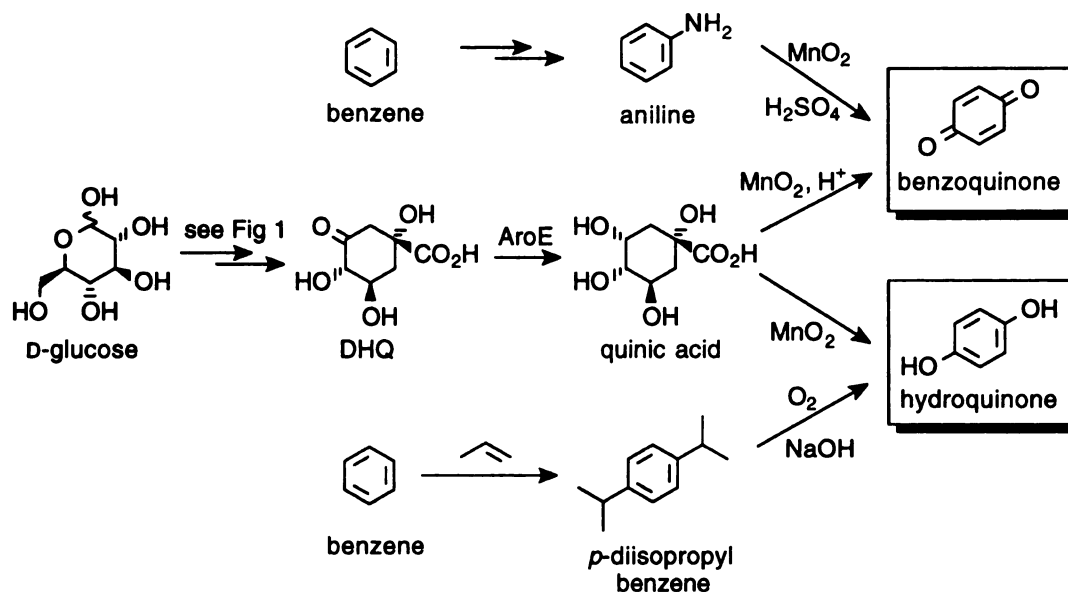


Figure 5. Comparison of benzoquinone and hydroquinone synthesis from glucose and benzene.

Hydroquinone is produced globally at volumes of $4.5 - 5.0 \times 10^7$ kg/year.³² The dominant route to manufacture hydroquinone is through hydroperoxidative synthesis.³² The *p*-diisopropylbenzene is synthesized by zeolite-catalyzed Friedel-Crafts reaction of benzene or cumene with propylene or isopropanol. Air oxidation of the *p*-diisopropylbenzene proceeds at 90 - 100 °C in an aqueous NaOH solution also containing organic bases along with cobalt or copper salts. Hydroperoxycarbinol and dicarbinol are produced along with the dihydroperoxide during air oxidation. Treatment with acid and H_2O_2 converts the hydroperoxycarbinol and dicarbonyl to the dihydroperoxide which is cleaved to form acetone and hydroquinone. During the acidic cleavage, explosive organic peroxide can form which presents a safety hazard.³² Benzoquinone is primarily manufactured from oxidation of aniline (Figure 5). The oxidant employed is MnO_2 in aqueous H_2SO_4 (Figure 5). Benzoquinone can also be reduced by Fe or hydrogenated to afford hydroquinone. Although accounting for approximately 10% of hydroquinone

production,³² this manufacturing route generates large quantities of MnSO_4 , $(\text{NH}_4)_2\text{SO}_4$, and iron oxide salts.³²

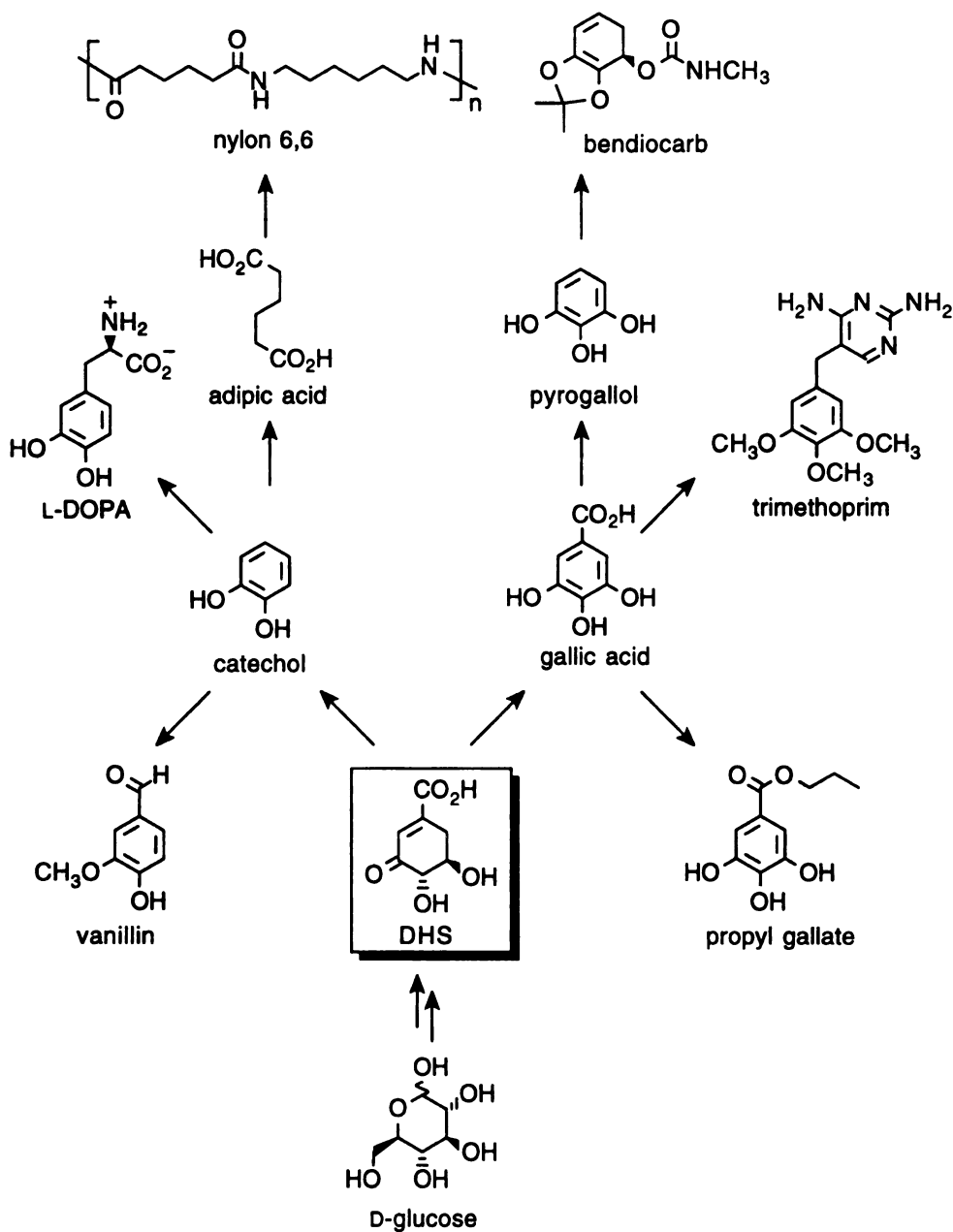


Figure 6. Value added-chemicals synthesized from glucose via DHS intermediacy.

3-Dehydroshikimic acid (DHS) is another important common pathway intermediate. It can be converted both biocatalytically and chemically into a variety of industrially useful molecules (Figure 6).³³ DHS itself is a potent antioxidant.³⁴ Chemical oxidation of DHS can afford gallic acid.³⁴ DHS can also be converted to gallic acid biocatalytically via protocatechuic acid (PCA) intermediary (Figure 7). DHS dehydratase, which is encoded by the *aroZ* locus isolated from *Klebsiella pneumoniae*, converts DHS to PCA. Hydroxylation of PCA catalyzed by a mutant isozyme of *p*-hydroxybenzoate hydroxylase isolated from *Pseudomonas fluorescens* then affords gallic acid. An *E. coli* biocatalyst has been constructed which synthesizes approximately 15 g/L of gallic acid from glucose under fed-batch fermentation conditions.³⁵ Derivatives of gallic acid include: propyl gallate, an important food-grade antioxidant; trimethoprim, an antibacterial agent; and pyrogallol, a product of chemical or enzymatic decarboxylation of gallic acid (Figure 6). Pyrogallol is used in photographic developing solutions and is a key building block in the manufacture of the insecticide bendiocarb (Figure 6).³⁶ Gallic acid is currently isolated from gall nuts and tara powder.

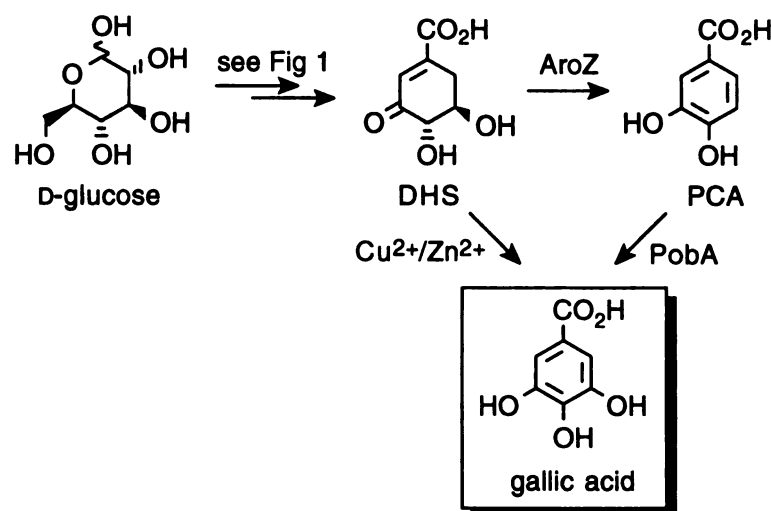


Figure 7. Synthesis of gallic acid from glucose.

Catechol is also microbially synthesized from DHS (Figure 8).³⁷ A genetically modified *E. coli* strain mediates the dehydration of DHS to form protocatechuic acid (Figure 8), which then undergoes an enzyme-catalyzed, non-oxidative decarboxylation to catechol. As in biocatalytic synthesis of gallic acid, dehydration of DHS to protocatechuic acid is catalyzed by *aroZ*-encoded DHS dehydratase. Conversion of protocatechuic acid into catechol is catalyzed by *aroY*-encoded PCA decarboxylase, which was isolated from *K. pneumoniae*. Although DHS and protocatechuic acid are intermediates in the conversion of glucose into catechol, only catechol accumulates in the culture supernatant.

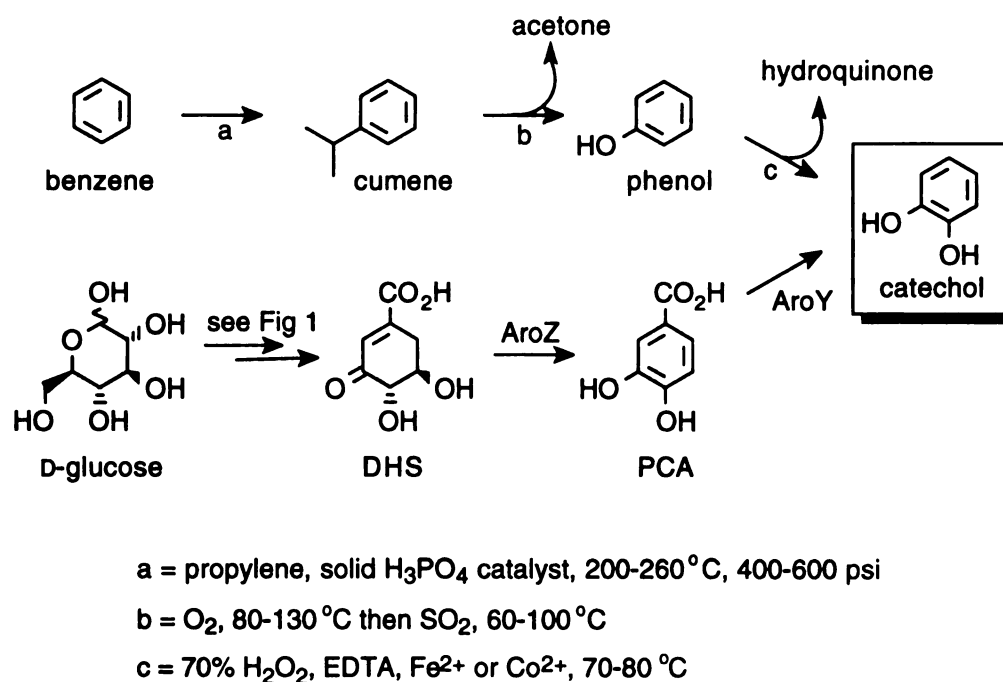


Figure 8. Comparison of microbial and chemical synthesis of catechol.

Global, non-captive production of catechol is approximately 25,000 tons per year.³⁸ Estimating total world production is difficult since catechol is often part of captive markets where it is produced and then converted into higher, value-added products before ever seeing the open market as catechol. Chemical products derived from catechol include pharmaceuticals (L-DOPA, adrenaline, papaverine), flavors (vanillin, eugenol, isoeugenol),

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agrochemicals (carbofuran, propoxur), and polymerization inhibitors and antioxidants (4-*tert*-butylcatechol, veratrol).³⁹ Most catechol production begins with Friedel-Crafts alkylation of benzene to afford cumene (Figure 8). Subsequent Hock-type, air oxidation of the cumene leads to formation of acetone and phenol. The phenol is then oxidized to a mixture of catechol and hydroquinone using 70% hydrogen peroxide either in the presence of transition metal catalysts or in formic acid solution where performic acid is the actual oxidant. Catechol and hydroquinone are separated by distillation (Figure 8).³⁹

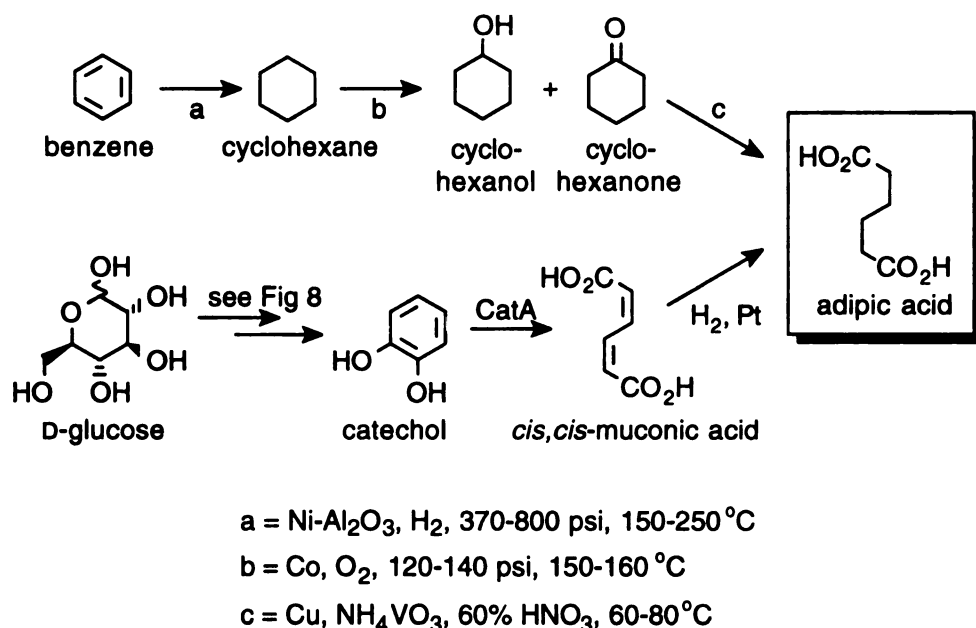


Figure 9. Comparison of microbial and chemical synthesis of adipic acid.

Inclusion of a *catA* gene encoding catechol 1,2-dioxygenase in the catechol-producing microbe converts catechol into *cis,cis*-muconic acid (Figure 9).⁴⁰ The *catA* gene was isolated from *Acinetobacter calcoaceticus*.⁴¹ Catalytic hydrogenation at 50 psi and room temperature using 10% Pt on carbon converts the *cis,cis*-muconic acid into adipic acid.⁴⁰ Primarily used in production of nylon-6,6, the annual global demand for adipic acid exceeds 1.9×10^9 kg.⁴² Benzene is the principal starting material from which adipic

acid is currently synthesized. Hydrogenation of benzene to produce cyclohexane is followed by air oxidation to yield a mixture of cyclohexenol and cyclohexanone. Nitric acid oxidation then yields adipic acid and nitrous oxide,⁴³ which is involved in ozone depletion and global warming.⁴⁴ Adipic acid production may account for some 10% of the annual increase in atmospheric nitrous oxide levels.⁴³

Construction of microbial catalysts typically involves disruption and/or overexpression of genes in a microbial host such as *E. coli*. Overexpressed genes are often not native to the host microbe and have been recruited from other microbes. This has been illustrated in the biocatalytic syntheses of Trp, Phe, PABA, *p*-hydroxybenzoic acid, shikimic acid, quinic acid, gallic acid, catechol, and adipic acid. In this thesis, similar strategies were used where *K. pneumoniae* and rat genes were recruited for synthesis of vanillic acid from glucose. A second enzymatic step is then used to reduce the vanillic acid to vanillin. The enzyme employed was aryl-aldehyde dehydrogenase purified from *Neurospora crassa*.⁴⁵

Metabolic Engineering of *E. coli* to Increase Product Titrers and Yields

To compete with chemical synthesis, microbial synthesis must produce value-added chemicals in a high yield (percent conversion) and high titer (product concentration) while taking advantage of abundant, inexpensive feedstocks. Metabolic engineering of biocatalytic syntheses utilizing the shikimate pathway has focused on the elimination of feedback inhibition and repression of gene expression, overexpression of enzymes leading to the desired product, and elimination of enzymes and pathways resulting in depletion of the desired product. For example, in commercial tryptophan-producing strains, the *tna*

gene encoding the tryptophan-degrading enzyme tryptophanase is deleted, and the genes in the terminal pathway are all overexpressed.⁴⁶

Alteration of central metabolism in *E. coli* to channel increased carbon flow into the common pathway has recently been the focus of considerable research activity.⁴⁷ In addition to overexpressing feedback-insensitive DAHP synthase, strategies have been evaluated to increase E4P and PEP availability. The rate of aromatic amino acid biosynthesis is controlled by modulation of the catalytic activity of DAHP synthase. There are three isozymes of DAHP synthase expressed in *E. coli*, each of which is sensitive to feedback inhibition by one of the three aromatic amino acids. The genes *aroF*, *aroG* and *aroH* encode tyrosine-sensitive, phenylalanine-sensitive, and tryptophan-sensitive DAHP synthase isozymes, respectively. These isozymes' in vivo activities are dictated by their expression levels, feedback inhibition by aromatic amino acids, and the availability of their E4P and PEP substrates.

One option to manipulate AroF activity is to introduce a mutation into the locus that encodes the aporepressor (TyrR) for *aroF* transcription. In lieu of TyrR, transcription of *aroF* is derepressed and the number of molecules of AroF increases.⁴⁸ Another way to increase the transcription of *aroF* is to replace the native promoter (P_{aroF}) with a strong promoter such as a *tac* promoter (P_{tac}). RNA polymerase binding to the strong *tac* promoter is not influenced by the TyrR repressor protein, resulting in more transcription. Expression of AroF can also be increased by increasing the number of *aroF* genes in a given microbe. This is often accomplished by extrachromosomally localizing *aroF* on a multi-copy plasmid. Such a strategy increases the number of *aroF* genes beyond the number of available TyrR repressor protein molecules thereby overriding transcriptional regulation of *aroF*.

Ultimately, the catalytic activity of DAHP synthase increases to a point where further amplification of even feedback-resistant DAHP synthase does not lead to improved synthesis of either aromatic amino acids or precursors to these end products. Historically, attention was focused on the in vivo availability of phosphoenolpyruvic acid (PEP) at this juncture. PEP is a high energy metabolite which provides 3 of the 7 carbons of DAHP (Figure 10). A number of different cellular processes and enzymes compete with DAHP synthase for PEP including pyruvate kinase, PEP carboxylase, and the PEP-dependent carbohydrate:phosphotransferase (PTS) system for uptake of glucose and structurally related sugars. Efforts to improve intracellular PEP availability began with mutational inactivation of PEP carboxylase^{47c, 52} and pyruvate kinase.⁵³ More recent efforts have focused on circumventing PTS-mediated uptake of glucose in microbes such as *E. coli*. Discovery that E4P availability is a critical limiting factor in aromatic amino acid biosynthesis separated these two periods of research focusing on intracellular PEP availability.^{47a}

Mutational inactivation of PEP carboxylase and pyruvate kinase did not lead to improvements in aromatic amino acid biosynthesis that were significant or practical. For example, mutational inactivation of *ppc*⁻ encoded PEP carboxylase results in a slow-growing *E. coli ppc*⁻ strain that requires succinic acid supplementation.^{47c} Although *E. coli ppc*⁻ produces 10-fold higher phenylalanine titers relative to *E. coli ppc*⁺, these phenylalanine titers are 10-fold lower than the amount of acetic acid which is produced. Also, the 1.5 g/L titers of phenylalanine produced by *E. coli ppc*⁻ are minuscule relative to the 46 g/L of phenylalanine which can be produced by *E. coli*.⁵⁴

In 1990, Frost and coworkers published the first work indicating that E4P availability was also an important factor limiting in vivo DAHP synthase activity (Figure 10).⁵⁵ As an aldose phosphate which can not exist in solution in a cyclic form, E4P is prone to dimerization, trimerization and polymerization. Dissociation of these E4P forms back to monomeric E4P is quite slow. This is probably the underlying reason why nature

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closely matches the rate of E4P synthesis with the rate of E4P utilization, thereby maintaining low, steady-state concentrations of E4P. Low steady-state concentrations of E4P likely limit this substrate's availability thereby limiting in vivo activity of DAHP synthase.

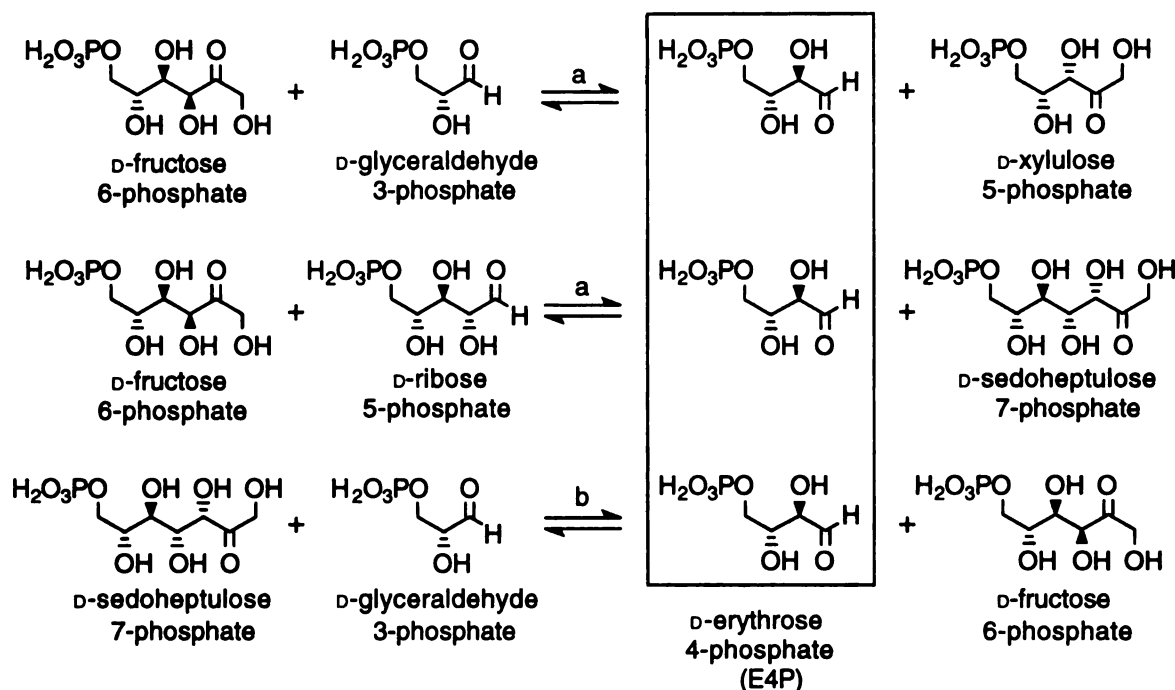


Figure 11. Reactions catalyzed by transketolase (a) and transaldolase (b).

Inspection of the enzymes which catalyze reactions where E4P is either a substrate or a product led to the pentose phosphate pathway and the enzyme transketolase (Figure 11). Amplified expression of transketolase increases the levels of E4P available to the cell for channeling into the common pathway.⁵⁵ Two of the three intracellular E4P-generating reactions are catalyzed by transketolase while the third reaction is catalyzed by transaldolase (Figure 11). Transketolase also serves to generate the substrate D-sedoheptulose-7-phosphate for the E4P-generating reaction catalyzed by transaldolase. Thus, transketolase plays a major role in increasing the levels of E4P available to the cell for aromatic production. DAHP synthase, which catalyzes the first irreversible reaction of the common

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pathway, commits elevated levels of carbon generated by transketolase to aromatic amino acid biosynthesis. Work combining expression of feedback-insensitive *aroG* with amplified expression of *tktA* has revealed that increased DAHP synthase catalytic activity in tandem with increased transketolase catalytic activity leads to a twofold increase in carbon flow directed into the common pathway above that achieved with amplified expression of DAHP alone.^{47a}

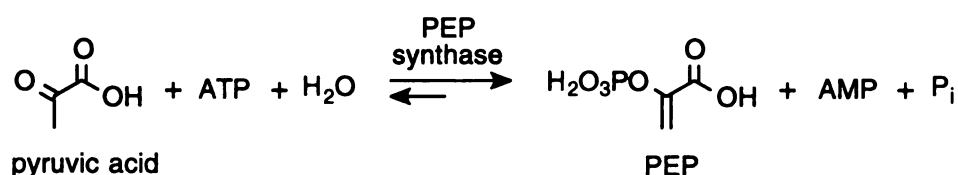


Figure 12. Substrates and products associated with PEP synthase (Pps) activity.

Liao and coworkers subsequently examined the impact of amplified expression of *pps*-encoded PEP synthase on aromatic synthesis in *E. coli aroB* (Figure 10).^{47d, e} PEP synthase catalyzes the reaction of pyruvate with adenosine triphosphate (ATP) resulting in formation of PEP, adenosine monophosphate (AMP) and inorganic phosphate (Figure 12). Overexpression of PEP synthase was designed to recycle the pyruvate generated from PEP during PTS-mediated glucose uptake back to PEP (Figure 10). Even though this might be expected to alleviate intracellular PEP limitations, no increase in carbon flow directed into the common pathway of aromatic amino acid biosynthesis was detected in *E. coli aroB* upon amplified expression of *pps* and *aroG^{FBR}*. However, expression (plasmid localization) of *tktA* along with *pps* and *aroG^{FBR}* resulted in a twofold improvement in carbon flow as measured by the yield of DAH synthesized by *E. coli aroB*.^{47d, e} These results suggest that E4P is the first limiting metabolite in aromatic amino acid biosynthesis. Once this limitation is removed, aromatic amino acid biosynthesis improves when PEP availability increases as is the case with PEP synthase-catalyzed recycling of pyruvic acid.

Amplified expression of transaldolase also relieves E4P limitation in the presence of amplified PEP synthase, but no further improvements in aromatic amino acid biosynthesis are observed relative to when transketolase is overexpressed.⁵⁶ While recycling of pyruvate back to PEP using overexpressed PEP synthase (in the presence of overexpressed transketolase and DAHP synthase) demonstrates that improved aromatic biosynthesis can be realized with improved PEP availability, amplified expression of PEP synthase might not be a long term solution. PEP synthase is a heavily regulated enzyme and the consequences of its overexpression on the metabolic viability of the microbial biocatalyst are unknown. Unfortunately, all experiments to date with amplified *pps* have been performed under shake-flask conditions. Corresponding data on the impact of overexpressed *pps* and *tktA* on aromatic biosynthesis under fed-batch fermentor conditions has never made its way into the literature. Indeed, prior to this dissertation, all research examining the impact of transketolase overexpression on carbon flow directed into the common pathway was performed under shake-flask and not fed-batch fermentor conditions.

Under typical shake-flask conditions, cells were first grown to stationary phase in rich medium using antibiotics as the selection pressure to maintain plasmids. These cells were then harvested and resuspended in minimal medium. Glucose in the minimal medium is then converted to the desired molecule with limited or no cell growth. Although this type of cell cultivation has been widely employed in evaluating pathway engineering, there are several inherent problems associated with such experimental procedures. For examples, the use of rich medium and antibiotics can dramatically increase the cost if such culturing methods were scaled up. Centrifugation and resuspension of cells also complicate large-scale manufacture. Under shake-flask conditions, because cells are grown in rich medium prior to resuspension in minimal medium, calculated yields do not really reflect the percent conversion of carbohydrate into product. DAH yields exceeding the theoretical maximum yield were repeatedly reported when *aroG^{FBR}*, *tktA* and *pps* were overexpressed in an *E.*

coli aroB cultured under shake-flask conditions.^{47a, d, e} In addition, under shake-flask conditions, cultures begin in glucose-rich environment and end in a glucose-deficient environment, and both oxygenation levels and pH are difficult to control.

In this thesis, fed-batch fermentor conditions were employed for DHS synthesis. One of the advantages of using fed-batch fermentor conditions is that it increases cell density at least 15-fold relative to the shake-flask cultivation. In addition, fed-batch fermentor conditions overcome most of disadvantages of shake-flask conditions. For examples, only minimal medium is used in the process, and the growth of the cells and the production of DHS occur "simultaneously" under fed-batch fermentor conditions. A strategy of using nutritional pressure to maintain the plasmid has also been developed. As a result, antibiotics are no longer used in the fermentor. In addition, under fed-batch fermentor conditions, variables such as temperature , pH, dissolved oxygen levels and the steady-state concentration of the glucose can be easily controlled and manipulated on line.

CHAPTER 2

SYNTHESIS OF VANILLIN FROM GLUCOSE

Background

Vanillin is the common name for 3-methoxy-4-hydroxybenzaldehyde (Figure 13). It is one of the most important aromatic compounds in the production of flavors for foods and fragrances for perfumes. As a food flavor, vanillin is second only to aspartame and well ahead of both citric acid and monosodium glutamate in terms of annual sales.¹⁵ About 12×10^6 kg of vanillin are globally manufactured annually.⁵⁷ Besides its popularity in the food and perfume industries, vanillin has many other useful applications. For examples, vanillin is used as a palatability enhancer to make animal feed more appetizing by flavor-masking minerals with off-taste.⁵⁸ Vanillin is also used in the pharmaceutical industry. Since 1970, the use of vanillin as a chemical intermediate in the production of pharmaceuticals has surpassed the quantities used for flavoring purposes.⁵⁸ The single largest use of vanillin is as a starting material for the manufacture of an antihypertensive drug having the chemical name of Methyldopa or L-3-(3,4-dihydroxyphenyl)-2-methylalanine.⁵⁸ Vanillin is also a potent antioxidant and an antimicrobial agent.^{58, 59}

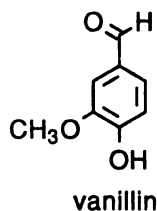


Figure 13. The structure of vanillin.

As the major flavor and aroma component in vanilla extract, the concentration of vanillin dictates the quality of vanilla extract.⁶⁰ Free, unconjugated vanillin does not exist in green vanilla beans. Natural vanillin is produced from glucovanillin when the beans of

the orchid *Vanilla planifolia* are submitted to a multi-step curing process.⁶¹ Free, unconjugated vanillin is formed during the curing process by β -glucosidase cleavage from the precursor glucovanillin which is biosynthesized from coniferol (Figure 14).⁶¹ The labor intensity associated with vine cultivation, bean harvesting, and hand pollination of flowers contributes to the difficulty in employing *V. planifolia* as a commercial source of vanillin.^{61, 62} In addition, the long and expensive curing process to produce vanilla extract from vanilla beans only yields a product that has an inconsistent quality. As a result, natural vanillin can supply only 0.2% of the demand for vanillin flavoring and the consumption of naturally-occurring vanilla has gradually given way to synthetic vanillin.⁵⁸

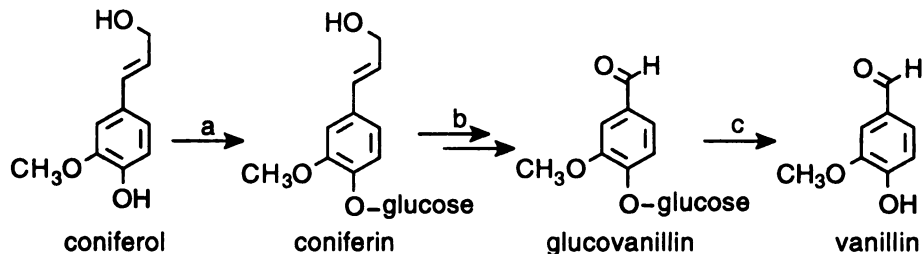


Figure 14. Biosynthesis of natural vanillin. (a) UDP-glucose:coniferyl alcohol glucosyltransferase; (b) unidentified enzymes; (c) β -glucosidase.

Synthetic vanillin can be produced either from the waste sulfite lye obtained from wood pulping operations or from guaiacol and glyoxylic acid (Figure 15).⁵⁸ To derive vanillin from the lignin content of sulfite waste, lignin is treated with sodium hydroxide or with calcium hydroxide solution under oxidative conditions. The reaction is generally run at 160-175 °C and 150-160 psi.⁵⁸ When the reaction is completed, the solid wastes are removed. Vanillin is extracted from the acidified solution with butanol or benzene, and then reextracted with sodium hydrogen sulfite solution. Reacidification with sulfuric acid followed by vacuum distillation yields technical-grade vanillin, which must be recrystallized several times to obtain food-grade vanillin. Although this process was widely used in the 1970s, it has faced serious problems since then. The availability of

sulfite waste is reduced because newer processes for making paper paste yields less liquor. The process's environmental impact is also problematic because over 160 ton of caustic waste are produced from every ton of vanillin manufactured.⁵⁸

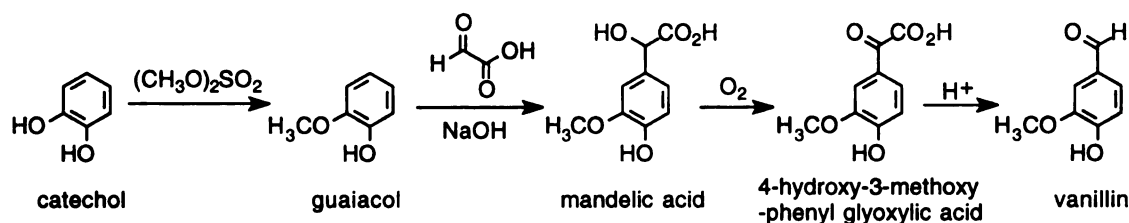


Figure 15. Chemical synthesis of vanillin.

The other synthetic route to produce vanillin begins with the condensation of guaiacol with glyoxylic acid (Figure 15). Air oxidation of the resulting mandelic acid affords phenylglyoxylic acid. Crude vanillin is obtained by acidification and decarboxylation of 4-hydroxyl-3-methoxyphenyl glyoxylic acid solution. Commercial grades are obtained by vacuum distillation and subsequent recrystallization.⁵⁸ Although this process is currently the major source for synthetic vanillin, it has several inherent problems. Guaiacol has historically been obtained from condensation of dimethyl sulfate with catechol. Dimethyl sulfate is classified as a highly toxic, cancer-suspect agent. Catechol is listed as being toxic and corrosive while guaiacol is a toxic irritant. Catechol is currently manufactured from benzene (Figure 8) which is carcinogenic and derived from nonrenewable petroleum.⁶³

The limited availability of vanilla beans has led to extensive research into the use of microorganisms to synthesize vanillin from ferulic acid. Such vanillin can be labeled as a natural or nature-equivalent flavoring.⁶⁴ Fungi, soil bacteria and plant cells are the main organisms that have been examined. A two-step process for synthesis of vanillin has been developed using *Aspergillus niger* to produce vanillic acid from ferulic acid followed by employment of *Pycnoporus cinnabrinus* to reduce vanillic acid to vanillin. However, the

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process was plagued by low overall yield. The process also produced many impurities because of the predominance of the vanillic acid oxidative system and the degradation route of vanillin in the filamentous fungi. Under optimal conditions, the *A. niger* and *P. cinnabrinus* route produced only 560 mg/L vanillin.⁶⁵ Several soil bacteria have also been carefully selected or mutagenized for their metabolic capacity to produce vanillin from ferulic acid. A selected *Streptomyce* strain can produce 650 mg/L to 1 g/L vanillin after at least three days' cultivation.^{64, 66} As another alternative, root cells from *V. planifolia* have been cultured to produce vanillin from ferulic acid. However, this method is handicapped by the fact that maintenance of plant cell cultures is rather expensive and time-consuming. The lack of an abundant, inexpensive supply of ferulic acid with its toxicity are key impediments to all biocatalytic approaches using ferulic acid as a starting material for vanillin synthesis.⁶⁷

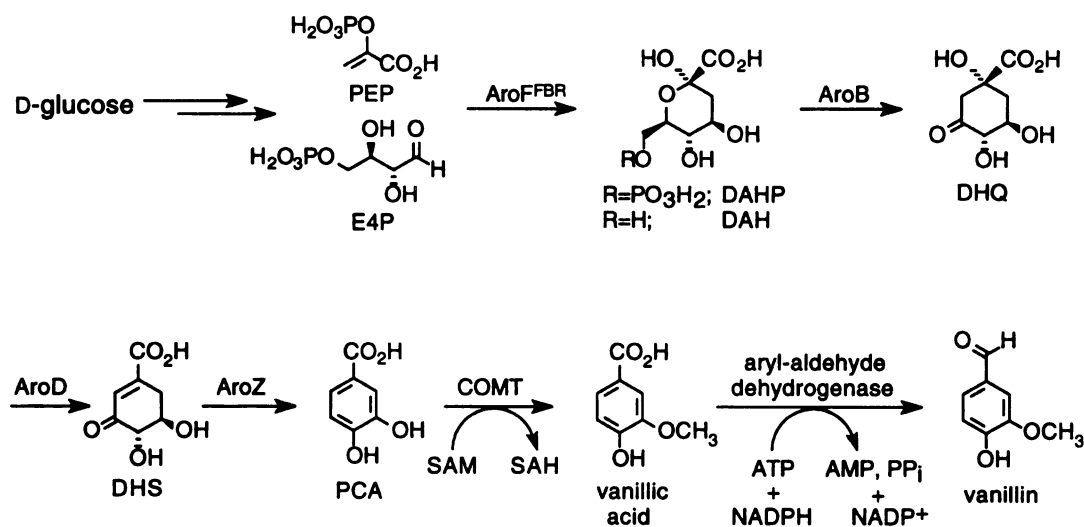


Figure 16. Biosynthesis of vanillin from glucose.

In this Chapter, a synthesis of vanillin from glucose will be elaborated. Glucose is converted into vanillic acid by a recombinant *Escherichia coli* biocatalyst under fed-batch fermentor conditions (Figure 16). Reduction of vanillic acid to vanillin is catalyzed by aryl-

aldehyde dehydrogenase isolated from *Neurospora crassa* (Figure 16). This synthesis qualifies both as a route to natural vanillin and as a first step towards large-scale, environmentally-benign manufacture of vanillin using biocatalysis.

Microbial Synthesis of Vanillic Acid from Glucose

A. The construction of the host strain

Overview

AB2834, an *E. coli aroE* strain,⁶⁸ was chosen as the parental strain for constructing a vanillate-producing biocatalyst. The lack of *aroE*-encoded shikimate dehydrogenase results in the synthesis of DHS when carbon flow is directed into the common pathway. An *aroBaroZ* cassette was synthesized and site-specifically inserted into the *serA* locus of *E. coli* AB2834 *aroE* via homologous recombination to generate *E. coli* KL7. Site-specific homologous recombination followed from *serA* sequences which flanked the cassette. The *serA* locus encodes D-3-phosphoglycerate dehydrogenase which is the first of the three enzymes responsible for L-serine biosynthesis. Insertion of *aroB* into the genome increases the activity of DHQ synthase, which is the only rate-limiting enzyme in the portion of the common pathway of aromatic amino acid biosynthesis required for vanillin synthesis.⁶⁹ DHS dehydratase, encoded by *aroZ*, catalyzes the conversion of DHS into PCA. Incorporation of *aroZ* into the genome was designed to economize on the number of genes which needed to be plasmid-localized. Since *serA*-encoded 3-phosphoglycerate dehydrogenase is an enzyme necessary for biosynthesis of L-serine, microbial growth in minimal salts medium lacking L-serine supplementation is only possible if plasmids containing *serA* inserts are stably maintained. This strategy employing nutritional pressure for plasmid maintenance obviates any need for use of antibiotics and plasmid-localized antibiotic resistance for plasmid maintenance.

Synthesis of *aroBaroZ* cassette

The *aroB* gene was obtained as a 1.6 kb fragment following digestion of pJB14 with *EcoRI*. Plasmid pJB14 is a 6.5 kb pKK223-3 derivative.⁷⁰ The 1.6-kb fragment included the native promoter and an inverted repeat downstream of the *aroB* gene capable

of forming a stem-loop structure characteristic of a *rho*-independent terminator. The *aroZ* gene was obtained as a 2.2-kb fragment through PCR amplification from pSU1-28. Plasmid pSU1-28 is a 5.8-kb plasmid which is a derivative of pSU19⁷¹ and contains a 3.5-kb *aroZ* fragment isolated from the genome of *K. pneumoniae*. The primers used for PCR were: 5'-CGGGATCCGCGCATACACATGC and 5'-CGGGATCCGGGTACAGAGGGTGTGT. Ligation of the 2.2-kb PCR product into the *Bam*HI site of pSU18 afforded pSK4.99A. The 2.2-kb *aroZ* fragment includes its native promoter and is transcribed in the same orientation relative to the *lac* promoter in pSK4.99A. The 1.6-kb *aroB* fragment was blunt ended through Klenow fragment treatment and subsequently inserted into the *Sma*I site of pSK4.99A to afford pKL4.237A (Figure 17). The *aroB* gene is transcribed in the same orientation as *aroZ* in plasmid pKL4.237A.

The purpose of employing pSK4.99A instead of pSU1-28 for pKL4.237A construction was to use a shorter *aroZ* fragment for the cassette. Surprisingly, the *Bam*HI site between the *aroB* and *aroZ* disappeared in pKL4.237A. However, the loci of *aroB* and *aroZ* were intact as were confirmed first by multiple restriction enzyme digestions. In addition, pKL4.237A was transformed into competent AB2834/pMF62A to afford AB2834/pMF62A/pKL4.237A. Plasmid pMF62A is a pBR322 derivative that bears a copy of *aroF^{FBS}* encoding feedback-sensitive DAHP synthase and a copy of *tktA*.^{54a} In shake-flask conditions, construct AB2834/pMF62A/pKL4.237A converted 56 mM glucose into 27.1 mM PCA, which was the sole product. As a control, construct AB2834/pMF62A/pSK4.99A was also tested under the same conditions. It afforded a mixture of 6.5 mM DAH(P) and 19.6 mM PCA (Table 1). Clearly, pKL4.237A overexpressed *aroB* compared to the parental plasmid pSK4.99A, while both plasmids conferred the active *aroZ* gene. The 3.9-kb *aroBaroZ* cassette was then isolated from pKL4.237A with the inclusion of *Bam*HI recognition sequences at the 5'- and 3'- ends using PCR amplification. The following primers were used for the PCR: 5'-

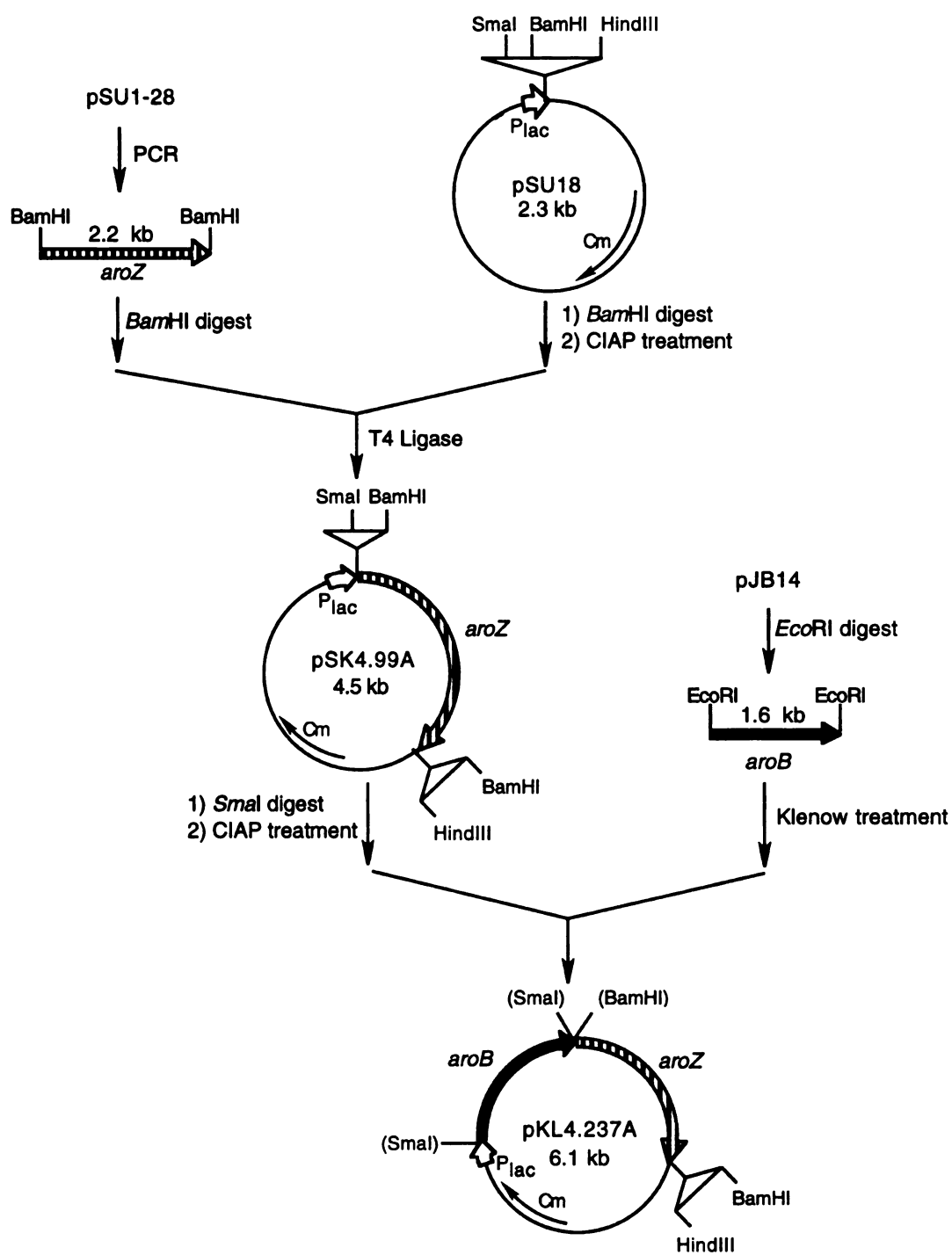


Figure 17. Preparation of Plasmid pKL4.237A

CGGGATCCATCGGAGCTGATGTGGG a n d 5' -
CGGGATCCGGGTACAGAGGGTGTGT.

Table 1. Confirmation of AroB and AroZ activities in pKL4.237A.

Construct	DAH(P)	DHS	PCA
AB2834/pMF62A/pSK4.99A	6.5 mM	0	19.6 mM
AB2834/pMF62A/pKL4.237A	0	0	27.1 mM

Genomic insertion of the *aroBaroZ* cassette into AB2834 to create KL7

Although plasmids are maintained in *E. coli* as extrachromosomal, circularized DNA, recombination events occasionally occur such that plasmid DNA is integrated into the chromosome of the host cell. Exploitation of this rare event provides a method for simple, site-specific insertion of a gene by flanking the gene with sequences homologous to the genomic region targeted for disruption. Identification of cells with integrated plasmid DNA is difficult since both freely replicating and integrated plasmids express resistance to drugs encoded by plasmid markers. By using a conditional non-replicative plasmids, selection of integrated plasmid DNA becomes possible since cells express drug resistance only if the plasmid resides in the genome.

Normal cloning and preparation of plasmids containing conditional origins of replication can be performed under permissive conditions whereas genomic insertions are accomplished under nonpermissive conditions where the replication machinery is inactive. Plasmids possessing a temperature-sensitive replicon are capable of being manipulated in this fashion. Plasmid pMAK705 contains a temperature-sensitive pSC101 replicon, a chloramphenicol resistance marker, and a convenient multiple cloning site.⁷² The plasmid is able to replicate normally when the host cell is grown at 30 °C but is unable to replicate when the host cell is cultured at 44 °C. Thus genetic manipulations of the plasmid are

carried out at 30 °C while the integration of the plasmid into the genome can be selected for at 44 °C.

The planned insertion of the synthetic cassette into the genomic *serA* locus in strain AB2834 necessitated construction of a plasmid containing the synthetic cassette flanked by *serA* DNA. The gene for *serA* was isolated from plasmid pD2625, which was digested with *EcoRV* and *DraI* to liberate a 1.9-kb *serA* fragment. Plasmid pMAK705 was digested with *Bam*HI and treated with Klenow fragment. Subsequent ligation of the *serA* fragment to pMAK705 afforded pLZ1.68A.⁷³ Since the original *Bam*HI site in pMAK705 was destroyed due to the Klenow treatment, the *Bam*HI site inside the *serA* gene becomes a unique restriction site, which facilitates the cloning of the synthetic cassette. Insertion of the cassette into the *Bam*HI site of *serA* in pLZ1.68A yielded pKL4.276B (Figure 18). Both *aroB* and *aroZ* are transcribed in the opposite orientation relative to the *lac* promoter. DH5 α /pKL4.276B was tested for AroB and AroZ activities before plasmid pKL4.276B was used for homologous recombination. Compared to DH5 α , DH5 α /pKL4.276B expressed 2.5-fold higher DHQ synthase activity. DH5 α /pKL4.276B also afforded a 0.01 U/mg specific activity of DHS dehydratase while no DHS dehydratase activity was detected in the *E. coli* DH5 α .

Table 2. Plate selection for characterization of genomic insertion strain.

Strain	M9/glucose/ Tyr/Phe/SA	M9/glucose/ Tyr/Phe/SA/ serine	LB/Cm	LB
AB2834	+	+	—	+
KL7	—	+	—	+

SA: shikimic acid

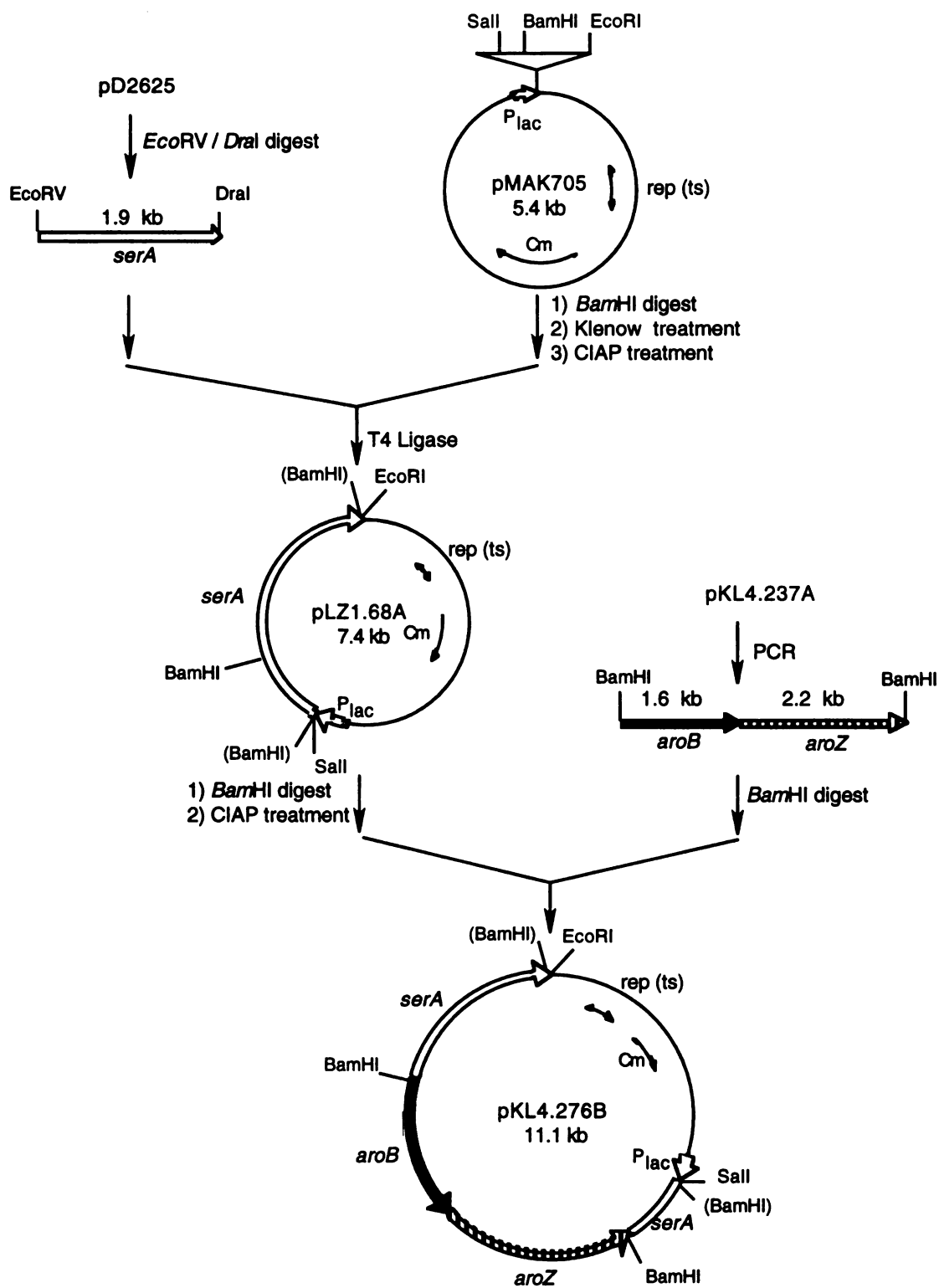


Figure 18. Preparation of Plasmid pKL4.276B.

Conditions for homologous recombination were based on those previously described.^{69b} The competent host strain was transformed with pKL4.276B. Following heat-shock treatment, cells were incubated in LB at 44 °C for 1 h and subsequently plated on LB plates containing Cm. Plates were incubated at 44 °C for approximately 20 h before colonies appeared. The resulting cointegrates were cultured in 5 mL of LB containing no antibiotics and were grown at 30 °C for 12 h to allow excision of the plasmid from the genome. Cultures were diluted (1:20000) in LB without antibiotics, and two more cycles of growth at 30 °C for 12 h were carried out to enrich cultures for more rapidly growing cells that had lost the temperature-sensitive replicon. Growth cultures were then diluted (1:20000) into LB and grown at 44 °C for 12 h to promote plasmid loss from the cells. Serial dilutions of each culture were spread onto LB plates and incubated at 30 °C overnight. The resulting colonies were screened on multiple plates to select the desired recombinants. *E. coli* KL7 was isolated based on the following growth characteristics: growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid and serine; no growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid; growth on LB; and no growth on LB containing Cm (Table 2).

B. Plasmid Constructions

Overviews

Three genes needed to be localized in a plasmid. These included *aroF^{FBR}*, *serA*, and *COMT*. The *aroF^{FBR}* insert encodes a 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase isozyme insensitive to feedback inhibition for increasing carbon flow into the common pathway. Plasmid-localized *serA* was necessary for complementation of the *serA* mutation in the KL7 genome. This provided the nutritional pressure for stable maintenance of plasmids carrying *serA* by KL7 when cultured in minimal salts medium lacking serine supplementation. Expression of the *COMT* gene encoding catechol-*O*-methyltransferase (COMT) was required for conversion of PCA into vanillic acid. The

activities of catechol-*O*-methyltransferase were primarily found in plant and mammalian tissue.⁷⁴ The cDNA encoding COMT from rat liver had been cloned and expressed in *E. coli*.⁷⁵ An *E. coli* promoter and ribosome binding site sequences were added in front of the open reading frame (ORF) of the COMT cDNA to ensure transcription and translation in *E. coli*. Because catechol-*O*-methyltransferase has a turnover number of only 24 per min,⁷⁶ the strong *P_{tac}* promoter was used to achieve adequate activity levels.

Construction of pKL5.26A and pKL5.97A

A 1.3-kb *aroF*^{FBR} fragment encoding a feedback-insensitive DAHP synthase AroF was obtained through UV mutagenesis. Ligation of the *aroF*^{FBR} fragment into the *EcoRI* site of pSU18 led to pKL4.20B. The *aroF*^{FBR} gene was transcribed in the opposite orientation relative to the *lac* promoter in pKL4.20B (Figure 19). Plasmid pKL4.20B was further digested with *SmaI*. Ligation of a 1.9-kb *DraI/EcoRV* fragment encoding *serA* obtained from pD2625 into the *SmaI* site of the pKL4.20B afforded pKL4.33B (Figure 20). The *serA* gene was transcribed in the same direction as *aroF*^{FBR}.

Plasmid pKK223-3⁷⁰ is a cloning vector with a pMB1 origin of replicon. It contains a strong *tac* promoter and a ribosome binding site (RBS) which can be utilized to express gene inserts. Plasmid pCRX-2⁷⁵ carries the open reading frame (ORF) of the *COMT* gene. Digestion of pRCX2 with *EcoRI/HindIII* was followed by isolation of a 0.7-kb fragment encoding the ORF of *COMT*. Plasmid pKK223-3 was also digested with *EcoRI/HindIII* to yield a 5.3-kb fragment. Ligation of these two fragments resulted in pKL3.272A, in which the *COMT* was behind the *tac* promoter and an *E. coli* RBS (Figure 21).

Digestion of pKL4.33B with *SphI* and *HindIII* afforded a 5.5-kb fragment, while the same digestion of pKL3.272A yielded a 1.1-kb *P_{tac}COMT* fragment. Ligation of these two purified fragments resulted in pKL5.26A (Figure 22). Plasmid pKL5.26A was digested with *HindIII/XbaI* and the 1.1 kb *P_{tac}COMT* fragment was isolated and treated

with Klenow fragment. Plasmid pKL5.26A was also digested with *Hind*III and treated with Klenow fragment. Subsequent ligation of the *P_{tac}COMT* to pKL5.26A afforded pKL5.97A. The two *P_{tac}COMT* gene are transcribed in the same orientation (Figure 23).

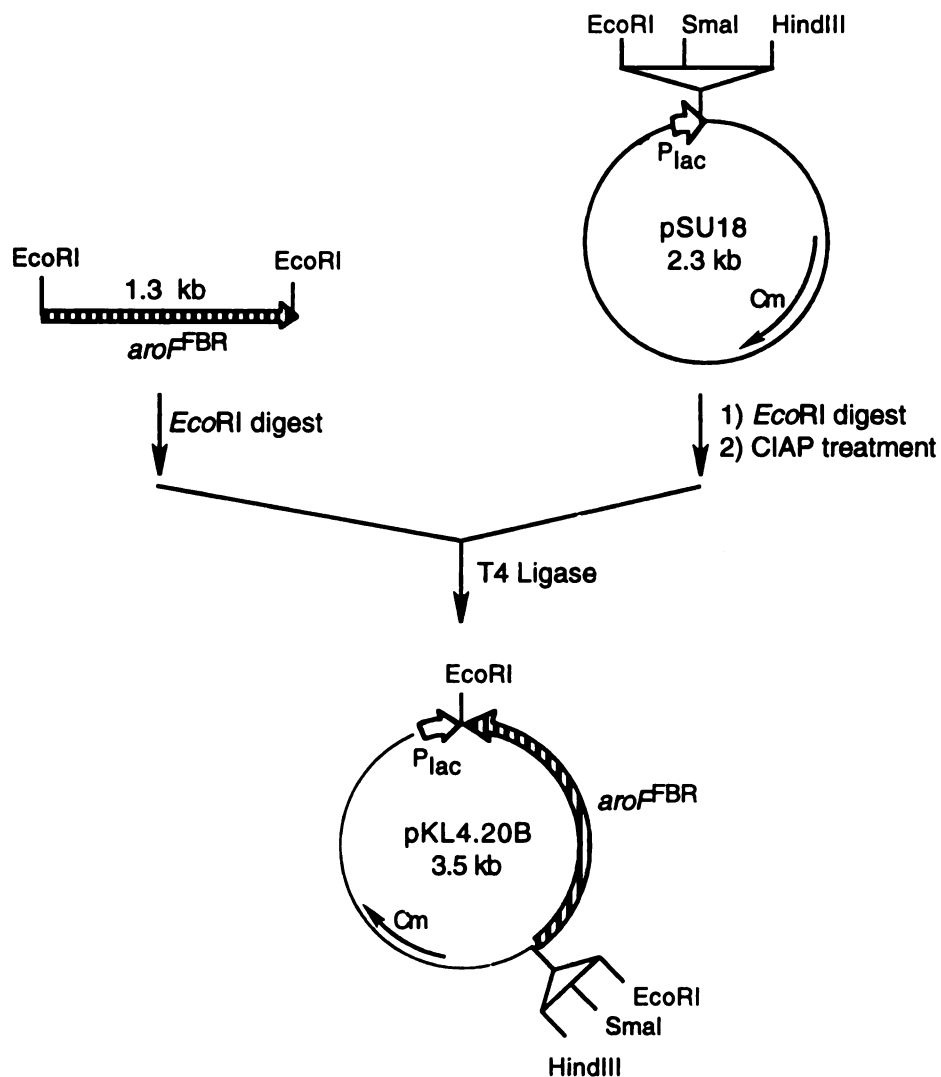


Figure 19. Preparation of plasmid pKL4.20B.

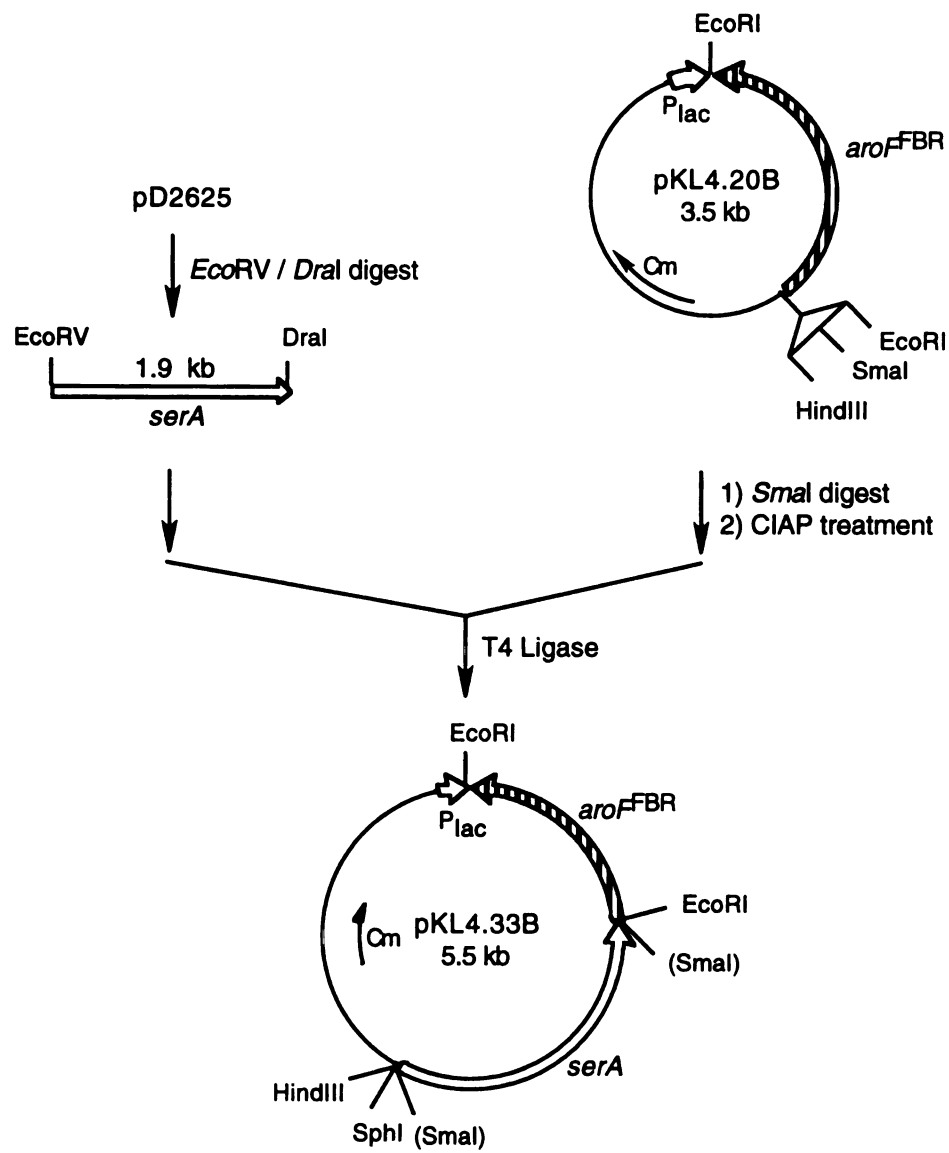


Figure 20. Preparation of plasmid pKL4.33B.

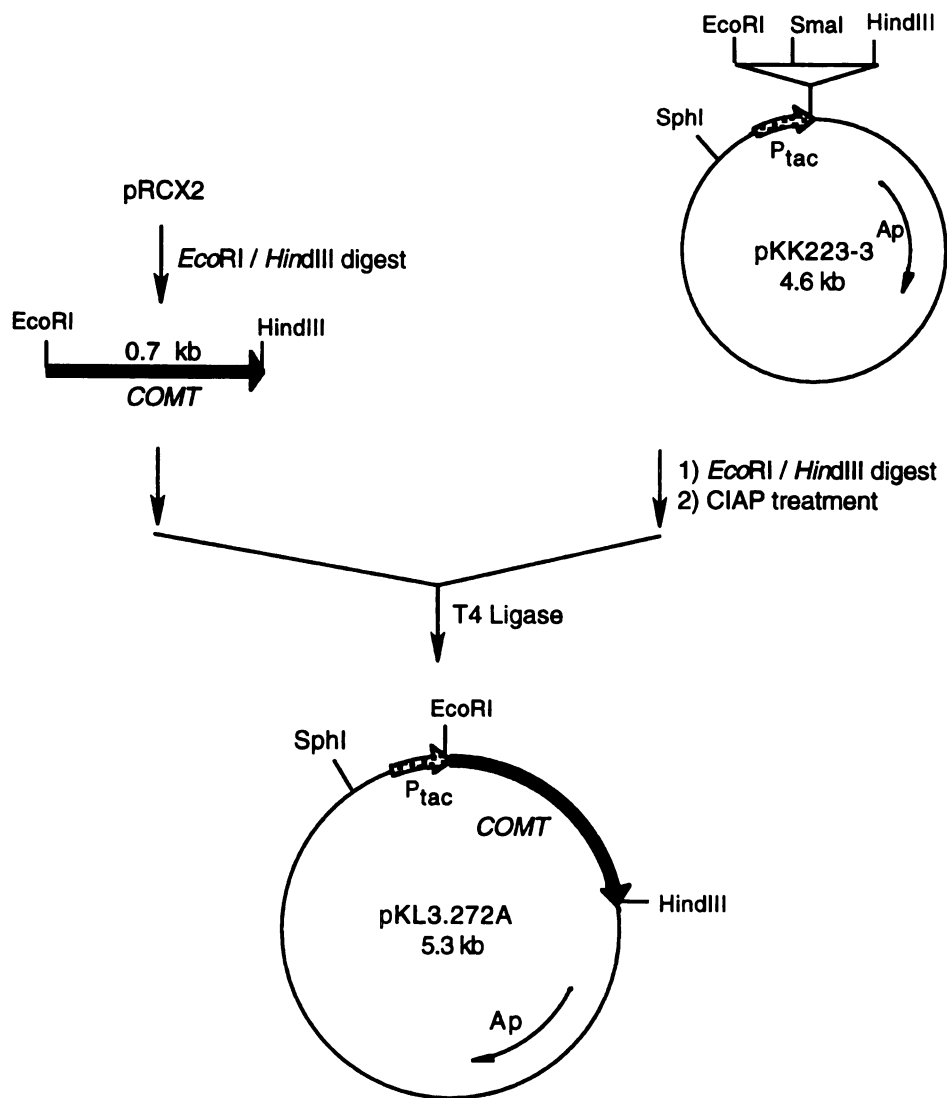


Figure 21. Preparation of plasmid pKL3.272A.

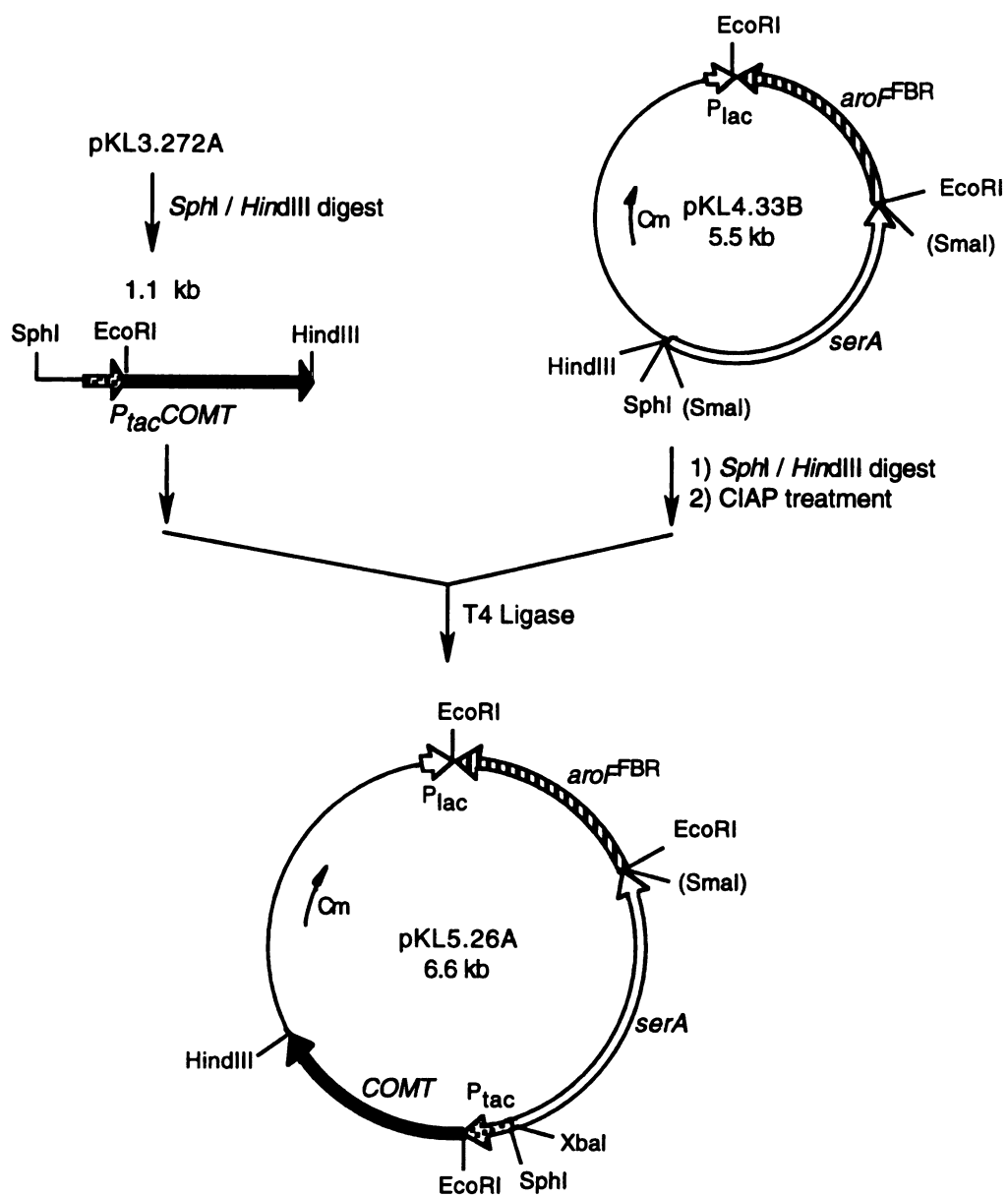


Figure 22. Preparation of Plasmid pKL5.26A.

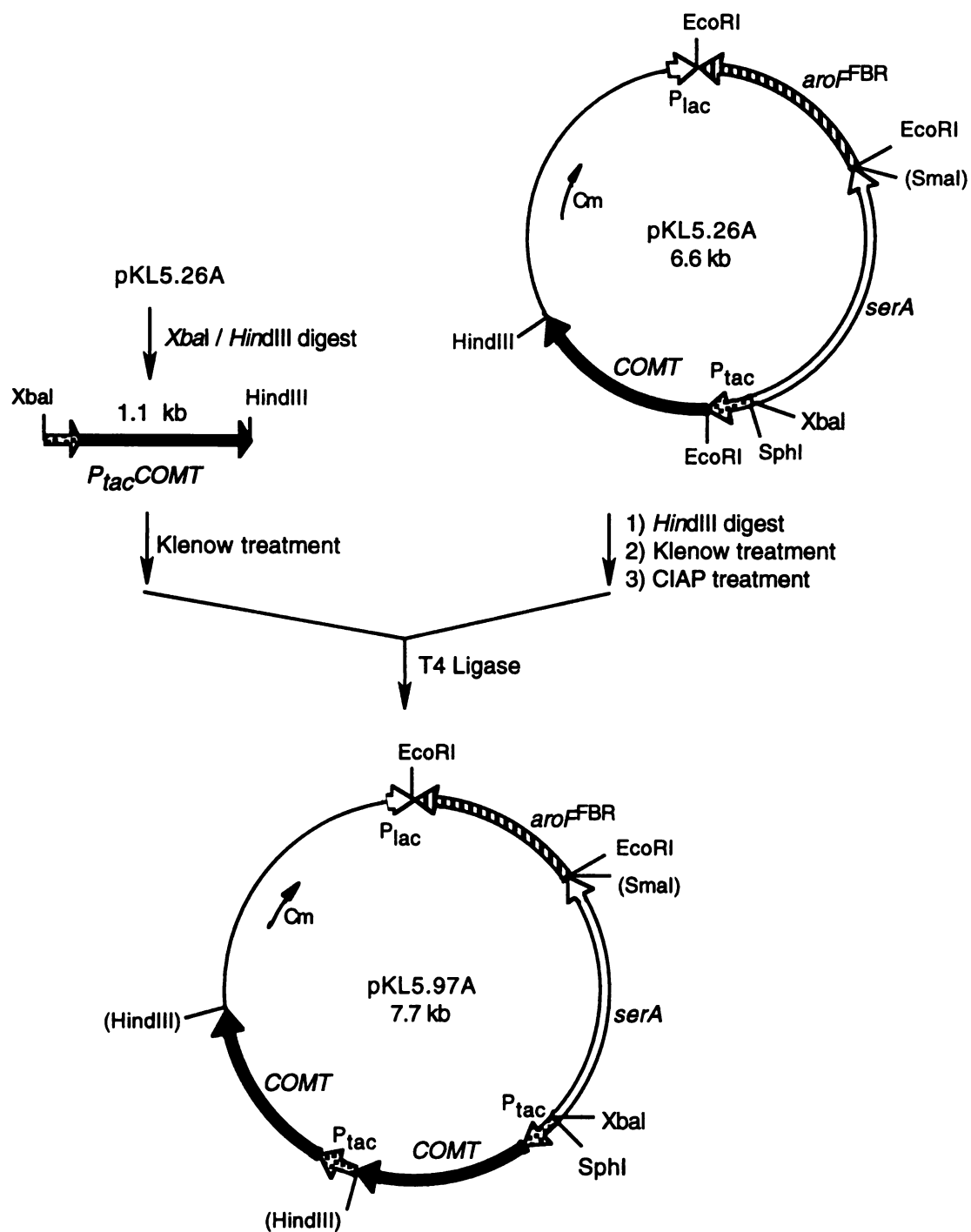


Figure 23. Preparation of Plasmid pKL5.97A.

C. Fed-Batch Fermentor Synthesis of Vanillic Acid

KL7/pKL5.26A were cultured for 48 h under fed-batch fermentor conditions at 37 °C, pH 7.0, and dissolved oxygen at 20% of air saturation.³³ Cells reached stationary phase at approximately 24 h after inoculation. Extracellular accumulation (Figure 24) of vanillic, isovanillic, protocatechuic, and 3-dehydroshikimic acids began in mid log phase of microbial growth. Upon cessation of the fermentation at 48 h, the production of vanillic acid, isovanillic acid, PCA and DHS were 2.5 g/L, 0.4 g/L, 9.7 g/L and 0.9 g/L, respectively. The 6-fold molar excess of vanillic acid synthesized relative to isovanillic acid (Table 3) is consistent with the reported selectivity of catechol-*O*-methyltransferase towards *meta*-hydroxyl group methylation. DHS usually constituted 6 mol% of the total product mixture indicating that the rates for its biosynthesis and dehydration were nearly equal. However, the molar dominance of protocatechuic acid relative to vanillic acid pointed to inadequate catechol-*O*-methyltransferase activity. In addition, little vanillic acid was produced during the stationary phase (Figure 24). A 25 mL of aliquot of fermentation broth was taken at 12 h and 36 h for determination of catechol-*O*-methyl transferase activity. COMT activity was apparently stable with a specific activity of 0.0064 U/mg and a specific activity of 0.0056 U/mg at 36 h.

Table 3. Products formed after 48 h under fed-batch fermentor conditions as a function of catechol-*O*-methyltransferase activity and L-methionine supplementation.

construct	COMT specific activity ^a	L-methionine addition ^b	vanillic acid (g/L)	isovanillic acid (g/L)	PCA (g/L)	DHS (g/L)
KL7/pKL5.26A	6.0 x 10 ⁻³	–	2.5	0.4	9.7	0.9
KL7/pKL5.26A	5.5 x 10 ⁻³	+	4.9	1.3	7.1	1.0
KL7/pKL5.97A	12.0 x 10 ⁻³	–	3.0	0.6	12.9	1.0
KL7/pKL5.97A	10.3 x 10 ⁻³	+	5.0	1.2	10.5	1.8

^a specific activity: μmol/min/mg. ^b 0.4 g/L added every 6 h beginning at 12 h.

To improve COMT activity, plasmid pKL5.97A was constructed, which contains two copies of $P_{tac}COMT$ as opposed to the single copy of $P_{tac}COMT$ in pKL5.26A. The specific activity of catechol-*O*-methyl transferase of 0.012 U/mg in KL7/pKL5.97A was twice the levels of the transferase activity in KL7/pKL5.26A (Table 3). Unfortunately, increased catechol-*O*-methyltransferase specific activity (Table 3) in KL7/pKL5.97A relative to KL7/pKL5.26A had little impact on the concentrations (Table 3, Figure 25) of synthesized vanillic acid. As with KL7/pKL5.26A, vanillic acid production was minimal during the stationary phase of KL7/pKL5.97A fermentation (Figure 25). This led to consideration of *S*-adenosylmethionine (SAM) availability as a possible factor limiting COMT activity.

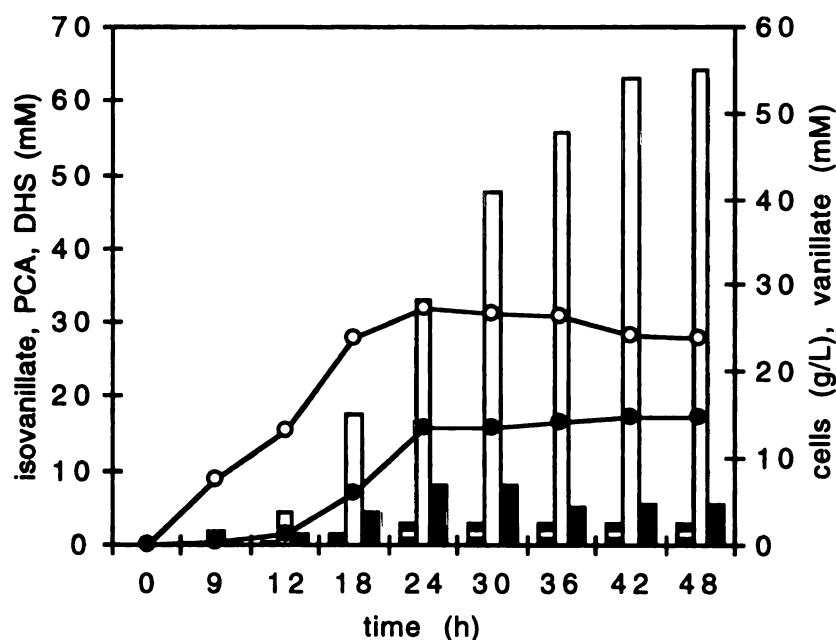


Figure 24. Fermentation culture of KL7/pKL5.26A without methionine supplementation. ● vanillic acid; □ isovanillic acid; □ protocatechuic acid (PCA); ■ 3-dehydroshikimic acid (DHS); ○ cell mass.

Catechol-*O*-methyltransferase catalyzes the methyl transfer reaction from SAM to PCA to form vanillic acid and isovanillic acid (Figure 16). SAM is a central metabolite of

E. coli. Synthesized from L-methionine and ATP by SAM synthetase, SAM is the major methyl donor in metabolism.⁷⁷

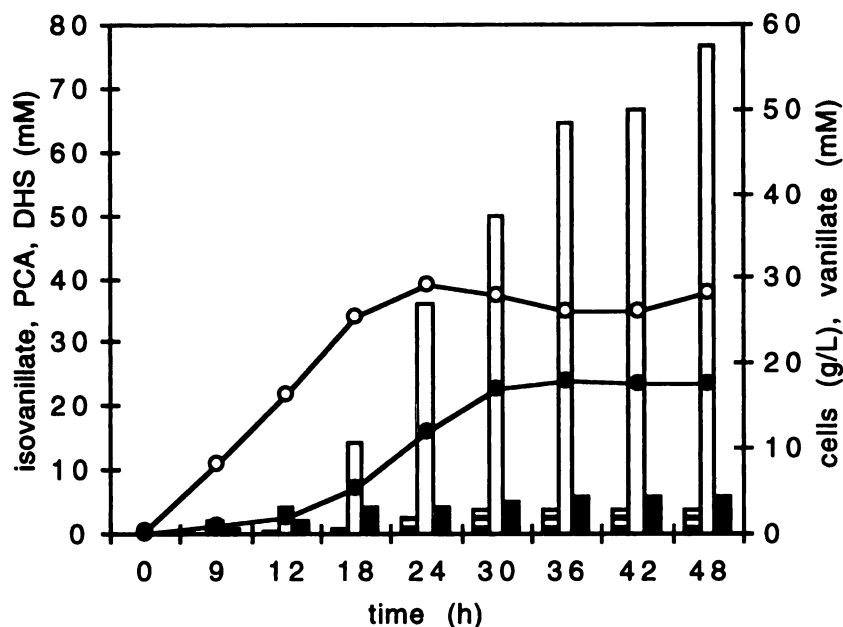


Figure 25. Fermentation culture of KL7/pKL5.97A without methionine supplementation. ●— vanillic acid; □— isovanillic acid; □— protocatechuic acid (PCA); ■— 3-dehydroshikimic acid (DHS); —○— cell mass.

Because supplementation with L-methionine had been reported to increase the intracellular SAM levels,^{77, 78} L-methionine was added to both KL7/pKL5.26A and KL7/pKL5.97A fermentations at timed intervals. In both cases, increased vanillic acid concentrations were synthesized as a result of L-methionine supplementation. With 0.4 g/L L-methionine addition every 6 h beginning at 12 h, KL7/pKL5.26A and KL3/pKL5.97A synthesized 4.9 g/L and 5.0 g/L of vanillic acid, respectively, after 48 h fermentation (Table 3, Figure 26a, b). Although PCA is still a major product in both cases, the titers of vanillic acid with L-methionine addition are almost twice as high as those of without L-methionine addition. The ratios of PCA/vanillic acid also decreased significantly while the specific COMT activities remained the same (Table 3). Of equal importance, vanillic acid synthesis

was observed during stationary phase as well as during exponential phase in both cases (Figure 26a, 26b). Clearly, methionine addition has an important impact on the conversion of PCA to vanillic acid. However, further increases in the amount of L-methionine added did not increase the concentrations or yields of synthesized vanillic acid.

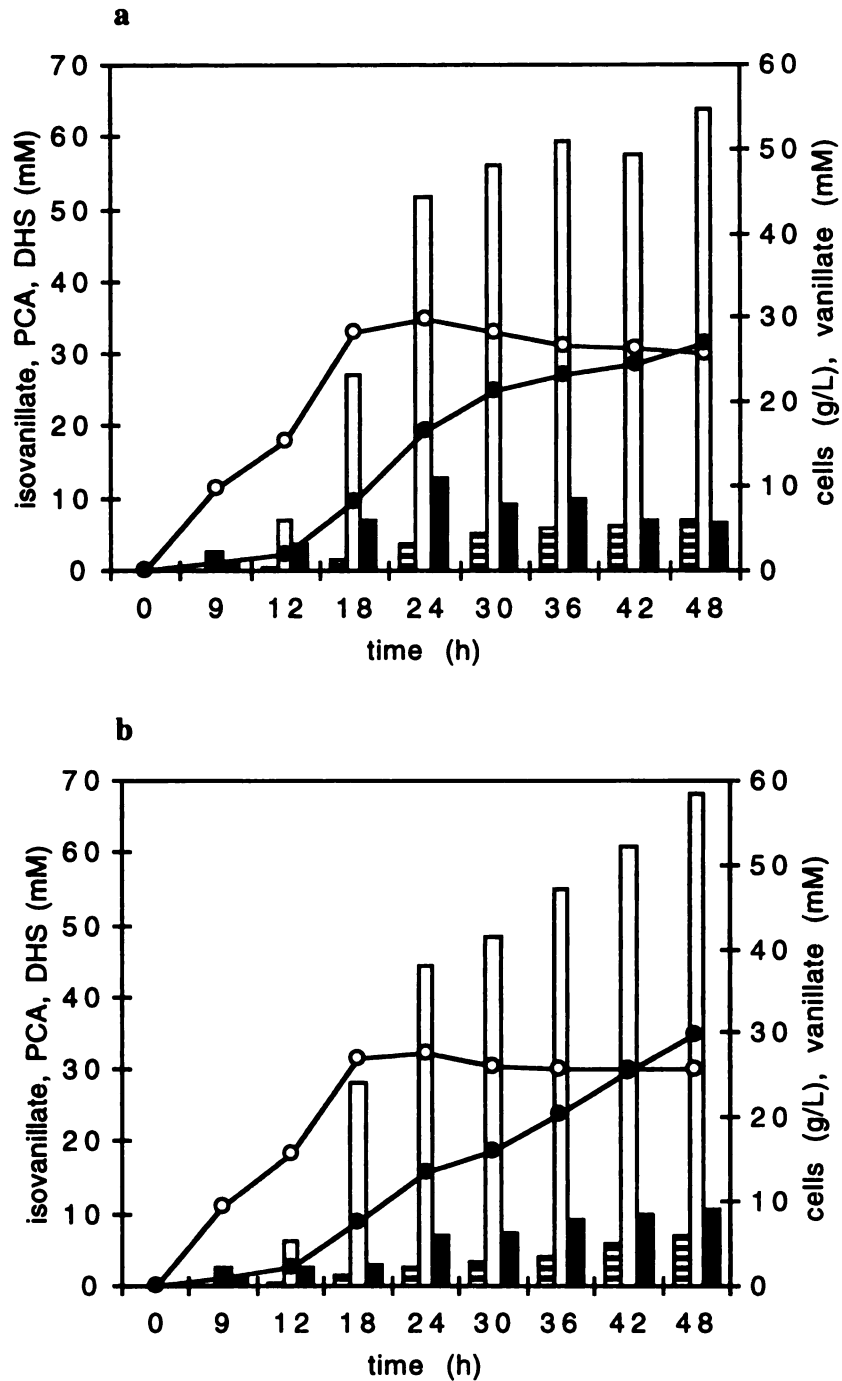


Figure 26. Fermentation cultures of (a) KL7/pKL5.26A and (b) KL7/pKL5.97A supplemented with methionine. —●— vanillic acid; ▨ isovanillic acid; □ protocatechuic acid (PCA); —■— 3-dehydroshikimic acid (DHS); —○— cell mass.

Enzymatic Reduction of Vanillic Acid to Vanillin

A. Purification of Aryl-Aldehyde Dehydrogenase from *Neurospora crassa*

Culturing of *Neurospora crassa*

Microbial reductions of aromatic carboxylic acids, usually to their corresponding alcohols, have been observed with whole cell biotransformations by a number of microorganisms, including *Actinomyces*, *Aspergillus niger*, *Neurospora crassa*, *Nocardia sp.* and *Corynespora melonis*.⁷⁹ Among them, *Neurospora crassa* was the first to be studied.⁸⁰ In the early 1970s, Gross and Zenk discovered that *Neurospora crassa* reduces aryl carboxylic acids to aryl alcohols via an intermediate aryl aldehyde. The enzyme that catalyzes the reduction of the carboxylic acid to aldehyde was identified and named aryl-aldehyde oxidoreductase or aryl-aldehyde dehydrogenase. Mycelia of the fungus *Neurospora crassa* has to be grown in culture medium supplemented with salicylate in order to form the enzyme. The aryl-aldehyde dehydrogenase can reduce a variety of aromatic acids including benzoic acid, salicylic acid, *p*-hydroxybenzoic acid and vanillic acid. The enzyme was purified and identified as a monomeric protein with an apparent molecular mass of 120,000 Da. The reduction required ATP, Mg²⁺ and NADPH as cofactors. Further work showed that this enzyme catalyzed the initial reaction between an aromatic acid and ATP to form an acyl-AMP intermediate which was subsequently reduced by NADPH to form the aldehyde (Figure 27).⁸⁰

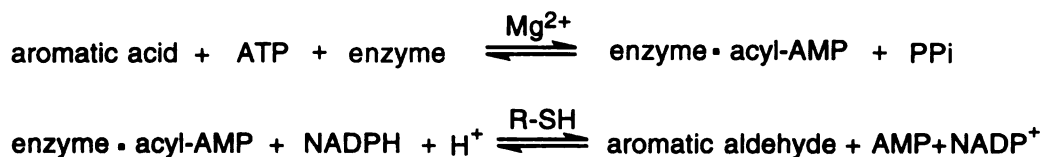


Figure 27. Mechanism of aryl-aldehyde dehydrogenase reduction of aromatic acids.

Neurospora crassa SY 7A was obtained from the American Type Culture Collection (ATCC 24740). It was grown on solid growth medium at 24 °C for 7 days and a mixture of mycelium and spores was obtained. After suspension in sterilized water and filtration of the mixture through sterilized glass wool, a spore suspension was obtained. The concentration of the spores in the suspension was estimated by measuring the absorbance at 650 nm (1 A₆₅₀ unit = 5.0 x 10⁶ spores/mL).⁸¹ Spores were then inoculated in liquid growth medium (2.5 x 10⁶ spores per liter medium) supplemented with 1.6 g/L sodium salicylate. The fungus starts to produce salicylic alcohol (saligenin) after a lag phase of about 50 h. Maximum aryl-aldehyde dehydrogenase activity was observed at 60 h when approximately 20% of salicylate were reduced to saligenin. The mycelium was harvested by filtration at this time and frozen at -20 °C. It is critical to use relatively fresh spores (stored at 4 °C less than two weeks) for the inoculation in order to obtain the optimal aryl-aldehyde dehydrogenase activity at 60 h.

Table 4. Purification of aryl-aldehyde dehydrogenase from *N. crassa*.

	total protein (mg)	total units	specific activity (μ mol/min/mg)	x-fold purification	yield
crude lysate	3600	\	\	\	\
DEAE	809	58	0.072	1	100%
RedA	107	55	0.52	7	96%

1 unit = 1 μ mol NADPH oxidized /min.

Purification of aryl-aldehyde dehydrogenase

Besides aryl-aldehyde dehydrogenase, a native enzyme called aryl-alcohol dehydrogenase also exists in *Neurospora crassa*. This aryl-alcohol dehydrogenase can reduce the aromatic aldehyde to the corresponding alcohol. Specifically, it can reduce vanillin to vanillyl alcohol.⁸⁰ Therefore, the primary purpose of the protein purification

was to purify away the unwanted aryl-alcohol dehydrogenase. In addition, other NADPH-consuming enzymes in *Neurospora crassa* needed to be purified away from aryl aldehyde dehydrogenase since these oxidoreductases can compete with the aryl-aldehyde dehydrogenase for cofactor NADPH.

Table 5. The process of reducing vanillic acid to vanillin.

	fermentation broth	EtOAc extraction	reprecipitation	reduction	CH ₂ Cl ₂ extraction
DHS (g)	0.17	0	0	0	0
PCA (g)	1.04	0.93	0.14	0.095	0
isovanillic acid (g)	0.12	0.098	0.062	0.007	0
vanillic acid (g)	0.50	0.44	0.38	0.03	0
protocatechu- -aldehyde (g)	—	—	—	0.043	0
isovanillin (g)	—	—	—	0.045	0.03
vanillin (g)	—	—	—	0.32	0.30
yield ^a (mol/mol)	100%	88%	76%	71%	66%

^a vanillic acid or vanillin recovery yield.

The purification was performed using a DEAE column followed by a RedA column. Aryl-aldehyde dehydrogenase activities were monitored by assaying for the reduction of benzoic acid using NADPH and ATP as cofactors. Benzoic acid was used for the assay as opposed to vanillic acid because benzoic acid is a better substrate for the aryl-aldehyde dehydrogenase. The specific activity of aryl-aldehyde dehydrogenase could not be determined in crude mycelium extract because of contaminating dehydrogenase activities. Aryl-aldehyde dehydrogenase purified through the two columns (Table 4) was not homogenous. However, contaminating aryl-alcohol dehydrogenase was no longer detectable. In addition, without addition of benzoic acid, NADPH was not consumed. This indicated that other NADPH-competing enzymes had also been purified away from

aryl-aldehyde dehydrogenase. Yields and specific activities for each step of the purification of aryl-aldehyde dehydrogenase are summarized in Table 4. This partially purified enzyme was used for the in vitro reduction of vanillic acid to vanillin.

B. Reduction of Vanillic Acid

KL7/pKL5.97A was cultured under fed-batch fermentor conditions with optimal L-methionine supplementation for 48 h. A portion (100 mL) of the fermentation broth was then acidified to pH 3.0 and the precipitated proteins were removed by centrifugation. Extraction of the protein-free broth with EtOAc separated DHS from vanillic, protocatechuic, and isovanillic acids which were in the organic layer after the extraction. Removal of the EtOAc afforded a solid mixture of vanillic acid, PCA and isovanillic acid with 88% (mol/mol) yield recovery. The extraction did not change the ratio of PCA and vanillic acid. A subsequent reprecipitation step increased the vanillic acid/protocatechuic acid ratio from 1:2 to 2.5:1 (mol/mol). This reprecipitation was accomplished by taking advantage of the solubility differences between PCA and vanillic acid. The solid mixture after the extraction and solvent removal was dissolved in water adjusted to pH 7.5 by NaOH addition. Subsequent dropwise addition of concentrated sulfuric acid acidified the solution to pH 1.8 and resulted in precipitation of a solid which was filtered and dried. The recovery of vanillic acid for this step was 86% (mol/mol). The resulting aromatic mixture was incubated with glucose 6-phosphate dehydrogenase (to recycle NADP⁺) and aryl-aldehyde dehydrogenase at 30 °C and pH 8.0 using 0.07 equiv. of NADP⁺ and 2 equiv. of ATP relative to vanillic acid. Reduction of vanillic acid to vanillin proceeded in 92% (mol/mol) yield in 7 h. Reduction of PCA was slower with a 33% (mol/mol) yield of protocatechualdehyde after 7 h. Vanillin was extracted from the enzymatic reduction with CH₂Cl₂ leaving protocatechualdehyde and protocatechuic acid in the aqueous phase. Removal of the solvent afforded vanillin as a solid with isovanillin present at 10 mol% as the only contaminant (Figure 35). Extraction of the fermentor broth, selective precipitation

to remove excess protocatechuic acid, aryl-aldehyde dehydrogenase reduction, and the final CH_2Cl_2 extraction led to a 66% overall yield (mol/mol) for conversion of vanillic acid into vanillin (Table 5).

Figure 28. Control ^1H NMR of vanillic acid. Resonances: δ 7.55 (d, 1H), 6.95 (d, 1H), 7.47 (dd, 1H), 3.92 (s, 3H).

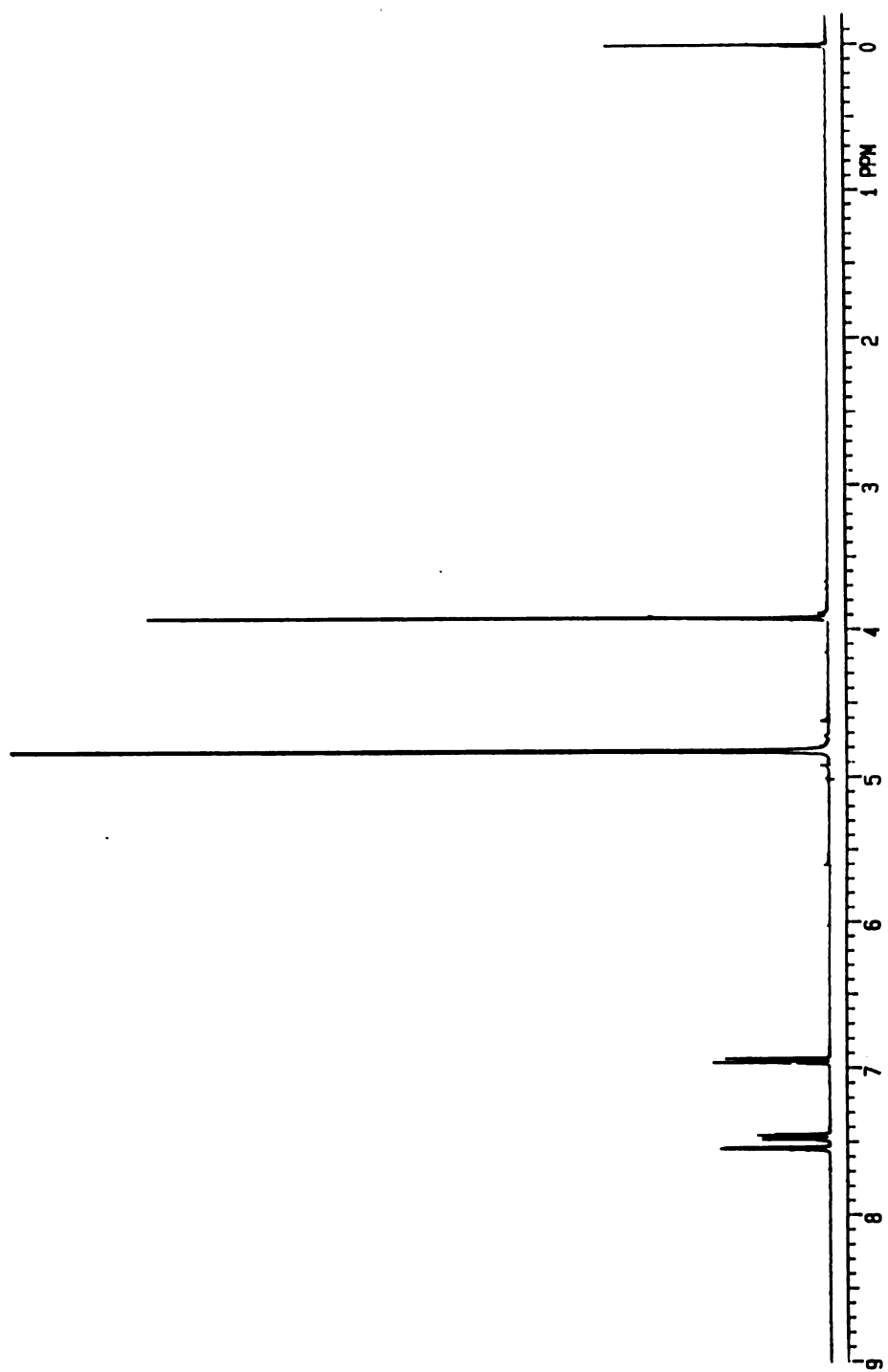


Figure 29. Control ^1H NMR of isovanillic acid. Resonances: δ 7.41 (d, 1H), 7.06 (d, 1H), 7.49 (dd, 1H), 3.91 (s, 3H).

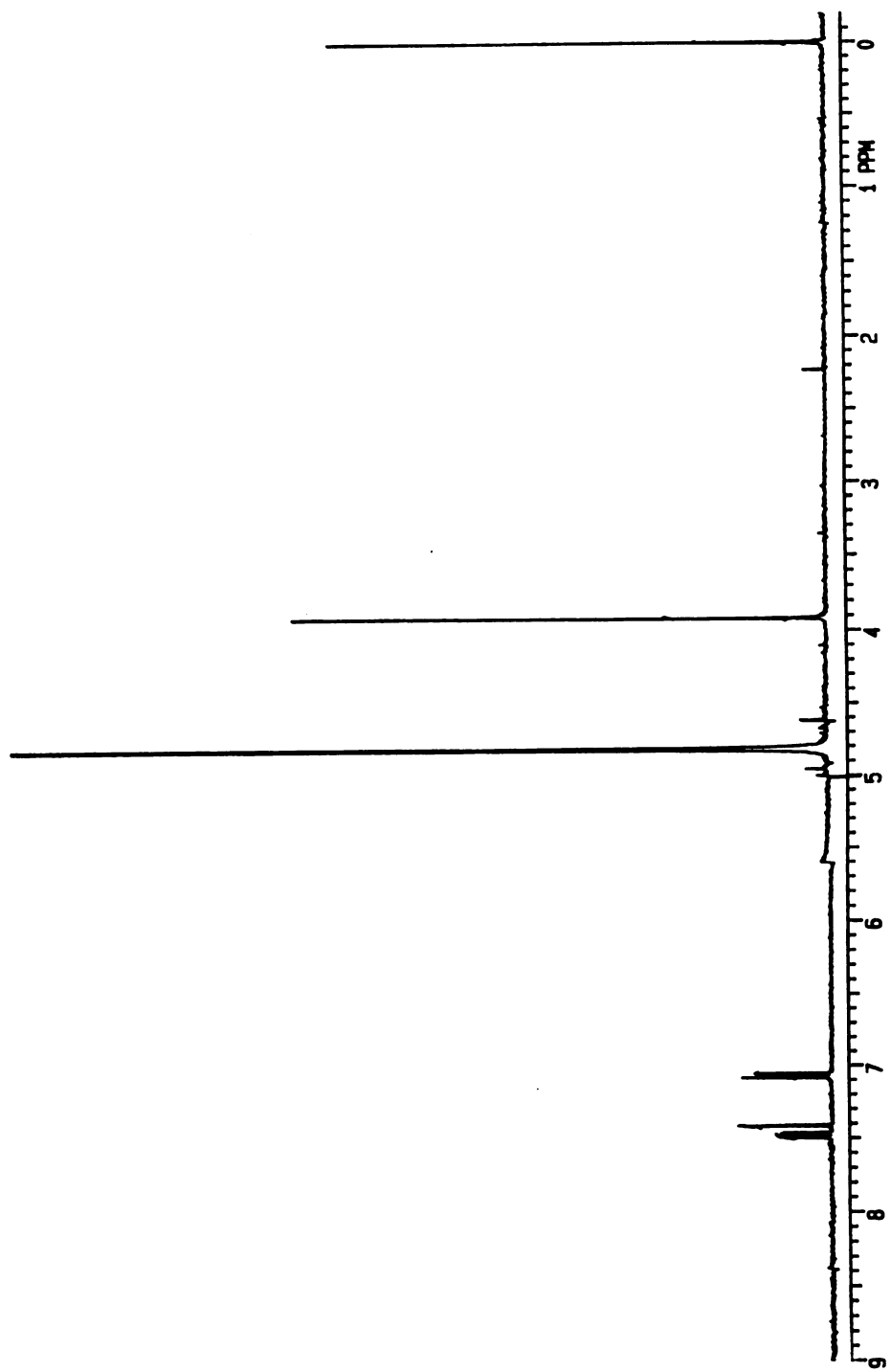


Figure 30. Control ^1H NMR of protocatechuic acid (PCA). Resonances: δ 7.45 (d, 1H), 6.95 (d, 1H), 7.40 (dd, 1H).

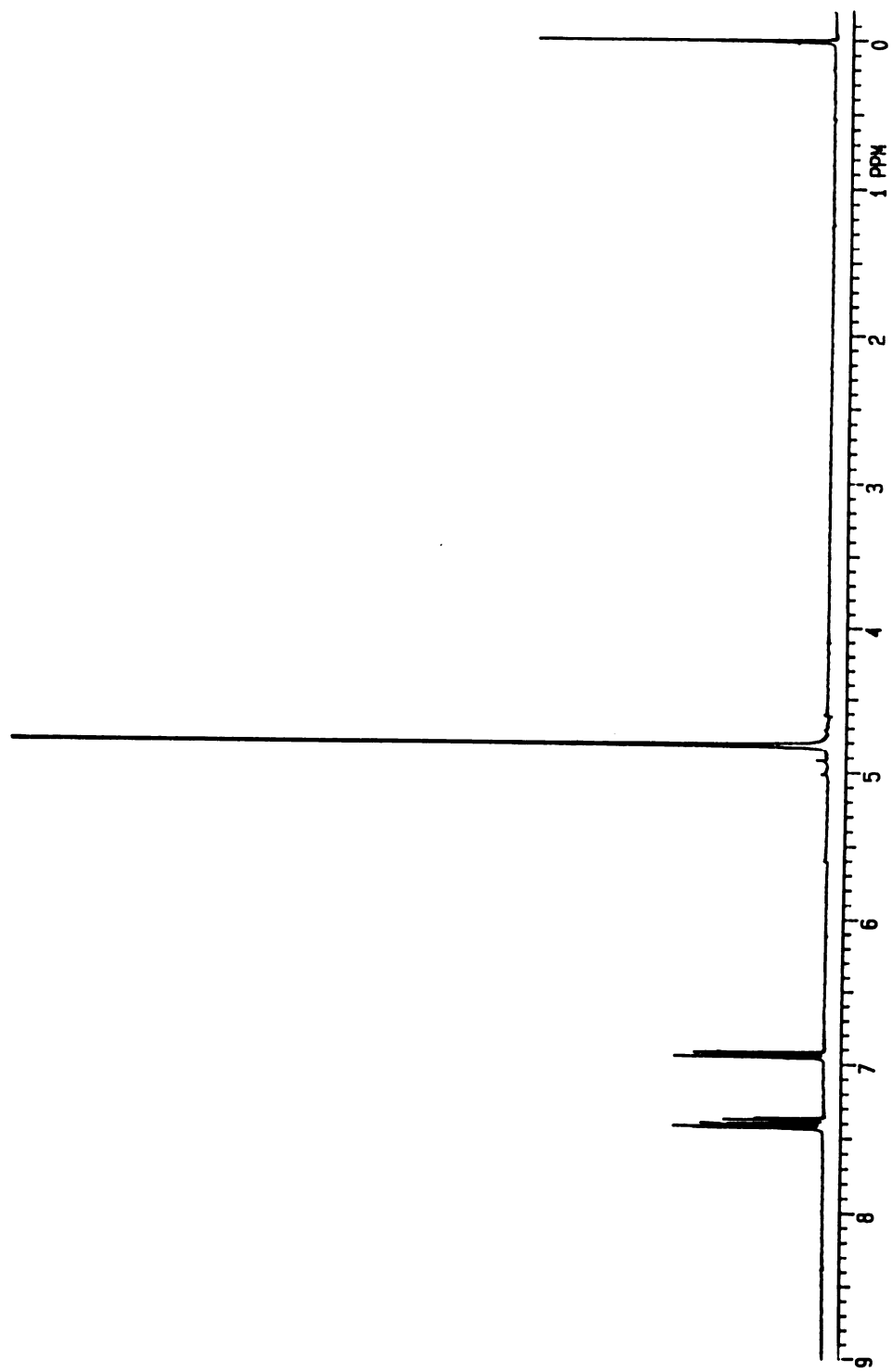


Figure 31. Control ^1H NMR of 3-dehydroshikimic acid (DHS). Resonances:
 δ 6.42 (d, 1H), 4.28 (d, 1H), 4.00 (ddd, 1H), 3.07 (dd, 1H), 2.66 (ddd, 1H).

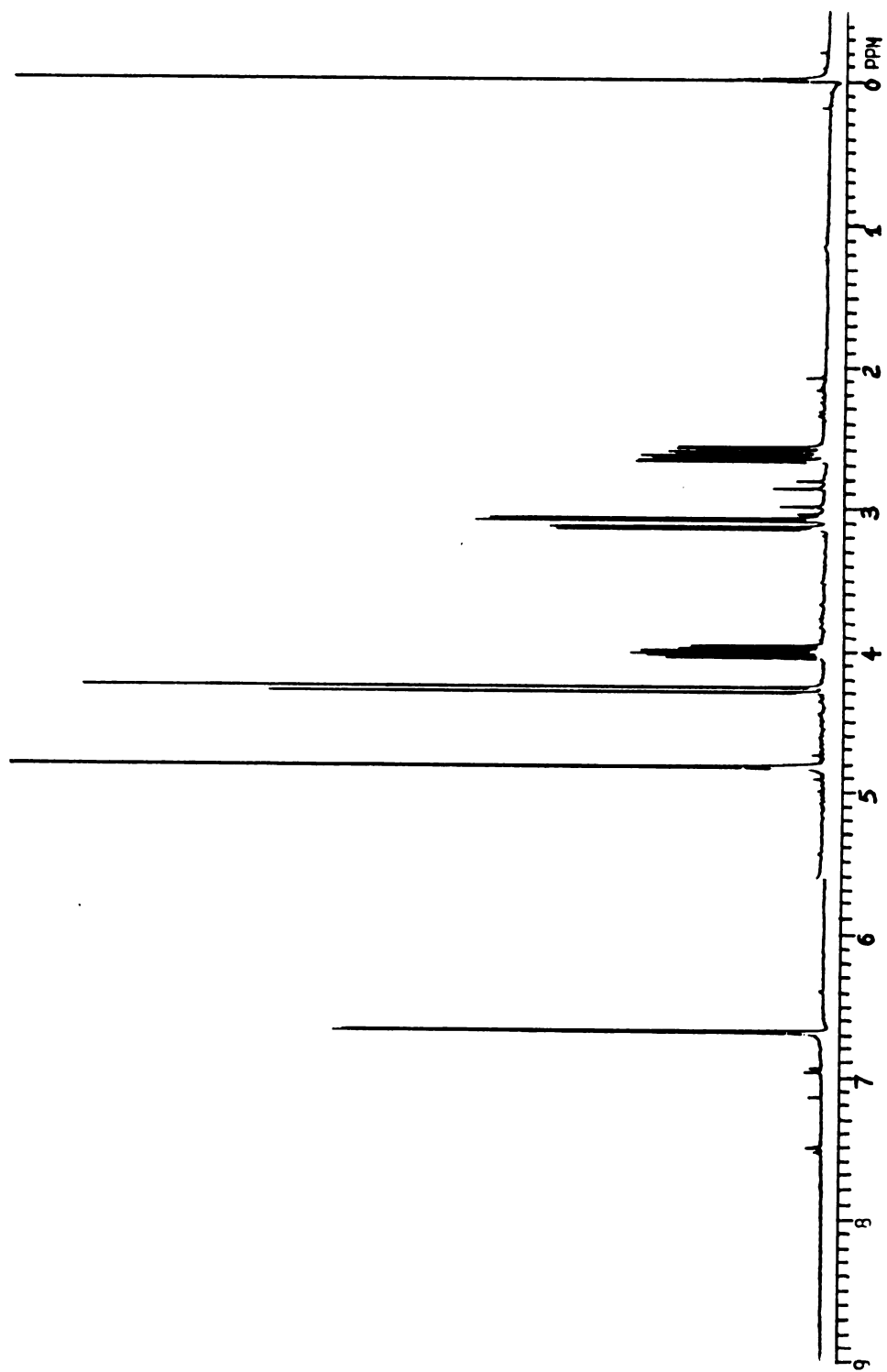


Figure 32. Control ^1H NMR of vanillin. Resonances: δ 7.39 (s, 1H), 6.98 (d, 1H), 7.46 (d, 1H), 3.88 (s, 3H), 9.63, (s, 1H).

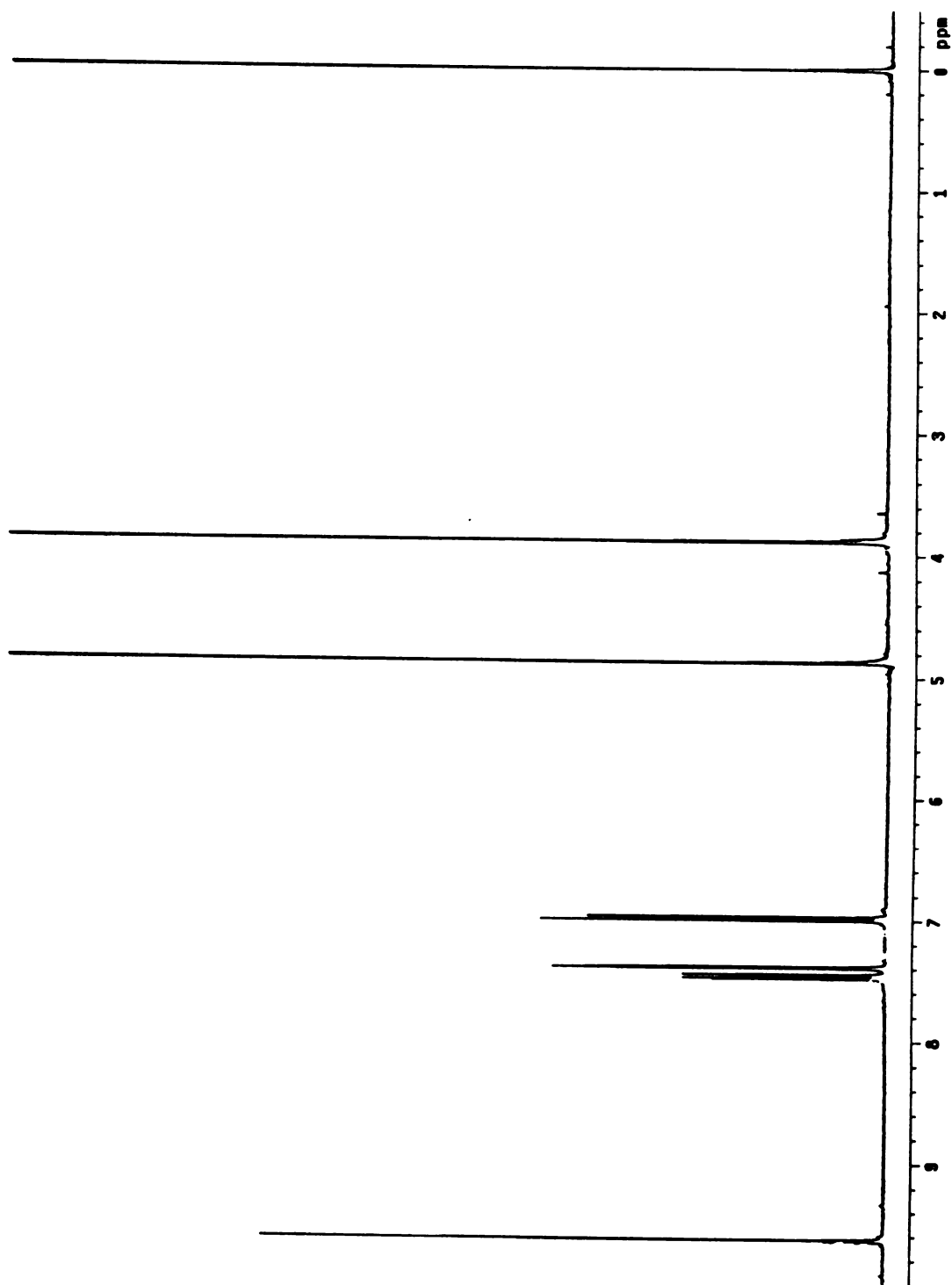
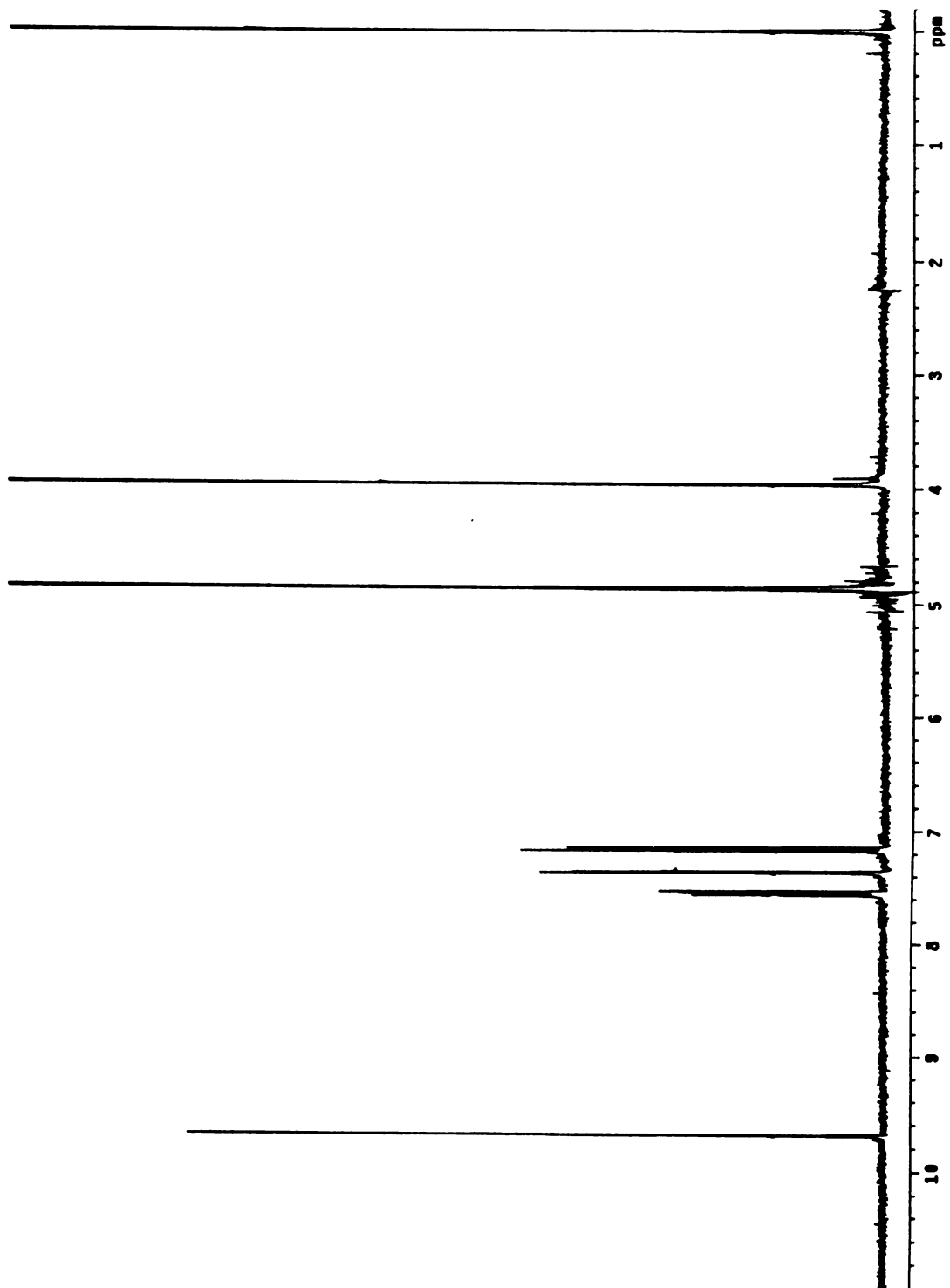


Figure 33. Control ^1H NMR of isovanillin. Resonances: δ 7.38 (s, 1H), 7.18 (d, 1H), 7.55 (d, 1H), 3.95 (s, 3H), 9.70 (s, 1H).



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Figure 34. ^1H NMR after EtOAc extraction of the KL3/pKL5.97A fermentaiton broth (with methionine supplementation). Vanillic acid resonances: δ 7.55 (d, 1H), 6.95 (d, 1H), 7.47 (dd, 1H), 3.92 (s, 3H). Isovanillic acid resonances: δ 7.41 (d, 1H), 7.06 (d, 1H), 7.49 (dd, 1H), 3.91 (s, 3H). PCA resonances: δ 7.45 (d, 1H), 6.95 (d, 1H), 7.40 (dd, 1H).

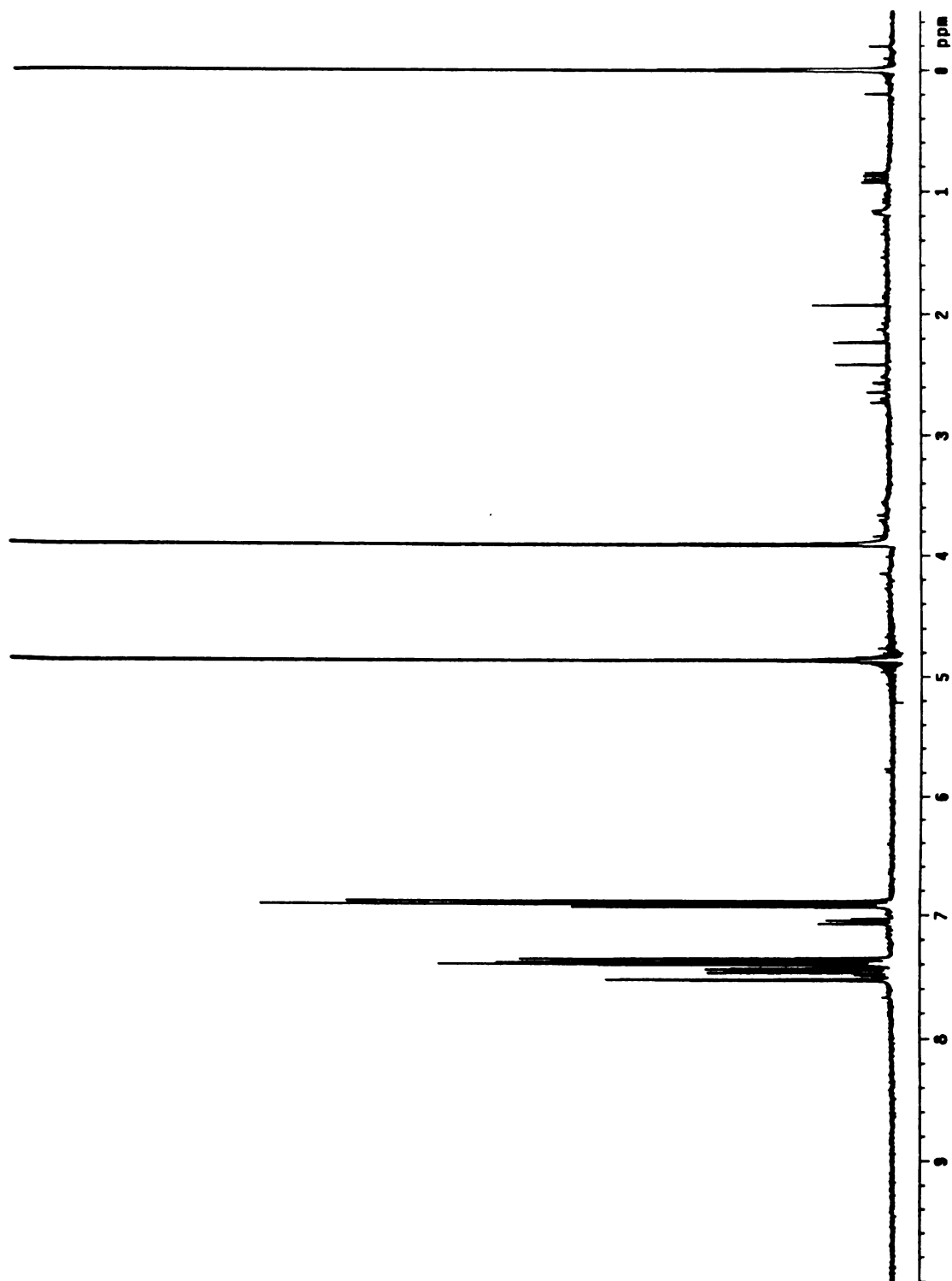
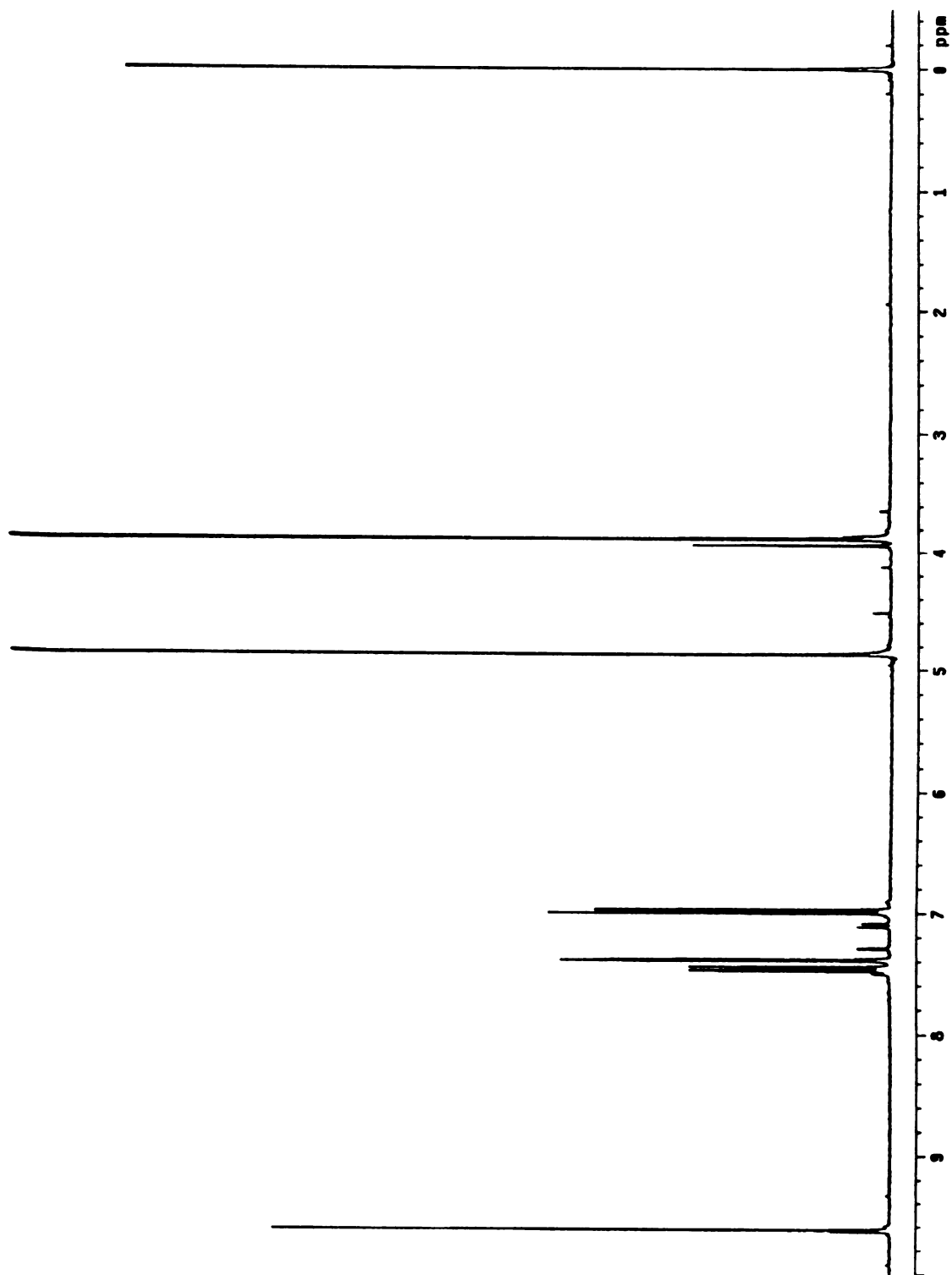


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Figure 35. ^1H NMR after CH_2Cl_2 extraction of the reduction reaction.
Vanillin resonances: δ 7.39 (s, 1H), 6.98 (d, 1H), 7.46 (d, 1H), 3.88 (s, 3H), 9.63 (s, 1H). **Isovanillin resonances:** δ 7.38 (s, 1H), 7.18 (d, 1H), 7.55 (d, 1H), 3.95 (s, 3H), 9.70 (s, 1H).



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Discussion

Biocatalytic synthesis of vanillin from glucose has a number of advantages relative to other biocatalytic vanillin syntheses. Coniferol, formed during phenylpropanoid biosynthesis, is converted into coniferin by a glucosyltransferase in *Vanilla planifolia*.⁶¹ Coniferin is then transformed into glucovanillin which is finally hydrolyzed to vanillin by a β -glucosidase. Synthesis of vanillin via 3-dehydroshikimic, protocatechuic, and vanillic acids circumvents phenylpropanoid biosynthesis and glucosylation/deglucosylation reactions. This substantially reduces the number of enzymes required to synthesize vanillin. Ferulic acid can also be microbially converted into vanillin. However, these routes produce vanillin titers below 1 g/L. Cultured plant tissue in medium supplemented with ferulic acid has also been used to produce vanillin although this conversion is slow and scale-up is relatively difficult. A problem with all vanillin syntheses from ferulic acid is the absence of low-cost, commercial production of this phenylpropanoid.

Biocatalytic synthesis of vanillin from glucose, although a longer term commercial prospect, also has advantages relative to synthetic vanillin manufacture (Scheme 1). Phenol and guaiacol are toxic and are derived from carcinogenic benzene. Nontoxic 3-dehydroshikimic, protocatechuic, and vanillic acids are derived from innocuous glucose. Corrosive H_2O_2 used for oxidation of phenol into catechol requires special handling precautions. By contrast, biocatalytically synthesized vanillin derives its oxygen atoms from the oxygen atoms of glucose. Dimethyl sulfate, a carcinogen, has historically been used to methylate catechol. Protocatechuic acid methylation employs *S*-adenosylmethionine generated and consumed intracellularly. Finally, synthetic vanillin manufacture is based on use of nonrenewable petroleum whereas glucose is derived from abundant, renewable starch. This difference in feedstock utilization is important given projected fierce international competition as global petroleum production diminishes.⁸²

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Future Work

Use of an intact microbe to reduce vanillic acid will be essential for future large-scale vanillin synthesis. Vanillic acid synthesized by one microbe from glucose could be reduced to vanillin by a second, different microbe. For example, *E. coli* can be used for vanillic acid synthesis and *Neurospora crassa* can be used to convert vanillic acid into vanillin. In active, growing *Neurospora crassa* cultures, it was observed that addition of vanillic acid (10 mM) resulted in formation of vanillyl alcohol in the culture supernatant after 60 h. All of the added vanillic acid and the initially formed vanillyl alcohol were eventually metabolized after cultivation for 5 days. This observation suggests that intact *Neurospora crassa* could be employed to reduce vanillic acid to vanillin. The primary challenge is to mutagenically inactivate the aryl-alcohol dehydrogenase, which converts vanillin into its alcohol form. Mutagenizing genes encoding other vanillin-utilizing enzymes may also be necessary.

Conversion of glucose into vanillin using a single vanillate-synthesizing microbe expressing aryl-aldehyde dehydrogenase may also be possible. For example, the *Neurospora crassa* gene encoding aryl-aldehyde dehydrogenase can be cloned, sequenced, and expressed in the vanillate-synthesizing *E. coli* biocatalyst. The clone of the gene encoding aryl-aldehyde dehydrogenase can be isolated either from a *Neurospora crassa* genomic or cDNA library. Direct isolation and expression of a gene from a *N. crassa* genomic DNA library in *E. coli* has been successful in the past.⁸³ However, this strategy is risky. Fungal promoters are not recognized in bacteria and introns, if present, cannot be excised from the primary transcript in *E. coli*, which results in expression of truncated protein.⁸⁴

To avoid problems with promoters and introns, a *Neurospora crassa* cDNA library can be constructed and screened with synthetic oligonucleotide probes designed from the determined amino acid sequence of aryl-aldehyde dehydrogenase. The aryl-aldehyde dehydrogenase will first need to be purified to homogeneity followed by determination of

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its amino acid sequence. Because an oligonucleotide probe containing fifteen to twenty nucleotides is sufficient to screen a library, only a small portion of the total protein needs to be sequenced.⁸⁵ The purified protein needs to be digested with trypsin and the resulting peptides separated. Once several peptides have been partially sequenced from their N-terminus, the six or seven amino acid stretch that can be encoded by the smallest number of possible DNA sequences will be determined and the corresponding degenerate probe synthesized. Radiolabelling of the probe can be carried out by standard procedure.⁸⁶

N. crassa poly(A⁺) mRNA will need to be isolated in order to construct the cDNA library. Two strategies can be used to reduce the size of the cDNA library needed for screening the desired aryl-aldehyde dehydrogenase gene. The aryl-aldehyde dehydrogenase is an induced enzyme, which is expressed upon addition of sodium salicylate. Subtractive hybridization can be used to differentiate the mRNA of induced gene from the large mRNA pool.⁸⁷ In addition, the aryl-aldehyde dehydrogenase has a molecular weight of 120 kDa, which is higher than the average *Neurospora crassa* protein. Its mRNA must therefore be larger in size than the bulk of the mRNA in *Neurospora crassa*. Size-based fractionation of the subtracted mRNA for aryl-aldehyde dehydrogenase will further reduce the size of the mRNA pool.⁸⁶ Subsequent synthesis of the first strand of cDNA with reverse transcriptase will be followed by replacement synthesis of the second strand of cDNA using RNAase H and the Klenow fragment from *E. coli*. After addition of synthetic linkers, the cDNA will be ligated into the bacteriophage λ ZAPII vector. The ligation mixture will be packaged in vitro and plated on XL1-Blue *E. coli* to obtain plaques.⁸⁸ The recombinant λ virions present in plaques on a lawn of *E. coli* will be transferred to a nylon membrane. Hybridization to the radiolabelled probes and autoradiography will be carried out until the positive clone is located. The identified gene can then be sequenced and plasmid-localized to provide a promoter and ribosome binding site for expression in *E. coli*.

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Improving protocatechuic acid methylation will also be essential. The lack of significantly improved protocatechuic acid methylation with increased catechol-*O*-methyltransferase activity and the improvement in methylation observed with L-methionine supplementation suggest that cosubstrate *S*-adenosylmethionine availability and/or feedback inhibition⁸⁹ may be limiting in vivo methyltransferase activity. Overexpression of *E. coli* SAM-synthase (MetK) along with use of L-methionine supplementation did not increase the ratio of vanillic acid/PCA.⁹⁰ This suggests that increasing SAM availability is a rather complicate issue. For example, supplementation with methionine represses MetK and most of the methionine biosynthetic enzymes.⁹¹

Ethionine-resistant *Saccharomyces cerevisiae* mutants have been reported to accumulate significant amounts of SAM⁹² while ethionine-resistant *E. coli* overproduces methionine.⁹³ Additional research has disclosed that a mutation in the *metJ* locus in *E. coli* is responsible for the ethionine resistance.⁹⁴ MetJ protein is a global regulator in methionine biosynthesis. Mutation of *metJ* results in derepression of methionine biosynthesis to afford increased in vivo synthesis of methionine (in *E. coli*) or SAM (in *S. cerevisiae*). Mutations in the *metJ* locus can be easily identified by the resistance these mutations impart towards ethionine. Creation of a KL7 *metJ* mutant could be a possible solution for increasing SAM availability. Alternatively, a *metJ* mutation could be combined with MetK overexpression. In the meantime, DNA shuffling can be used to obtain a feedback-insensitive COMT and/or to improve the properties of COMT. The gene product of *COMT* has a K_m of 136 μM and a V_{max} of 4.6 $\mu\text{mol L}^{-1} \text{min}^{-1}$ for vanillic acid⁹⁵ and a turnover number of 24 per min.

CHAPTER 3

FED-BATCH FERMENTOR SYNTHESIS OF 3-DEHYDROSHIKIMIC ACID USING RECOMBINANT ESCHERICHIA COLI

Introduction

Microbe-catalyzed conversion of D-glucose into bioproducts such as L-phenylalanine and L-tryptophan are intimately related to microbe-catalyzed syntheses of industrial chemicals such as adipic acid and catechol from D-glucose (Figure 36).⁹⁶ For example, the carbon flow which is directed into the common pathway of aromatic amino acid biosynthesis is of central importance to product yields and titers in each of these syntheses. Carbon flow directed into this common pathway can be conveniently measured by the accumulation of 3-dehydroshikimic acid (DHS) in the culture supernatants of microbial mutants lacking shikimate dehydrogenase activity (Figure 36). DHS is of particular importance since it is the most advanced common pathway intermediate shared by aromatic amino acid biosynthesis and biocatalytic syntheses of adipic acid, catechol, vanillin and gallic acid.^{37, 40, 45, 35} DHS synthesis is also important in its own right given the potent antioxidant activity which has been reported for this hydroaromatic.³⁴

As the first enzyme in the common pathway of aromatic amino acid biosynthesis, DAHP synthase activity dictates the amount of cellular carbon directed into DHS synthesis. Historically, transcriptional repression and feedback inhibition of DAHP synthase by aromatic amino acids have been viewed as the regulatory mechanisms that control the in vivo catalytic activity of this enzyme. More recently, the intracellular concentrations of substrates phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) have come under scrutiny as critical determinants of in vivo DAHP synthase activity. Intracellular E4P availability was been reported to be markedly influenced by expression levels of the enzyme transketolase.^{47a, 55a} Overexpression of PEP synthase recycles pyruvic acid back

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to PEP resulting in increased carbon flow directed into the common pathway as long as transketolase is overexpressed.⁴⁷

Biocatalytic syntheses of common pathway intermediates have been examined under shake-flask conditions. Such culturing conditions are problematic. Cells are in a glucose-rich environment at the beginning of shake-flask cultures but experience glucose-deficient conditions by the end of shake-flask cultures. Oxygenation levels and pH are also difficult to control under standard shake-flask conditions.⁴⁷ Cultivation of *E. coli* under fed-batch fermentor conditions offers a number of important advantages. Variables such as temperature, pH, dissolved oxygen levels and the steady-state concentration of glucose can be controlled. Greater aeration is provided by the fermentor stir sparger and the impeller. This increased aeration results in much higher cell densities and growth rates for *E. coli* cultured under fed-batch fermentor conditions relative to shake-flask conditions.⁹⁷

In this chapter, the synthesis of DHS is examined by a series of recombinant *E. coli* biocatalysts under fed-batch fermentor conditions where oxygenation levels and pH are controlled and where glucose availability is maintained at a constant, limiting level. Titer and yields for synthesized DHS are compared with previously reported syntheses of common pathway intermediates in shake flasks. Transketolase overexpression was observed to have a pronounced impact on the yields and titers of DHS synthesized from glucose under fed-batch fermentor conditions even in lieu of PEP synthase overexpression. Theoretical maximum yields for product DHS and cell mass formation are also discussed.

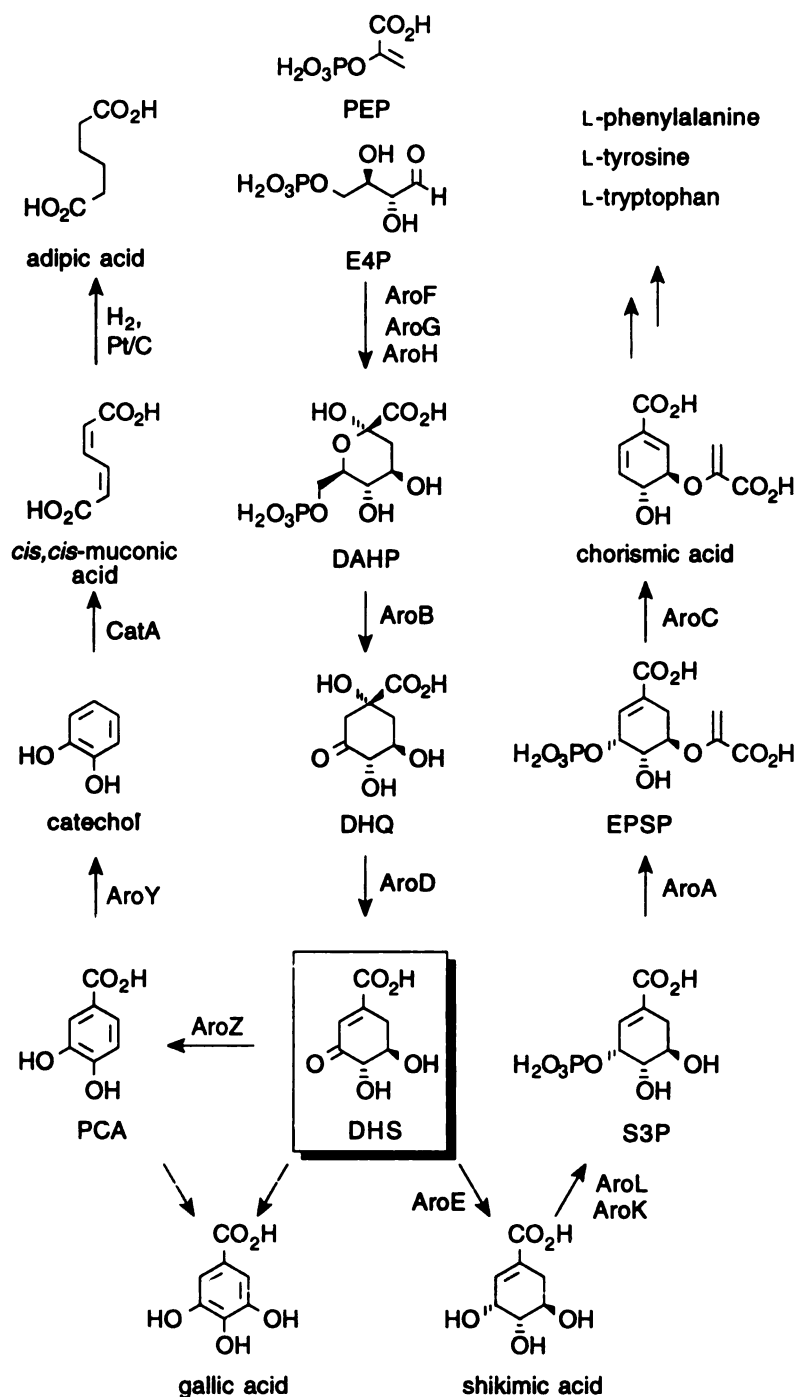


Figure 36. DHS as the most advanced intermediate shared by aromatic amino acid biosynthesis and biocatalytic synthesis of adipic acid and catechol.

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Biocatalysts and Fed-Batch Fermentor Conditions for DHS Synthesis

A. Shared Genomic and Plasmid Elements

All of the DHS-synthesizing biocatalysts shared several genetic and recombinant elements including a mutation in the genomic *aroE* locus, a second copy of the *aroB* gene inserted into the genomic *serA* locus, plasmid-localized *serA* and plasmid-localized *aroF^{FBR}*. *E. coli* AB2834, an *aroE* mutant lacking shikimate dehydrogenase activity, was the ancestral strain used to construct the KL3 host used in all of the DHS syntheses. The absence of catalytically-active, *aroE*-encoded shikimate dehydrogenase, which catalyzed the conversion of DHS into shikimic acid, resulted in the accumulation of DHS in the culture supernatants of *E. coli* KL3. Growth of *E. coli* AB2834 requires supplementation with aromatic amino acids for protein biosynthesis along with supplementation with aromatic vitamins for biosynthesis of folic acid, coenzyme Q, and enterochelin. Aromatic amino acids supplements include L-phenylalanine, L-tyrosine, and L-tryptophan while aromatic vitamin supplements consisted of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid.

Because of the increased carbon flow directed into the common pathway resulting from increased in vivo activity of DAHP synthase, wild-type expression levels of *aroB*-encoded DHQ synthase is inadequate in *E. coli* AB2834 for conversion of substrate DAHP into product DHQ at a rate which is sufficiently rapid to avoid substrate accumulation. DAHP undergoes dephosphorylation to 3-deoxy-D-*arabino*-heptulosonic acid (DAH) which accumulates in the culture supernatant resulting in reductions in the titer, yield, and purity of synthesized DHS. An approximately 2-fold increase in DHQ synthase activity, which can be accomplished by introduction of a second copy of *aroB* into the genome of *E. coli* AB2834, is required to eliminate DAH accumulation.⁶⁹

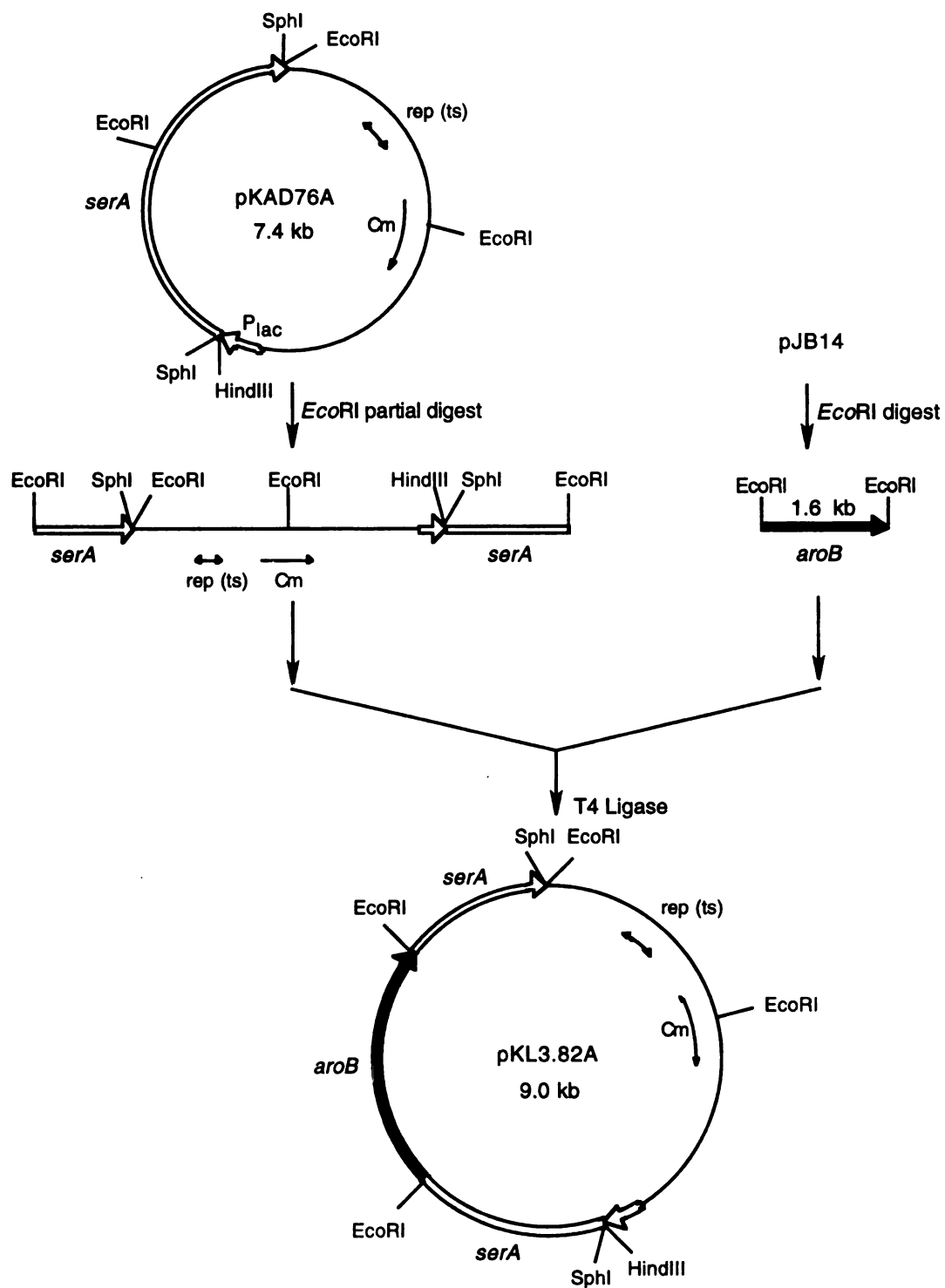


Figure 37. Preparation of Plasmid pKL3.82A.

The genomic modification in *E. coli* KL3 responsible for increased DHQ synthase expression resulted from site-specific insertion of a cassette consisting of *aroB* with flanking *serA* nucleotide sequences into the *serA* locus of *E. coli* AB2834. This was achieved through homologous recombination. Localization of the *serA* gene in temperature-sensitive plasmid pMAK705 followed by insertion of the *aroB* into an *EcoRI* site internal to the *serA* directed recombination of the *aroB* into the *serA* locus of the genome (Figure 37). Digestion of pKAD63 with *SphI* liberated a 1.9-kb *serA* fragment, which was subsequently inserted into the *SphI* site of pMAK705 to afford pKAD76A. The *aroB* gene was obtained as a 1.6-kb fragment following digestion of pJB14 with *EcoRI*. Insertion of the *aroB* fragment into the *EcoRI* site of *serA* was complicated by two additional *EcoRI* sites in pKAD76A. Following *EcoRI* partial digestion of pKAD76A, the resulting DNA fragments were resolved on an agar gel and the 7.4-kb fragment corresponding to the linearized plasmid was isolated. Ligation of the linearized plasmid to the 1.6-kb *EcoRI* fragment of *aroB* afforded pKL3.82A (Figure 37). Temperature-sensitive plasmid pKL3.82A was used for homologous recombination. Conditions for homologous recombination⁹⁸ were identical to those described in Chapter 2 of this thesis. KL3 was isolated based on the following growth characteristics: growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid and serine; no growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid; growth on LB; and no growth on LB containing Cm (Table 6).

Disruption of the genomic *serA* locus was also the basis for plasmid maintenance. Gene *serA* encodes D-3-phosphoglycerate dehydrogenase, which is the first of the three enzymes responsible for serine biosynthesis⁹⁹ (Figure 38). Since KL3 is a serine auxotroph, growth in medium lacking L-serine supplementation required expression of *serA* localized in all plasmids carried by KL3 biocatalysts. Using this nutritional pressure for plasmid maintenance avoided the necessity of using antibiotics in fermentation medium. However, all of the DHS-synthesizing plasmids carried markers encoding antibiotics

because rich medium with added antibiotic was used as the inoculum for all the fermentations discussed in this Chapter.

Table 6. Plate selection for characterization of genome insertion strain.

Strain	M9/glucose/ Tyr/Phe/SA	M9/glucose/ Tyr/Phe/SA/ serine	LB/Cm	LB
AB2834	+	+	—	+
KL3	—	+	—	+

SA: shikimic acid

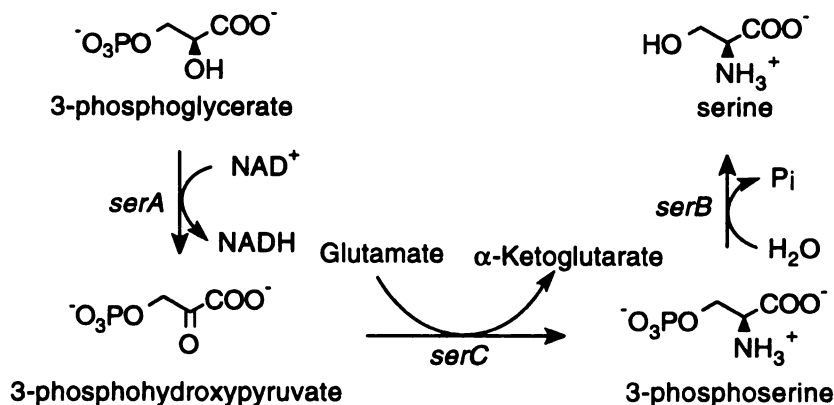


Figure 38. Serine biosynthesis. Genetic loci are as follows: *serA*, 3-phosphoglycerate dehydrogenase; *serC*, 3-phosphoserine aminotransferase; *serB*, 3-phosphoserine phosphatase.

In *E. coli*, the most important regulation of DAHP synthase is feedback inhibition of the enzymes by aromatic amino acids.¹⁰⁰ All DHS-synthesizing constructs therefore employed a mutant isozyme of DAHP synthase, designated as *aroF^{FBR}*, which was insensitive to feedback inhibition by aromatic amino acids. This mutant isozyme was obtained by photochemical mutagenesis of an *E. coli* AB2.24, which expressed only the genome-encoded, tyrosine-sensitive isozyme (AroF) of DAHP synthase. The *aroF^{FBR}*

gene was isolated from a mutant selected as a result of its more rapid growth in a diffusion gradient chamber against an increasing concentration of *m*-flurotyrosine.¹⁰¹ Sequencing of the isolated *aroF^{FBR}* gene revealed a Pro-148 to Leu-148 point mutation which corresponds to a previously reported AroF mutant isozyme insensitive to feedback inhibition by L-tyrosine.¹⁰²

B. Fed-batch fermentor conditions

Fed-batch fermentation was performed in a 2.0 L capacity Biostat MD B-Braun fermentor connected to a DCU system and a Compaq computer equipped with B-Braun MFCS software for data acquisition and automatic process monitoring (Figure 39). The temperature, pH and glucose feeding were controlled with a PID controller. The temperature was maintained at 37 °C, and pH was maintained at 7.0 by addition of concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen was maintained at 20% air saturation throughout the fermentation process using a Braun polarographic probe. Antifoam (Sigma 204) was added manually as needed.

Inoculants were grown in 100 mL LB medium (enriched with 2 g glucose) containing the appropriate antibiotic for 12 h to 14 h at 37 °C with agitation at 250 rpm. The inoculants were then transferred to the fermentor. The initial glucose concentration in the fermentation varied from 18 g/L to 23 g/L according to the growth rates of different constructs. Three different methods were used to maintain dissolved oxygen (D.O.) level at 20% air saturation during the course of each fed-batch fermentor run. After inoculation of the fermentor solution containing inorganic salts, aromatic amino acids, aromatic vitamins, and a quantity of glucose, D.O. was maintained by increasing the impeller stirring rate until a preset maximum value (940 rpm) was reached. Approximately 10 h were required before the impeller reached its maximum stirring rate. The mass flow controller then maintained D.O. levels at 20% saturation at the constant impeller stirring rate by increasing the airflow rate until a preset maximum value (3.0 L/L/min) was reached.

Approximately 2 h were needed for the airflow to increase to its maximum rate. At a constant impeller stirring rate (940 rpm) and constant airflow (3 L/L/min), D.O. levels were then maintained at 20% saturation by oxygen-sensor-controlled glucose feeding for the rest of the fermentation. At the beginning of this stage, dissolved oxygen levels fell below 20% saturation due to residual glucose in the medium. This lasted for approximately 1 h before all residual glucose was consumed and the glucose feeding started.

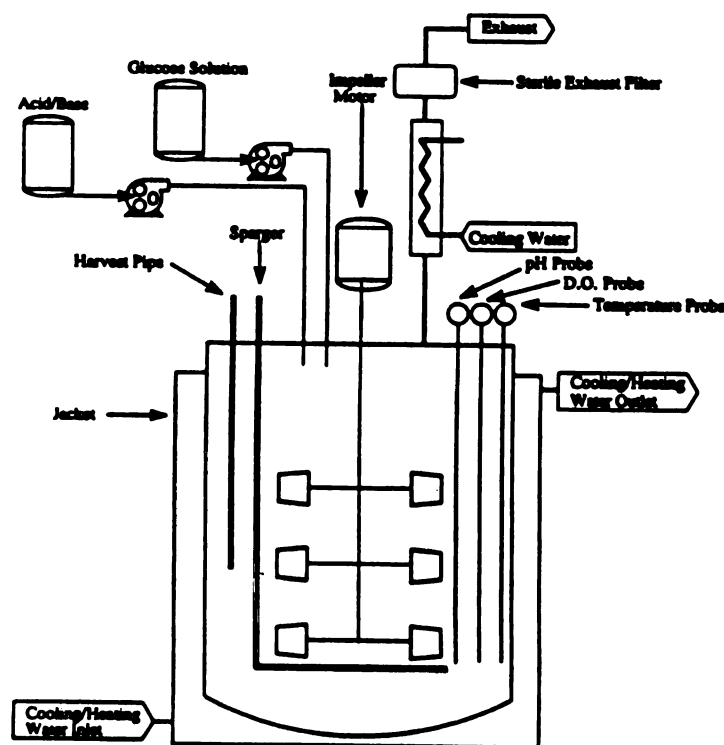


Figure 39. B. Braun Fermentor.

The concentration of the glucose added to fermentor runs was 60% (w/v). The PID control parameters 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). Oxygen sensor control of glucose usually became too difficult to maintain at about 48 h into

a fermentation run. Loss of oxygen sensor control was characterized by unregulated addition of glucose to the fermentor culture.

Fermentor runs typically entered logarithmic growth 6 h after inoculation. After approximately 24 h, fermentor cultures moved from a logarithmic to a stationary growth phase. Microbial cell density normally reached a maximum of 25-30 g/L dry cell weight. Over the course of the fermentations, the culture solution turned progressively darker. By the end of all of the fermentor runs, the culture solutions were always a deep black color. Acetic acid accumulation was observed at 6 h and at the conclusion of fermentor runs which corresponds to early logarithmic and late stationary microbial growth phases, respectively. Concentrations of acetic acid declined or were absent for most of the logarithmic and early stationary microbial growth phases. Maximum productivity in DHS synthesis generally started at 12 h and continued until 48 h. DHS synthesis typically did not continue beyond 48 h.

Carbon Flow Directed into the Common Pathway as a Function of DAHP Synthase Expression

The key determinant of carbon flow directed into a biosynthetic pathway is often the *in vivo* activity of the first enzyme in the pathway. For the common pathway of aromatic amino acid biosynthesis, the *in vivo* activity of DAHP synthase is dictated by feedback inhibition, transcription repression, and the availability of substrates E4P and PEP. *E. coli* uses three different isozymes of DAHP synthase encoded by *aroF*, *aroG*, and *aroH* which are feedback-inhibited, respectively, by L-tyrosine, L-phenylalanine, and L-tryptophan. Feedback inhibition was circumvented in the DHS synthesizing strains by use of *aroF^{FBR}* which is obtained via photochemical mutagenesis of *aroF*. Choice of *aroF^{FBR}*, as opposed to use of feedback-insensitive mutants of other DAHP synthases, followed from previous employment of *aroF^{FBR}* to achieve the highest titers thus far reported for microbial synthesis of L-phenylalanine under fed-batch fermentor conditions.⁵⁴

The first DHS-synthesizing biocatalyst was created by transforming plasmid pKL4.33B into the KL3 host. Plasmid pKL4.33B includes a copy of *aroF^{FBR}* under the transcription control of its native promoter and a copy of *serA* in a pSU18 vector (Figure 19, 20).⁷¹ This vector has a *p15A* origin of replicon with a copy number of approximately 12 per cell. Vector pSU18 also contains a *lac* promoter and a genetic marker encoding Cm resistance. Plasmid pKL4.33B was created as follows: After UV mutagenesis and chemotactic selection using a Diffusion Gradient Chamber, the *aroF^{FBR}* locus was amplified by PCR with its native promoter to yield a 1.3-kb fragment. Inclusion of *EcoRI* recognition sequences at the 5'- and 3'- ends of the *aroF^{FBR}* fragment facilitated its insertion into the *EcoRI* site of pCL1920 to afford pCL2-13A. Plasmid pCL2-13A was digested with *EcoRI* and the 1.3-kb *aroF^{FBR}* fragment was isolated and ligated into the *EcoRI* site of pSU18 to create pKL4.20B. The *aroF^{FBR}* gene is transcribed in the opposite orientation relative to the *lac* promoter in pKL4.20B (Figure 19). Ligation of a 1.9-kb *DraI/EcoRV* fragment encoding *serA* obtained from pD2625 into the *SmaI* site of the

pKL4.20B afforded pKL4.33B. The *serA* gene is transcribed in the same orientation as *aroF*^{FBR} (Figure 20).

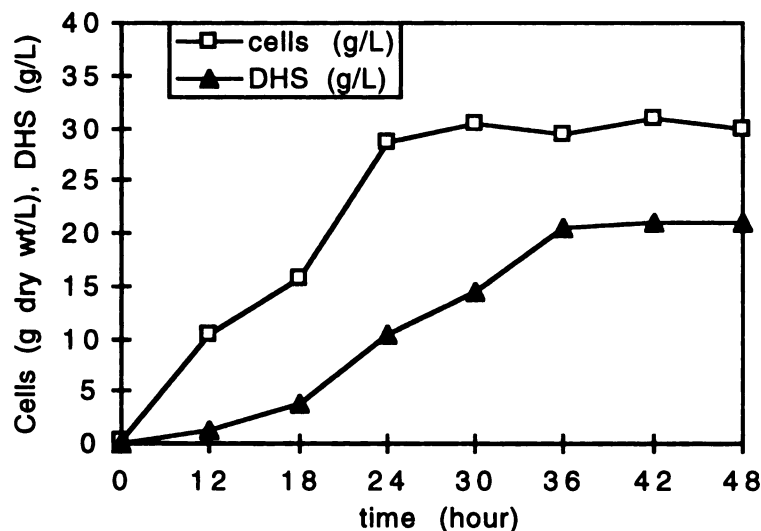


Figure 40. KL3/pKL4.33B synthesis of DHS under fed-batch fermentor conditions.

KL3/pKL4.33B was examined under fed-batch fermentor conditions. The fermentation reached stationary phase at around 24 h. DHS was synthesized from 12 h to 36 h after which DHS production leveled off (Figure 40). After 48 h, KL3/pKL4.33B produced 20.3 g/L DHS in a 17% (mol of DHS synthesized /mol of glucose consumed) yield. DHQ (1.0 g/L) and gallic acid (0.5 g/L) also accumulated in the fermentation medium. A total yield, which includes all of the accumulated common pathway intermediates and their derivatives, was used to gauge carbon flow directed into the common pathway. The total yield of KL3/pKL4.33B fermentation is 18% (mol/mol) including DHS, DHQ and gallic acid (Table 7). DAHP synthase specific activity was also monitored at four different time points during the 48 h fermentation (Table 7). It started at 0.118 U/mg at 12 h and increased to 0.228 U/mg at 24 h. At 36 h, DAHP synthase specific activity decreased significantly to 0.014 U/mg and 0.018 U/mg at 48 h (Table 7). The coincidence between the low DAHP synthase activity and the leveling off of DHS

synthesis after 36 h indicated that DAHP synthase activity might be the limiting factor in achieving higher DHS titers and yields.

Table 7. Product titers and yield after 48 h fermentation for KL3/pKL4.33B

[DHS] (g/L)	DHS yield (mol/mol)	[DHQ] (g/L)	[GA] (g/L)	Total yield (mol/mol)	DAHP synthase specific activity			
					12h	24h	36h	48h
20.3	17%	1.0	0.5	18%	0.118	0.228	0.014	0.018

One approach taken to increase the levels of DAHP synthase expression entailed replacement of the native promoter of *aroF^{FBR}* with a strong *P_{tac}* promoter. Use of a stronger promoter and the attendant tighter binding of RNA polymerase leads to increased transcription. However, DAHP synthase overexpression can reach a level where cell growth and metabolism are compromised. Inclusion of *lacI^Q* in the same plasmid was designed to control this trade-off. The *lacI^Q* gene product is the Lac repressor protein, which binds to the *lac* operator DNA sequence. Since the *lac* operator is included in the *P_{tac}* promoter region, Lac repressor encoded by plasmid-localized *lacI^Q* will repress the transcription of the gene controlled by the *P_{tac}* promoter. However, binding of lactose to the Lac repressor can cause a conformational change of the repressor so that the repressor no longer binds to the operator. Transcription of the gene under the control of *P_{tac}* is thus derepressed. Clearly, the advantage of this *P_{tac}/lacI^Q* system is the ability to modulate the concentration of the inducible gene products in the cell by varying the lactose inducer concentration. The frequently used inducer is the nonhydrolyzable analog of lactose, isopropyl β-D-thiogalactopyranoside (IPTG). AroF specific activity was thus controlled by the amount and frequency of IPTG added to the fermentor medium.

Plasmid pKL4.79B was created for the inducible, controlled expression of *aroF^{FBR}*. First, the open reading frame (ORF) of the *aroF^{FBR}* was amplified from

pKL4.33B using following primers: 5'- GGAATTCATGCAAAAAGACGCGCTGA and 5'- GGAATTCTTAAGCCACGCGAGCCGT. Localization of the resulting 1.1-kb fragment in pJF118EH afforded pKL4.71A (Figure 41). Cloning vector pJF118EH contains a *tac* promoter and *lacIQ* gene.¹⁰³ With the copy number of about 15 per cell, it also contains a *pMB1* origin of replicon and an Ap resistant marker. In pKL4.71A, the ORF of *aroF^{FBR}* is transcribed in the same orientation as the *tac* promoter to ensure the proper transcription and translation of *aroF^{FBR}* (Figure 41). Further ligation of a 1.9-kb *DraI/EcoRV serA* fragment obtained from pD2625 into the *SmaI* site of the pKL4.71A afforded pKL4.79B. The *serA* gene in pKL4.79B is transcribed in the opposite orientation relative to the *aroF^{FBR}* gene (Figure 42).

Table 8a. DAHP synthase activities ($\mu\text{mol}/\text{min}/\text{mg}$) when *aroF^{FBR}* expression is controlled by *P_{tac}*.

Entry	IPTG addition	DAHP synthase specific activity			
		12h	24h	36h	48h
^a 1	^b 0.0	0.009	0.011	0.012	0.007
^a 2	^b 0.32	0.11	0.098	0.13	0.048
^a 3	^b 1.6	0.10	0.33	0.13	0.12
^a 4	^b 4.8	0.24	0.30	0.27	0.42
^a 5	^b 8.0	4.0	2.8	1.2	1.1
^a 6	^b 40.0	1.5	1.5	0.91	1.2
^a 7	^c 1000	3.0	2.2	0.29	0.21

^a KL3/pKL4.79B

^b Amount of IPTG (mg) added at 12, 18 24, 30, 36, and 42 h.

^c Amount of IPTG (mg) added at 4 h.

Optimization of IPTG addition of KL3/pKL4.79B fermentation led to several trends. During addition of IPTG at timed intervals, a maximum specific activity of DAHP was achieved after which an additional increase in IPTG concentration resulted in a decline in DAHP synthase specific activity (entry 5, Table 8a). A high specific activity for DAHP

synthase could also be achieved by a single addition of a relatively large quantity of IPTG early in the fermentor run (entry 7, Table 8a).

Table 8b. Product titers and yields when *aroF^{FBR}* expression is controlled by *P_{tac}*.

Entry	[DHS] (g/L)	DHS yield (mol/mol)	[DHQ] (g/L)	[GA] (g/L)	Total yield (mol/mol)
^a 1	24.3	13%	1.4	0.6	14%
^a 2	29.1	16%	2.6	1.5	18%
^a 3	34.0	21%	2.0	1.4	23%
^a 4	52.0	20%	6.3	3.7	24%
^a 5	26.8	12%	1.8	1.7	14%
^a 6	22.0	11%	1.0	1.4	12%
^a 7	17.4	12%	0.1	0.2	13%

^a KL3/pKL4.79B

AroF is preceded¹⁰⁴ to be labile to protease activity during the stationary phase of *E. coli* growth. However, DAHP synthase specific activities were stable over the course of most of the fed-batch fermentations where IPTG was added at timed intervals (entries 2-4 and 6, Table 8a). This sharply contrasts with the decline in DAHP synthase activity observed overtime with KL3/pKL4.33B where *aroF^{FBR}* expression was under its native promoter (Table 7). These differences may suggest that a *P_{tac}* but not a *P_{aroF}* promoter allows for *aroF^{FBR}* transcription during stationary phase. Genes expressed from a *P_{tac}* promoter and a *P_{trp}* promoter continue to be transcribed during stationary phase in *E. coli*.¹⁰⁵ There does appear to be a ceiling above which AroF specific activities can not be maintained even when *aroF^{FBR}* is under *P_{tac}* promoter control. This is evident in the pronounced decline in enzyme activity observed for the two IPTG addition regimes resulting in the highest DAHP synthase activities (entries 5 and 7, Table 8a) measured at 12 h into the fermentation runs.

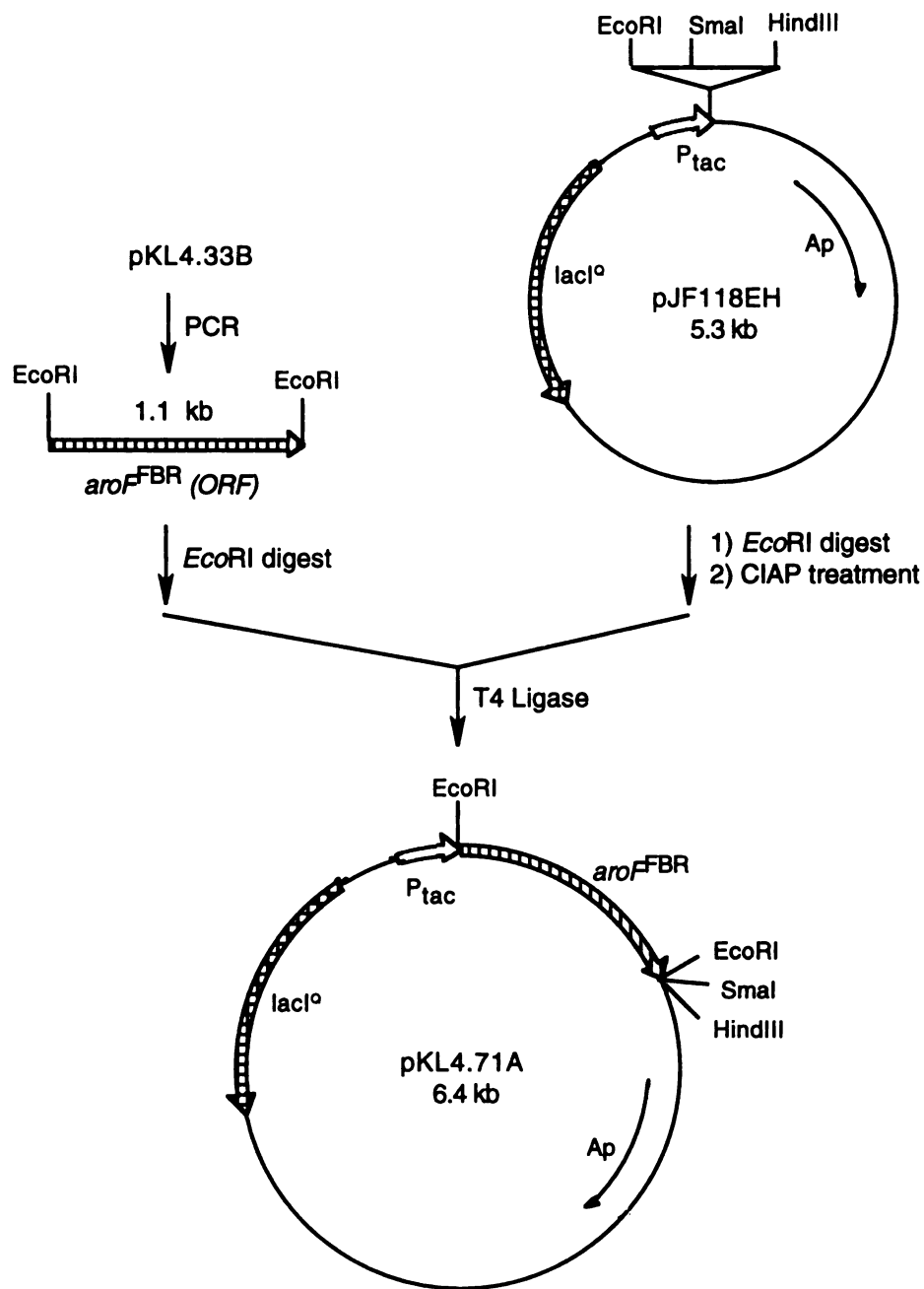


Figure 41. Preparation of Plasmid pKL4.71A.

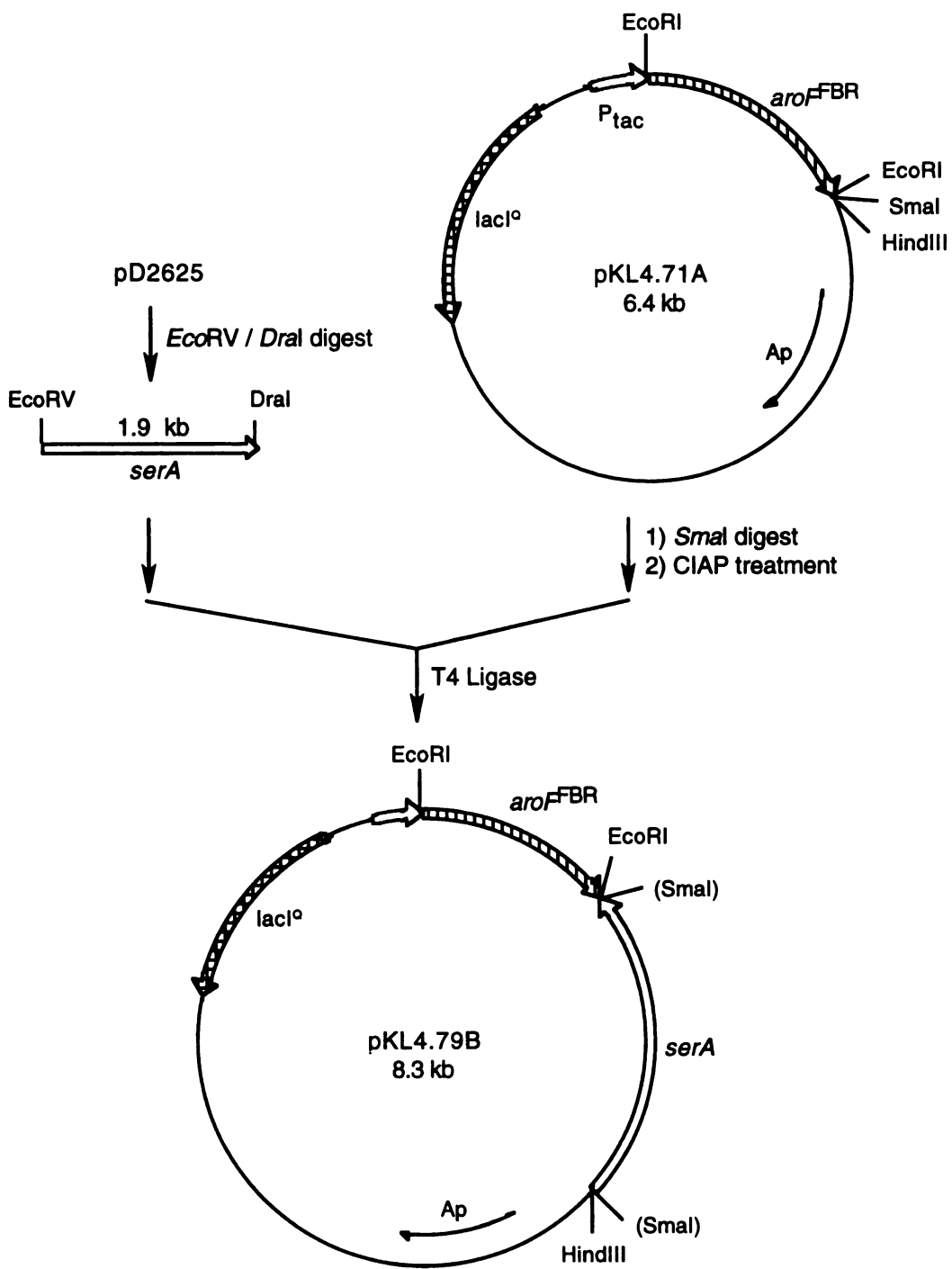


Figure 42. Preparation of Plasmid pKL4.79B.

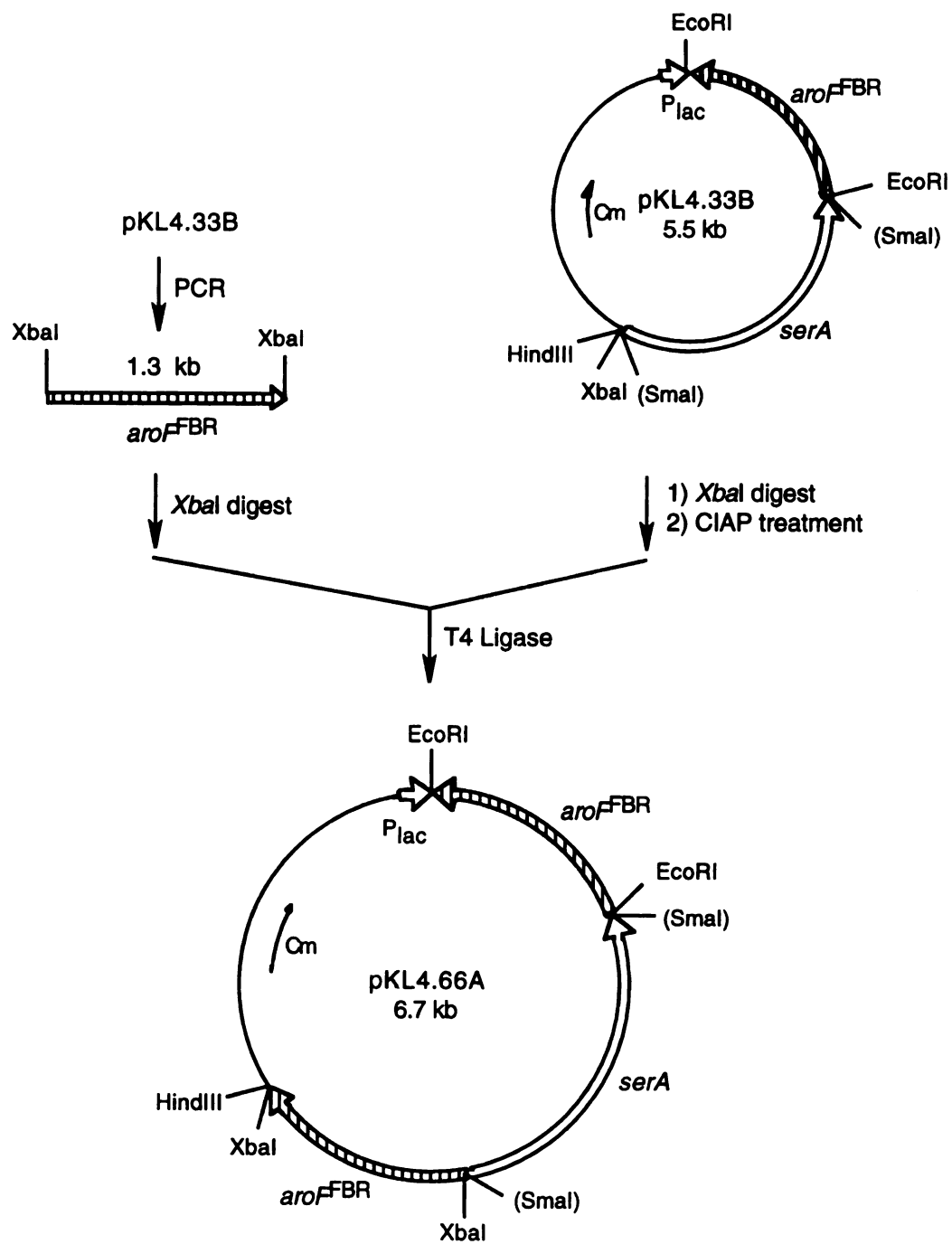


Figure 43. Preparation of Plasmid pKL4.66A.

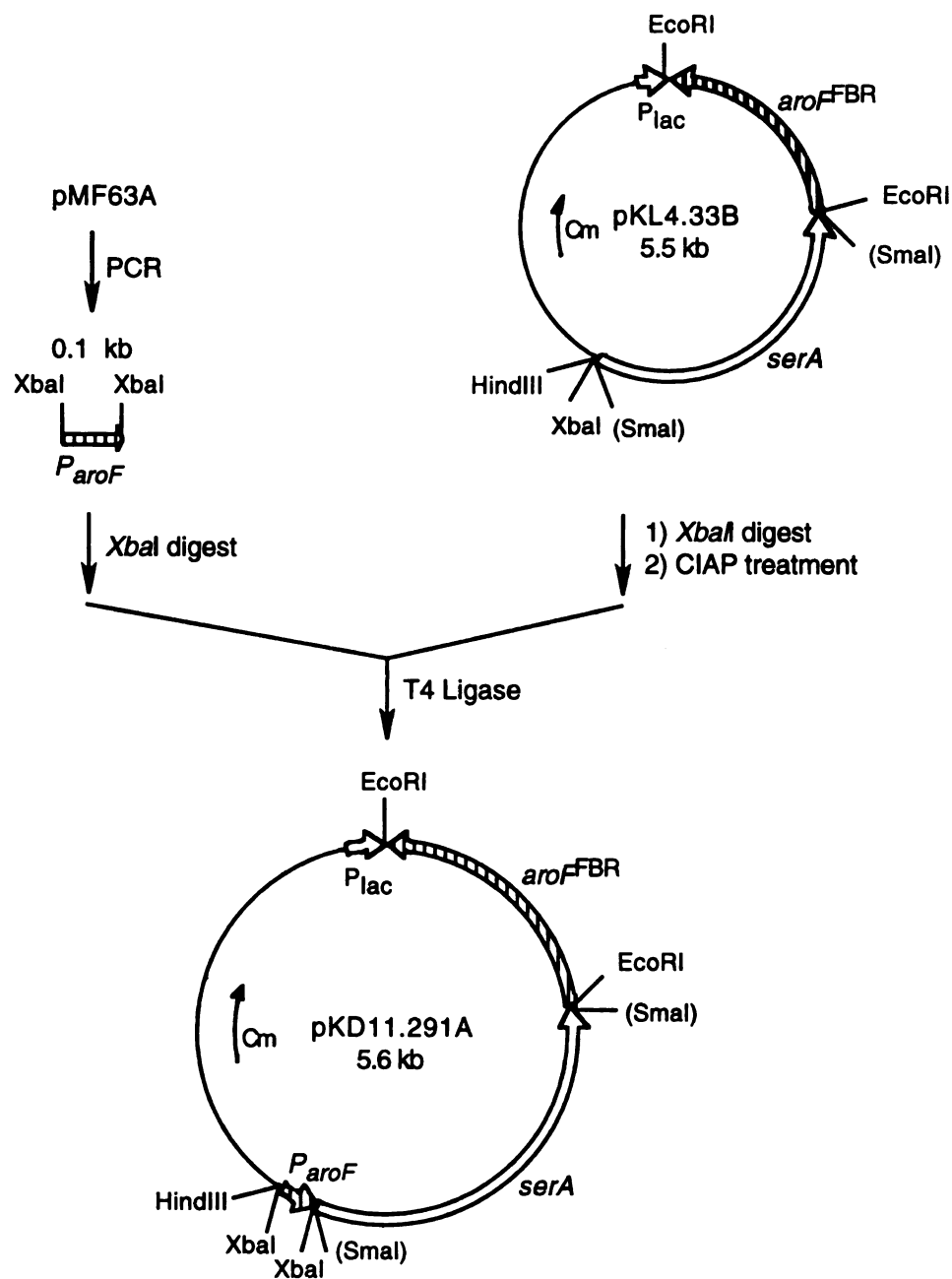


Figure 44. Preparation of Plasmid pKD11.291A.

Incremental increases in added IPTG concentration led to corresponding improvements in DHS titers and yields (Table 8a). The highest DHS titer and yield was achieved when 4.8 mg of IPTG was added at 12, 18, 24, 36, and 42 h. KL3/pKL4.79B synthesized 52.0 g/L DHS in a 20% (mol/mol) yield (entry 4, Table 8a and 8b). DHQ (6.3 g/L) and gallic acid (3.7 g/L) were also synthesized leading to a total yield of hydroaromatic and aromatic products of 24% (mol/mol). Beyond this optimal IPTG addition, addition of higher IPTG concentration resulted in a pronounced decrease in DHS titers and yields (entries 5 and 6, Table 8a and 8b). This cut-off point appears to correspond to a ceiling in DAHP synthase activity above which further increases in DAHP synthase activity levels may diminish the biocatalyst's ability to synthesize DHS.

As shown in Table 8a and Table 8b, higher than optimal DAHP synthase expression levels results in a significant decrease in DHS titers and yields. This is probably due to the competition of PEP availability between DAHP synthase and phosphotransferase (PTS) mediated glucose uptake. Too much DAHP synthase drives PEP into the common pathway, thereby leading to a diminish rate of glucose uptake by the cell. This is consistent with the observation that cells grow slower when IPTG additions exceed 4.8 mg per time point. The increased metabolic burden imposed on the cell's metabolism by excessive enzyme overexpression may also be responsible for the decline in DHS synthesis observed when DAHP synthase expression exceeds an optimal level.

Because of the anticipated need to use strong promoters such as P_{tac} for amplified expression of enzymes other than DAHP synthase, options were explored for exploiting the native promoter of *aroF^{FBR}* for amplified expression of this gene. In *E. coli*, the *tyrR* gene product represses transcription of *aroF*. Upon plasmid localization, the increased copies of *aroF^{FBR}* with its unmodified native promoter should titrate away the cellular supply of TyrR which binds to the operator region of *aroF^{FBR}*. Some percentage of the *aroF^{FBR}* promoters thus evade TyrR binding and are derepressed. This was the reason for comparing localization of one *aroF^{FBR}* locus per plasmid, two *aroF^{FBR}* loci per plasmid,

and one *aroF^{FBR}* locus accompanied by one *P_{aroF}* promoter per plasmid. The strategy where only *P_{aroF}*, the promoter region of *aroF^{FBR}*, was inserted into the multicopy plasmid along with a single copy of *aroF^{FBR}* was designed to achieve derepression of *aroF^{FBR}* while reducing the amount of DNA contained in the multicopy plasmid.

Table 9. Product titers and yield synthesized by KL3/pKL4.66A after 48 h

[DHS] (g/L)	DHS yield (mol/mol)	[DHQ] (g/L)	[GA] (g/L)	Total yield (mol/mol)	DAHP synthase specific activity			
					12h	24h	36h	48h
38.5	16%	2.2	3.2	18%	0.41	0.14	0.077	0.11

Table 10. Product titers and yield synthesized by KL3/pKD11.291A after 48 h

[DHS] (g/L)	DHS yield (mol/mol)	[DHQ] (g/L)	[GA] (g/L)	Total yield (mol/mol)	DAHP synthase specific activity			
					12h	24h	36h	48h
41.2	18%	2.9	2.6	21%	0.059	0.06	0.072	0.054

Plasmid pKL4.66A was created to contain two copies of *aroF^{FBR}*. The *aroF^{FBR}* locus was amplified by PCR from pKL4.20B with *XbaI* ends. Localization of the 1.3-kb *aroF^{FBR}* into the *XbaI* site of pKL4.33B resulted in pKL4.66A. Transcription of the *aroF^{FBR}* locus in the *XbaI* site of pKL4.66A proceeds in the opposite orientation of *serA* (Figure 43). Plasmid pKD11.291A was created to contain a copy of *aroF^{FBR}* and a copy of *P_{aroF}*. This 5.6-kb plasmid was constructed by inserting a fragment encoding the promoter region of *aroF* into the *XbaI* site of pKL4.33B. *P_{aroF}* was amplified from pMF63A using following primers: 5'- GCTCTAGAGAATTCAAAGGGAGTGTA and 5'-

GCTCTAGACCTCAGCGAGGATGACGT. Transcription from *P_{aroF}* is in the same orientation as the *serA* gene (Figure 44).

Both KL3/pKL4.66A and KL3/pKD11.291A were examined using the same fermentation conditions. KL3/pKL4.66A expressed significantly higher DAHP synthase specific activities relative to KL3/pKL4.33B where only a single copy of *aroF^{FBR}* is plasmid-localized (Table 7, 9). DAHP synthase specific activity was lower for KL3/pKD11.291A compared to that for KL3/pKL4.66A (Table 10). However, this biocatalyst's DAHP synthase specific activities remained reasonably constant over time (Table 10). Both KL3/pKL4.66A and KL3/pKD11.291A produces much higher DHS titers and similar DHS yield relative to KL3/pKL4.33B. KL3/pKL4.66A synthesized 38.5 g/L of DHS in 16% (mol/mol) yield while KL3/pKD11.291A synthesized 41.2 g/L of DHS in 18% (mol/mol) yield. Taking into consideration the synthesized DHQ and gallic acid, total yields of hydroaromatic and aromatic products were 18% (mol/mol) and 21% (mol/mol) for KL3/pKL4.66A and KL3/pKD11.291A, respectively (Table 9, 10).

Overexpression of Transketolase under Fed-Batch Fermentor Conditions

As described in the previous section, several different strategies were explored for circumventing transcriptional repression including placing plasmid-localized *aroF^{FBR}* under *P_{tac}* control, employing two plasmid-localized copies of *aroF^{FBR}*, and insertion of *P_{aroF}* in the same plasmid containing *aroF^{FBR}*. However, higher DAHP synthase expression levels did not necessarily translate into higher DHS yields or titers as evidenced by the relationship between DAHP synthase specific activity and DHS synthesized when plasmid-localized *aroF^{FBR}* was under *P_{tac}* control (Table 8a, b). Even though DAHP synthase specific activity and DHS synthesis initially increased with increased IPTG concentration, a point was reached where further increases in IPTG concentrations and associated improvements in DAHP synthase activity did not result in increased DHS titers or yields. Indeed, the IPTG addition regimens leading to the three highest DAHP synthase specific activities resulted in DHS titers which were no more than 52% (mol/mol) of the DHS titers observed for the best DHS synthesis when *aroF^{FBR}* was under *P_{tac}* control (Table 8a, b).

E4P availability is another factor likely limiting in vivo DAHP synthase activity. Using shake-flask experiments, Draths et al. discovered that the E4P availability was an important limiting factor in aromatic amino acid biosynthesis.^{47a} Steady-state concentrations of E4P are low even in the absence of amplified DAHP synthase activity.¹⁰⁶ E4P is prone to dimerization, trimerization, and polymerization.¹⁰⁷ Dissociation of these E4P forms back to monomeric E4P is quite slow. By closely matching the rate of E4P synthesis with the rate of E4P utilization, the resulting low, steady-state concentration of E4P may be Nature's in vivo strategy favoring the monomeric form of E4P.

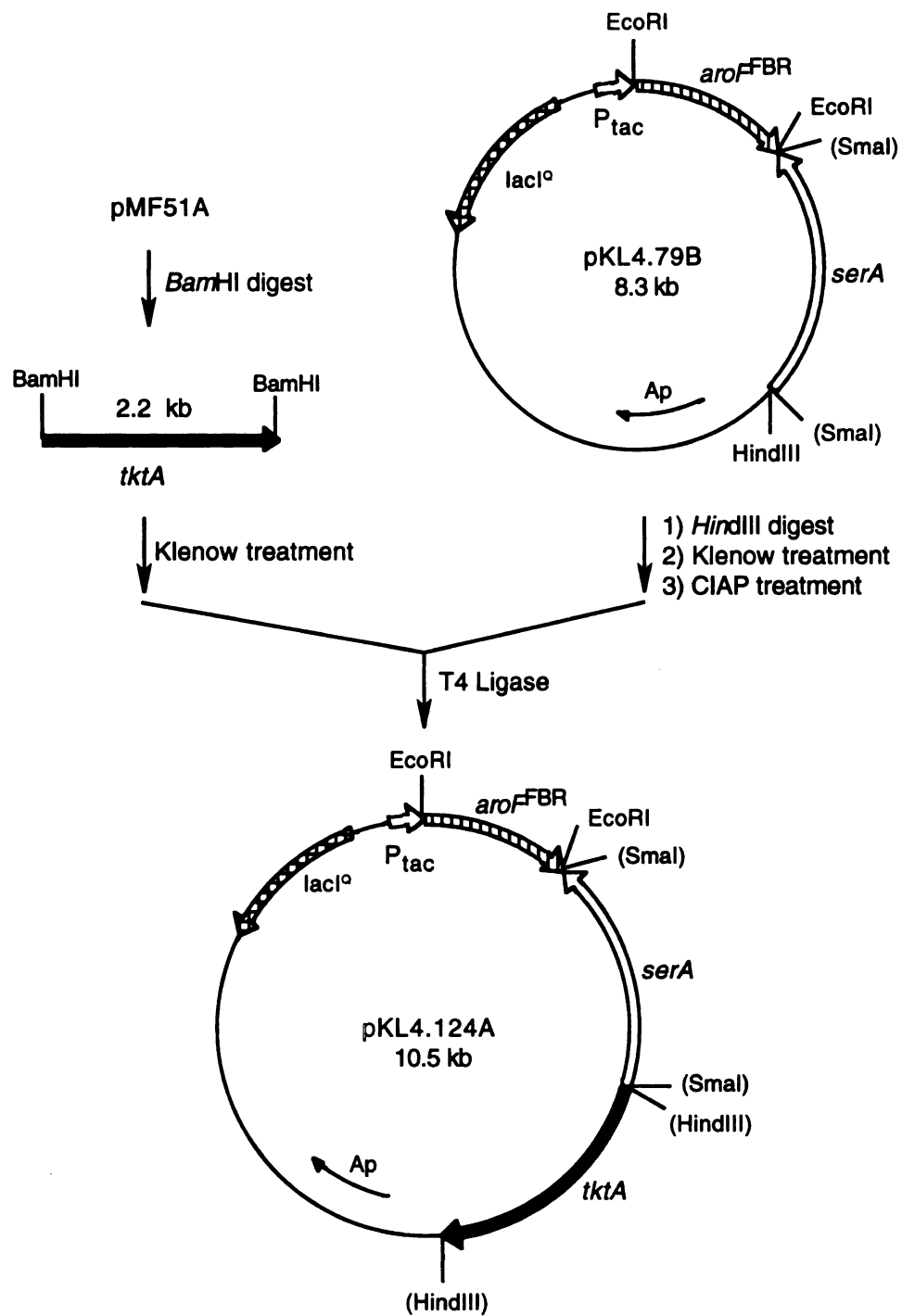


Figure 45. Preparation of Plasmid pKL4.124A.

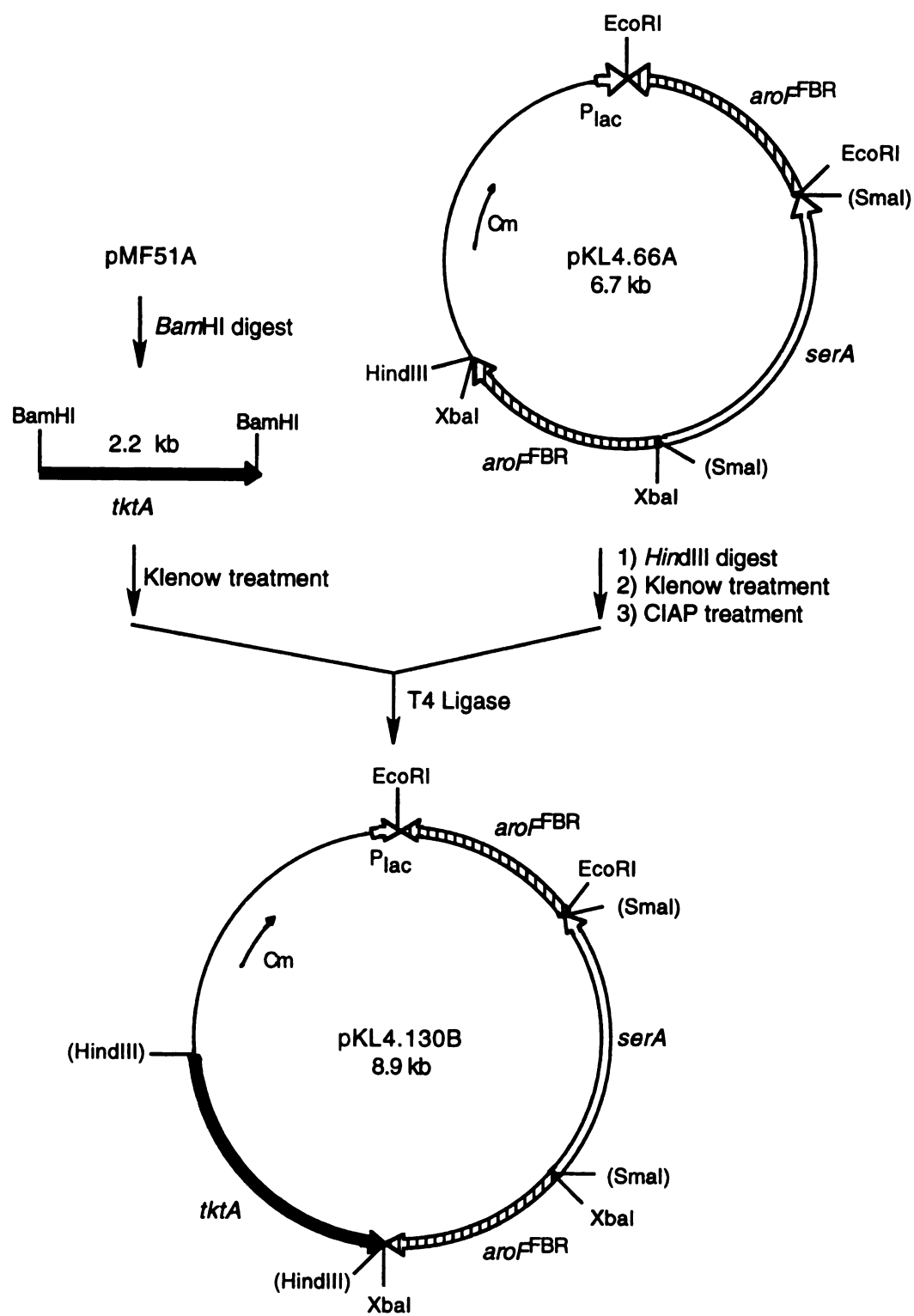


Figure 46. Preparation of Plasmid pKL4.130B.

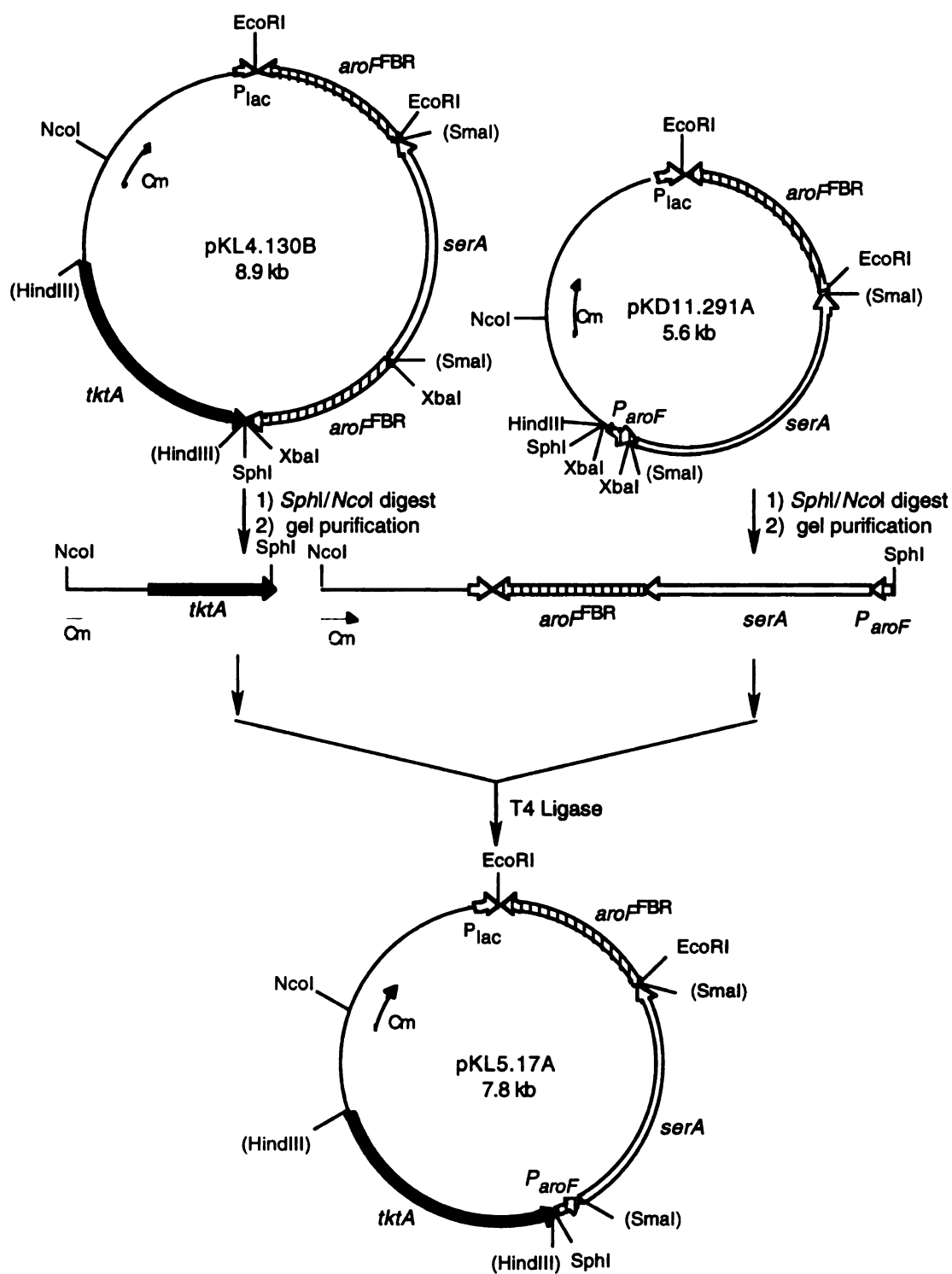


Figure 47. Preparation of Plasmid pKL5.17A.

Amplified expression of either transketolase or transaldolase¹⁰⁸ has been demonstrated to increase the in vivo availability of E4P in *E. coli* cultured under shake-flask conditions. Transketolase and transaldolase are enzymes in the nonoxidative pentose phosphate pathway which facilitate the interconversion of C-7, C-6, C-5, and C-4 aldoses and ketoses. In order to test whether E4P is a limiting factor at optimized DAHP synthase expression levels under fed-batch fermentor conditions, the *tktA* gene was inserted in the plasmid pKL4.79B to overexpress transketolase. Plasmid pKL4.124A was created for this purpose. This 10.5-kb plasmid was constructed by inserting the *tktA* fragment from pMF51A into pKL4.79B. Following digestion of pMF51A with *Bam*HI, the 2.2-kb *tktA* fragment was blunt ended using Klenow fragment. Plasmid pKL4.79B was digested with *Hind*III and also treated with Klenow fragment. Subsequent ligation of the *tktA* fragment with linearized pKL4.79B afforded pKL4.124A. The *tktA* gene is transcribed in the same orientation as the *aroF*^{FBR} gene (Figure 45).

Table 11a. DAHP synthase specific activities (μmol/min/mg) in TktA-overexpression constructs

Construct	DAHP synthase specific activity			
	12h	24h	36h	48h
KL3/pKL4.124A ^a	0.27	0.12	0.092	0.062
KL3/pKL4.130B	0.23	0.11	0.08	0.075
KL3/pKL5.17A	0.037	0.031	0.033	0.025

^a 4.8 mg IPTG added at 12, 18, 24, 30, 36, 42h.

KL3/pKL4.124A synthesis of DHS was examined under fermentation conditions where 4.8 mg IPTG were added at 12, 18, 24, 36, and 42 h. DAHP synthase activities were also monitored during the fermentation run and were observed to decrease relative to those measured for the non-*tktA* KL3/pKL4.79B (Table 8a, Table 11a). This was

probably due to the metabolic burden imposed by the expression of the additional gene (*tktA*) localized in pKL4.124A. However, the titer and yield of DHS increased significantly. KL3/pKL4.124A synthesized 66.3 g/L of DHS in 28% (mol/mol) yield. DHQ (6.0 g/L) and gallic acid (6.0 g/L) were also produced. The total yield of DHS, DHQ, and gallic acid synthesized from glucose was 33% (mol/mol) (Table 11b).

Table 11b. Product titers and yield in TktA-overexpression constructs

Construct	[DHS] (g/L)	DHS yield (mol/mol)	[DHQ] (g/L)	[GA] (g/L)	Total yield (mol/mol)
KL3/pKL4.124A ^a	66.3	28%	6.0	6.0	33%
KL3/pKL4.130B	69.0	30%	6.8	6.6	36%
KL3/pKL5.17A	58.1	24%	3.6	4.6	27%

^a 4.8 mg IPTG added at 12, 18, 24, 30, 36, 42h.

The impact on synthesized DHS by amplified transketolase was also examined when *aroF^{FBR}* expression was under the transcription control of its native promoter. Insertion of *tktA* gene into pKL4.66A and pKD11.291A resulted in pKL4.130B and pKL5.17A, respectively. Plasmid pMF51A was digested with *Bam*HI and the resulting 2.2-kb *tktA* fragment was treated with Klenow fragment. Plasmid pKL4.66A was digested with *Hind*III and treated with Klenow fragment. Subsequent ligation of the *tktA* fragment with linearized pKL4.66A afforded pKL4.130B. The *tktA* gene is transcribed in the same orientation as the *serA* gene (Figure 46). The 7.8-kb pKL5.17A plasmid was created by replacing the 1.0-kb *Nco*I/*Sph*I fragment of pKD11.291A with a 3.2-kb *Nco*I/*Sph*I fragment from pKL4.130B that included *tktA*. Digestion of pKD11.291A with *Nco*I and *Sph*I afforded a 4.6-kb fragment while similar digestion of pKL4.130B yielded a

3.2-kb DNA fragment. Ligation of these two purified fragments resulted in pKL5.17A (Figure 47).

When two copies of plasmid-localized *aroF^{FBR}* system were utilized for overexpressing transketolase, DHS yields and titers went from a titer of 39 g/L and 16% yield to a titer of 69 g/L and 30% yield upon plasmid-localization of *tktA* (KL3/pKL4.66A versus KL3/pKL4.130B). A DHS titer of 41 g/L synthesized in 18% (mol/mol) yield improved to a titer of 58 g/L synthesized in 24% (mol/mol) yield upon plasmid localization of *tktA* along with *aroF^{FBR}* and *P_{aroF}* (KL3/pKD11.291A versus KL3/pKL5.17A). The highest concentrations of synthesized gallic acid (6.6 g/L) and DHQ (6.6 g/L) were again observed for the biocatalyst that also synthesized the highest concentration of DHS (Table 11b).

Figure 48 and Figure 49 compare the profiles of the fermentations for KL3/pKL4.66A and KL3/pKL4.130B. Both fermentations yield similar cell mass. However, compared to non-transketolase overexpressing construct KL3/pKL4.66A, the transketolase overexpressing KL3/pKL4.130B produced DHS, DHQ and gallic acid at a much higher rate. With transketolase overexpression, more acetic acid accumulated in both the log phase and the late stationary phase of the fermentation.

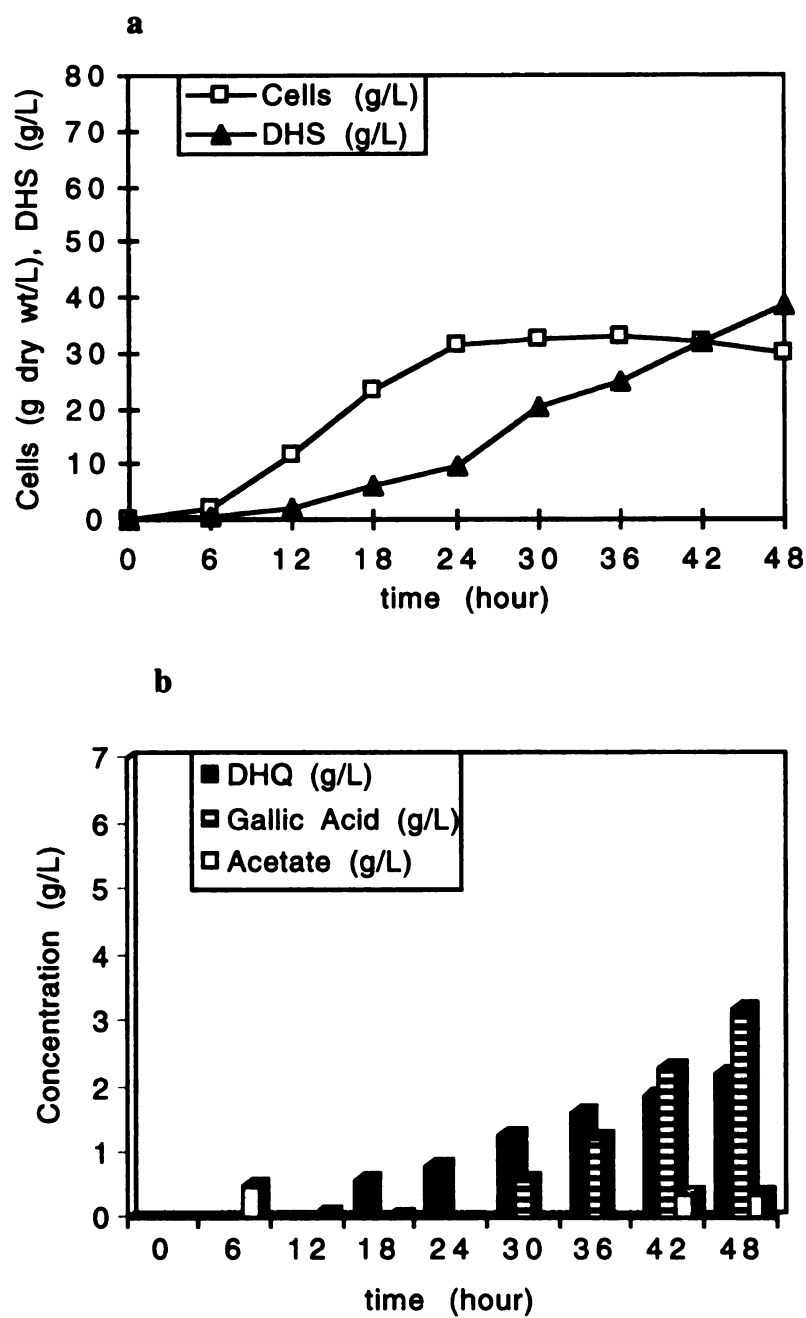


Figure 48. KL3/pKL4.66A (a) DHS production and cell growth. (b) DHQ, gallic acid, and acetic acid production.

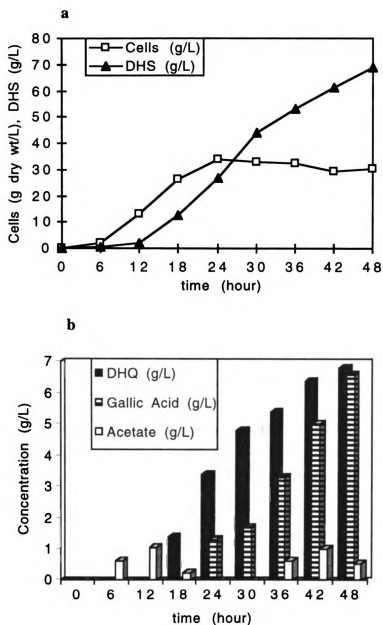
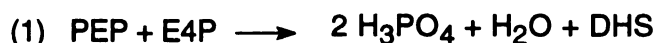


Figure 49. KL3/pKL4.130B (a) DHS production and cell growth. (b) DHQ, gallic acid, and acetic acid production.

Discussion

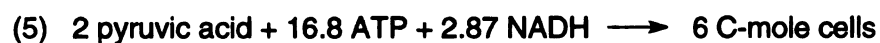
A. Comparison of Titters and Yields

Determining¹⁰⁹ the theoretical maximum yield for biocatalytic synthesis of DHS begins with balancing (Eq. (1)) the PEP and E4P inputs with DHS product and byproducts. The PEP and E4P inputs are then equated to the amount of D-glucose which is required to form these substrates (Eq. (2a)). Because *E. coli* relies on the carbohydrate phosphotransferase (PTS) system for glucose uptake, a pyruvic acid term is included in Eq. (2a) to reflect the conversion of one molecule of PEP into pyruvic acid for each molecule of glucose transported into the cytoplasm as glucose 6-phosphate. Eq. (2a) reflects the apparent absence of pyruvic acid recycling back to PEP which would be catalyzed by the enzyme PEP synthase. PTS-generated pyruvic acid is considered to be the primary carbon source for the anabolism and catabolism required for generation of *E. coli* cellular biomass.



A coefficient is determined (Eq. (2b)) to balance the number of carbon atoms in the glucose starting material with the total number of carbon atoms formed in PEP, E4P, and pyruvic acid. The determined coefficient leads to a maximum theoretical yield of 43% (mol DHS/mol glucose) for synthesis of DHS from glucose. KL3/pKL4.130B, the construct producing the highest yields and titers of DHS when *aroF^{FBR}* was expressed from its native *P_{aroF}* promoter, synthesized DHS at 70% of the theoretical maximum yield. DHS was synthesized at 66% of theoretical maximum yield by KL3/pKL4.124A, which produced the highest titer and yield of DHS when two copies of *aroF^{FBR}* were expressed

from their P_{tac} promoters. In considering the performance of KL3/pKL4.130B and KL3/pKL4.124A, synthesis of DHQ and gallic acid must also be taken into consideration. DHQ is a metabolic precursor to DHS and gallic acid is a metabolite derived from DHS. Including DHQ and gallic acid synthesis, KL3/pKL4.130B and KL3/pKL4.124A channeled a total of 83% and 77%, respectively, of the theoretical maximum amount of carbon which can be directed into aromatic amino acid biosynthesis.



A similar balancing approach was used to estimate the theoretical maximum *E. coli* cell yield from the pyruvic acid, ATP, and electron equivalents generated by this pathway. For every seven glucose molecules entering the pathway, 7 pyruvic acid, 10 reduced NADH, and 4 ATP molecules are produced. The stoichiometries for production of pyruvic acid from glucose via glycolysis (Eq. (3)) and cell mass production from glucose¹¹⁰ (Eq. (4)) can be summed to estimate the stoichiometry to cell mass production from pyruvic acid (Eq. (5)). A fraction of the pyruvic acid produced must be converted to acetyl CoA and then catabolized to CO₂ in the Krebs cycle to provide energy and electrons needed for cell synthesis. Each FADH₂ produced in the Krebs cycle was assumed to yield two ATP in the electron transport chain, and each NADH was assumed to yield three ATP. Balanced equations for pyruvic acid, cells, FADH₂, NADH, and ATP were written, based on Eq. (5) and the known stoichiometry of pyruvic acid oxidation via the Krebs cycle. The pseudo-steady-state assumption was invoked for non-secreted intermediates.¹¹⁰ Solving the balances simultaneously resulted in a cell yield of 15 C-mols of cells per seven moles of glucose consumed. This number corresponds to 28% of the pyruvic acid derived from the PTS being catabolized, and 72% being used for cell synthesis. The mass of one C-mol of

E. coli cells is 24.6 g, based on a measurement of the cell's elemental composition. Using this value, the theoretical maximum cell yield on a mass basis is 0.29 g cell/g glucose. Typically, the cell concentrations obtained in these fermentations were about 25 g/L, and the glucose consumption levels were about 250 g/L. Thus, the experimental cell yields were about 0.10 g cells/g glucose, which is well below the theoretical maximum value.

Microbial synthesis of DHS leading to a titer of 5.2 g/L and a yield of 54% (mol/mol) has previously been realized under shake-flask conditions.¹¹¹ These experiments employed overexpressed native *aroF* and *tktA*. Accumulation in the culture supernatant of DAH, which results from dephosphorylation of DAHP, has been more frequently employed as the measure of carbon flow directed into the common pathway of aromatic amino acid biosynthesis. DAH has been synthesized under shake flask conditions at a titer of 6.2 g/L and a yield of 63% (mol/mol) by an *E. coli* construct overexpressing the isozyme of DAHP synthase encoded by *aroG^{FBR}* which is insensitive to feedback inhibition by L-phenylalanine.^{47d, e} Overexpression of transketolase and PEP synthase in addition to overexpression of *aroG^{FBR}* led to a titer of 12.5 g/L of DAH synthesized in a yield of 94% (mol/mol).^{47d, e} All of these reported yields of DHS and DAH are substantially in excess of the theoretical maximum yield of 43% (mol/mol) and 86% (mol/mol) for DAH and DHS synthesis in the absence and presence, respectively, of PEP synthase-catalyzed recycling of pyruvate back to PEP. Such yields reflect the shake flask conditions employed for DHS and DAH syntheses where the *E. coli* construct is initially grown in rich medium, harvested, and then resuspended in minimal salts medium containing glucose where synthesis of DHS proceeds. Using fed-batch fermentor conditions similar to those employed in this Chapter for DHS synthesis, an *E. coli* construct overexpressing *aroF^{FBR}* synthesized 46.8 g/L of L-phenylalanine in 20% (mol/mol) yield.⁵⁴

B. DHQ and Gallic Acid Formation

Substantial concentrations of 3-dehydroquinic acid (DHQ) and gallic acid accumulated during DHS synthesis. Various common pathway metabolites have been reported to accumulate in the culture supernatants of *E. coli* constructs in which carbon flow directed into the common pathway of aromatic amino acid biosynthesis has been substantially increased.⁶⁹ These metabolites result from rate-limiting common pathway enzymes which can not catalyze the conversion of substrate into product at a sufficiently rapid rate to avoid substrate accumulation and subsequent export of these metabolites into the culture supernatant. However, DHQ accumulation has not previously been reported for *E. coli* biocatalyst despite the detailed scrutiny this microbe has received for rate-limiting, common pathway enzymes.⁶⁹ Gallic acid formation during DHS synthesis is also surprising. Beyond its occurrence in plants¹¹² and reported biosynthesis in the filamentous fungus *Phycomyces blakesleeanus*,¹¹³ gallic acid synthesis has not previously been observed in *E. coli* or any other bacterium. For constructs KL3/pKL4.130B and KL3/pKL4.124A, which synthesized DHS in the highest yields and titers, substantial quantities of DHQ (6-7 g/L) and gallic acid (6-7 g/L) were produced.

Metabolite accumulation has typically been attributed to impeding, rate-limiting enzymes when the common pathway is operating in the forward direction towards biosynthesis of aromatic amino acids. For DHQ accumulation, it is possible that the *aroD* encoded, DHQ dehydratase is rate-limiting under the fed-batch fermentor conditions. However, an alternative explanation may be that synthesized DHS accumulating in the fermentor culture medium is transported back into the cytoplasm where DHQ dehydratase catalyzes the conversion of the DHS back into DHQ. DHS uptake by *E. coli* may be mediated by protein(s) encoded by the *shiA* locus¹¹⁴ which catalyze uptake of the structurally similar shikimic acid. In addition, DHQ dehydratase is known to catalyze DHS hydration.¹¹⁵ Further conversion of DHQ is unlikely since the next common pathway enzyme, DHQ synthase, catalyzes an irreversible reaction which precludes conversion of

DHQ back into DAHP. Equilibration of DHS with DHQ is supported by the fact that molar ratio of [DHS]/[DHQ] synthesized by DHS producer is similar in magnitude to the known equilibrium constant ($K_{eq} = 15$) for the reversible DHQ dehydration/DHS hydration catalyzed by DHQ dehydratase. If DHQ accumulation is due to rate-limiting DHQ dehydratase, expression of the *aroD* locus needs to be amplified. Increasing DHQ dehydratase activity, however, would not reduce DHQ formation if this byproduct is the result of microbial equilibration of initially synthesized DHS. Eliminating transport of DHS from the culture medium into the cytoplasm would likely be a more productive strategy. By cloning another copy of *aroD* in the plasmid and testing the new plasmid under the same conditions, the reason of DHQ accumulation would be clarified according to the changed (or unchanged) ratio of [DHS]/[DHQ].

Several different mechanisms can be offered for formation of gallic acid. Abiotic, inorganic phosphate-catalyzed conversion of DHS into gallic acid is precedented.³² Alternatively, DHS could be enzymatically converted into gallic acid by DHS dehydratase-catalyzed conversion of DHS into protocatechuic acid (PCA) followed by hydroxylation of the PCA to form gallic acid. Oxidoreductase-catalyzed dehydrogenation of either the C-4 or C-5 hydroxyl group of DHS could also lead to gallic acid formation since subsequent aromatization would be expected to be spontaneous and rapid. Despite repeated attempts, no conversion of DHS into gallic acid was observed in cell-free extracts to which a variety of different cofactors were added. This lack of assayable enzyme activity could be consistent with either an abiotic route for gallic acid formation or indicative of an enzymatic process where the responsible enzymes do not survive cell lysis. Abiotic, inorganic phosphate-catalyzed conversion of DHS into gallic acid is always accompanied by PCA formation. The absence of PCA when gallic acid is formed during microbial synthesis of DHS is thus not consistent with an abiotic conversion. Furthermore, much higher concentrations of inorganic phosphate are required to catalyze abiotic conversion of DHS into gallic acid than were employed during the fed-batch fermentor syntheses of DHS.³⁴

The absence of PCA formation appears to be more consistent with oxidoreductase oxidation of the C-4 or C-5 alcohol of DHS rather than dehydration of DHS and hydroxylation of the intermediate PCA. However, the possibility remains that PCA might be present in undetected low, steady-state concentrations.

C. E4P Limitation versus PEP Limitation

E4P and PEP availabilities are two possible limiting factors for directing more carbon into the common pathway after DAHP synthase has been overexpressed. Historically, examination of small molecule limitations to aromatic amino acid biosynthesis has focused on PEP availability.¹¹⁶ Competition between PTS-mediate glucose uptake and DAHP synthase for PEP is an obvious problem during biocatalytic synthesis of aromatics and hydroaromatics. Other enzymes which employ PEP as substrate such as PEP carboxylase, pyruvate kinase, PEP carboxykinase, and 3-deoxy-D-manno-octulosonate 8-phosphate synthase add to this intracellular competitive fray. Although various strategies have been examined for increasing in vivo PEP availability, amplified expression of PEP synthase is particularly effective. PEP synthase catalyzes the conversion of pyruvic acid into PEP at the expense of both of ATP's high-energy phosphodiester (Figure 12). Overexpressed PEP synthase enables PTS generated pyruvate to be recycled back into PEP thereby ameliorating the competition between glucose uptake and aromatic amino acid biosynthesis for intracellular PEP concentrations.

The impact of both E4P and PEP availability on the in vivo activity of DAHP synthase has been examined by Liao and coworkers under shake-flask conditions.^{47d, e} This was accomplished by using amplified expression of *tktA*-encoded transketolase and *pps*-encoded PEP synthase activities. These experiments demonstrated that, when *aroG^{FBR}*, *pps* and *tktA* were all overexpressed, yields close to or exceeding the theoretical maximum value were achieved for a common pathway intermediate used to gauge carbon flow directed into the common pathway. Overexpression of feedback-insensitive *aroG^{FBR}*

and *pps*-encoded PEP synthase in lieu of *tktA* overexpression did not increase the flow of carbon directed into the common pathway of aromatic amino acid biosynthesis relative to overexpression of just *aroG^{FBR}*. Only a modest increase in common pathway carbon flow was detected when *aroG^{FBR}* and *tktA* were overexpressed in lieu of amplified *pps* expression. These observations suggested that even in a biocatalyst environment possessing ample PEP concentrations and overexpressed, feedback-insensitive DAHP synthase, E4P availability was a critical factor which limited aromatic amino acid biosynthesis.

DHS synthesis under fed-batch fermentor conditions clearly demonstrates the pronounced effect of *tktA* overexpression even in the absence of PEP synthase overexpression. This contrasts with the report^{47d, e} by Liao that carbon flow directed into the common pathway was only modestly increased by transketolase overexpression in the absence of amplified PEP synthase expression. The impact of *tktA* overexpression on DHS synthesis is an important observation since overexpression of PEP synthase inhibits growth of *E. coli*. In actively growing *E. coli* cultures, this growth inhibition negates the positive impact PEP synthase has on titers and yields of synthesized common pathway intermediate.^{47d, e}

An important difference between this study and the previous Liao study may be D.O. control of glucose addition during fed-batch fermentor synthesis of DHS relative to the shake flask culturing conditions employed by Liao. The steady-state concentration of glucose was maintained at approximately 200 μ M during most of the fed-batch fermentor synthesis of DHS as a result of employing D.O. to control glucose concentration. By contrast, glucose concentrations are not maintained at a constant level during shake-flask cultures. Such cultures begin in a glucose-rich environment and end in a glucose-deficient environment. D.O. levels are also maintained at a constant value over the entire course of the fed-batch fermentor runs, whereas D.O. levels are uncontrolled in shake-flask cultures. Titrers and yields of DHS synthesized in this Chapter may therefore highlight a fundamental

difference in the in vivo availability of PEP in *E. coli* cultured under fed-batch fermentor conditions relative to culturing of *E. coli* under shake-flask conditions.

Figure 50. Control ^1H NMR of gallic acid. Resonances: δ 7.06 (s, 2H).

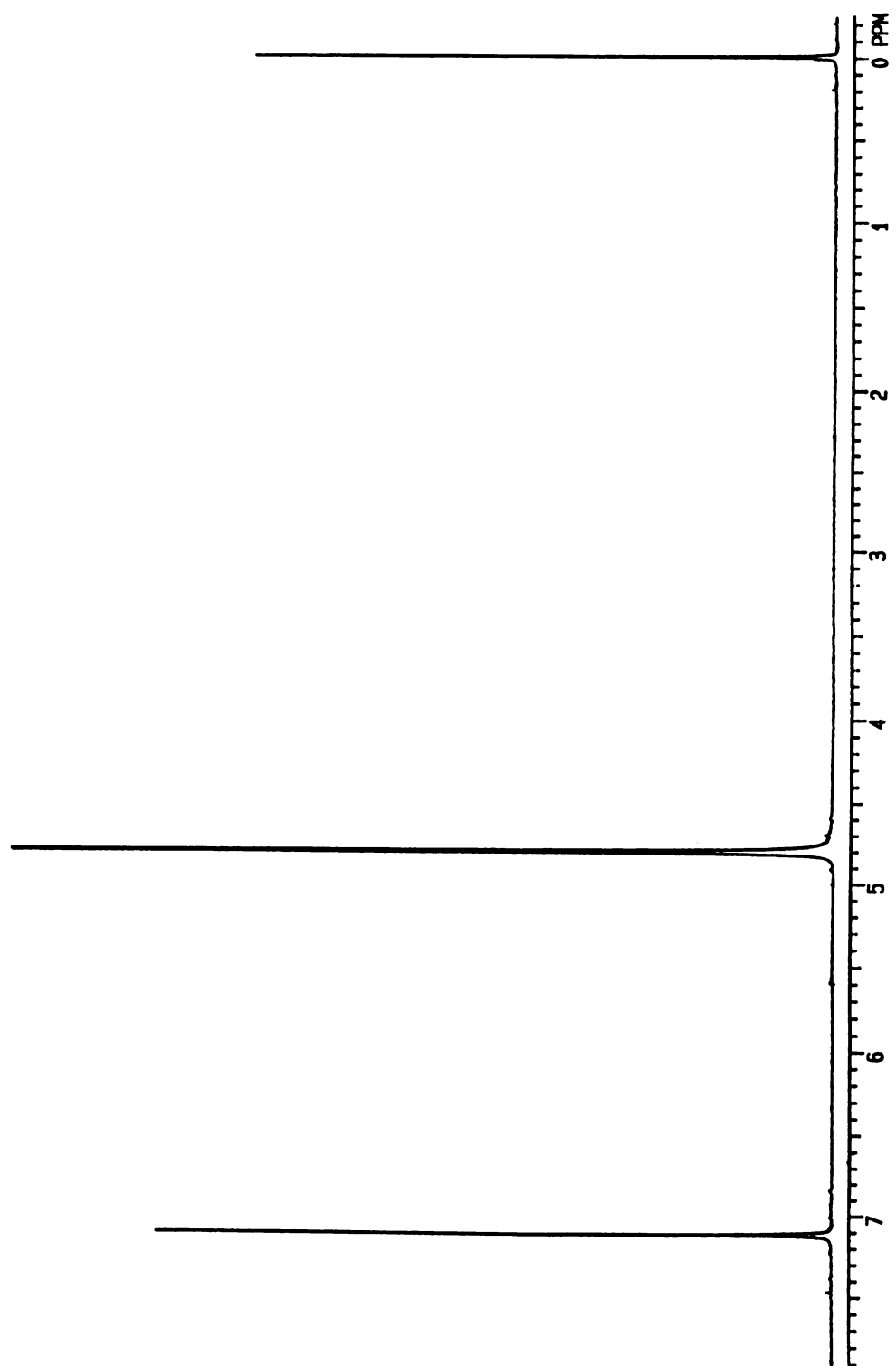


Figure 51. Control ^1H NMR of 3-dehydroquinic acid (DHQ). Resonances: δ 2.35 (d, 1H), 2.39 (d, 1H), 2.60 (dd, 1H), 3.21 (d, 1H), 3.95 (ddd, 1H), 4.36 (d, 1H).

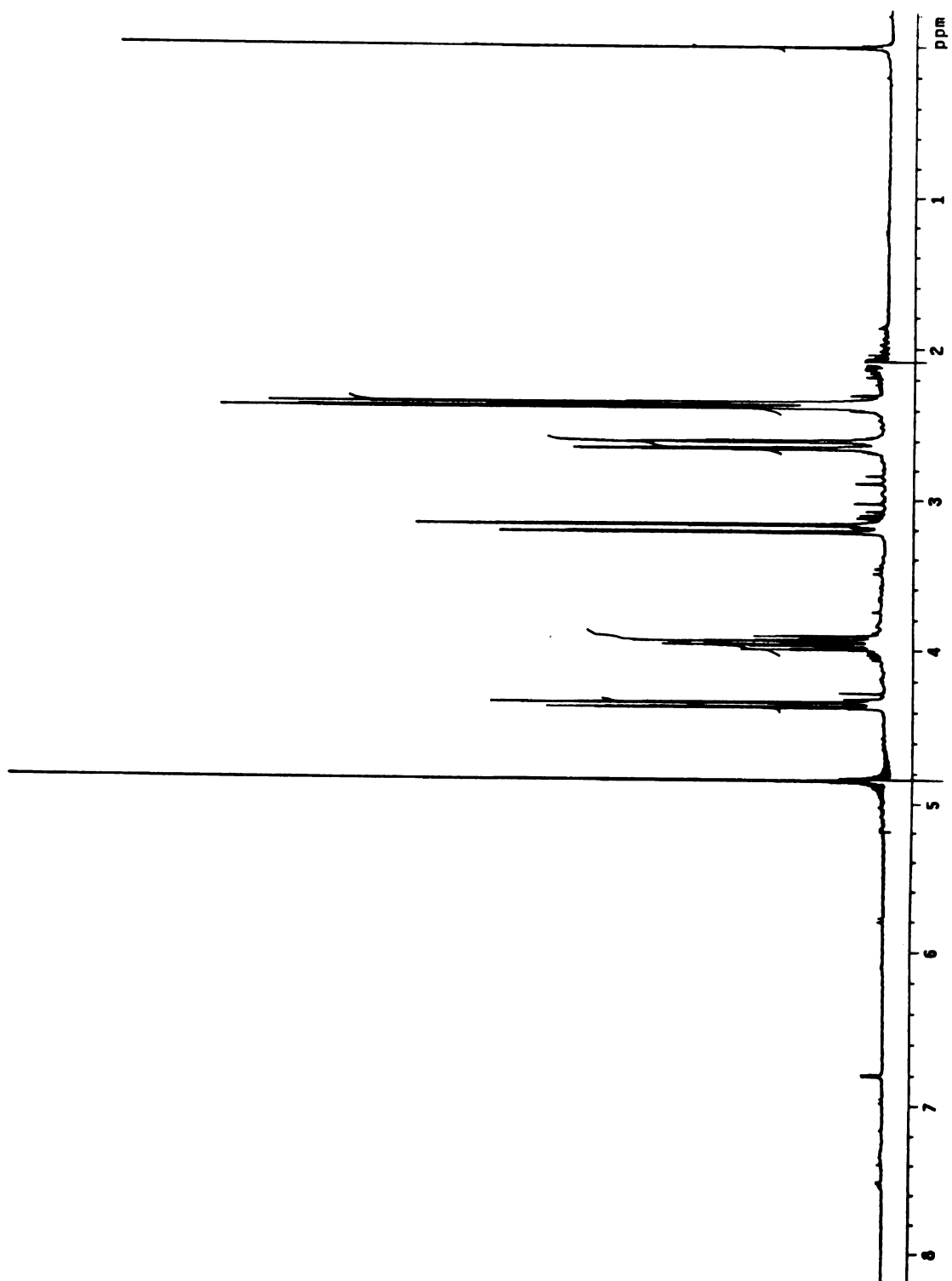
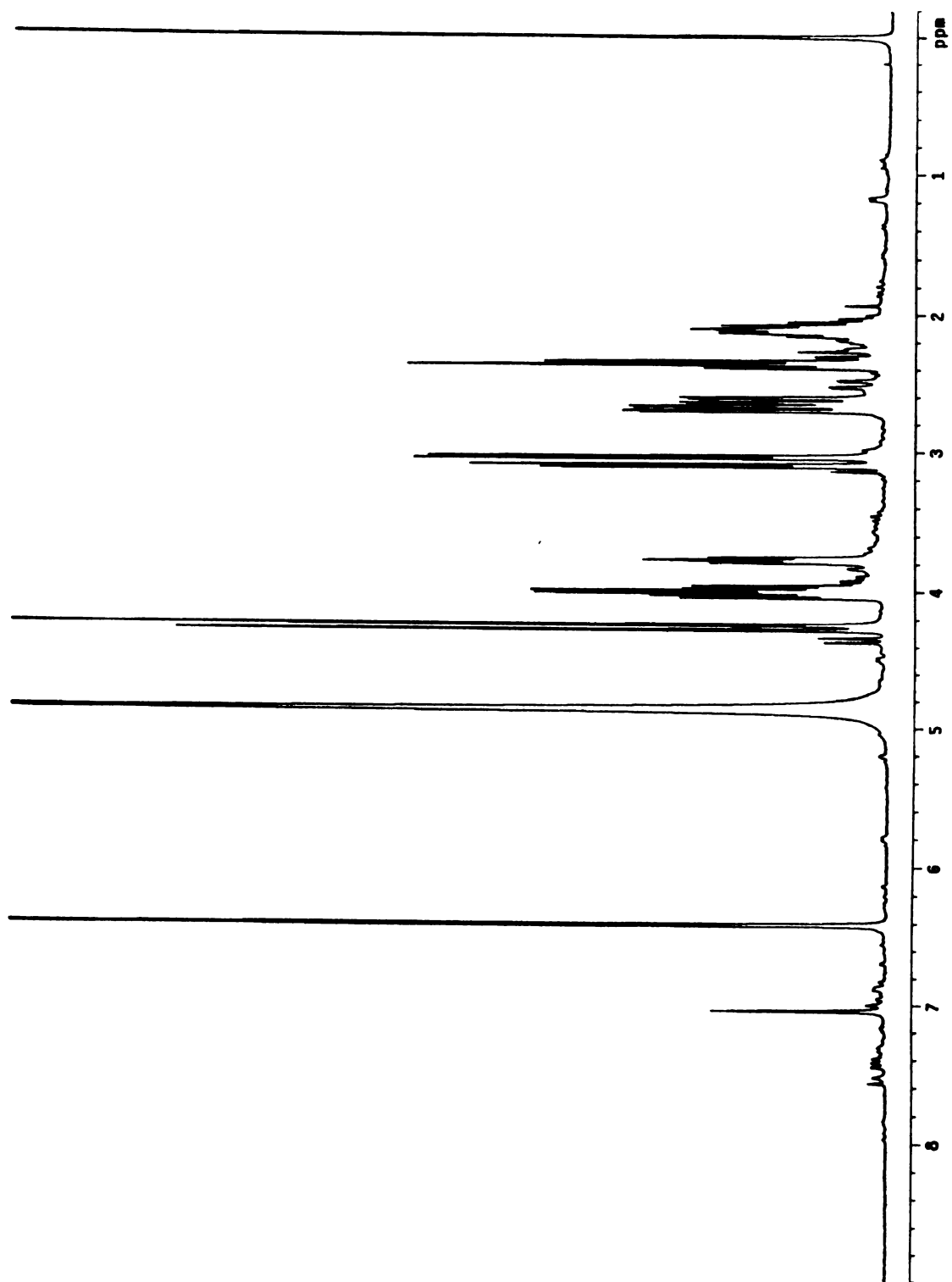


Figure 52. ^1H NMR of a typical DHS fermentation after 48 h. (This specific NMR was obtained using KL3/pKL5.17A). DHS Resonances: δ 6.42 (d, 1H), 4.28 (d, 1H), 4.00 (ddd, 1H), 3.07 (dd, 1H), 2.66 (ddd, 1H).



CHAPTER 4

CARBON SOURCE SELECTION: IS D-GLUCOSE THE BEST CARBON SOURCE FOR MICROBIAL SYNTHESIS?

Introduction

Carbon source selection is one of the most important considerations confronting microbe-catalyzed chemical synthesis. Although D-glucose derived from corn starch has historically been used for microbial synthesis of chemicals under fed-batch fermentor conditions, complete dependence on glucose may not be advisable. Microbes such as *Escherichia coli* and *Bacillus subtilis* employ a phosphoenolpyruvate (PEP)-expending phosphotransferase system (PTS) for glucose transport. When glucose serves as the sole carbon source for synthesis of chemicals via the common pathway of aromatic amino acid biosynthesis, limitations imposed on PEP availability reduce titer and yield of synthesized product.^{47d, e}

Corn fiber, an inexpensive byproduct of wet milling, constitutes 10% of the dry weight of processed corn.¹¹⁷ Fiber is separated from corn, mixed (typically) with steeping liquor, dried, and sold as gluten feed.¹¹⁸ This use of corn fiber constitutes a waste of a valuable synthetic starting material. Corn fiber is a polymer composed of D-glucose, D-xylose, and L-arabinose monomers which is end-capped with phenylpropanoids such as ferulic acid. Besides its price and availability, the corn fiber's D-xylose and L-arabinose content are particularly attractive in terms of serving as carbon sources for microbial synthesis. D-Xylose and L-arabinose are transported into *E. coli* by ATP-based permeases.¹¹⁹ Therefore, they should not suffer, in theory, from the pronounced yield limitation associated with PTS-driven glucose utilization. Compared to microbe-catalyzed synthesis of chemicals from glucose, microbe-catalyzed syntheses of chemicals using

xylose and particularly arabinose as the carbon source have received comparatively little attention.

Succinic acid is manufactured via succinic anhydride and maleic anhydride from butane whose principal source is petroleum refining.¹²⁰ Succinic acid availability benefits from the wide availability of butane and the simple process to convert butane into succinic acid. In the biological world, succinic acid is an intermediate in the tricarboxylic (TCA) cycle. The TCA cycle is the common mode of oxidative metabolism in both eukaryotes and prokaryotes. In *E. coli*, the TCA cycle is connected with the common pathway of aromatic amino acid biosynthesis through reactions catalyzed by PEP carboxylase (Ppc)^{116c} and PEP carboxykinase (Pck).¹²¹ While Ppc catalyzes an irreversible condensation of PEP with bicarbonate to form oxaloacetate, Pck catalyzes conversion of PEP into oxaloacetate in a reversible reaction.

This Chapter will explore the potential use of both corn fiber hydrolysate and a mixture of glucose and succinic acid as synthetic starting materials in the microbial synthesis of value-added chemicals. The focus is again on synthesis of DHS using *E. coli* as the microbe. In the first part of this Chapter, xylose and arabinose are evaluated as carbon sources individually and as a 3/3/2 molar mixture of glucose/xylose/arabinose. The 3/3/2 molar mixture of glucose/xylose/arabinose simulates the carbohydrate composition of corn fiber hydrolysate. In the second part, a 1/1 molar ratio of glucose/succinic acid was used as a carbon source in conjunction with overexpression of Pck. For both pentose and glucose/succinic acid mixtures, the DHS yields and/or titers are significantly increased relative to the data obtained using glucose as a sole carbon source.

Fermentation Conditions

Fed-batch cultures were performed in a 2.0 L capacity Biostat MD B-Braun fermentor connected to a DCU system and a Dell Optiplex Gs⁺ 5166M personal computer equipped with B-Braun MFCS/win software for data acquisition and automatic process monitoring. The temperature, pH and substrate feeding were controlled with a PID controller. The temperature was maintained at 36 °C and pH 7.0 was maintained by addition of concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen was measured using a Braun polarographic probe. Antifoam (Sigma 204) was added manually as needed.

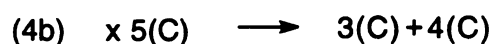
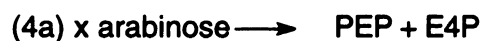
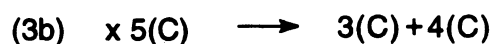
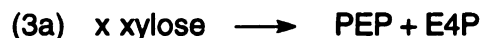
The major differences in the fermentation conditions used in the study of this Chapter relative to those of Chapter 3 are inoculum and the preset maximum airflow rate. As in the research summarized in Chapter 3, a minimal medium containing inorganic salts, aromatic amino acids, aromatic vitamins, and a quantity of carbon source was used for the fermentation medium. Previously, rich medium (LB medium) supplemented with antibiotics was used as the inoculum. In this Chapter, minimal medium without antibiotic supplementation was used as the inoculum. Inoculants were typically started by introduction of a single colony into 5 mL of M9 medium supplemented with L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid. The culture was grown at 37 °C with agitation at 250 rpm for 18 h and subsequently transferred to 100 mL of M9 medium. After growth at 37 °C and 250 rpm for an additional 12 h, the inoculant was ready for transferring into the fermentation vessel. The fermentation process was similar as the one reported in Chapter 3. However, the maximum air flow was decreased from 3.0 L/L/min to 1.0 L/L/min.

A Comparative Analysis of D-Xylose, L-Arabinose, and D-Glucose Carbon
Sources for Microbial Synthesis of DHS

A. Background

D-Glucose is transported into the *E. coli* cytoplasm by the phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS), while xylose and arabinose are transported by ATP-based permease systems. These different transport mechanisms are reflected in the different theoretical maximum yield for biocatalytic synthesis of DHS from glucose versus xylose or arabinose.

Determining the theoretical maximum yield begins with balancing the PEP and E4P inputs with DHS product and byproducts (Eq. (1)). The PEP and E4P are then equated to the amount of D-glucose or D-xylose or L-arabinose which is required to form these substrates (Eq. (2a), (3a), and (4a)). A pyruvate acid term is included in Eq. (2a) to reflect the conversion of one molecule of glucose transported into the cytoplasm as glucose 6-phosphate. A coefficient is determined (Eq. (2b)) to balance the number of carbon atoms in the glucose starting material with the total number of carbon atoms formed in PEP, E4P and pyruvic acid. The determined coefficient leads to a maximum theoretical yield of 43% (mol of DHS/mol of glucose). Similar calculations (Eq. (3b) and Eq (4b)) result in a maximum theoretical yield of 71% (mol of DHS/mol of xylose or arabinose) for both xylose and arabinose. In the case of glucose, PTS-generated pyruvic acid is considered to be the carbon source for the anabolism and catabolism required for generation of *E. coli* cellular biomass.³³ However, when xylose or arabinose was used as the sole carbon source, some of the carbon sources must be diverted to biomass production. As a result, the calculated yield of 71% (mol/mol) must be considered to be the upper limit for the theoretical maximum yield for both xylose and arabinose.



As previously mentioned, corn fiber represents a cheap, renewable resource that is abundantly available from the corn wet milling industry.¹²² A number of pretreatment processes have been developed in order to utilize the corn fiber as a low-cost feedstock for production of fuel ethanol. All processes yield a mixture of glucose, xylose and arabinose, although the ratio of the components varies depending on the treatment condition. For example, when the corn fiber was treated with dilute sulfuric acid at pH 1.0 and 140 °C for 20 min, the hydrolysate obtained had a glucose, xylose and arabinose molar ratio of 3.3 : 3.6 : 2;¹²³ When corn fiber was pretreated with dilute sulfuric acid at pH 2.0 and 160 °C for 20 min, neutralized and then hydrolyzed with an enzyme mixture, the resulting sugar mixture had a glucose, xylose and arabinose molar ratio of 5.1 : 3.5 : 2.¹²³ Saccharification of alkaline hydrogen peroxide (AHP) pretreated corn fiber using crude enzyme from *Aureobasidium sp.* NRRL Y-2311-1 afforded a mixture of glucose, xylose and arabinose at a molar ratio of 6.6 : 2.2 : 2.¹²⁴ It has also been reported that no difference is observed between using corn fiber hydrolysate and mixtures of pure carbohydrates that simulate the carbohydrate composition of hydrolysate for production of ethanol through fermentation.¹²² In this section, the performance of individual carbohydrates including glucose, xylose and arabinose will be compared in terms of

synthesized product titers and yields. The utilization of a 3 : 3 : 2 molar ratio of glucose/xylose/arabinose to simulate the composition of the carbohydrate mixture resulting from mild acid hydrolysate of corn fiber¹²⁵ will also be reported.

B. Biocatalyst

KL3/pKL4.33B was the first biocatalyst tested using xylose as a sole carbon source. However, this biocatalyst grew slowly on xylose. The starter inoculum (5 mL) had to be grown for 24 h at 37 °C and subsequently transferred into 100 mL of culture for an additional 24 h growth at 37 °C in order to reach a cell density appropriate for inoculating a fermentation run. The fermentation also had a long initial lag phase and did not reach the third stage and stationary phase until after 31 h and 48 h, respectively. By contrast, KL3/pKL4.33B cultured on glucose required only 18 h and 12 h, respectively, for growth of the 5 mL and 100 mL starter cultures. KL3/pKL4.33B cultured on glucose under fed-batch fermentor conditions reached the third stage and stationary phase at 13 h and 24 h, respectively. Therefore, KL3/pKL4.33B was not used for this study due to the significant growth rate difference when xylose versus glucose was employed as a carbon source.

DAHP synthase expression levels are known to affect *E. coli* growth rates. A slower growth rate is typically observed with higher DAHP synthase overexpression levels. Plasmid pKL4.79B has a $P_{tac}aroF^{FBR}/lacIQ$ system which allows DAHP synthase overexpression to be controlled by IPTG addition. Without IPTG addition, DAHP synthase (AroF) expression is minimal due to the repression of transcription by the Lac repressor. KL3/pKL4.79B was thus expected to show faster growth without IPTG addition relative to KL3/pKL4.33B in which AroF is expressed through its native promoter. For KL3/pKL4.79B cultured on xylose or glucose in the absence of IPTG, only 18 h and 12 h, respectively, were required for the 5 mL and 100 mL starter cultures. IPTG was not added until the beginning of the third stage of the fermentation. The xylose

fermentation reached the third stage and stationary phase at about 14 h and 30 h, respectively. For comparison, the third stage and stationary phase were reached at approximately 13 h and 24 h, respectively, when glucose was the carbon source. Therefore, KL3/pKL4.79B was used as the model biocatalyst for the research summarized in this section. In all fermentations, 4.8 mg IPTG were added at the beginning of stage 3, at 18 h, and after every subsequent 6 h time interval until completion of the fermentor run.

C. Glucose Fermentation Versus Xylose or Arabinose Fermentation.

As a control, KL3/pKL4.79B was first examined under the new fed-batch fermentor conditions using glucose as a sole carbon source (Figure 53a). The fermentation was again divided into three stages according to the three different methods used to control dissolved oxygen (D.O.) levels at 20% air saturation. After inoculation, D.O. levels were maintained by increasing the impeller stirring rate until a preset maximum value was reached. Approximately 11 hours was required before the impeller reach its maximum stirring rate (940 rpm). The mass flow controller then maintained D.O. levels at 20% air saturation at the constant impeller stirring rate (940 rpm) by increasing the airflow until a preset maximum value (1.0 L/L/min) was reached. Approximately 2.5 h was needed for the airflow to increase to its maximum rate. At a constant impeller stirring rate (940 rpm) and constant airflow rate (1.0 L/L/min), D.O. levels were then maintained at 20% saturation by oxygen sensor-controlled glucose feeding for the rest of the fermentation. The initial glucose concentration in the fermentation culture medium was 18 g/L. The concentration of the glucose solution added under D.O. control was 60% (w/v).

When KL3/pKL4.79B was cultivated under the same conditions using xylose or arabinose as a sole carbon source (Figure 53b, c), fermentations behaved the same during the first two stages as when glucose was used as the carbon source. The initial xylose and arabinose concentrations in the fermentation culture medium was 23 g/L. Approximately 14 h was needed for completion of the first two stages of D.O. control. However, after the

residual xylose or arabinose was completely consumed at the beginning of the third stage of the fermentation, the D.O. increased to 40% and could not subsequently be reduced back to 20% even with aggressive addition of pentose. Addition of pentose was consequently set to a constant rate (10 mL/h) in order to feed xylose (65% w/v) or arabinose (61% w/v) in excess of what was consumed in the fermentation to avoid cell starvation. As a result, the xylose or arabinose slowly accumulated in the fermentation medium. The accumulation continued until 36 h when the pump controlling pentose addition was shut off. Microbial metabolism was then maintained on the excess of xylose or arabinose present in the culture medium until the end of the fermentor run at 48 h (Figure 53 b, c). During the entire third stage, the D.O. level slowly increased from about 40% up to about 60% air saturation.

All fermentor runs entered logarithmic growth 6 h after inoculation (Figure 53). Glucose fermentation reached stationary phase at approximately 24 h while xylose and arabinose fermentation reached stationary phase at about 30 h. Glucose fermentation also afforded a slightly higher cell mass than xylose and arabinose fermentation (Figure 53). Despite the accumulation of sugars in the xylose and arabinose fermentation, no acetic acid was observed during most of the logarithmic phase and the entire stationary phase. No acetic acid was observed during the same period of time in the glucose fermentation.

Glucose fermentation of KL3/pKL4.79B afforded 36.4 g/L of DHS in a yield of 22% (Table 12). DHQ (3.6 g/L) and gallic acid (2.6 g/L) were also formed. Use of xylose as a carbon source for culturing KL3/pKL4.79B afforded 41.7 g/L of DHS in a 32% yield while use of arabinose as a carbon source afforded 41.1 g/L of DHS synthesized in a 35% yield (Table 12). Along with DHQ and gallic acid, use of both xylose and arabinose as a carbon source produced significant amounts of DAH (9.7 g/L and 9.5 g/L for xylose and arabinose fermentation, respectively) (Table 12). This is different from glucose fermentation, where no detectable DAH was formed throughout the fermentation run.

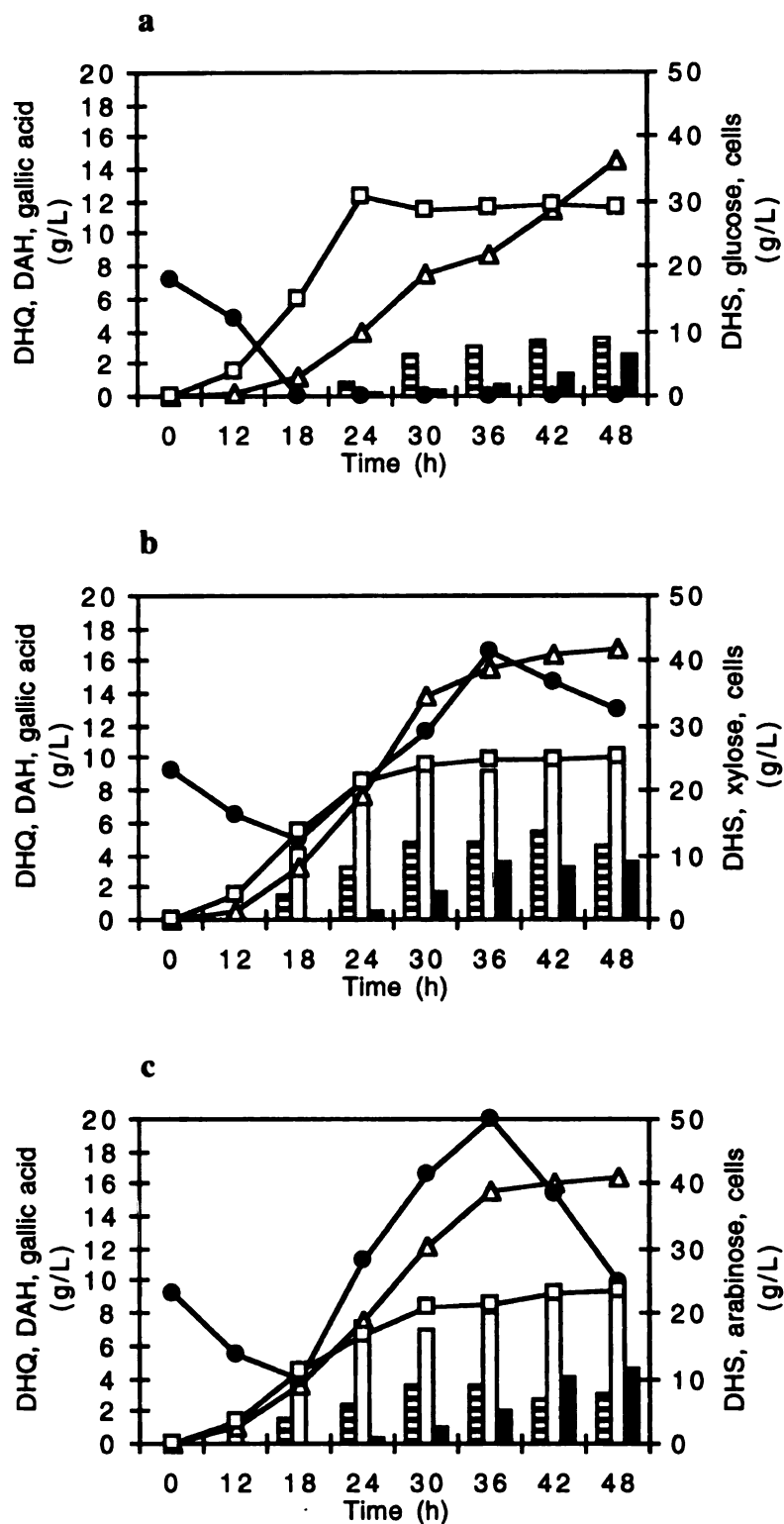


Figure 53. Synthesis of DHS by KL3/pKL4.79B. (a) D-glucose (b) D-xylose (c) L-arabinose: —□— cell dry wt; —△— DHS; —●— glucose or xylose or arabinose; ▨ DHQ; ▩ DAH; — gallic acid;

Along with the increased DHS, DHQ, and gallic acid production, the DAH accumulation results in a higher total yield of hydroaromatics and aromatics synthesized from xylose and arabinose relative to use of glucose as the carbon source (Table 12). A total yield of 44% (mol/mol) for xylose and 47% (mol/mol) for arabinose was achieved relative to 26% (mol/mol) for use of glucose as the carbon source.

Table 12. KL3/pKL4.79B synthesis of DHS using different carbon sources.

products	glucose	xylose	arabinose	mix ^c
DHS ^a	36.4	41.7	41.1	53.0
DHS yield ^b	22%	32%	35%	36%
DAH ^a	0	9.7	9.5	7.3
DHQ ^a	3.6	4.8	3.0	7.8
GA ^a	2.6	4.0	4.7	2.8
total yield ^b	26%	44%	47%	45%
theoretical yield ^b	43%	71%	71%	60%

^a g/L; ^b mol/mol; ^c 3/3/2 molar ratio of glucose/xylose/arabinose.

D. Glucose, Xylose and Arabinose as a Mixed Carbon Source

Culturing *E. coli* KL3/pKL4.79B under fed-batch fermentor conditions at 36 °C and pH 7.0 was also examined when a 3/3/2 molar mixture of glucose/xylose/arabinose was used as the carbon source. With the carbohydrate mixture as the initial substrate in the first two stages of the fermentation, the cells exhibits diauxic growth (Figure 54). Glucose is the first carbohydrate consumed due to the inhibition of both arabinose and xylose transport by catabolite repression. Arabinose consumption did not begin until the cells

consumed all of the available glucose. Unexpectedly, xylose utilization did not start until arabinose was exhausted, which suggests that arabinose may repress xylose uptake.

At 10 h, when the cells exhausted glucose, the fermentation typically was at the beginning of second stage with the air flow at approximately 0.15 L/L/min. Because the cells must divert their energies from growth to “retool” for the new carbon supply, an air flow rate of 0.15 L/L/min at the maximum stir rate (940 rpm) was adequate for maintenance of the D.O. level at 20% air saturation. At the transition between glucose and arabinose consumption, the airflow rate decreased sharply to its minimum value (0.06 L/L/min) followed by a decrease in the stirring rate to around 850 rpm. Upon initiation of arabinose consumption, the D.O. level was again maintained at 20% air saturation first by increasing the impeller rate and then by increasing the airflow rate. About 1 h was needed before all the arabinose was consumed. A sudden decrease in airflow rate followed by a drop in the stirring rate to about 800 rpm was again observed at the transition between arabinose and xylose consumption. The beginning of xylose consumption corresponded with an increase of the stirring rate followed by an increase in the air flow rate. About 3 h was required for the cells to consume all the remaining xylose when the fermentation was in its second stage with the air flow rate at about 0.3 L/L/min. Approximately 2 mL of the carbohydrate mixture was added to the fermentation to drive the airflow rate up to its preset maximum instantly due to the presence of the glucose. Only several minutes was required to consume all of the added carbohydrate mixture.

D.O. sensor-controlled addition of the carbohydrate mixture began immediately after the aliquot of the carbohydrate mixture was consumed. The D.O. level was maintained at 20% air saturation throughout the fermentation. The initial carbon source concentrations in the fermentation culture medium were 7.6/6.3/4.1 g/L for glucose/xylose/arabinose mixture. The concentrations (w/v) of the individual carbohydrates in the carbohydrate mixture were 26.3%, 21.9%, and 14.4% for glucose, xylose, and arabinose, respectively.

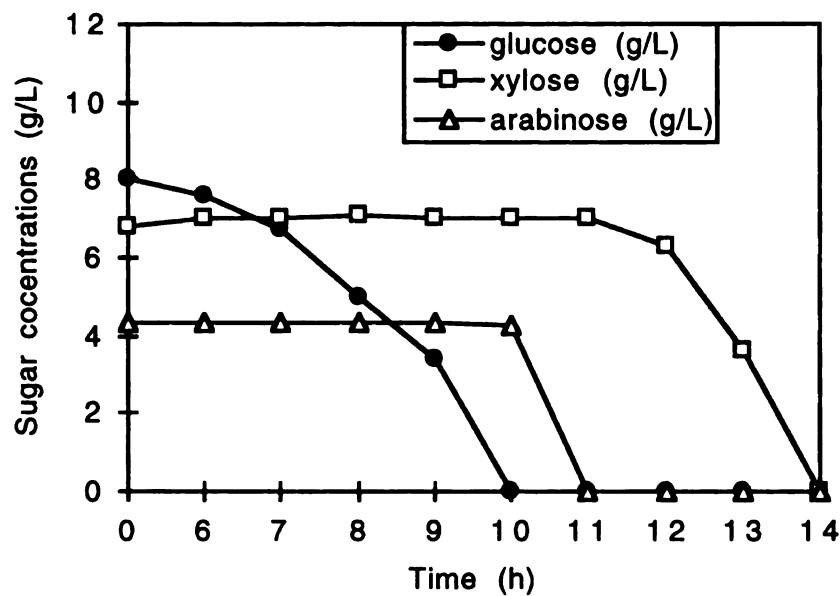


Figure 54. KL3/pKL4.79B cultivation prior to initiation of oxygen sensor-controlled addition of the glucose : xylose : arabinose mixture.

The steady-state concentration of glucose was maintained below 0.5 mM (not detectable by 300 MHz NMR) while D.O. levels were used to control addition of the carbohydrate mixture. This glucose concentration was sufficiently low that catabolite repression of xylose and arabinose utilization was avoided.¹²⁶ As a result, no accumulation of pentoses was observed until 42 h when about 6 g of arabinose accumulated in the culture (Figure 55). Fermentations were typically stopped at 42 h because running beyond 42 h resulted in both xylose and arabinose accumulation (Figure 55).

Cultivation of KL3/pKL4.79B on the mixed carbohydrates as a carbon source was similar to cultivation on glucose in terms of time required to reach the stationary phase and the final cell mass which was produced (Figure 52a, Figure 55a). At no time during cultivation on the glucose/xylose/arabinose mixture was acetic acid accumulation detected. The concentration of DHS synthesized by KL3/pKL4.79B when cultivated on the mixed carbohydrate as a carbon source was substantially in excess of the concentration of DHS

synthesized when any of the individual carbohydrates were employed as the carbon source. A DHS titer of 53 g/L was synthesized in 36% yield (mol/mol). Taking into consideration (Table 12) the concentrations of DHQ (7.8 g/L), DAH (7.3 g/L) and gallic acid (2.8 g/L) synthesized, the total yield for the synthesis of hydroaromatics and aromatics was 45% (mol/mol).

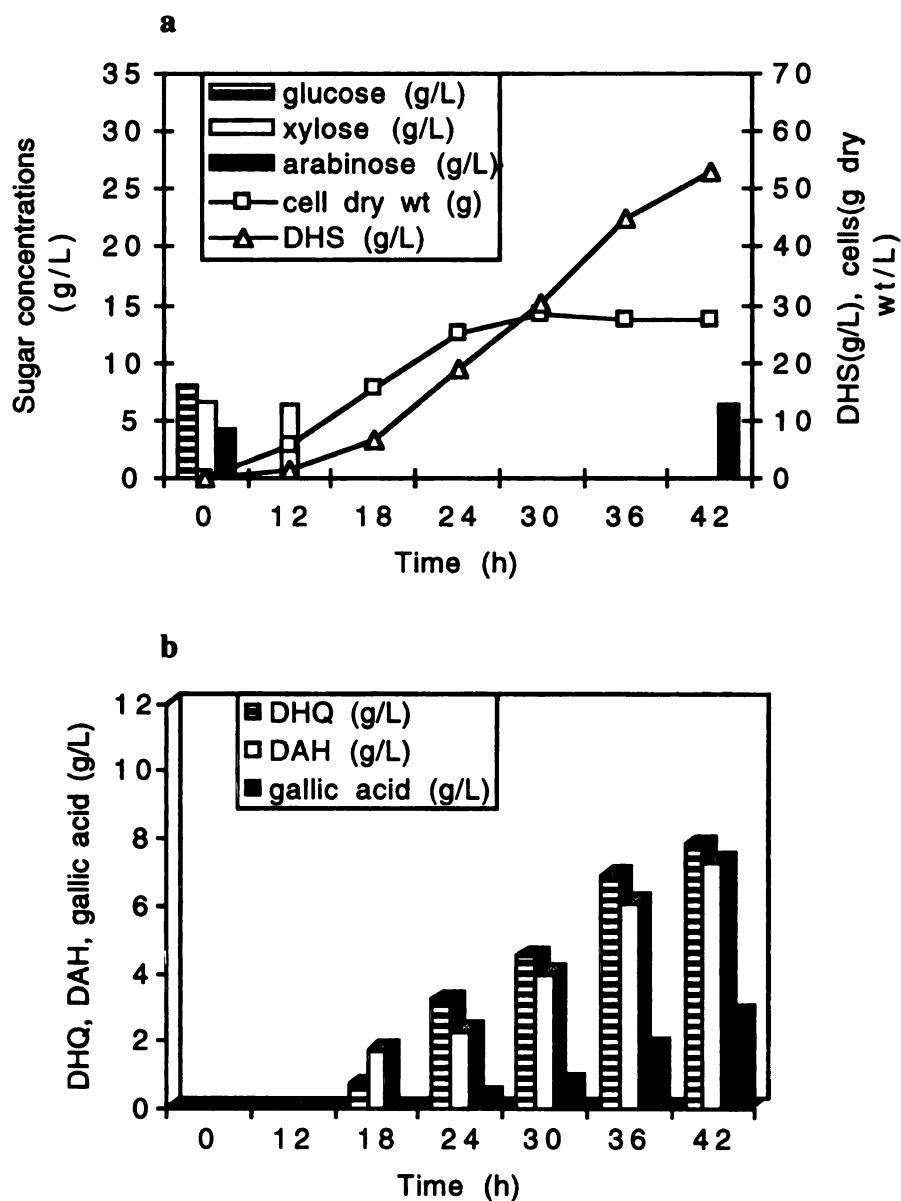


Figure 55. Cultivation of KL3/pKL4.79B under fed-batch fermentor conditions using a mixture of glucose, xylose, and arabinose at a molar ratio of 3 : 3 : 2. (a) DHS production, cell growth and carbon source concentrations (b) DHQ, DAH, and gallic acid concentrations.

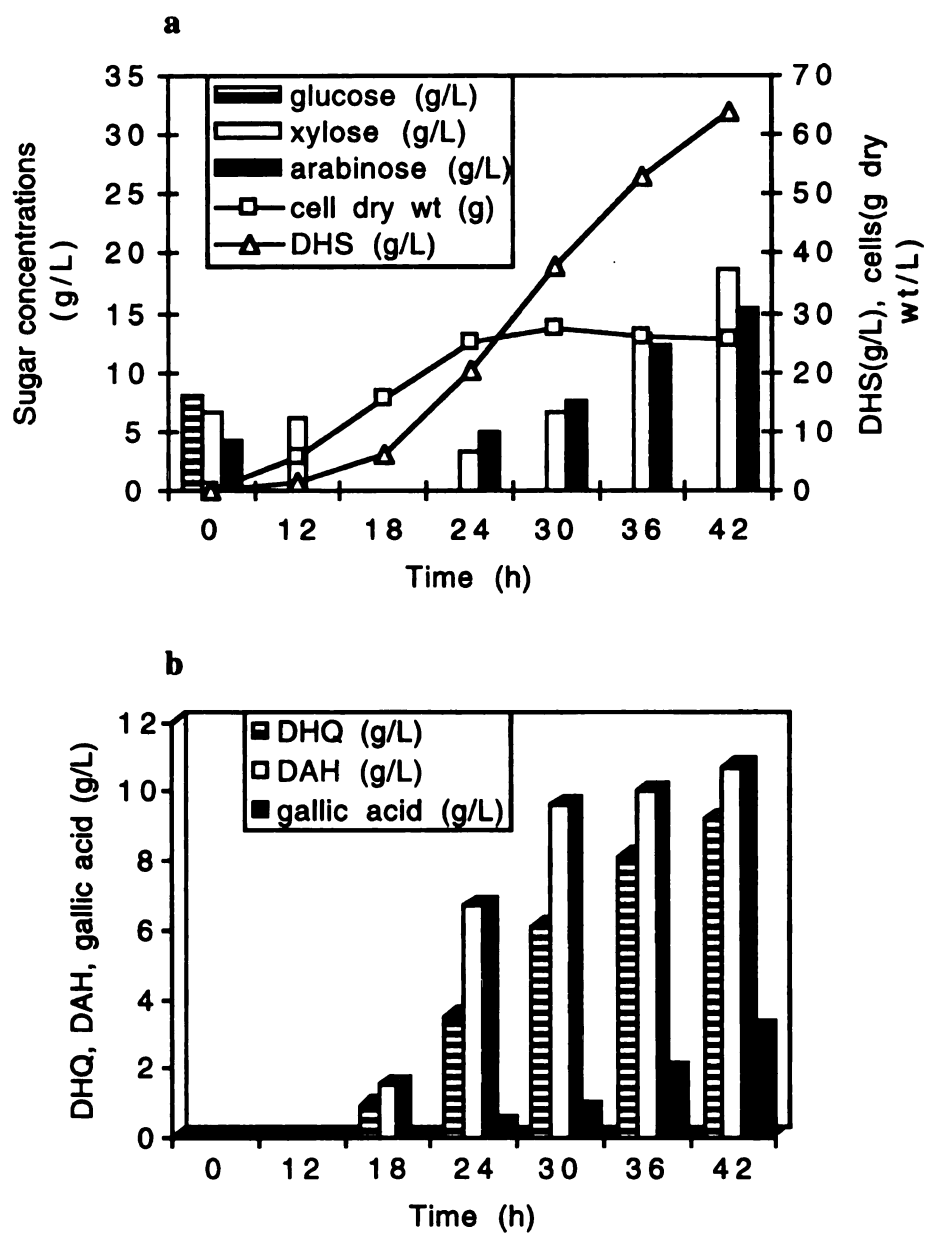


Figure 56. Cultivation of KL3/pKL4.124A under fed-batch fermentor conditions using a mixture of glucose, xylose, and arabinose at a molar ratio of 3 : 3 : 2. (a) DHS production , cell growth and carbon source concentrations (b) DHQ, DAH, and gallic acid concentration ns.

E. The Impact of Transketolase

KL3/pKL4.124A, which was obtained by cloning *tktA* gene into pKL4.79B (Figure 45), was also examined using individual carbohydrate as well as the carbohydrate mixture as carbon sources in order to compare the effect of transketolase overexpression. Arabinose was not tested as a carbon source given the similar results when KL3/pKL4.79B was cultured on arabinose or xylose. Despite the report that overexpression of transketolase only modestly increased the carbon flow directed into the common pathway of aromatic amino acid biosynthesis when glucose was employed as the carbon source under shake-flask conditions,^{45d, e} Chapter 3 clearly demonstrated that transketolase can significantly improve the flow of carbon directed into the common pathway under fed-batch fermentor conditions when glucose was employed as the carbon source.

KL3/pKL4.124A was examined under the conditions described above. IPTG (4.8 mg) was added at the beginning of stage 3, at 18 h, and after each subsequent 6 h time interval to realize optimal DAHP synthase expression levels. Results are listed in Table 13. Using glucose as a carbon source, KL3/pKL4.124A produced DHS at a titer of 46.0 g/L with a yield of 28% (mol/mol), which sharply contrasts with a titer of 36.4 g/L and a yield of 22% (mol/mol) that was observed when KL3/pKL4.79B was cultured under the same culture conditions (Table 12, 13). Although both DHQ and gallic acid production increased, DAH was not detected in the fermentation supernatant when glucose was used as the carbon source. Fed-batch fermentor cultivation using xylose as the carbon source did not show a significant change upon overexpression of transketolase. KL3/pKL4.124A produced DHS, DHQ, and gallic acid at similar levels relative to KL3/pKL4.79B (Table 12, 13). The production of DAH was slightly increased which led to a small increase in total yield. The yield of DHS synthesis remained unchanged. For the fermentation utilizing a 3 : 3 : 2 molar ratio of glucose/xylose/arabinose, overexpression of transketolase showed a significant impact on both titer and yield. A DHS titer of 64.0 g/L was synthesized by KL3/pKL4.124A after 42 h of cultivation. The yield of DHS was 41%

(mol/mol) relative to 36% (mol/mol) in KL3/pKL4.79B. Combination of the DHQ (9.2 g/L), DAH (10.7 g/L), gallic acid (3.1 g/L) and DHS synthesis results in a total yield of synthesized products of 55% (mol/mol) (Table 13).

Table 13. KL3/pKL4.124A synthesis of DHS with different carbon source.

products	glucose	xylose	mix ^c
DHS ^a	46.0	43.1	64.0
DHS yield ^b	28%	33%	41%
DAH ^a	0	12.8	10.7
DHQ ^a	4.5	4.8	9.2
GA ^a	3.8	3.8	3.1
total yield ^b	33%	47%	55%
theoretical yield ^b	43%	71%	58%

^a g/L; ^b mol/mol; ^c 3/3/2 molar ratio of glucose/xylose/arabinose.

When glucose and xylose were used as carbon sources, KL3/pKL4.124A showed the same sugar accumulation of carbohydrates as did KL3/pKL4.79B. KL3/pKL4.124A did not accumulate any glucose when glucose was the sole carbon source. However, when cultivated in xylose, KL3/pKL4.124A accumulated xylose up to approximately 40 g/L at 36 h. When the carbohydrate mixture was used as a carbon source, a noteworthy difference between KL3/pKL4.124A and KL3/pKL4.79B was the accumulation of both xylose and arabinose by transketolase-overexpressing KL3/pKL4.124A beginning at 24 h (Figure 56) while the fermentation was under D.O. sensor control. By 42 h, 18.6 g/L of xylose and 15.4 g/L of arabinose had accumulated in the fermentation medium. Table 14 compares the total amount of individual carbohydrates consumed by KL3/pKL4.79B and KL3/pKL4.124A when the carbohydrate mixture was the source of carbon.

KL3/pKL4.79B and KL3/pKL4.124A consumed approximately the same amount of xylose and arabinose. On the other hand, overexpression of transketolase in KL3/pKL4.124A significantly increased the amount of glucose consumed. Transketolase overexpression apparently increased the glucose utilization rate but did not increase the utilization rate of xylose and arabinose.

Table 14. Consumption of the individual components of the mixed carbon source.

	KL3/pKL4.79B	KL3/pKL4.124A
glucose consumed	82.5 g	108.0 g
xylose consumed	64.9 g	63.6 g
arabinose consumed	37.0 g	37.2 g
molar ratio ^a	3.7 : 3.5 : 2	4.8 : 3.4 : 2

^a glucose/xylose/arabinose

F. Discussion

In considering the performance of DHS-producers under different conditions, synthesis of DAH, DHQ and gallic acid must also be taken into consideration. Both DAH and DHQ are metabolic precursors to DHS and gallic acid is a metabolite derived from DHS. The theoretical maximum yield of DHS, DAH, DHQ and gallic acid is 43% (mol/mol) for glucose and 71% (mol/mol) for xylose. KL3/pKL4.79B directed a total of 63% and 62% of the theoretical maximum amount of carbon which can be channeled into aromatic amino acid biosynthesis when glucose and xylose, respectively, were used as carbon sources. For KL3/pKL4.124A, the total yield corresponds to 76% and 66% of the

theoretical maximum yield when glucose and xylose, respectively, were used as carbon sources.

Determining the theoretical maximum yield for biocatalytic synthesis of DHS from the carbohydrate mixture takes into account the consumption ratio of each individual sugar. For KL3/pKL4.79B, the glucose, xylose and arabinose was consumed at a ratio of 3.7 : 3.5 : 2. Calculation according to Eq. (5a) leads to a theoretical maximum yield of 60% (mol/mol). For KL3/pKL4.124A, since the actual sugar consumption is 4.8 : 3.4 : 2 for glucose, xylose and arabinose, respectively, a similar calculation results in a theoretical yield of 58% (mol/mol) (Eq. (5b)). Thus, KL3/pKL4.79B channeled 75% of the theoretical maximum amount of carbon which can be directed into aromatic amino acid biosynthesis. With the help of transketolase, KL3/pKL4.124A channeled 94% of the theoretical maximum amount of carbon which can be directed into aromatic amino acid biosynthesis.

$$(5a) \quad \frac{3.7}{3.7 + 3.5 + 2} \times 43\% + \frac{3.5}{3.7 + 3.5 + 2} \times 71\% + \frac{2}{3.7 + 3.5 + 2} \times 71\% = 60\%$$

$$(5b) \quad \frac{4.8}{4.8 + 3.4 + 2} \times 43\% + \frac{3.4}{4.8 + 3.4 + 2} \times 71\% + \frac{2}{4.8 + 3.4 + 2} \times 71\% = 58\%$$

KL3/pKL4.124A, cultivated on glucose, consumed approximately 240 g of glucose which generated 1.3 mol of pyruvic acid for biomass synthesis. When the carbohydrate mixture was the carbon source, KL3/pKL4.124A consumed only 108 g of glucose (Table 14) which translates into 0.6 mol of pyruvic acid. Since both fermentations produced roughly the same amount of cell mass, the efficiency for conversion of pyruvic acid to biomass when using the carbohydrate mixture as the carbon source is more than two times higher than when glucose was the sole carbon source.

In the Chapter 3, it was demonstrated that introduction of a second *aroB* copy into the genome of *E. coli* constructs expressing elevated levels of DAHP synthase activity eliminated accumulation of DAH in the culture supernatant due to the attendant increase in DHQ synthase activity. However, use of xylose, arabinose, or the glucose/xylose/arabinose mixture as a carbon source for KL3/pKL4.79B or KL3/pKL4.124A cultivation results in DAH becoming a major byproduct. This accumulation of DAH is an indication of the basic success in exploiting pentoses to alleviate the competition between DAHP synthase and PTS-mediated glucose transport for in vivo PEP supplies. Since xylose and arabinose do not require PEP expenditure for their transport into the cytoplasm, aromatic amino acid biosynthesis is not limited by the availability of PEP. An increase in the rate of aromatic amino acid biosynthesis is reflected in the accumulation of DAH. Further amplification in the expression of *aroB* could reduce or even eliminate DAH accumulation.

KL3/pKL4.124A afforded the highest DHS titer and yield when cultivated on a mixture of glucose, xylose and arabinose. Nevertheless, the accumulation of xylose and arabinose in the medium represents a waste of material and may also affect the purification of the target molecule. One strategy to explore for eliminating pentose accumulation might entail changing the ratio of glucose/xylose/arabinose added to the fermentor medium to reflect the 4.8/3.4/2 molar ratio of glucose/xylose/arabinose consumed by KL3/pKL4.124A during DHS synthesis. Depending on the different method employed in the pretreatment of corn fiber, the ratio of these three carbohydrates in the corn fiber hydrolysate varies.^{122,123,124} A number of pretreatment methods can yield mixtures with higher glucose contents. For example, the enzymatic hydrolysate of ammonia fiber explosion (AFEX) pretreated corn fiber gives a mixture of glucose, xylose and arabinose at a molar ratio of 12.6 : 1.6 : 2.¹²² Whatever the sugar ratio is in the corn fiber hydrolysate, the pure sugar can always be added to the corn fiber hydrolysate to make the ratio more suitable for the fermentation. An alternative strategy to avoid the accumulation of xylose

and arabinose is to shut the feeding pump off at 42 h. The fermentation will end upon exhaustion of the xylose and arabinose in the fermentation culture medium.

Utilizing Succinic Acid as a Glucose Adjunct in Fed-Batch Fermentation:

Is Butane a Feedstock Option in Microbial-Catalyzed Synthesis?

A. Introduction

PEP limitation is an inherent problem associated with the synthesis of industrial chemicals from glucose using recombinant *E. coli*. A severe price is exacted in terms of reduced titers of synthesized products because of PTS-mediated glucose transport. As reported in the pervious section, PEP limitation can be resolved by switching the carbon source from D-glucose to D-xylose and L-arabinose, which are transported into the *E. coli* cytoplasm by ATP-driven permeases. Alternatively, a strategy can be explored to allow *E. coli* to continue to use PEP-expending, PTS-mediated glucose transport while supplementing the glucose-containing medium with metabolic precursors to PEP. The primary focus of the supplementation strategy explored in this section is the TCA cycle intermediate succinic acid. Succinic acid is readily available. It is manufactured via hydrogenation of maleic anhydride and hydrolysis of the resulting succinic anhydride (Figure 57). Maleic anhydride is produced by reaction of butane (derived from liquefied petroleum gas) with oxygen using a vanadium phosphorus oxide catalyst. Succinic acid can also be biosynthetically derived from renewable carbon sources such as glucose.¹²⁷ DHS synthesis was again used as the model system for this study.

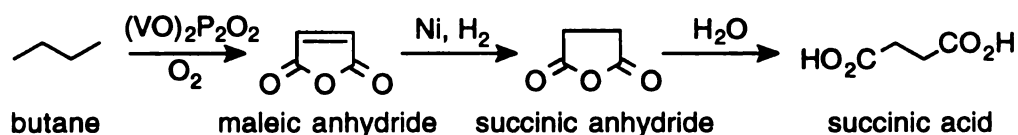


Figure 57. Manufacture of succinic acid from butane.

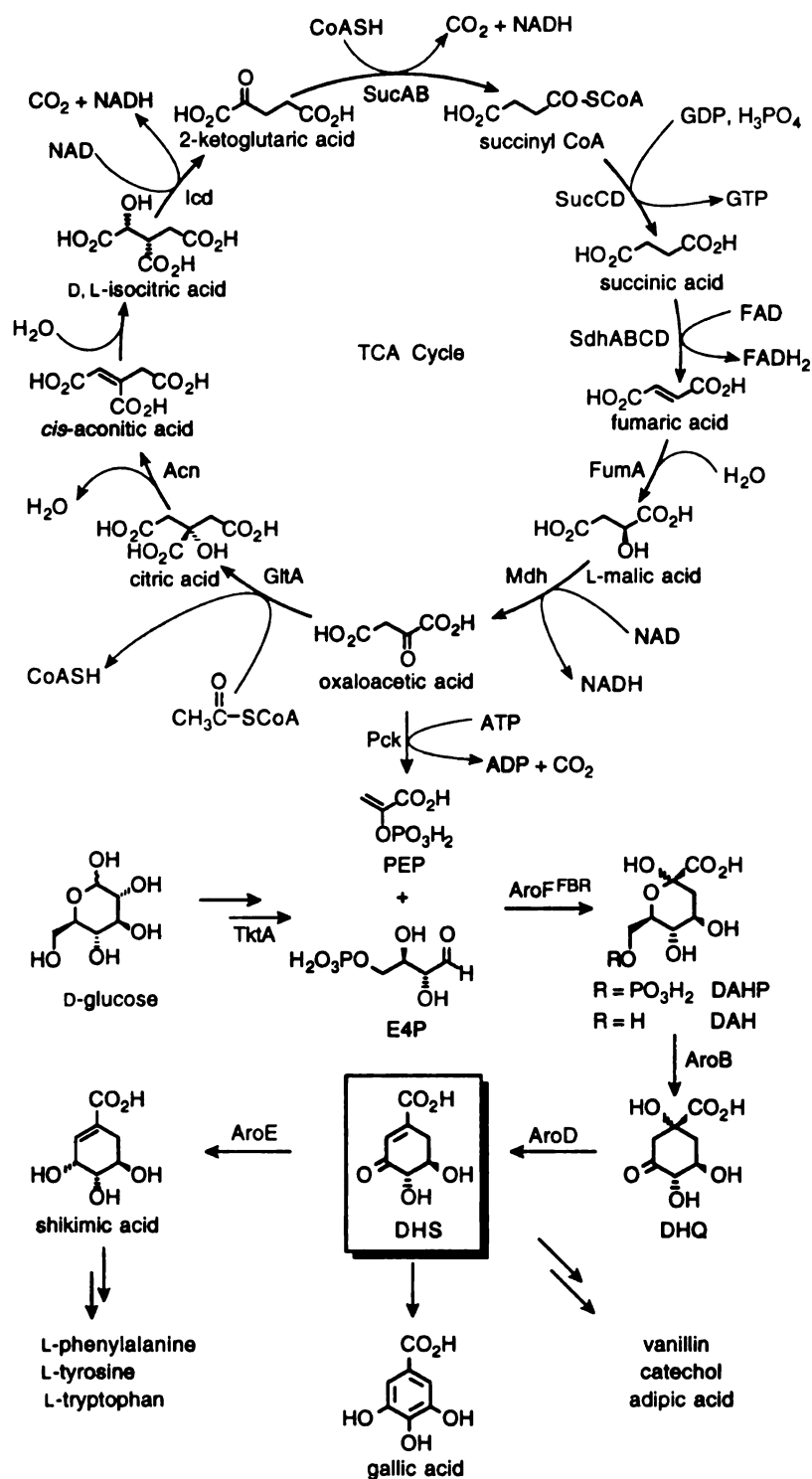


Figure 58. Aromatic amino acid biosynthesis and the TCA cycle. TktA: transketolase; AroF^{FBR}: feedback-insensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; AroB: 3-dehydroquinase synthase; AroD: 3-dehydroquinone dehydratase; AroE: shikimate dehydrogenase; Pck: PEP carboxykinase; GltA: citrate synthase; Acn: aconitase; Icd: isocitrate dehydrogenase; SucAB: 2-ketoglutarate dehydrogenase; SucCD: succinyl-CoA synthetase; SdhABCD: succinate dehydrogenase; FumA: fumarase; Mdh: malate dehydrogenase.

B. Biocatalyst Construction

E. coli can employ dicarboxylic acid intermediates of the TCA cycle such as succinic, fumaric, and L-malic acids as sole sources of carbon for growth. The transport system consists of an inner membrane component encoded by *dctA* and *dctB* and a binding protein component encoded by *cbt*. Upon transport into the cytoplasm, these dicarboxylates can be converted into oxaloacetate, which leads to an increase in turnover of the TCA cycle. Oxaloacetate is an anaplerotic metabolite whose concentration is maintained at a low, constant value. If growth on dicarboxylates such as succinic acid leads to an increase in oxaloacetate concentrations, various enzymes can catalyze reactions with TCA cycle intermediates withdrawing carbon flow from the TCA cycle. The reaction between oxaloacetate and ATP catalyzed by *pck*-encoded PEP carboxykinase, which results in formation of PEP, ADP, and CO₂, was of particular interest to our research. With *pck* overexpression, the increased carbon flow moving into the TCA cycle due to supplementation of glucose with succinic acid will result in increased synthesis of PEP, which was designed to translate into increased DHS synthesis (Figure 58).

Plasmid pKL4.79B was used as a parental plasmid for cloning of the *pck* gene (Figure 60). The choice of pKL4.79B for the study was to obtain data which could be compared with data collected in pentose studies, which employed this same plasmid. As a source of the *pck* gene, plasmid pHG26¹²⁸ was fully digested with *EcoRI* and partially digested with *EcoRV* to isolate a 2.6-kb fragment containing the *pck* gene. Subsequent ligation of the *pck* fragment into the *EcoRI/SmaI* digested pSU18 afforded pKL2.222 (Figure 59). Following digestion of pKL2.222 with *EcoRI/BamHI*, the 2.6-kb *pck* fragment was modified to blunt ends using Klenow fragment. Plasmid pKL4.79B was digested with *SalI* and treated with Klenow fragment. Subsequent ligation of the *pck* fragment into pKL4.79B afforded pKL6.198A. The *pck* gene is transcribed in the same orientation as the *aroF*^{FBR} gene (Figure 60). Plasmid pMF51A was digested with *BamHI* and the resulting 2.2-kb *tktA* fragment was treated with Klenow fragment. Plasmid

pKL6.198A was digested with *Hind*III and treated with Klenow fragment. Subsequent ligation of the *tktA* fragment to pKL6.198A afforded pKL6.218A. The *tktA* gene is transcribed in the same orientation as the *aroF*^{FBR} gene (Figure 61).

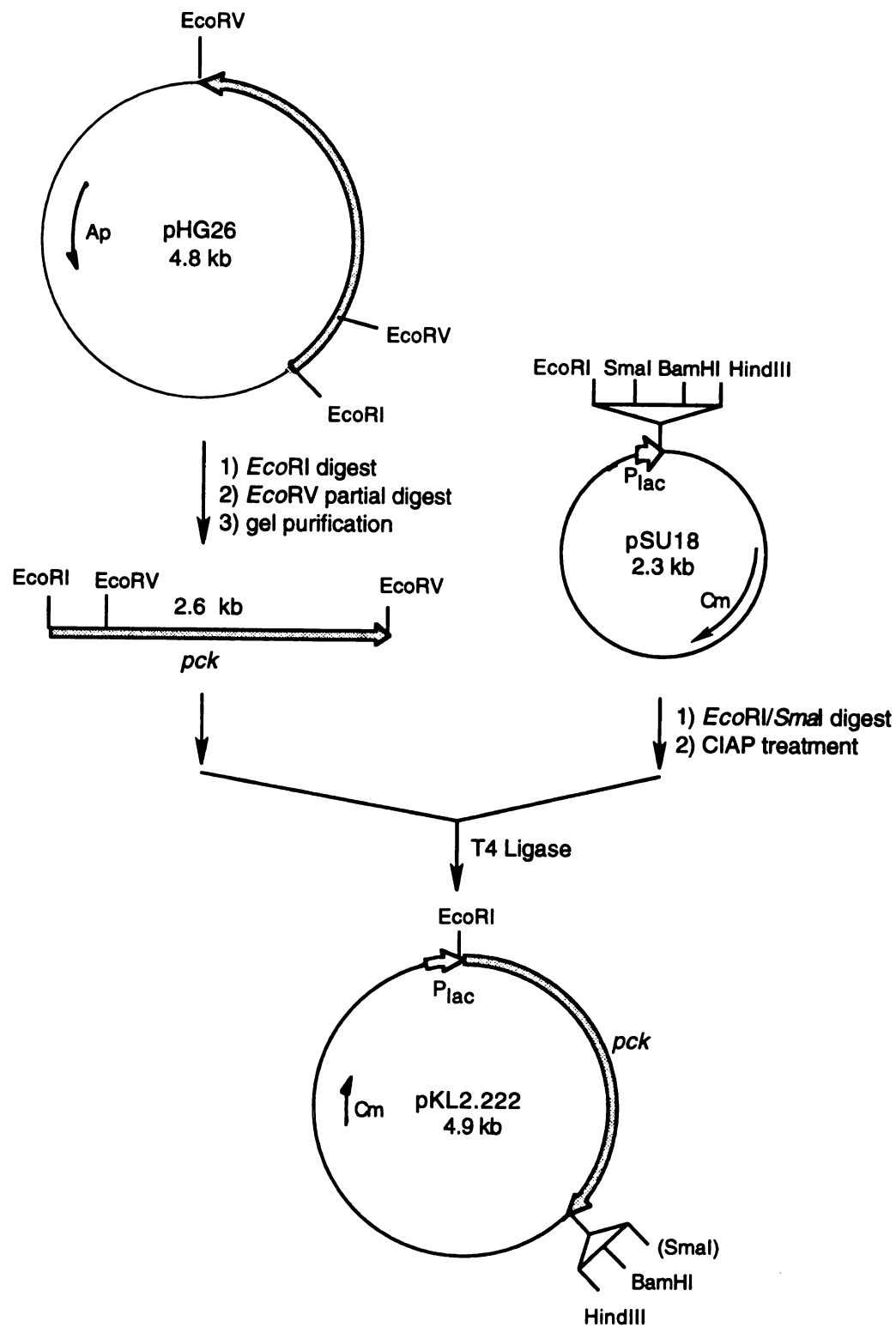


Figure 59. Preparation of Plasmid pKL2.222.

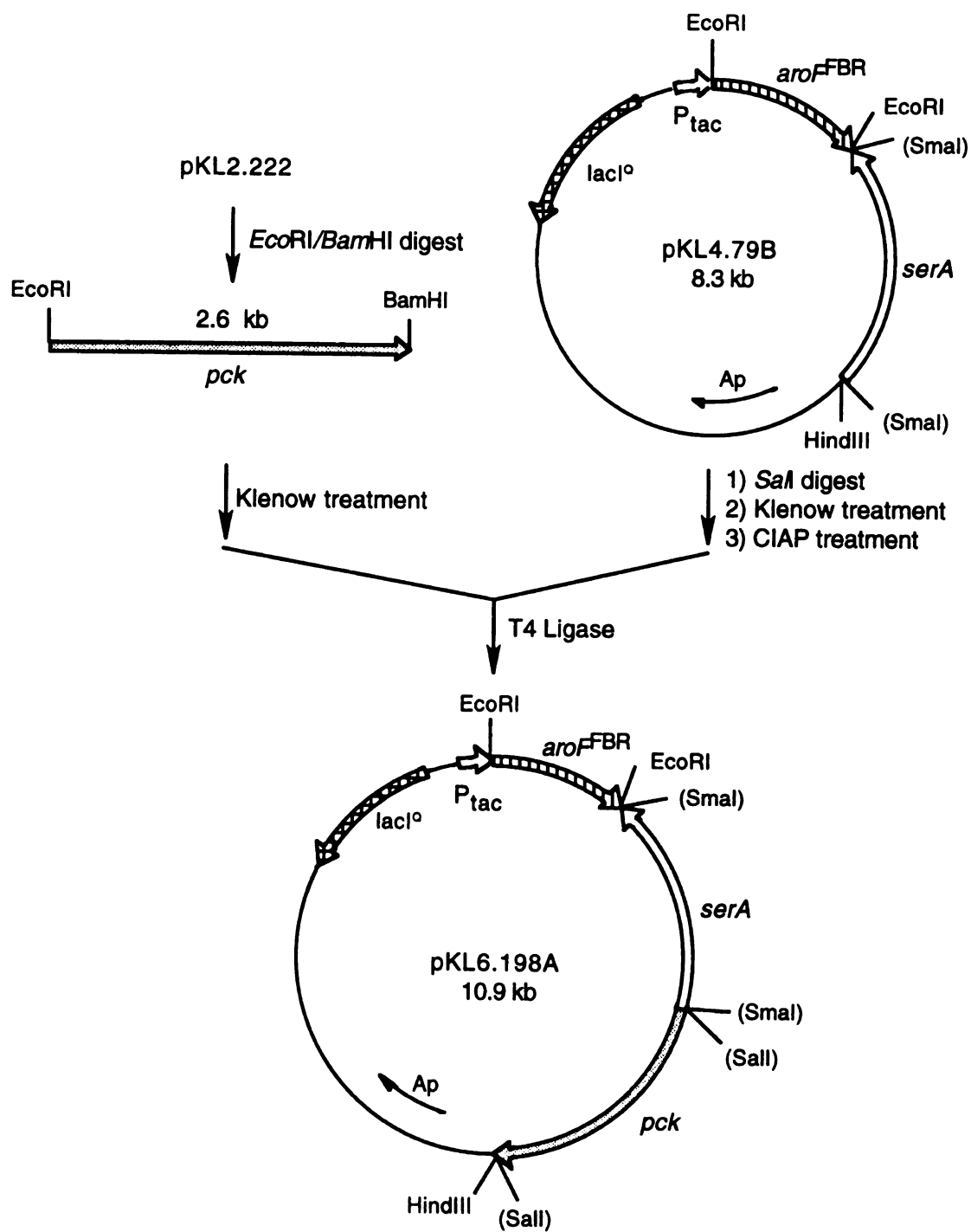


Figure 60. Preparation of Plasmid pKL6.198A.

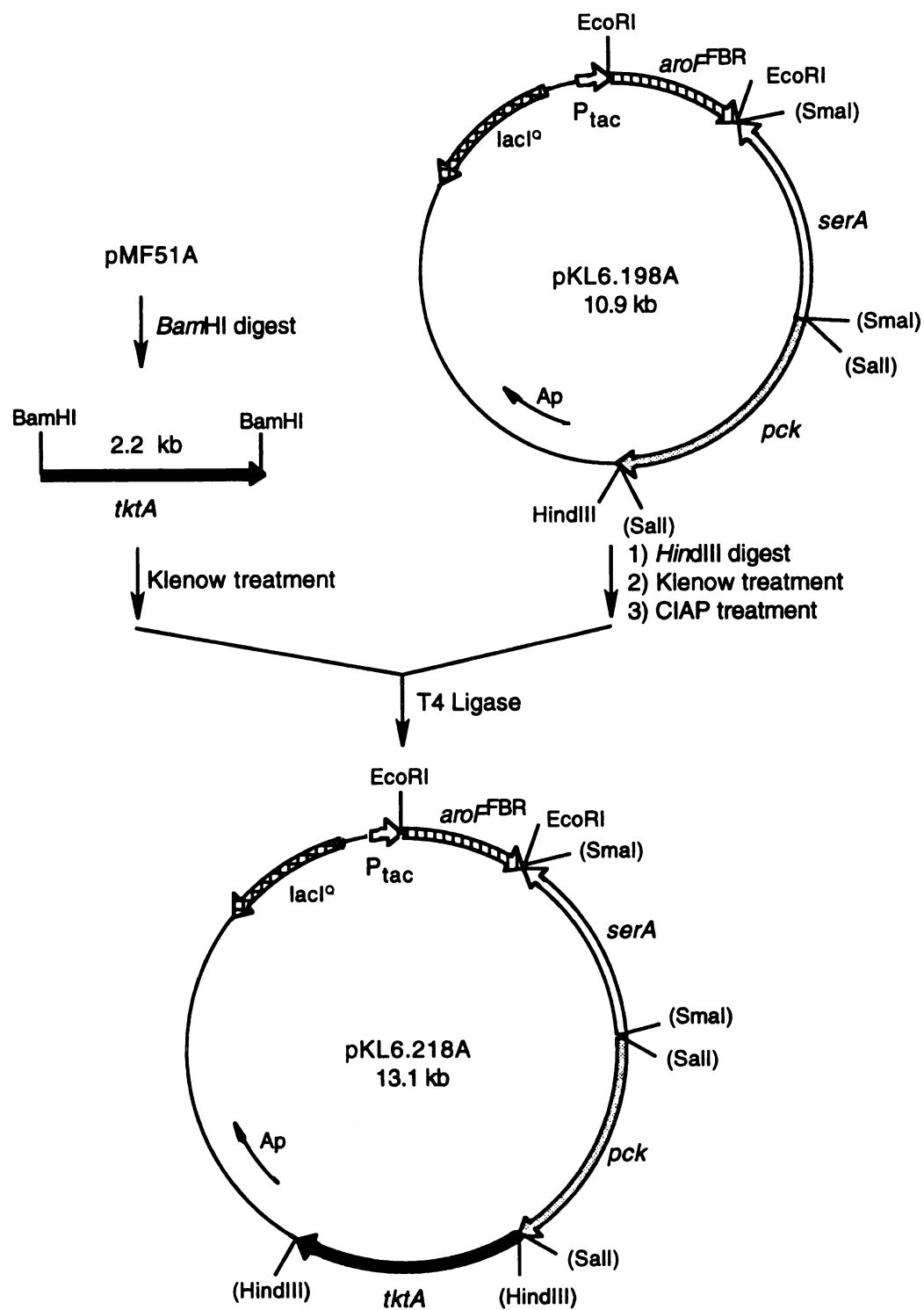


Figure 61. Preparation of Plasmid pKL6.218A.

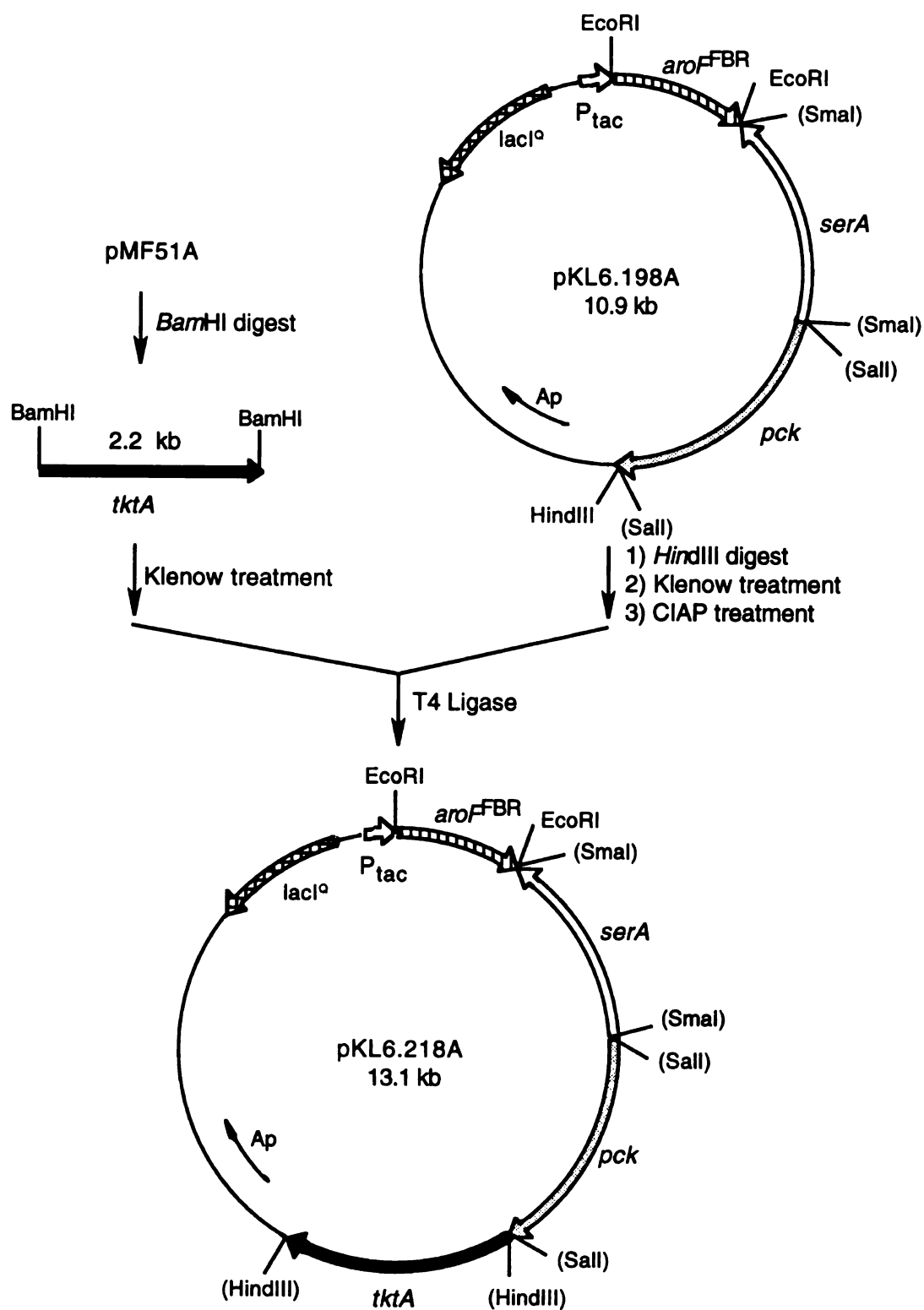


Figure 61. Preparation of Plasmid pKL6.218A.

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C. Channeling Carbon from Succinic Acid into DHS Synthesis

The basic design for use of succinic acid as a glucose supplement entailed cultivation of a DHS-synthesizing *E. coli* construct, which overexpresses *pck*, under fed-batch fermentor conditions where equimolar of succinic acid and glucose were used as the source of carbon. Glucose could be viewed as a specialized source of E4P while succinic acid was the specialized source of PEP. Conversion of glucose into E4P requires fewer enzymes than conversion of succinic acid into E4P, which would require both TCA cycle and gluconeogenesis enzymes. On the other hand, processing of succinic acid into PEP requires fewer enzymatic steps than processing of glucose through glycolysis to obtain PEP.

All fermentations were cultured for 48 h under fed-batch fermentor conditions at 36°C, pH 7.0, and dissolved oxygen maintained at 20% air saturation. Induction of DAHP synthase overexpression was accomplished by incremental addition of IPTG (4.8 mg) during the fermentation run. The initial carbon sources and their concentrations in the fermentation culture medium were 18 g/L when glucose was employed as the sole source of carbon, and 9.0 g/L of glucose and 5.9 g/L of succinic acid when the 1 :1 molar ratio of glucose/succinic acid was used as the carbon source. Diauxic growth was observed during the first two stages of D.O. control when the glucose/succinic acid mixture was used as the carbon source. This likely reflects catabolite repression of succinic acid transport.¹²⁹ After cells exhausted the glucose supply at 11-12 h, the airflow dropped sharply to its minimal value. Succinic acid then became the only carbon source and the D.O. level was again maintained at 20% air saturation by increasing the air flow rate. Cells consumed all the succinic acid in the culture medium in about 1 h. At this juncture, 2 mL of the mixed carbon source was added to the fermentation to allow the airflow rate to increase to its preset maximum at which time D.O. sensor-controlled addition of the carbon source began. The concentration for added glucose solution was 60% (w/v). The concentration of added glucose/succinic acid mixture was 31.3/20.5% (w/v).

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Table 15. Product titers and yields synthesized by KL3 as a function of plasmid and carbon source.

	pKL4.79B	pKL4.124A	pKL6.198A	pKL6.218A
<i>tktA</i>	—	+	—	+
<i>pck</i>	—	—	+	+
		glucose		
[DHS], g/L	36	46	N.D.	44
DHS yield ^a	22%	28%	N.D.	28%
total yield ^b	26%	33%	N.D.	32%
activity ^c	0.04	0.04	N.D.	0.48
		glucose/succinate ^d		
[DHS], g/L	N.D.	46	34	57
DHS yield ^a	N.D.	24%	21%	29%
total yield ^b	N.D.	29%	25%	37%
activity ^c	N.D.	0.04	0.77	0.44

^a mol/mol; ^b mol of (DHS + DHQ + DAH + gallic acid)/ mol of carbon sources consumed; ^c μ mol/min/mg (Since stable Pck activities were observed through the fermentation run, the number reported is the average of 18 h and 36 h.); ^d molar ratio: 1 : 1.

With glucose serving as a sole carbon source, KL3/pKL4.79B synthesized 36.4 g/L of DHS in a 22% (mol/mol) yield (Table 15). As demonstrated previously, KL3/pKL4.124A, which overexpresses transketolase, significantly increased the DHS titer and yield with the synthesis of 46.0 g/L DHS in 28% (mol/mol) yield from glucose (Table 15). For comparison, use of a 1/1 molar mixture of glucose/succinic acid in conjunction with overexpression of Pck in KL3/pKL6.198A resulted in a DHS titer and yield similar to that observed for KL3/pKL4.79B cultured on glucose (Table 15). Since growth on succinic acid increases the carbon flow moving into the TCA cycle, the *in vivo* synthesis of PEP is believed to be increased when the Pck is also amplified. The difference between titers and yields of DHS synthesized by KL3/pKL4.124A from glucose and

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KL3/pKL6.198A from glucose/succinic acid mixture is consistent with E4P, rather than PEP, being the first limiting metabolite in aromatic amino acid biosynthesis when DAHP synthase is overexpressed. After E4P limitation was alleviated by overexpressing transketolase, overexpression of Pck coupled with the succinic supplementation further increased the titer of DHS. KL3/pKL6.218A synthesized 57.0 g/L DHS in 29% yield from the glucose/succinic mixture (Table 15). However, neither KL3/pKL6.218A using glucose only or KL3/pKL4.124A using glucose/succinic acid mixture was able to achieve the same increase, indicating that the in vivo PEP availability can only be increased through a combination of Pck overexpression and succinic acid supplementation (Table 15).

Substantial concentrations of 3-dehydroquinic acid (DHQ) and gallic acid were also synthesized in all cases. DAH accumulation was only observed in KL3/pKL6.218A fermentation when the glucose/succinic acid mixture was used. The observation of DAH in the culture indicates again that two copies of *aroB* in the KL3 genome are insufficient to avoid substrate accumulation. This further demonstrates that the carbon flow into the common pathway was significantly increased by the combination of Pck overexpression and succinic acid supplementation after E4P limitation was relieved with transketolase overexpression. While KL3/pKL4.124A synthesized 4.5 g/L of DHQ and 3.8 g/L of gallic acid using glucose as a sole carbon source, KL3/pKL6.218A synthesized 7.1 g/L of DHQ and 4.4 g/L of gallic acid in addition to 4.2 g/L of DAH when the glucose/succinic acid mixture was used as a carbon source (Figure 62).

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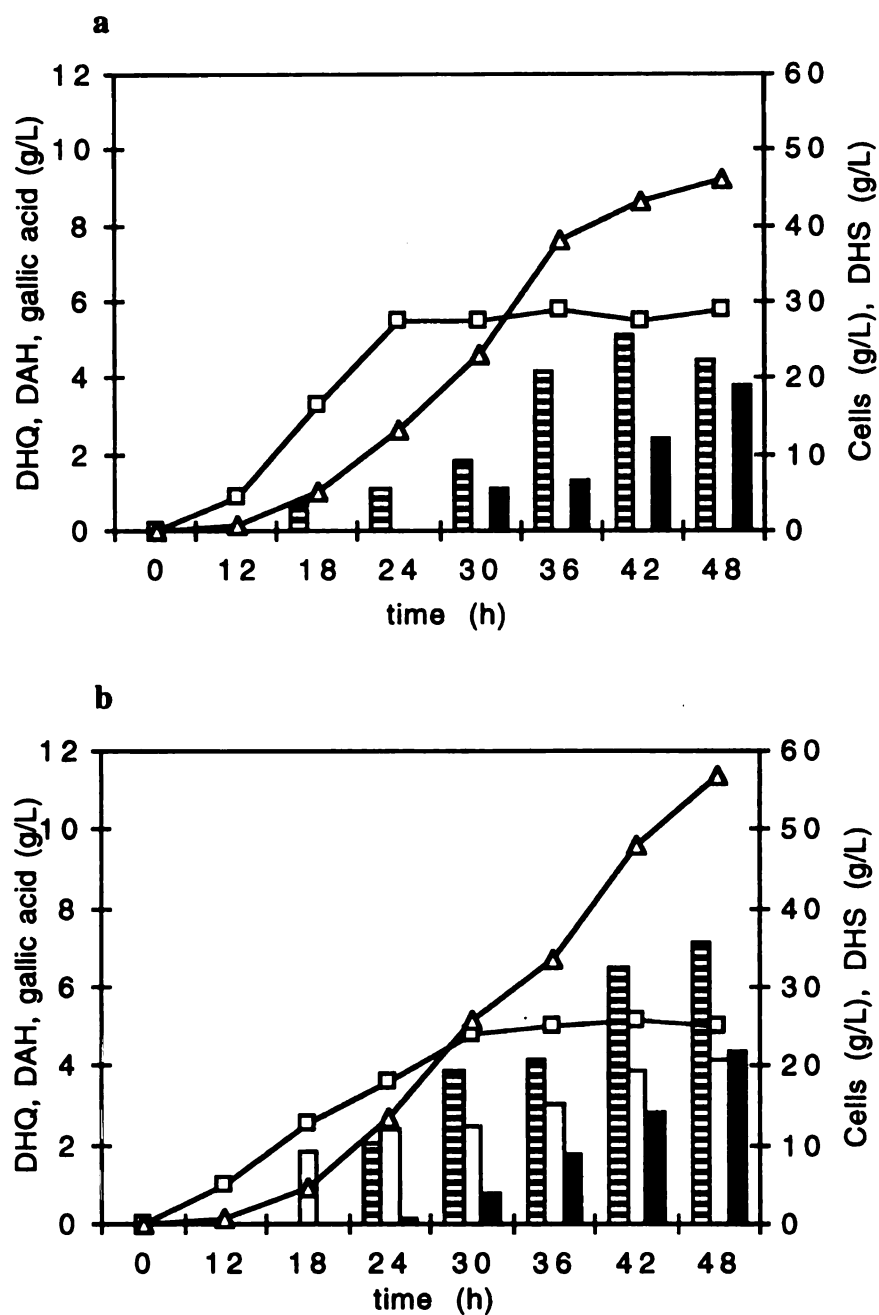


Figure 62. Products and cells produced during fed-batch fermentation.
 —□— cell dry wt; —△— DHS; ▨ DHQ; ▤ DAH; — gallic acid. (a) KL3/pKL4.124A cultured on glucose (b) KL3/pKL6.218A cultured on a 1/1 molar mixture of glucose/succinic acid.

Succinic acid transport is preceded to be subject to catabolite repression in the presence of glucose.¹²⁹ In cultures where glucose concentrations are relatively high, cyclic adenosine monophosphate (cAMP) levels are low, resulting a repression of succinic acid

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uptake. In our study, diauxic growth has been observed prior to use of the D.O. sensor to control addition of the glucose/succinic acid mixture. Before the initial glucose is exhausted, the uptake of succinic acid is minimal although the detection of trace fumaric acid suggests there is some level of transport (Figure 63). When addition of the glucose/succinic acid mixtures subsequently is controlled by a dissolved oxygen sensor, the steady-state glucose concentration is maintained at approximately 200 μ M. Under these glucose-limited conditions, an elevated cAMP level has been reported.¹³⁰ This accounts for the absence of succinic acid accumulation during D.O. sensor controlled addition of the glucose/succinic acid mixture.

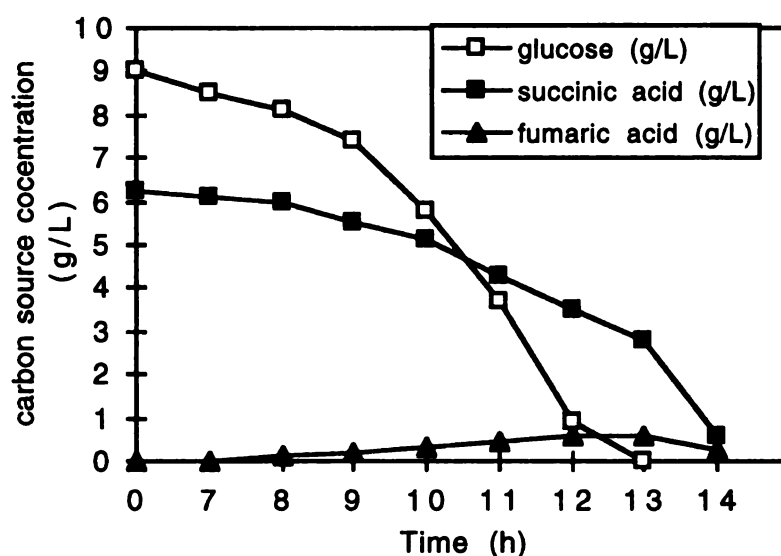


Figure 63. KL3/pKL6.218A cultivation prior to initiation of oxygen sensor-controlled addition of the glucose/succinic acid mixture.

D. Replacing succinic acid with other TCA cycle intermediates

To further demonstrate that supplementation with TCA cycle dicarboxylates can elevate carbon flow directed into the TCA cycle, which ultimately results in increased PEP synthesis, 1/1 molar mixtures of glucose/L-malic acid and glucose/2-ketoglutaric acid were

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also examined using KL3/pKL6.218A as the catalyst. The glucose/L-malic acid fermentation was run in similar fashion to the glucose/succinic acid fermentation. Carbon source concentrations were 9.0 g/L of glucose and 6.7 g/L of L-malic acid for malic acid supplementation. For 2-ketoglutaric acid supplementation, it was observed that D.O. levels were difficult to control at 20% air saturation in the beginning of the third stage of fermentation when a glucose/2-ketoglutaric acid mixture was used as an initial carbon source. Therefore, 18 g/L of glucose was used as the initial carbon source prior to initiation of D.O. sensor-controlled addition of the glucose/2-ketoglutaric acid mixture. The concentration of glucose/L-malic acid was 32%/24% (w/v), and the concentration of glucose/2-ketoglutaric acid was 31%/26% (w/v).

Table 16. Product titers and yields synthesized by KL3/pKL6.218A as a function of glucose supplement.

	glucose	glucose/ succinate	glucose/ malate	glucose/ 2-ketoglutarate
[DHS] ^a	44	57	62	58
DHS yield ^b	28%	29%	30%	29%
[DAH] ^a	0	4.2	5.4	4.8
[DHQ] ^a	4.5	7.1	9.1	7.9
[gallic acid] ^a	3.8	4.4	4.6	4.3
total yield ^c	32%	37%	38%	37%

^a g/L; ^b mol/mol; ^c mol of (DHS + DHQ + DAH + gallic acid)/ mol of carbon sources consumed.

Similar increases in titers of DHS and co-products were observed with malic acid and 2-ketoglutaric acid supplementation. KL3/pKL6.218A synthesized 61.9 g/L of DHS, 5.4 g/L of DAH, 9.1 g/L of DHQ and 4.6 g/L of gallic acid from the glucose/L-malic acid mixture. The DHS yield was 30% (mol/mol) and the total yield of hydroaromatic and

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aromatic products was 38% (mol/mol). KL3/pKL6.218A synthesized 57.6 g/L of DHS, 4.8 g/L of DAH, 7.9 g/L of DHQ, 4.3 g/L of gallic acid from the glucose/2-ketoglutaric acid mixture. The DHS yield was 29% (mol/mol) and the total yield of hydroaromatic and aromatic products was 37% (mol/mol) (Table 16).

E. Discussion

Determining the theoretical yield for DHS synthesis when glucose is supplemented with succinic acid begins with balancing (Eq. (1)) the substrate and cosubstrate inputs with the products and byproducts. The PEP and E4P input required for DHS synthesis is then equated to the amount of D-glucose and succinic acid that is required to form these substrates (Eq. (2a)). A pyruvic acid term is then added (Eq. (2a)) to take into consideration the operation of the PEP phosphotransferase system in *E. coli*. A carbon dioxide term is also added because the conversion of one molecule of succinic acid (through oxaloacetic acid) to one molecule of PEP produces one molecule of carbon dioxide. Coefficients are determined to balance the number of carbon atoms in the glucose and succinic acid input with the total number of carbon atoms constituted by PEP, E4P, pyruvic acid and carbon dioxide formation. Since equal moles of glucose and succinic acid were consumed, the same coefficient for the glucose term and the succinic acid term is used in the carbon balance (Eq. (3b)) for overall conversion (Eq. (3a)). A requirement of 1.17 molecule of glucose and 1.17 molecule of succinic acid for synthesis of each molecule of DHS leads to a maximum theoretical yield of 43% [mol of DHS/(mol of glucose + mol of succinic acid)] for synthesis of DHS from glucose and succinic acid.

The basic strategy to direct carbon from TCA cycle intermediate succinic acid into aromatic amino acid biosynthesis pathway to alleviate the PEP limitation has been proven to be feasible. DHS titers were improved but DHS yields did not improved significantly when both the moles of glucose and the moles of added succinic acid are taken into consideration. KL3/pKL4.124A synthesized DHS and associated byproducts from

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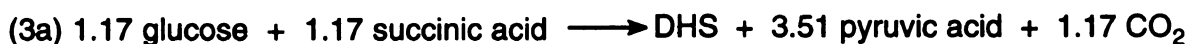
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glucose at 77% of the theoretical maximum yield. KL3/pKL6.218A synthesis of DHS and associated byproducts from glucose and succinic acid was 86% of the theoretical maximum yield.



In relation to the aforementioned strategies for improving product titers in microbe-catalyzed synthesis, use of succinic acid as a glucose adjunct is distinguished by the combination of genetic manipulation and carbon source modification. Under fermentor conditions, normal growth rates of the *E. coli* constructs are observed, and carbon flow into aromatic amino acid biosynthesis is increased as reflected by DHS titers as well as by DAH and DHQ titers. Pure succinic acid is also commercially available. Although current pricing of succinic acid restricts its use to microbe-catalyzed synthesis of higher, value-added chemicals, economies of scale remain to be fully exploited in succinic acid manufacture. In light of the abundant availability of butane from liquefied petroleum gases,¹³¹ use of butane-derived succinic acid as a glucose supplement demonstrates how a fossil fuel feedstock can be combined with a renewable feedstock to create a carbon source for microbe-catalyzed chemical synthesis, which is superior to glucose.

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CHAPTER 5

EXPERIMENTAL

General Methods

Chromatography

HPLC analyses employed a Rainin HPLC interfaced with a Rainin Dynamax UV-Vis detector (Model UV-1). A C18 column (5 μ m, Rainin Microsrobe-MVTM, 4.6 x 250 mm) was used for all HPLC separations. Diethylaminoethyl cellulose (DE52) was purchased from Whatman. Dye Matrex Red A was obtained from Amicon.

Spectroscopic Measurements

¹H NMR spectra were recorded on a Varian VX-300 FT-NMR spectrometer (300 MHz). Chemical shifts were reported in parts per million (ppm) downfield from internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-*d*₄ (TSP, δ = 0.00) with D₂O 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 or on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with HP 89532A UV-Visible Operating Software.

Bacterial Strains

E. coli DH5 α [*F'* *endA1 hsdR17(r⁻km⁺K) supE44 thi-1 recA1 gyrA relA1 ϕ 80lacZ Δ M15 Δ (*lacZYA-argF*)_{U169}] and RB791 (W3110 *lacL8Iq*) were obtained previously by this laboratory.¹³² AB2834⁶⁸ [*tsx-352 supE42 λ^- aroE353 malA352 (λ^-)*] was obtained from the *E. coli* Genetic Stock Center at Yale University. AB3248¹³³ [*F'-aroF363 aroG365 aroH367 proA2 argE3 ilv-7 his-4 lac gal-2 tsx-358 thi (λ)*] was obtained*

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from the laboratory of Professor C. Yanofsky. *Neurospora crassa* SY 7A was obtained from the American Type Culture Collection (ATCC 24740). Plasmid pCRX-2⁷⁵ carrying the open reading frame of catechol-*O*-methyltransferase cDNA from rat liver was obtained from Orion Corporation.

Storage of Bacterial Strains and Plasmids

All bacterial strains were stored at -78 °C in glycerol. Plasmids were transformed into DH5 α for long-term storage. Glycerol samples were prepared by adding 0.75 mL of an overnight culture to a sterile vial containing 0.25 mL of 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 medium¹³⁴ (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). TB medium contained (1 L) tryptone (10 g) and NaCl (5 g). After autoclaving and directly before use, MgSO₄ (10 mL of 1 M stock per L) was added to the TB medium. M9 salts (1 L) contained Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NH₄Cl (1 g), and NaCl (0.5 g). M9 medium contained D-glucose (10 g), MgSO₄ (0.12 g), and thiamine (0.001 g) in 1 L of M9 salts. M63 medium (1 L) contained KH₂PO₄ (13.6 g), (NH₄)₂SO₄ (2 g), FeSO₄·7H₂O (0.0005 g), D-glucose (2 g), MgSO₄ (0.12 g), and thiamine (0.001 g). The pH of M63 inorganic salts was adjusted to 7.0 with 1 N KOH before autoclaving. Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed. Fermentation medium (1 L) contained K₂HPO₄ (7.5 g), ammonium iron(III) citrate (0.3 g), citric acid monohydrate (2.1 g), L-phenylalanine (0.7 g), L-tyrosine (0.7 g), and L-tryptophan (0.35 g), and concentrated H₂SO₄ (1.2 mL). The culture medium was adjusted to pH 7.0 by addition of concentrated NH₄OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: glucose (18 g,

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20 g or 23 g), MgSO_4 (0.24 g), aromatic vitamins *p*-aminobenzoic acid (0.01 g), 2,3-dihydroxybenzoic acid (0.01 g), and *p*-hydroxybenzoic acid (0.01 g), and trace minerals $(\text{NH}_4)_6(\text{Mo}_7\text{O}_{24}) \cdot 4\text{H}_2\text{O}$ (0.0037 g), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0029 g), H_3BO_3 (0.0247 g), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0025 g), and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.0158 g). D-Glucose and MgSO_4 were autoclaved separately while aromatic vitamins and trace minerals were sterilized through 0.22- μm membranes prior to addition to the medium. Antibiotics were added where appropriate to the following final concentrations: chloramphenicol, 20 $\mu\text{g/mL}$; ampicillin, 50 $\mu\text{g/mL}$; kanamycin, 50 $\mu\text{g/mL}$; tetracycline, 12.5 $\mu\text{g/mL}$; and spectinomycin, 50 $\mu\text{g/mL}$. Stock solution of antibiotics were prepared in water with the exception of chloramphenicol which was prepared in 95% ethanol and tetracycline which was prepared in 50% aqueous ethanol. L-Phenylalanine, L-tyrosine, shikimic acid, L-histidine, L-isoleucine, L-valine, L-proline, L-arginine, and L-serine were added to M9 medium or M63 medium where indicated to a final concentration of 0.04 g/L. Antibiotics, thiamine and amino acid supplements were sterilized through 0.22- μm membranes prior to addition to M9 or M63 medium. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to the medium.

Conditions for Shake-Flask Cultivation

For analysis of product accumulation in shake flasks, bacterial strains were cultivated as follows. One liter of LB (4 L Erlenmeyer flask) containing the appropriate antibiotics and if necessary, IPTG, was inoculated with 10 mL of an overnight culture grown in LB with the appropriate antibiotics. Cultures were grown at 37 °C in a gyratory shaker at 250 rpm for 10 h. Cells were collected by centrifugation (4000 $\times g$, 5 min) and directly resuspended in 1 L of M9 medium (4 L Erlenmeyer flask) containing the appropriate antibiotics and if necessary, IPTG. Cultures were then returned to the shaker (37 °C, 250 rpm). Samples were taken at timed intervals for product analysis.

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General Fed-Batch Fermentor Conditions

Fed-batch cultures were grown in a 2.0 L capacity Biostat MD B-Braun fermentor connected to a DCU system and a Compaq computer equipped with B-Braun MFCS software or a Dell Optiplex Gs⁺ 5166M personal computer equipped with B-Braun MFCS/win software. The temperature, pH and substrate feeding were controlled with a PID controller. The temperature was maintained at 36 °C or 37 °C as specified. pH was maintained at 7.0 by addition of concentrated NH₄OH or 2 N H₂SO₄. Dissolved oxygen was measured using a Braun polarographic probe and was set at 20% air saturation. Antifoam (Sigma 204) was pumped in manually as needed.

Two types of inoculants were prepared for the fermentation experiments described in this thesis. For the data reported in Chapter 2 and 3, inoculants were started by introduction of a single colony into 100 mL LB medium (enriched with 2 g glucose) containing the appropriate antibiotic. After growth for 12 h to 14 h at 37 °C with agitation at 250 rpm, the inoculant was ready for transfer into the fermentation vessel. For the data reported in Chapter 4, inoculants were started by introduction of a single colony into 5 mL of M9 medium supplemented with L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid. The culture was grown at 37 °C with agitation at 250 rpm for 18 h and subsequently transferred to 100 mL of M9 medium. After growth at 37 °C and 250 rpm for an additional 12 h, the inoculant was ready for transfer into the fermentation vessel.

The typical fermentation can be divided into three stages according to three different methods used to maintain dissolved oxygen (D.O.) at the set point of 20% air saturation. The dissolved oxygen concentration was first maintained by gradually increasing the impeller speed from a minimum value of 50 rpm to a maximum setting of 940 rpm. After the impeller reached 940 rpm, the mass flow controller governing airflow maintained D.O. levels at 20% saturation by increasing the airflow rate while the impeller was maintained at 940 rpm. Airflow ranged from 0.06 L/L/min to a preset maximum value. Two preset

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maximum values for air flow rate were used in this thesis. For data reported in Chapter 2 and Chapter 4, the value was 1.0 L/L/min. For data reported in Chapter 3, the value was 3.0 L/L/min. Approximately 1-2 h were needed for the airflow to increase to its maximum rate. Once the impeller speed and airflow rate reached their pre-set maximum values, D.O. levels were maintained at 20% saturation by oxygen sensor-controlled substrate feeding. At the beginning of this stage, dissolved oxygen levels fell below 20% saturation due to residual substrate in the medium. This period lasted anywhere from several minutes to approximately 1 h before all residual substrate was consumed and the substrate feeding was started. The PID control parameters 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). Typical fermentations lasted for 48 h.

^1H NMR Analysis of Culture Supernatant

For strains being evaluated in shake flasks, samples (20 mL) of the culture were taken at timed intervals, and the cells were removed by centrifugation (4000 x g, 5 min). For strains being evaluated in a fermentor, samples (5 mL) were taken at timed intervals and the cells were removed using a Beckman microfuge. A portion (0.5-2.0 mL) of the supernatant was concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D_2O , and then redissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid (TSP). Concentrations of metabolites in the supernatant were determined by comparison of integrals corresponding to each metabolite with the integral corresponding to TSP ($\delta = 0.00$ ppm) in the ^1H NMR.

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Genetic Manipulations

General

Recombinant DNA manipulations generally followed methods described in Sambrook *et al.*⁸⁷ Restriction enzymes were purchased from Gibco BRL or New England Biolabs. T4 DNA ligase was obtained from Gibco BRL. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. Agarose (electrophoresis grade) was obtained from Gibco BRL. Phenol was prepared by addition of 0.1 % (w/v) 8-hydroxyquinoline to distilled, liquefied phenol. Extraction with an equal volume of 1 M Tris-HCl pH 8.0 (two times) was followed by extraction with 0.1 M Tris-HCl pH 8.0 until the pH of the aqueous layer was greater than 7.6. Phenol was stored at 4 °C under an equal volume of 0.1 M Tris-HCl pH 8.0. SEVAG was a mixture of chloroform and isoamyl alcohol (24:1 v/v). TE buffer contained 10 mM Tris-HCl (pH 8.0) and 1 mM Na₂EDTA (pH 8.0). Endostop solution (10X concentration) contained 50% glycerol (v/v), 0.1 M Na₂EDTA, pH 7.5, 1% sodium dodecyl sulfate (SDS) (w/v), 0.1% bromophenol blue (w/v), and 0.1% xylene cyanole FF (w/v) and was stored at 4 °C. Prior to use, 0.12 mL of DNAase-free RNAase was added to 1 mL of 10X Endostop solution. DNAase-free RNAase (10 mg mL⁻¹) was prepared by dissolving RNAase in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl. DNAase activity was inactivated by heating the solution at 100 °C for 15 min. Aliquots were stored at -20 °C. PCR amplifications were carried out as described by Sambrook.⁸⁸ Each reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), dTTP (0.2 mM), template DNA, 0.5 µM of each primer, and 2 units of Vent polymerase. Initial template concentrations varied from 0.02 µg to 1.0 µg.

Large Scale Purification of Plasmid DNA

Plasmid DNA was purified on a large scale using a modified alkaline lysis method described by Sambrook *et al.*⁸⁶ In a 2 L Erlenmeyer flask, LB (500 mL) containing the

appropriate antibiotics was inoculated from a single colony, and the culture was incubated in a gyratory shaker (250 rpm) for 14 h at 37 °C. Cells were harvested by centrifugation (4000 x g, 5 min, 4 °C) and then resuspended in 10 mL of cold GETL solution [50 mM glucose, 20 mM Tris-HCl (pH 8.0), 10 mM Na₂EDTA (pH 8.0)] into which lysozyme (5 mg mL⁻¹) had been added immediately before use. The suspension was stored at room temperature for 5 min. Addition of 20 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH was followed by gentle mixing and storage on ice for 15 min. Fifteen milliliters of an ice cold solution containing 3 M KOAc (prepared by combining 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of H₂O) was added. Vigorous shaking resulted in formation of a white precipitate. After the suspension was stored on ice for 10 min, the cellular debris was removed by centrifugation (48000 x g, 20 min, 4 °C). The supernatant was transferred to two clean centrifuge bottles and isopropanol (0.6 volumes) was added to precipitate the DNA. After the samples were left at room temperature for 15 min, the DNA was recovered by centrifugation (20000 x g, 20 min, 4 °C). The DNA pellet was then rinsed with 70% ethanol and dried.

Further purification of the DNA sample involved precipitation with polyethylene glycol (PEG). The DNA was dissolved in TE (3 mL) and transferred to a Corex tube. Cold 5 M LiCl (3 mL) was added and the solution was gently mixed. The sample was then centrifuged (12000 x g, 10 min, 4 °C) to remove high molecular weight RNA. The clear supernatant was transferred to a clean Corex tube and isopropanol (6 mL) was added followed by gentle mixing. The precipitated DNA was collected by centrifugation (12000 x g, 10 min, 4 °C). The DNA was then rinsed with 70% ethanol and dried. After redissolving the DNA in 0.5 mL of TE containing 20 µg/mL of RNAase, the solution was transferred to a 1.5 mL microcentrifuge tube and stored at room temperature for 30 min. 500 µL of 1.6 M NaCl containing 13% PEG-8000 (w/v) (Sigma) was added to this sample. The solution was mixed and centrifuged (microcentrifuge, 10 min, 4 °C) to recover the precipitated DNA. The supernatant was removed and the DNA was then

redissolved in 400 μL of TE. The sample was extracted sequentially with phenol (400 μL), phenol and SEVAG (400 μL each), and finally SEVAG (400 μL). Ammonium acetate (10 M, 100 μL) was added to the aqueous DNA solution. After thorough mixing, 95% ethanol (1 mL) was added to precipitate the DNA. The sample was left at room temperature for 5 min and then centrifuged (microcentrifuge, 5 min, 4 $^{\circ}\text{C}$). The DNA was rinsed with 70% ethanol, dried, and then redissolved in 200-500 μL of TE.

The concentration of DNA in the sample was determined as follows. An aliquot (10 μL) of the DNA was diluted to 1 mL in TE and the absorbance at 260 nm was measured relative to the absorbance of TE. The DNA concentration was calculated based on the fact that the absorbance at 260 nm of a 50 $\mu\text{g mL}^{-1}$ of plasmid DNA is 1.0.

Small Scale Purification of Plasmid DNA

An overnight culture (5 mL) of the plasmid-containing strain was grown in LB containing the appropriate antibiotics. Cells from 3 mL of the culture were collected in a 1.5 mL microcentrifuge tube by centrifugation. The resulting cell pellet was liquefied by vortexing (30 sec) and then resuspended in 0.1 mL of cold GETL solution into which lysozyme (5 mg mL^{-1}) had been added immediately before use. The solution was stored on ice for 10 min. Addition of 0.2 mL of 1% sodium dodecyl sulfate (w/v) in 0.2 N NaOH was followed by gentle mixing and storage on ice for 5-10 min. To the sample was added 0.15 mL of cold KOAc solution. The solution was shaken vigorously and stored on ice for 5 min before centrifugation (15 min, 4 $^{\circ}\text{C}$). The supernatant was transferred to another microcentrifuge tube and extracted with equal volumes of phenol and SEVAG (0.2 mL). The aqueous phase (approximately 0.5 mL) was transferred to a fresh microfuge tube, and DNA was precipitated by the addition of 95% ethanol (1 mL). The sample was left at room temperature for 5 min before centrifugation (15 min, room temperature) to collect the DNA. The DNA pellet was rinsed with 70% ethanol, dried, and redissolved in

50 -100 μ L TE. DNA isolated from this method was used for restriction enzyme analysis, and the concentration was not determined by spectroscopic methods.

Restriction Enzyme Digestion of DNA

Restriction enzyme digests were performed using buffer solutions supplied by BRL or New England Biolabs. A typical digest contained approximately 0.8 μ g of DNA in 8 μ L TE, 2 μ L of restriction enzyme buffer (10X concentration), 1 μ L of restriction enzyme, and TE to a final volume of 20 μ L. Reactions were incubated at 37 °C for 1 h. Digests were terminated by addition of 2.2 μ L of Endostop solution (10X concentration) and subsequently analyzed by agarose gel electrophoresis. When DNA was required for subsequent cloning, restriction digests were terminated by addition of 1 μ L of 0.5 M Na₂EDTA (pH 8.0) followed by extraction of the DNA with equal volumes of phenol and SEVAG and precipitation of the DNA. DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2) followed by thorough mixing and addition of 3 volumes of 95% ethanol. Samples were stored for at least 2 h at -78 °C. Precipitated DNA was recovered by centrifugation (15 min, 4 °C). To the DNA pellet was added 70% ethanol (100 μ L), and the sample was centrifuged again (15 min, 4 °C). DNA was dried and redissolved in TE.

Agarose Gel Electrophoresis

Agarose gels were run in TAE buffer containing 40 mM Tris-acetate and 2 mM EDTA (pH 8.0). Gels typically contained 0.7% agarose (w/v) in TAE buffer. Higher concentrations of agarose (1%) were used to resolve DNA fragments smaller than 1 kb. Ethidium bromide (0.5 μ g mL⁻¹) was added to the agarose to allow visualization of DNA fragments over a UV lamp. The size of the DNA fragments were determined by using two sets of DNA standards: λ DNA digested with *Hind*III (23.1-kb, 9.4-kb, 6.6-kb, 4.4-kb,

2.3-kb, 2.0-kb, and 0.6-kb) and λ DNA digested with *EcoRI* and *HindIII* (21.2-kb, 5.1-kb, 5.0-kb, 4.3-kb, 3.5-kb, 2.0-kb, 1.9-kb, 1.6-kb, 1.4-kb, 0.9-kb, 0.8-kb, and 0.6-kb).

Isolation of DNA from Agarose

The band of agarose containing DNA of interest was excised from the gel and chopped thoroughly with a razor in a plastic weighing tray. The agarose was then transferred to a spin column consisting of a 500 μ L microfuge tube packed tightly with glass wool and with an 18 gauge hole in its bottom. The spin column was then centrifuged for 5 min using a Beckman microfuge to separate the DNA solution from the agarose. The DNA-containing aqueous phase was collected during centrifugation in a 1.5 mL microfuge tube. The DNA was precipitated with 3 M NaOAc and 95% ethanol as previously described and redissolved in TE.

Treatment of Vector DNA with Calf Intestinal Alkaline Phosphatase

Plasmid vectors digested with a single restriction enzyme were dephosphorylated to prevent self-ligation. Digested vector DNA was dissolved in TE (88 μ L). To this sample was added 10 μ L of dephosphorylation buffer (10X concentration) and 2 μ L of calf intestinal alkaline phosphatase (2 units). The reaction was incubated at 37 °C for 1 h. The phosphatase was inactivated by addition of 1 μ L of 0.5 M Na₂EDTA (pH 8.0) followed by heat treatment (65 °C, 20 min). The sample was extracted with phenol and SEVAG (100 μ L each) to remove the protein, and the DNA was precipitated as previously described and redissolved in TE.

Treatment of DNA with Klenow fragment

DNA fragment with recessed 3' termini was modified to blunt-ended fragment by treatment with the Klenow fragment of *E. coli* DNA polymerase I. After the DNA (0.8-2 μ g) restriction digestion was completed in a 20 μ L reaction, a solution (1 μ L) containing

each of the desired dNTPs was added to a final concentration of 1 mM. Addition of 1-2 units of the Klenow fragment was followed by incubation of the mixture at room temperature for 20-30 min. Since the Klenow fragment works well in the common buffers used for restriction digestion of DNA, there was no need to purify the DNA after restriction digestion and prior to filling recessed 3' termini. Klenow reactions were quenched by extraction with equal volumes of phenol and SEVAG. DNA was recovered by DNA precipitation.

Ligation of DNA

DNA ligations were designed so that the molar ratio of insert to vector was 3 to 1. A typical ligation reaction contained 0.03 to 0.1 µg of vector and 0.05 to 0.2 µg of insert in a total volume of 7 µL. To this sample was added 2 µL of ligation buffer (5X concentration) and 1 µL of T4 DNA ligase (2 units). The reaction was incubated at 16 °C for at least 4 h and then used to transform competent cells.

Preparation and Transformation of Competent Cells

Competent cells were prepared using a procedure modified from Sambrook *et al.*⁸⁸ 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 of growth (judged from the absorbance at 600 nm reaching 0.4-0.6). The culture was poured into a large centrifuge bottle that had been previously sterilized with bleach and rinsed with sterile water. The cells were collected by centrifugation (4000 x g, 5 min, 4 °C) and the culture medium was discarded. All manipulations were carried out on ice during the remaining portion of the procedure. The cell pellet was washed with 100 mL of cold 0.9% NaCl (w/v) and then resuspended in 50 mL of cold 100 mM CaCl₂. The suspension was stored on ice for a minimum of 30 min and then centrifuged (4000 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 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen. Competent cells were stored at $-78\text{ }^\circ\text{C}$ with no significant decrease in transformation efficiency over a period of six months.

Frozen competent cells were thawed on ice for 5 min before transformation. A small aliquot (1 to 10 μL) of plasmid DNA or a ligation reaction was added to the thawed competent cells (0.1 mL). The solution was gently mixed and stored on ice for 30 min. The cells were then heat shocked at $42\text{ }^\circ\text{C}$ for 2 min and placed on ice briefly (1 min). LB (0.5 mL, no antibiotics) was added to the cells, and the sample was incubated at $37\text{ }^\circ\text{C}$ (no agitation) for 1 h. Cells were collected in a microcentrifuge (30 s). If the transformation was to be plated onto LB plates, the cells were resuspended in a small volume of LB medium (0.1 mL), and then spread onto plates containing the appropriate antibiotics. If the transformation was to be plated onto minimal medium plates, the cells were washed once with the same minimal medium. After resuspension in fresh minimal medium (0.1 mL), the cells were spread onto the plates. A sample of competent cells with no 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.

Purification of Genomic DNA

Genomic DNA was purified using a modified method described by Silhavy.¹³⁵ A single colony of the strain was inoculated into 100 mL of TB medium (500 mL Erlenmeyer flask). The cells were cultured in a gyratory shaker ($37\text{ }^\circ\text{C}$, 250 rpm) for 12 h. Centrifugation ($4000 \times g$, 5 min, $4\text{ }^\circ\text{C}$) of the culture was followed by resuspension of the cell pellet in 5 mL of buffer [50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0)] and storage at $-20\text{ }^\circ\text{C}$ for 20 min to freeze the suspension. To the frozen cells was added 0.5 mL of 0.25 M Tris-HCl (pH 8.0) that contained 5 mg of lysozyme. The suspension was

thawed at room temperature in a water bath with gentle mixing and then stored on ice for 45 min. The sample was then transferred to a Corex tube. After addition of 1 mL of STEP solution [25 mM Tris-HCl (pH 7.4), 200 mM EDTA (pH 8.0), 0.5% SDS (w/v), and proteinase K (1 mg mL⁻¹, Sigma), prepared just before use], the mixture was incubated at 50 °C for at least 1 h with gentle, periodic mixing. The solution was then divided into two Corex tubes, and the contents of each tube were extracted with phenol (4 mL). The organic and aqueous layers were separated by centrifugation (1000 x g, 15 min, room temperature), and the aqueous layer was transferred to a fresh Corex tube. All transfers of the aqueous layer were carried out using wide bore pipette tips to minimize shearing of the genomic DNA. The contents of each tube were extracted again with a mixture of phenol (3 mL) and SEVAG (3 mL). Extractions with phenol/SEVAG were repeated (approximately 6 times) until the aqueous layer was clear.

Genomic DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2), gentle mixing, and addition of 2 volumes of 95% ethanol. Threads of DNA were spooled onto a sealed Pasteur pipette and transferred to a Corex tube that contained 5 mL of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), and 1 mg of RNAase. The mixture was stored at 4 °C overnight to allow the DNA to dissolve completely. The solution was then extracted with SEVAG (5 mL) and centrifuged (1000 x g, 15 min, room temperature). The aqueous layer was transferred to a fresh Corex tube and the genomic DNA was precipitated as described above. The threads of DNA were spooled onto a Pasteur pipette and redissolved in 2 mL of 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0). Genomic DNA was stored at 4 °C.

Enzyme Assays

After collected and resuspended in proper resuspension buffer, the cells were disrupted by two passages through a French pressure cell (SLM Aminco) at 16000 psi. Cellular debris was removed from the lysate by centrifugation (48000 x g, 20 min, 4 °C).

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Protein was quantified using the Bradford dye-binding procedure. A standard curve was prepared using bovine serum albumin. The protein assay solution was purchased from Bio-Rad.

DAHP synthase assay

DAHP synthase was assayed according to the procedure described by Schoner.¹³⁶ Harvested cells were resuspended in 50 mM potassium phosphate (pH 6.5) that contained 10 mM PEP and 0.05 mM CoCl_2 . The cells were disrupted using a French press as described above. Cellular lysate was diluted in potassium phosphate (50 mM), PEP (0.5 mM), and 1,3-propanediol (250 mM), pH 7.0. A dilute solution of E4P was first concentrated to 12 mM by rotary evaporation and neutralized with 5 N KOH. Two different solutions were prepared and incubated separately at 37 °C for 5 min. The first solution (1 mL) contained E4P (6 mM), PEP (12 mM), ovalbumin (1 mg/mL), and potassium phosphate (25 mM), pH 7.0. The second solution (0.5 mL) consisted of the diluted lysate. After the two solutions were mixed (time = 0), aliquots (0.15 mL) were removed at timed intervals and quenched with 0.1 mL of 10% trichloroacetic acid (w/v). Precipitated protein was removed by centrifugation, and the DAHP in each sample was quantified using thiobarbituric acid assay¹³⁷ as described below.

An aliquot (0.1 mL) of DAHP containing sample was reacted with 0.1 mL of 0.2 M NaIO_4 in 8.2 M H_3PO_4 at 37 °C for 5 min. The reaction was quenched by addition of 0.8 M NaAsO_2 in 0.5 M Na_2SO_4 and 0.1 M H_2SO_4 (0.5 mL) and vortexed until a dark brown color disappeared. Upon addition of 3 mL of 0.04 M thiobarbituric acid in 0.5 M Na_2SO_4 (pH 7), the sample was heated at 100 °C for 15 min. Samples were cooled (2 min), and the pink chromophore was then extracted into distilled cyclohexanone (4 mL). The aqueous and organic layers were separated by centrifugation (2000 x g, 15 min). The absorbance of the organic layer was recorded at 549 nm ($\epsilon = 68000 \text{ L mol}^{-1} \text{ cm}^{-1}$). One

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unit of DAHP synthase activity was defined as the formation of 1 μmol of DAHP per min at 37 °C.

DHQ synthase

DHQ synthase activity was determined by measuring the disappearance of DAHP over time using the thiobarbituric acid.¹³⁷ Harvested cells were resuspended in Buffer A (100 mL) consisting of 50 mM potassium 3-(N-morpholino)propanesulfonate (MOPS), pH 7.5, and 0.25 mM CoCl_2 . After centrifugation, the cells were resuspended in Buffer A (2 mL g^{-1} cells) and disrupted using a French press as previously described. An assay solution was prepared containing MOPS (50 mM), pH 7.5, NAD^+ (0.25 mM), CoCl_2 (0.25 mM), and DAHP (0.2 mM). The assay solution (1.5 mL) was preincubated at 37 °C for 10 min. An aliquot (0.2 mL) of appropriately diluted enzyme (diluted with buffer A) was added ($t = 0$) and the sample was incubated at 37 °C. Aliquots (0.2 mL) were removed at timed intervals (15 s) and quenched with 10% trichloroacetic acid (0.1 mL). Precipitated protein was removed by centrifugation using a Beckman microfuge and DAHP was quantified using the thiobarbituric acid (TBA) assay as described above. One unit of DHQ synthase activity is defined as the loss of one μmol of DAHP per min at 37 °C.

DHS dehydratase

DHS dehydratase activity was assayed by measuring formation of protocatechuic acid at 290 nm. Harvested cells were resuspended in a solution containing 100 mM Tris-HCl and 2.5 mM MgCl_2 , pH 7.5 and disrupted as previously described. The assay solution contained 0.1 M Tris-HCl (pH 7.5), 25 mM MgCl_2 , and 1 mM DHS in a total volume of 1 mL. The reaction was initialized upon the addition of the enzyme. The absorbance at 290 nm was monitored for 5 min at room temperature. The specific activity was expressed as μmol of protocatechuic acid generated per min per mg protein at room

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temperature. The molar extinction coefficient for protocatechuic acid at 290 nm is 3890 L mol⁻¹ cm⁻¹.¹³⁸

Catechol-*O*-methyltransferase

A modification of the method of Reenilä was used to assay catechol-*O*-methyltransferase activity.¹³⁹ Buffer A containing 10 mM NaH₂PO₄, pH 7.4 and 0.5 mM dithiothreitol was used for washing and resuspending the cells and diluting the cellular lysate. The cells were washed twice and then resuspended in buffer A. The cells were disrupted as previously described. Two solutions were prepared separately and incubated at 37 °C for 3 min. The first solution (4 mL) contained NaH₂PO₄ buffer (125 mM) pH 7.4, MgCl₂ (6.25 mM), *S*-adenosyl-L-methionine (0.75 mM), and protocatechuic acid (0.5 mM). The second solution (1 mL) contained the diluted lysate. The two solutions were combined, vortexed briefly and incubated at 37 °C (time = 0). Aliquots (0.5 mL) were removed at timed intervals (1 min) and quenched with 40 µL of ice-cold 4 M HClO₃. Precipitated protein was removed by centrifugation using a Beckman microfuge. The resulting supernatant was either analyzed immediately by HPLC as described below or stored at -78 °C for up to two days.

The protocatechuic acid, vanillic acid and isovanillic acid components in the supernatant were quantitated by HPLC. Individual component was separated by isocratic elution (17:2:1 H₂O/CH₃/CH₃CO₂H v/v) on a C18 column. The absorbance of the eluent at 250 nm was monitored. Samples were quantitated by comparison of the peak area corresponding to each component with a standard curve. One unit of catechol-*O*-methyltransferase activity is defined as the formation of 1 µmol of the sum of vanillic acid and isovanillic acid per min at 37 °C.

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Aryl-aldehyde dehydrogenase

Aryl-aldehyde dehydrogenase assay solution (1 mL) contained Tris-HCl (100 mM), pH 8.0, MgCl₂ (10 mM), dithiothreitol (20 mM), NADPH (0.15 mM), ATP (20 mM), and benzoic acid (4 mM), and was pre-incubated at 30 °C. After addition of solution containing aryl-aldehyde dehydrogenase, reduction of benzoic acid was monitored by following the loss of absorbance at 340 nm. One unit of aryl-aldehyde dehydrogenase activity is defined as the loss of 1 μmol of NADPH ($\epsilon = 6220 \text{ L mol}^{-1} \text{ cm}^{-1}$) per min at 30°C.

PEP carboxykinase

PEP carboxykinase was assayed by modification of a procedure previously described by Goldie.¹²⁸ Harvested cells were resuspended in 100 mM Tris-HCl (pH 7.5) that contained 1 mM EDTA and 0.1 mM DTT. The cells were disrupted as previously described. A buffer solution at pH 7.5 was prepared containing Tris-HCl (100 mM), PEP (10 mM), NaHCO₃ (50 mM), ADP (4 mM), MgCl₂ (80 mM). Lysate (0.1 mL) and the buffer solution (16 mL) were incubated separately at 31°C for 3 min. After addition of the lysate solution to the buffer solution (time = 0), aliquots (2 mL) were removed at timed intervals (1 min) and quenched with 0.75 mL absolute ethanol. Following the addition of 100 μL of freshly prepared Fast Violet B Salt (2% w/v in H₂O), the mixture was allowed to stand at 31°C for 10 min followed by addition of 250 μL of 1 M HCl to stop the color development. After a 1:5 dilution of each sample with H₂O, the absorbance was recorded at 520 nm. Oxaloacetic acid formation was determined by comparison to a standard curve prepared using oxaloacetic acid purchased from Sigma. One unit of activity is defined as the formation of 1 μmol of oxaloacetic acid per min in 31°C.

Chapter 2

Purification of *Neurospora crassa* Aryl-Aldehyde Dehydrogenase

Buffers

Buffers used for purification of aryl-aldehyde dehydrogenase included buffer A: Tris-HCl (100 mM) and L-cystine (10 mM), pH 7.6; buffer B: Tris-HCl (50 mM), EDTA (1 mM), DTT (1 mM), and PMSF (0.4 mM), pH 7.6; buffer C: Tris-HCl (50 mM), EDTA (1 mM), DTT (1 mM), and PMSF (0.4 mM), and KCl (400 mM), pH 7.6; buffer D: Tris-HCl (20 mM), EDTA (0.4 mM), DTT (0.4 mM), and PMSF (0.15 mM), pH 7.5; buffer E: Tris-HCl (20 mM), EDTA (0.4 mM), DTT (0.4 mM), PMSF (0.15 mM), and KCl (2.5 M), pH 7.5.

***Neurospora crassa* cultivation**

Growth medium for cultivation of *N. crassa* was prepared in distilled, deionized water. Solid growth medium (1 L) contained sucrose (20 g), sodium citrate dihydrate (2.5g), KH_2PO_4 (5.0 g), NH_4NO_3 (2.0 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g), MgSO_4 (0.1 g), biotin (5.0 μg), and trace elements including citric acid monohydrate (5.0 mg), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5.0 mg), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (1.0 mg), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25 mg), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.05mg), H_3BO_3 (0.05 mg), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.05 mg). Difco agar was added to a final concentration of 2% (w/v). The liquid growth medium (1 L) differed from solid growth medium only in the addition of Difco yeast extract (2.0 g) and sodium salicylate (1.6 g). *N. crassa* SY 7A was grown on solid growth medium in a petri dish (dimension: 100 mm x 80 mm) at 24 °C for 7 days and a mixture of mycelium and spores was obtained, which was subsequently suspended in 20 mL of sterile water and filtered through glass wool that had been previously sterilized by autoclaving. The resulting spore suspension can be stored at 4 °C for up to two weeks. The concentration of spores in the suspension was estimated by measuring the absorbance at 650 nm ($A_{650} = 1.0 = 5.0 \times 10^6$ spores/mL).

Spores were inoculated into 2 L liquid growth medium in a 4 L Erlenmeyer flask to give a final concentration of 2.5×10^6 spores/L. After culturing at room temperature on a gyratory shaker at 200 rpm for 60 h, the mycelium was harvested by filtration through Whatman filter paper on a Buchner funnel. The mycelium can be stored at -20 °C for at least 6 months with minimal loss of the aryl-aldehyde dehydrogenase activity.

Purification of aryl-aldehyde dehydrogenase

All protein purification manipulations were carried out at 4 °C. Frozen mycelium (400 g, wet weight) was thawed in 900 mL buffer A, and disrupted in a Waring blender for 20 min at 4 °C. To prevent overheating the lysate, the blending time was divided into 10 two-minute sessions with three-minute cooling time between two consecutive sessions. The debris was removed by centrifugation at 18000 x g for 30 min followed by concentration of the supernatant to 200 mL through ultrafiltration (PM-10 Diaflo membranes from Amicon). After dialysis against buffer B (3x), the mycelium extract was applied to a DEAE column (5 x 23 cm) equilibrated with buffer B. The column was washed with 500 mL of buffer B followed by elution with a linear gradient (1.5 L + 1.5 L, buffer B/buffer C). Fractions containing aryl-aldehyde dehydrogenase were combined and concentrated to 30 mL. After dialysis against buffer D (3x), the protein was loaded onto a RedA column (2.5 x 8 cm) equilibrated with buffer D. The column was washed with 200 mL buffer C and eluted with a linear gradient (150 mL + 150 mL, buffer D/buffer E). Fractions containing aryl-aldehyde dehydrogenase were concentrated, frozen in liquid nitrogen, and stored at -78 °C.

Strain Constructions

KL7

E. coli KL7 was prepared by homologous recombination of an *aroBaroZ* cassette into the *serA* locus⁶⁹ (*serA::aroBaroZ*) of AB2834.⁶⁸ Localization of the *serA* gene in

pMAK705⁷² followed by insertion of the *aroBaroZ* cassette into a *Bam*HI site internal to *serA* directed recombination of the *aroBaroZ* cassette into the *serA* locus of the genome. Plasmid pMAK705 contains a temperature-sensitive pSC101 replicon. Since derivatives of pMAK705 replicate at 30 °C but are unstable at 44 °C, isolation of all pMAK705 derivatives required culturing of cells at 30 °C.

Digestion of pD2625^{69b} with *Eco*RV and *Dra*I liberated a 1.9-kb *serA* fragment. Plasmid pMAK705 was digested with *Bam*HI and treated with Klenow fragment. Subsequent ligation of *serA* fragment to pMAK705 afforded pLZ1.68A.⁷³ The *aroBaroZ* cassette was created as follows: The *aroB* gene was obtained as a 1.7-kb fragment following digestion of pJB14 with *Eco*RI. After treatment with the Klenow fragment, the *aroB* fragment was inserted into the *Sma*I site of pSK4.99A³⁵ which was constructed by ligation of 2.1-kb *aroZ* fragment into *Bam*HI site of pSU18 to afford pKL4.237A. The 3.9-kb *aroBaroZ* cassette was amplified from pKL4.237A with the inclusion of *Bam*HI recognition sequences at the 5'- and 3'- ends. Insertion of the cassette into the *Bam*HI site of *serA* in pLZ1.68A yielded pKL4.276B. Both *aroB* and *aroZ* are transcribed in the opposite orientation relative to the *lac* promoter.

Conditions for homologous recombination were based on those previously described.^{69b} Competent AB2834 was transformed with pKL4.276B. Following heat-shock treatment, cells were incubated in LB at 44 °C for 1 h and subsequently plated onto LB plates containing Cm. Plates were incubated at 44 °C for approximately 20 h before colonies appeared. The resulting cointegrates were inoculated into 5 mL of LB containing no antibiotics, and the cells were grown at 30 °C for 12 h to allow excision of the plasmid from the genome. Cultures were diluted (1:20000) in LB without antibiotics, and two more cycles of growth at 30 °C for 12 h were carried out to enrich cultures for more rapidly growing cells that had lost the temperature-sensitive replicon. Cultures were then diluted (1:20000) into LB and grown at 44 °C for 12 h to promote plasmid loss from the cells. Serial dilutions of each culture were spread onto LB plates and incubated at 30 °C

overnight. The resulting colonies were screened on multiple plates to select the recombined ones. *E. coli* KL7 was isolated based on the following growth characteristics: growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid and serine; no growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid; growth on LB; and no growth on LB containing Cm.

Plasmid pKL3.272A

Plasmid pCRX-2⁷⁵ carries the open reading frame (ORF) of the *COMT*. Digestion of pCRX-2 with *EcoRI/HindIII* was followed by isolation of a 0.7-kb fragment encoding the ORF of *COMT*. Plasmid pKK223-3⁷⁰ was also digested with *EcoRI/HindIII* to yield a 5.3-kb fragment. Ligation of these two fragments resulted in pKL3.272A in which the *COMT* gene was transcribed from a *tac* promoter.

Plasmid pKL5.26A

This 6.6-kb plasmid was constructed by inserting the *P_{tac}COMT* fragment from pKL3.272A into pKL4.33B. Digestion of pKL4.33B with *SphI* and *HindIII* afforded a 5.5-kb fragment while similar digestion of pKL3.272A yielded a 1.1-kb *P_{tac}COMT* fragment. Ligation of these two purified fragments resulted in pKL5.26A.

Plasmid pKL5.97A

Plasmid pKL5.26A was digested with *HindIII/XbaI* and the 1.1-kb *P_{tac}COMT* fragment was isolated and treated with Klenow fragment. Plasmid pKL5.26A was also digested with *HindIII* and treated with Klenow fragment. Subsequent ligation of the *P_{tac}COMT* to pKL5.26A afforded pKL5.97A. The two *P_{tac}COMT* genes are transcribed in the same orientation in pKL5.97A.

Biocatalytic Synthesis of Vanillic Acid

Fermentation process was performed as described previously. The initial glucose concentration in the fermentation medium was 20 g/L. The glucose feed was 60% (w/v). A solution (500 mL) of glucose feed was prepared by autoclaving a mixture of 300 g of glucose and 300 mL of water. L-Methionine, when employed, was added as an autoclaved solution (40 g/L) in timed intervals (6 h) starting at 12 h after initiation of a fermentor run.

Fermentation samples (6 mL) were removed at 6 h intervals. A portion (1 mL) was used to measure the cell density. The remaining 5 mL of each fermentation sample was centrifuged and the DHS, protocatechuic acid, vanillic acid and isovanillic acid components in the supernatant were quantitated by HPLC. Individual components were separated by isocratic elution (17:2:1 H₂O/CH₃/CH₃CO₂H v/v) on a C18 column. The absorbance of the eluent at 250 nm was monitored. Samples were quantitated by comparison of the peak area corresponding to each component with a standard curve. Separate aliquots (25 mL) of fermentation broth were taken at 12 h and 36 h for assay of catechol-*O*-methyl transferase activity. After the fermentation was complete, the entire broth was centrifuged at 16000 x g for 10 min, and the resulting supernatant was stored at 4 °C.

Reduction of Vanillic Acid to Vanillin

Fermentation broth (100 mL) containing DHS, protocatechuic acid, vanillic acid, and isovanillic acid was acidified to pH 3.1 by addition of concentrated HCl and the resulting precipitated protein was removed by centrifugation at 16000 x g for 10 min. After three extractions of the resulting clear brown supernatant with EtOAc (100 mL), the organic extracts were combined and the solvent was removed under reduced pressure. The resulting solid containing protocatechuic acid, vanillic acid and isovanillic acid was dissolved in 12 mL of water, and adjusted to pH 7.5 by addition of NaOH (10 N). Subsequent dropwise addition of concentrated H₂SO₄ to pH 1.8 resulted in precipitation of a solid which was filtered and dried. The collected precipitate containing protocatechuic

acid, vanillic acid and isovanillic acid was dissolved in a solution (100 mL) containing Tris-HCl (200 mM), pH 8.0, MgCl₂ (100 mM), DTT (10 mM), ATP (60 mM), NADP⁺ (2 mM), glucose 6-phosphate (60 mM), 2,000 units of glucose 6-phosphate dehydrogenase and 200 units of the partially purified aryl-aldehyde dehydrogenase. The reaction was incubated at 30 °C and monitored by HPLC. After 7 h, 92% of the starting vanillic acid and 34% of the protocatechuic acid had been reduced. The reaction mixture was then extracted with 100 mL CH₂Cl₂ (3x). The combined organic extracts were washed one time with an equal volume of water. Concentration of the organic layers afforded a yellow powder consisting of vanillin (0.30 g) and isovanillin (0.03 g). ¹H NMR (D₂O) for vanillin were: δ 3.88 (s, 3H), 6.98 (d, J = 8 Hz, 1H), 7.39 (s, 1H), 7.46 (d, J = 8 Hz, 1H), 9.63 (s, 1H); ¹H NMR (D₂O) for isovanillin were: δ 3.95 (s, 3H), 7.18 (d, J = 8 Hz, 1H), 7.38 (s, 1H), 7.55 (d, J = 8 Hz, 1H), 9.70 (s, 1H).

Chapter 3

Strain Constructions

Strain KL3

E. coli KL3 was prepared from AB2834⁶⁸ by homologous recombination of the *aroB* gene into the *serA* locus⁶⁹ (*serA::aroB*). Localization of the *serA* gene in pMAK705⁷³ followed by insertion of the *aroB* into an *EcoRI* site internal to the *serA* directed recombination of the *aroB* into the *serA* locus of the genome. Digestion of pKAD63¹⁴⁰ with *SphI* liberated a 1.9-kb *serA* fragment, which was subsequently inserted into the *SphI* site of pMAK705 to afford pKAD76A.¹³⁹ The *aroB* gene was obtained as a 1.7-kb fragment following digestion of pJB14¹³⁹ with *EcoRI*. Insertion of the *aroB* fragment into the *EcoRI* site of *serA* was complicated by two additional *EcoRI* sites in pKAD76A. Following *EcoRI* partial digestion of pKAD76A, the resulting DNA fragments were resolved on an agarose gel and the 7.4-kb fragment corresponding to the linearized plasmid was isolated. Ligation of the linearized plasmid to the 1.7-kb *EcoRI* fragment of *aroB* afforded pKL3.82A. Following homologous recombination of the *serA::aroB* locus of pKL3.82A into AB2834, *E. coli* KL3 was isolated based on the following growth characteristics: growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid and serine; no growth on M9 containing L-tyrosine, L-phenylalanine, shikimic acid; growth on LB; and no growth on LB containing Cm.

Strain AB2.24

E. coli AB2.24 was constructed to facilitate mutagenesis of *aroF* to a feedback insensitive isozyme of DAHP synthase (*aroF^{FBR}*). AB2.24 was derived from AB3248¹³³ by homologous recombination of an *aroFkan^R* locus into the *serA* gene. The *aroF* locus was obtained as a 1.3-kb *EcoRI/BamHI* fragment following PCR amplification from pKD136,¹¹¹ while a 1.2-kb *BamHI/EcoRI kan^R* fragment was obtained from pKAD62A.⁶⁹

Ligation of *aroF* and *kan^R* to pSU18 which had been linearized by *EcoRI* afforded pKD10.156. Digestion of pKD10.156 with *EcoRI* yielded the 2.5-kb *aroFkan^R* fragment which was subsequently localized in the *EcoRI* site internal to the *serA* gene in pKAD76A to afford pKD10.186A. Following homologous recombination of the *serA::aroFkan^R* locus of pKD10.186A into AB3248, *E. coli* AB2.24 was isolated based on the following growth characteristics: no growth on M63 containing histidine, isoleucine, valine, proline, and arginine; no growth on M63 containing histidine, isoleucine, valine, proline, arginine, and shikimic acid; growth on M63 containing histidine, isoleucine, valine, proline, arginine, and serine; growth on LB containing Kan; and no growth on LB containing Cm.

Strain AC2-13A

AB2.24 was subjected to in situ UV mutagenesis, and chemotactic selection was performed in a Diffusion Gradient Chamber¹⁰¹ to select mutant strains bearing the *aroF^{FBR}*. Strain AC2-13A was isolated using the process previously reported.⁹⁷ In vitro assay of DAHP synthase in the presence of 125 μ M tyrosine confirmed the *aroF* locus in AC2-13A was feedback insensitive. The *aroF^{FBR}* locus was amplified from AC2-13A with its native promoter to yield a 1.3-kb fragment. Inclusion of *EcoRI* recognition sequences at the 5'- and 3'- ends of the *aroF^{FBR}* fragment facilitated its insertion into the *EcoRI* site of pCL1920 to afford pCL2-13A. Plasmid pCL2-13A was used to sequence the *aroF^{FBR}* locus.

Plasmid pKL4.20B

Plasmid pCL2-13A was digested with *EcoRI* and the 1.3-kb *aroF^{FBR}* fragment was isolated and ligated into the *EcoRI* site of pSU18 to create pKL4.20B. The *aroF^{FBR}* gene is transcribed in the opposite orientation relative to the *lac* promoter in pKL4.20B.

Plasmid pKL4.33B

This 5.5-kb plasmid was created by ligation of a 1.9-kb *DraI/EcoRV* fragment encoding *serA* obtained from pD2625^{69b} into the *SmaI* site of the pKL4.20B. The *serA* gene is transcribed in the same orientation as *aroF^{FBR}*.

Plasmid pKL4.66A

The *aroF^{FBR}* locus was amplified by PCR from pKL4.20B with *XbaI* ends. Localization of the 1.3-kb *aroF^{FBR}* into the *XbaI* site of pKL4.33B resulted in pKL4.66A. Transcription of the *aroF^{FBR}* locus in the *XbaI* site of pKL4.66A proceeds in the opposite orientation of *serA*.

Plasmid pKL4.130B

This 8.9-kb plasmid was created by inserting the *tktA* fragment from pMF51A⁵⁶ into pKL4.66A. Plasmid pMF51A was digested with *BamHI* and the resulting 2.2-kb *tktA* fragment was treated with Klenow fragment. Plasmid pKL4.66A was digested with *HindIII* and treated with Klenow fragment. Subsequent ligation of the *tktA* fragment to pKL4.66A afforded pKL4.130B. The *tktA* gene is transcribed in the same orientation as the *serA* gene.

Plasmid pKL4.71A

This 6.4-kb plasmid was constructed by ligating the open reading frame (ORF) of *aroF^{FBR}* into the *EcoRI* site of pJF118EH.¹⁰³ The *aroF^{FBR}* ORF was amplified from pKL4.33B using following primers: 5'- GGAATTCATGCAAAAAGACGCGCTGA and 5'- GGAATTCTTAAGCCACGCGAGCCGT. Localization of the resulting 1.1-kb fragment into pJF118EH afforded pKL4.71A. The ORF of *aroF^{FBR}* is transcribed in the same orientation as the *tac* promoter in pKL4.71A.

Plasmid pKL4.79B

This 8.3-kb plasmid was created by ligation of a 1.9-kb *DraI/EcoRV serA* fragment obtained from pD2625 into the *SmaI* site of the pKL4.71A. The *serA* gene is transcribed in the opposite orientation relative to the *aroF*^{FBR} gene.

Plasmid pKL4.124A

This 10.5-kb plasmid was constructed by inserting the *tktA* fragment from pMF51A into pKL4.79B. Following digestion of pMF51A with *Bam*HI, the 2.2-kb *tktA* fragment was treated with Klenow fragment. Plasmid pKL4.79B was digested with *Hind*III and treated with Klenow fragment. Subsequent ligation of the *tktA* fragment to pKL4.79B afforded pKL4.124A. The *tktA* gene is transcribed in the same orientation as the *aroF*^{FBR} gene.

Plasmid pKD11.291A

This 5.6-kb plasmid was constructed by inserting a fragment encoding the promoter region of *aroF* into the *Xba*I site of pKL4.33B. *P*_{aroF} was amplified from pMF63A using following primers: 5'- GCTCTAGAGAATTCAAAGGGAGTGTA and 5'- GCTCTAGACCTCAGCGAGGATGACGT. Transcription from *P*_{aroF} is in the same orientation as the *serA* gene.

Plasmid pKL5.17A

This 7.8-kb plasmid was created by replacing the 1.0-kb *NcoI/SphI* fragment of pKD11.291A with a 3.2-kb *NcoI/SphI* fragment from pKL4.130B that included *tktA*. Digestion of pKD11.291A with *NcoI* and *SphI* afforded a 4.6-kb fragment while similar digestion of pKL4.130B yielded a 3.2-kb DNA fragment. Ligation of these two purified fragments resulted in pKL5.17A.

Fed-Batch Fermentation

Fermentation process was performed as described previously. The initial glucose concentration in the fermentation was either 18 g/L (for KL3/pKL4.79B, KL3/pKL4.33B, and KL3/pKD11.291A) or 23 g/L (for KL3/pKL4.124A, KL3/pKL4.66A, KL3/pKL4.130B, and KL3/pKL5.17A). The glucose feed concentration was 60% (w/v) which was prepared as described previously. As needed, IPTG was added every 6 h from 12 h into the fermentation until the end of the fermentation

Sample (5 mL) of fermentation broth were taken at timed intervals. A portion (1 mL) was used to determine the cell density by measuring the absorption at 600 nm (OD_{600}). The remaining 4 mL of each fermentation broth sample was centrifuged using a Beckman microfuge. A portion (0.5-3.0 mL) of the culture supernatant was used for ^1H NMR analysis. The OD_{600} was converted to the cell dry weight by using a conversion coefficient determined as follows: Fermentation broth with a known OD_{600} was centrifuged, and all the cells were collected. After washing three times with fresh M9 salts (400 mL), the cells were transferred to a container and dried in a 100 °C oven until the weight was constant. The dry cell weight was determined and a conversion coefficient (dry cell weight / OD_{600}) of 0.43 was obtained using an average value of three experiments.

Chapter 4

Strain Constructions

Plasmid pKL2.222

Plasmid pHG26¹²⁸ was fully digested with *EcoRI* and partially digested with *EcoRV* to isolate the 2.6-kb *pck* fragment. Subsequent ligation of the *pck* fragment into the *EcoRI/SmaI* digested pSU18 afforded pKL2.222.

Plasmid pKL6.198A

This 10.9-kb plasmid was constructed by inserting the *pck* fragment from pKL2.222 into pKL4.79B. Following digestion of pKL2.222 with *EcoRI/BamHI*, the 2.6-kb *pck* fragment was treated with Klenow fragment. Plasmid pKL4.79B was digested with *SalI* and treated with Klenow fragment. Subsequent ligation of the *pck* fragment to pKL4.79B afforded pKL6.198A. The *pck* gene is transcribed in the same orientation as the *aroF*^{FBR} gene.

Plasmid pKL6.218A

This 13.1-kb pKL6.218A was created by inserting the *tktA* fragment from pMF51A into pKL6.198A. Plasmid pMF51A was digested with *BamHI* and the resulting 2.2-kb *tktA* fragment was treated with Klenow fragment. Plasmid pKL6.198A was digested with *HindIII* and treated with Klenow fragment. Subsequent ligation of the *tktA* fragment to pKL6.198A afforded pKL6.218A. The *tktA* gene is transcribed in the same orientation as the *aroF*^{FBR} gene.

Fermentation Conditions

Conditions for the Study of "A Comparative Analysis of D-Xylose, L-Arabinose, and D-Glucose Carbon Sources for Microbial Synthesis of DHS"

The fermentation was performed as described previously. The inoculum carbon sources were either 4 g/L when glucose, xylose, and arabinose were used individually or a combination of 1.67 g/L of glucose, 1.4 g/L of xylose, and 0.92 g/L of arabinose for sugar mixture fermentations. Initial sugar concentrations in the fermentor culture medium were 18 g/L for glucose, 23 g/L for xylose or arabinose, and 7.6/6.3/4.1 g/L for the glucose/xylose/arabinose mixture.

The feeding method differed depending on the carbon substrate. For both glucose and the sugar mixture fermentations, at a constant impeller speed and a constant airflow, D.O. was maintained at 20% air saturation by oxygen sensor-controlled substrate feed. The concentration of the substrate feed for glucose fermentation was 60% (w/v) as was prepared as described previously. The feed concentration for sugar mixture fermentations was 26.3% (w/v) glucose, 21.9% (w/v) xylose, and 14.4% (w/v) arabinose. A typical sugar mixture feeding solution (480 mL) was prepared by autoclaving a mixture of glucose (126 g), xylose (105 g), arabinose (69 g) in water (300 mL). When xylose or arabinose were used as carbon sources, the D. O. could not be controlled at 20% air saturation in the third stage of the fermentation. In these cases, the carbon substrate was added at a constant rate of 10.0 mL/h after the initial substrate was exhausted. A typical xylose feed solution (460 mL) was prepared by autoclaving a mixture of xylose (300 g) and water (280 mL) (65% w/v). A typical arabinose feed solution (490 mL) was prepared by autoclaving a mixture of arabinose (300 g) and water (300 mL) (61% w/v). IPTG (4.8 mg) was added to all the fermentations at the beginning of the third stage, and then every 6 h started from 18 h until the end of the fermentation.

Conditions for the Study of "Utilizing Succinic Acid as a Glucose Adjunct in Fed-Batch Fermentation: Is Butane a Feedstock Option in Microbial-Catalyzed Synthesis?"

The fermentation was performed as described previously. The carbon source for the inoculum was 4 g/L of glucose in all cases. Initial carbon sources and their concentrations in the fermentation culture medium were 9.0 g/L of glucose and 5.9 g/L of succinic acid for glucose/succinic acid mixture, and 9.0 g/L glucose and 6.7 g/L of L-malic acid for glucose/L-malic acid mixture. D.O. could be maintained at 20% air saturation during the third stage of fermentation when 2-ketoglutaric acid was used as the glucose adjunct only if glucose (at an initial concentration of 18 g/L) was used as the sole carbon source for the first two stages.

The concentration of glucose/succinic acid feed mixture was 31.3/20.5% (w/v). A typical glucose/succinic acid feed solution (480 mL) was prepared as follows: An ammonium succinate solution was prepared by dissolving succinic acid (98.3 g) in H₂O (130 mL) with the addition of concentrated NH₄OH (52 mL). A glucose solution was prepared by mixing glucose (150 mL) with H₂O (140 mL). The two solutions were autoclaved separately and mixed after both were cooled to room temperature. The concentration of glucose/L-malic acid feed mixture was 31.9/23.8% (w/v). A typical glucose/L-malic acid feed solution (470 mL) was prepared as follows: A L-malic acid solution was prepared by dissolving L-malic acid (111.7 g) in H₂O (200 mL). A glucose solution was prepared by mixing glucose (150 g) with H₂O (100 mL). The two solutions were autoclaved separately and mixed after both were cooled to room temperature. The concentration of added glucose/2-ketoglutaric acid mixture was 31.3/25.4% (w/v). A typical glucose/2-ketoglutaric acid feed solution (480 mL) was prepared as follows: A 2-ketoglutaric acid solution was prepared by dissolving 2-ketoglutaric acid (121.8 g) in H₂O (160 mL). A glucose solution was prepared by mixing glucose (150 g) with H₂O (140 mL). The two solutions was autoclaved separately and mixed after both were cooled to

room temperature. IPTG (4.8 mg) was added for all the fermentations at the beginning of the third stage, at 18 h and every 6 h increment after 18 h until the end of the fermentation.

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