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SYNTHESIS OF VALUE-ADDED CHEMICALS FROM GLUCOSE USING CHEMICAL AND MICROBIAL CATALYSIS: GALLIC ACID, PROTOCATECHUIC ACID, PYROGALLOL AND CATECHOL

by

Spiros Kambourakis

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ABSTRACT

SYNTHESIS OF VALUE-ADDED CHEMICALS FROM GLUCOSE USING CHEMICAL AND MICROBIAL CATALYSIS: GALLIC ACID, PROTOCATECHUIC ACID, PYROGALLOL AND CATECHOL

By

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Thesis research focused on synthesis of gallic acid (3,4,5-trihydroxybenzoic acid), pyrogallol (1,2,3-trihydroxybenzene), and protocatechuic acid (3,4-dihydroxybenzoic acid) and catechol (1,2-dihydroxybenzene) from glucose using both chemical and microbial catalysis. Esterified gallic acids are potent antioxidants while the widely used antibiotic trimethoprim is synthesized from gallic acid. Pyrogallol is the starting material for the synthesis of insecticide bendiocarb and is used in adhesives and as a corrosion inhibitor. Protocatechuic acid is an antioxidant and an intermediate in the biocatalytic synthesis of vanillin, catechol, and adipic acid. Catechol is used in the synthesis of pharmaceticals (L-Dopa, adrenaline), flavors (vanillin, eugenol), insecticides (carbofuran) and polymerization inhibitors (veratrol).

Gallic acid provided an opportunity to compare direct synthesis of an aromatic using a recombinant *Escherichia coli* construct with a hybrid strategy employing both microbial and chemical catalysis. For the hybrid strategy, 3-dehydroshikimic acid was synthesized from glucose under fed-batch fermentor conditions. Chemical oxidation methodology was then elaborated whereby 3-dehydroshikimic acid was oxidized in high yield using catalytic amounts of Cu^{+2} and Zn^{+2} with O_2 as the cooxidant. Detailed mechanistic studies of the solution chemistry of 3-dehydroshikimc acid provided the foundation for elaboration of the chemical oxidative methodology. An intriguing

outgrowth of these studies was the discovery that 3-dehydroshikimic acid possesses commercially-relevant antioxidant activity.

Microbe catalyzed, direct conversion of glucose into gallic acid followed a route where 3-dehydroshikimic acid was converted into protocatechuic acid followed by hydroxylation catalyzed by a mutant isozyme of *p*-hydroxybenzoate hydroxylase. The toxicity of gallic acid towards microbes required fine tuning of the expression of a variety of genes and extensive optimization of fermentor conditions. Recombinant *E. coli* synthesized 20 g/L of gallic acid and 22 g/L of protocatechuic acid when cultured under fed-batch fermentor conditions. Conversion of 3-dehydroshikimic acid and protocatechuic acid into gallic acid using intact *E. coli* constructs was also examined. An abiotic aromatization of microbially-produced 3-dehydroshikimic acid and 3-dehydroquinic acid to protocatechuic acid was also examined.

Protocatechuate decarboxylase encoded by the *aroY* locus cloned out of *Klebsiella pneumoniae* was discovered to decarboxylate gallic acid in addition to protocatechuic acid. Decarboxylation of gallic acid to pyrogallol and protocatechuic acid to catechol was accomplished under carefully controlled fermentor conditions using native *K. oxytoca* or recombinant *E. coli* expressing *aroY*-encoded protocatechuate decarboxylase. Microbe-catalyzed decarboxylation of gallic acid and protocatechuic acid provided pyrogallol and catechol, respectively, which was amenable to straightforward isolation and purification. The yields of pyrogallol and catechol microbially synthesized from gallic acid and protocatechuic acid were essentially quantitative.

To my parents for their love and support
To my friends in Lansing for their friendship
And encouragement.

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The love and support of my family in Greece gave me the courage and dedication needed for finishing this Ph.D. I know how proud of me they feel now, but they must know that this achievement is half theirs. For the way they brought me up, and the

support and help of any type (moral or financial) they so generously provided with their love to me as the only motive.

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LIST OF ABBREVIATIONS

ADC	4-amino-4-deoxychorismate
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-*O*-methyltransferase
DAH	3-deoxy-D-*arabino*-heptulosonic acid
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DCU	digital control unit
DEAE	diethylaminoethyl
2, 3-DHB	2,3-dihydroxybenzoic acid
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
L-DOPA	L-3,4-dihydroxyphenylalanine
DO	dissolved oxygen
E4P	D-erythrose 4-phosphate

EPSP	5-enolpyruvoylshikimate 3-phosphate
FBR	feed-back resistant
FBS	feed-back sensitive
GA	gallic acid
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase
Km	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	*p*-aminobenzoic acid
PCA	protocatechuic acid
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PHB	*p*-hydroxybenzoic acid

PID	proportional-integral-derivative
Pck	PEP carboxykinase
PCR	polymerase chain reaction
Phe	L-phenylalanine
PMSF	phenylmethylsufonyl fluride
PobA	*p*-hydroxybenzoate hydroxylase wild type
PobA*	*p*-hydroxybenzoate hydroxylase mutant
Ppc	PEP carboxylase
Pps	PEP synthase
PTS	phosphotransferase system
QA	quinic acid
RBS	ribosome binding site
rpm	round per minute
SA	shikimic acid
SAM	*S*-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-d*$_4$
Tyr	L-tyrosine
UDP	uridine diphosphate
UV	ultraviolet

CHAPTER 1

I. 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 figure prominently in industrial chemical manufacturing. 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 this thesis, syntheses of the value-added industrial chemicals 3,4,5-trihydroxybenzoic acid (gallic acid, GA), 1,2,3-trihydroxybenzene (pyrogallol, PGL), 3,4-dihydroxybenzoic acid (protocatechuic acid, PCA) and 1,2-dihydroxybenzene (catechol) from glucose were evaluated (Figure 1). Chapters 2 and 5, describe the biosynthetic routes to protocatechuic and gallic acids from glucose that were developed through metabolic engineering of the common pathway of aromatic amino acid biosynthesis (the shikimate pathway, Figure 2) in *Escherichia coli*. Various biocatalysts and fermentation conditions were tested and optimized in Chapter 5, before gallic acid could be synthesized in 12% yield from glucose in titers of 20 g/L. A hybrid chemical and biocatalytic strategy for the synthesis of protocatechuic acid relied on the abiotic, thermal dehydration of the early common pathway intermediates 3-dehydroquinic acid (DHQ, Figure 1) and 3-dehydroshikimic acid (DHS, Figure 1) as discussed in Chapter 2. Chapter 3 describes the development of novel catalytic methodology for the chemical oxidation of 3-dehydroshikimic acid into gallic acid. Potent commercially-relevant antioxidant activity of 3-dehydroshikimic acid was also discovered as a result of the detailed mechanistic studies of its chemical oxidation. Biocatalytic oxidation of 3-dehydroshikimic acid or protocatechuic acid into gallic acid using recombinant *E. coli* cultured under fermentor

conditions is discussed in Chapter 4 and compared with the methodology developed
Chapter 3 for the chemical oxidation of 3-dehydroshikimic acid to gallic acid (Figure
Chapter 6 describes how *Klebsiella oxytoca M5a1* and recombinant *E. coli* were cultur
under fermentor conditions for the high-yielding decarboxylation of both gallic acid a
protocatechuic acid into pyrogallol and catechol, respectively (Figure 1).

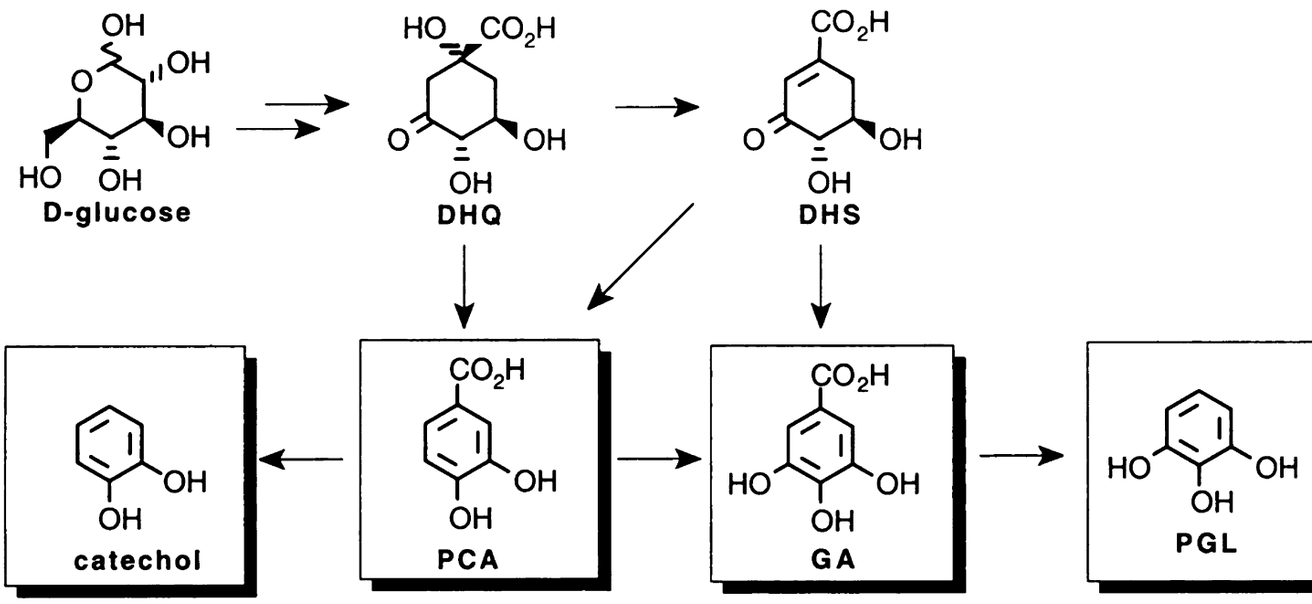


Figure 1. Synthetic pathways and intermediates of the syntheses of PCA, GA, PG and catechol.

Comparison of the one-step versus the hybrid two-step synthesis of gallic ac
and protocatechuic acid demonstrated the power and limitations of the use of microb
cultured under fermentor conditions to synthesize industrial chemicals from renewab
glucose feedstocks. When the targeted end products limit microbe-catalyzed synthes
due to their toxicity towards the microbial catalyst, combining chemical synthesis wi
biocatalysis can serve as a potential solution. Using biocatalysis, high titers (60-70 g/
of non-toxic microbial metabolites (such as 3-dehydroshikimic acid and 3-dehydroquin

acid) can be synthesized in moderate yields (20% to 40%) from glucose, and then chemically transformed into the desired industrial product. These hybrid processes can increase the number of products that can be practically synthesized from glucose using biocatalysis. Some of the potential problems that can be encountered with direct, one-step synthesis of aromatics from glucose using microbial catalysts are discussed in detail in this thesis, such as the case of genetically engineering *E. coli* to synthesize protocatechuic acid and gallic acid.

Utilizing microbes to catalyze single organic reactions is another useful comparison aspect of this thesis. The high reaction rates, substrate specificity, as well as the regioselectivity and stereoselectivity are some of the known advantages of enzymatic reactions.[5] However, their industrial use is limited by enzymatic stability and requirements for cofactors which are expensive and difficult to regenerate. Using intact microbes expressing native or amplified levels of a desired enzyme can circumvent the aforementioned problems. However, its practical usefulness is not always guaranteed. In this thesis, microbial oxidation of 3-dehydroshikimic acid leads to incomplete reactions, while the decarboxylation of both gallic acid and protocatechuic acid is fast, high-yielding, and the product purification is straightforward. Employment of fermentors for the cultivation of the microbial catalysts of interest allows reactions to be catalyzed under conditions where temperature, pH and dissolved O_2 (D.O.) levels can be controlled.

II. 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.[6] It consists of seven, enzyme-catalyzed reactions converting phosphoenolpyruvic acid (PEP) and erythrose 4-phosphate (E4P) into chorismic acid (Figure 2). 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 2).[6] Folic acid-derived coenzymes are frequently involved in the biosynthetic transfer of one carbon fragments. Ubiquinones are involved in electron transport. Enterochelin is an iron chelator responsible for iron uptake in numerous microorganisms.

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 2) 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

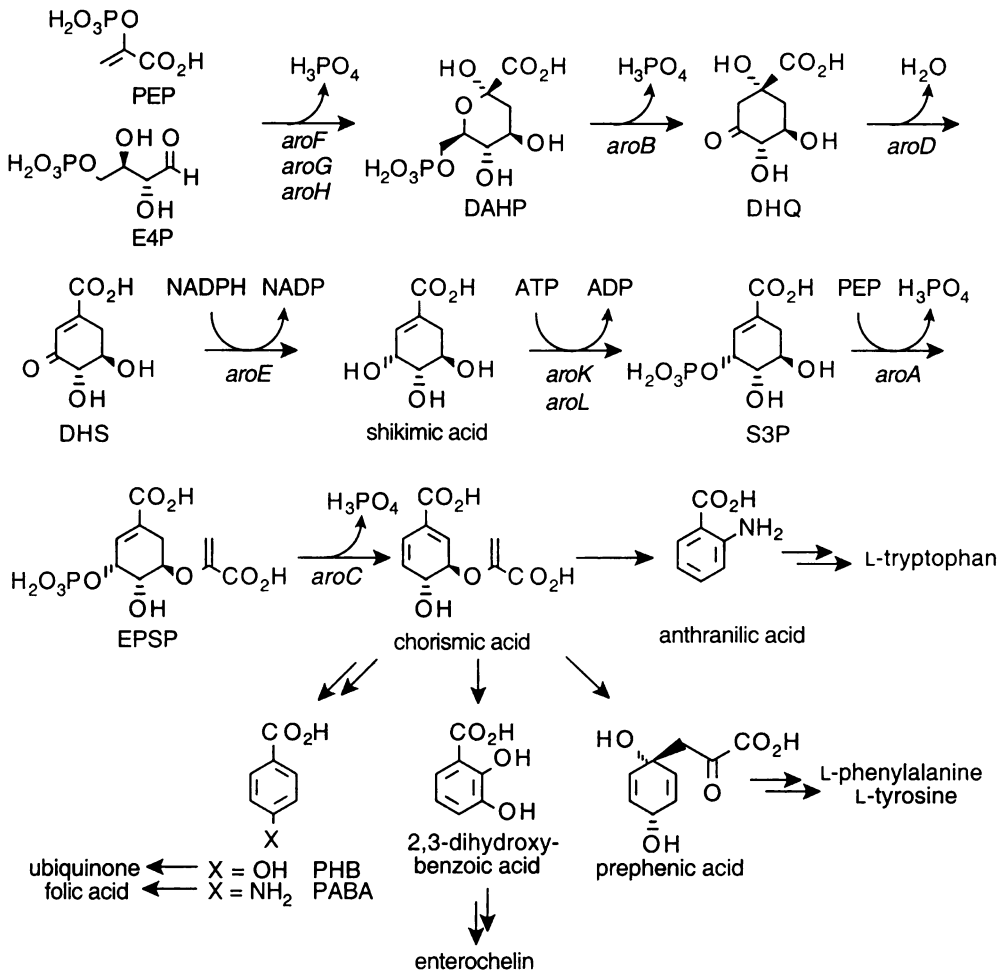


Figure 2. The common pathway of aromatic amino acid biosynthesis. Genetic loci are as follows: *aroF aroG aroH*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroL aroK*, shikimate kinase; *aroA*, EPSP synthase; *aroC*, chorismate synthase.

DAHP synthase.[6] 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*[7] in a complex reaction where active site NAD is used as a catalytic active site residue.[7] A *syn* elimination of water from DHQ affords 3-dehydroshikimic acid (DHS).[8] 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.[9] 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*[10] and *aroK*.[11] 5-Enolpyruvoylshikimate 3-phosphate (EPSP) synthase, encoded by *aroA*,[12] 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.[13] Encoded by *aroC*, it catalyzes the elimination of inorganic phosphate from EPSP to afford chorismic acid.

III. MICROBIAL SYNTHESIS 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.[14] 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 3),[15] which has the largest sales volume of all food additives.[16] Introduction of naphthalene dioxygenase into a tryptophan-synthesizing microbe that also expresses tryptophanase results in biocatalytic synthesis of indigo (Figure 3),[17] 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 3),[18] the mammalian pigmentation that is responsible for hair coloring as well as protection from solar irradiation.

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.[19] Because the current isolation of shikimic acid from the fruit of *Illicium* plants[20] precludes its use in kilogram-level synthesis, an *E. coli* biocatalyst has been constructed which synthesizes 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.[21]

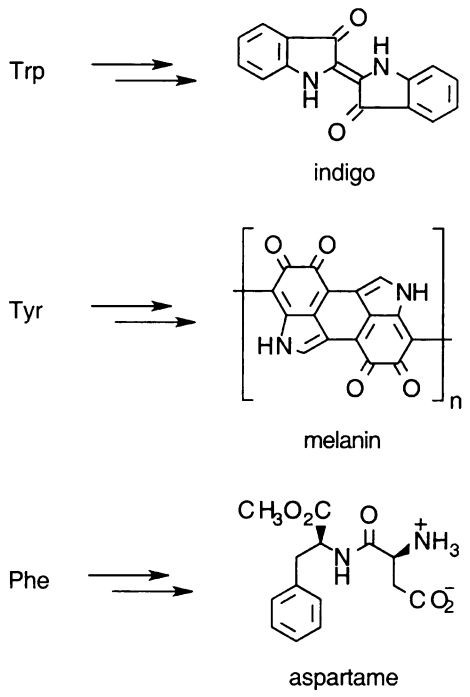


Figure 3. Chemicals synthesized from L-tryptophan, L-tyrosine, and L-phenylalanine.

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 4). 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 sequenced.[22] 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 b esterified to form the local anesthetic known as benzocaine.[23]

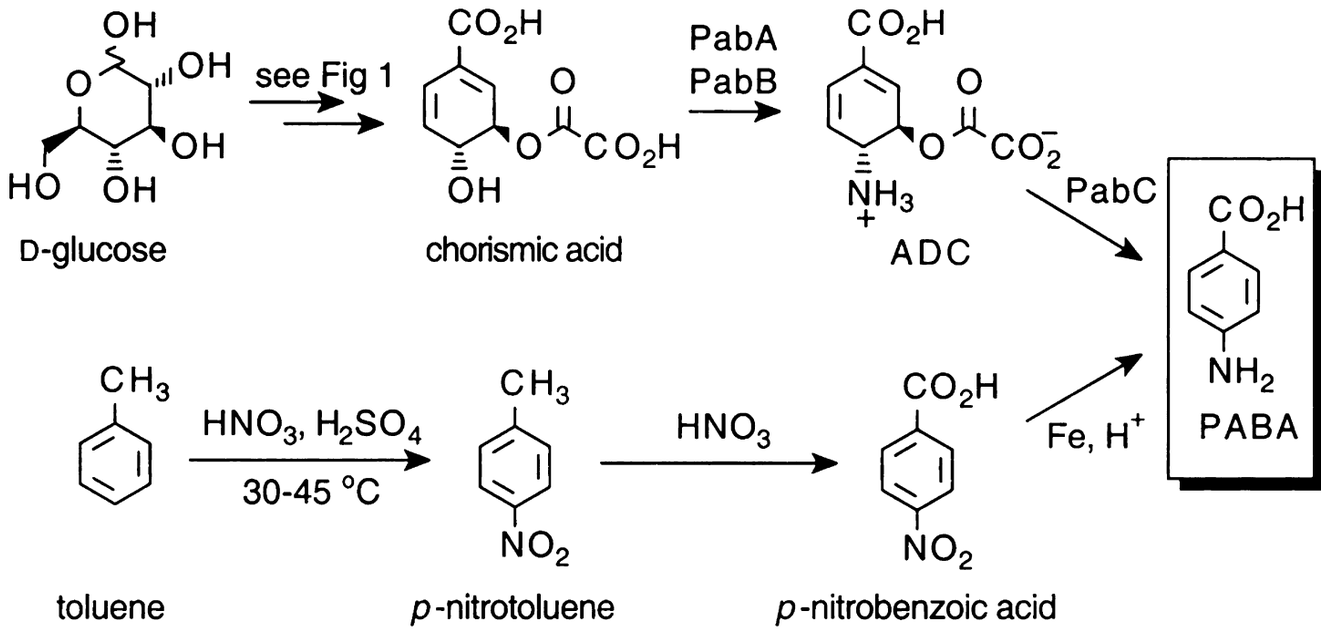


Figure 4. Comparison of microbial and chemical synthesis of *p*-aminobenzoic aci (PABA).

PABA is currently industrially synthesized from toluene (Figure 4). The firs step entails reaction of toluene with a 20/60 (v/v) of nitric acid/sulfuric acid at 30-45 °C.[2] *p*-Nitrotoluene is then separated from the other nitrotoluene isomers by sequentia distillation and crystallization. Oxidation of the methyl group of *p*-nitrotoluene wit nitric or chromic acids is followed by reduction to afford PABA. Almost every step o this manufacturing route has to contend with a health/safety hazard. These range fro strong acid solutions to manipulation of toxic, flammable toluene and highly toxi *p*-nitrotoluene.[24]

Chorismic acid can also be converted directly into *p*-hydroxybenzoic acid in a reaction catalyzed by *ubiC*-encoded[25] chorismate-pyruvate lyase (Figure 5). 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 2). The biocatalyst can synthesize 12 g/L of *p*-hydroxybenzoic acid from glucose under fed-batch fermentor conditions.[26] *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 5).[26] *p*-Hydroxybenzoic acid is a component of liquid crystal polymers such as Xydar,[27] 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.[28]

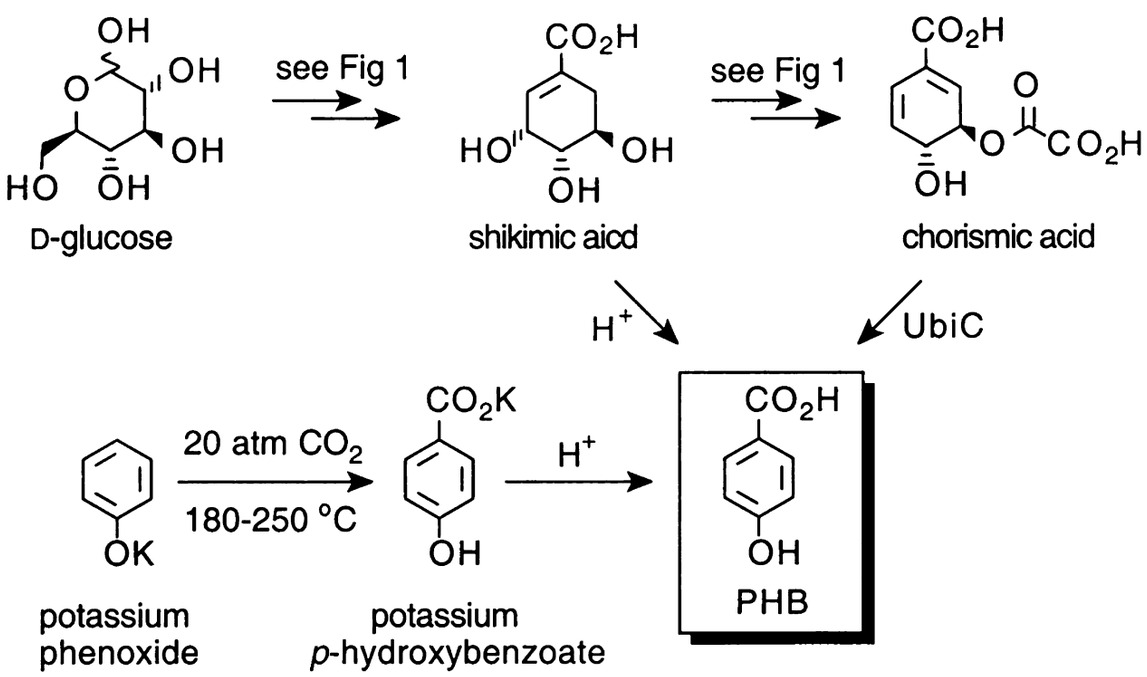


Figure 5. Comparison of *p*-hydroxybenzoic acid (PHB) synthesis from glucose versus synthesis from potassium phenoxide.

p-Hydroxybenzoic acid is currently manufactured by Kolbe-Schmitt reaction o dried potassium phenoxide with 20 atm dry carbon dioxide at 180-250 °C (Figure 5).[2] Product *p*-hydroxybenzoate potassium salt is converted to its free acid upon addition o mineral acid. Besides the required temperatures and pressures, *p*-hydroxybenzoic aci manufacture has to contend with handling of phenol, which is listed as a highly toxic corrosive chemical.[24]

Quinic acid is another useful molecule that can be microbially synthesized throug the utilization of the shikimate pathway (Figure 6).[29] Quinic acid is a widely used chira starting material in multi-step chemical synthesis.[30] It is currently obtained by a expensive isolation from plant sources. An *E. coli* biocatalyst has been constructed vi mutational inactivation of *aroD*-encoded DHQ dehydratase, overexpression of $aroF^{FBR}$ encoded feedback-insensitive DAHP synthase and overexpression of *aroE*-encode shikimate dehydrogenase. The biocatalyst can synthesize up to 80 g/L of quinic acid.[3] Quinic acid can be converted into either benzoquinone or hydroquinone through simpl chemical oxidation (Figure 6).[29] Hydroquinone's selective reduction of photoactivate silver ion is the basis for this organic's widespread use in photography. Benzoquinone i an important chemical precursor in the manufacture of various chemicals.[32]

Hydroquinone is produced globally at volumes of 4.5 - 5.0 x 10^7 kg/year.[33] Th dominant route to manufacture hydroquinone is through hydroperoxidative synthesis.[3] The *p*-diisopropylbenzene is synthesized by Zeolite-catalyzed Friedel-Crafts reaction o benzene or cumene with propylene or isopropanol. Air oxidation of th *p*-diisopropylbenzene proceeds at 90 - 100 °C in an aqueous NaOH solution als containing

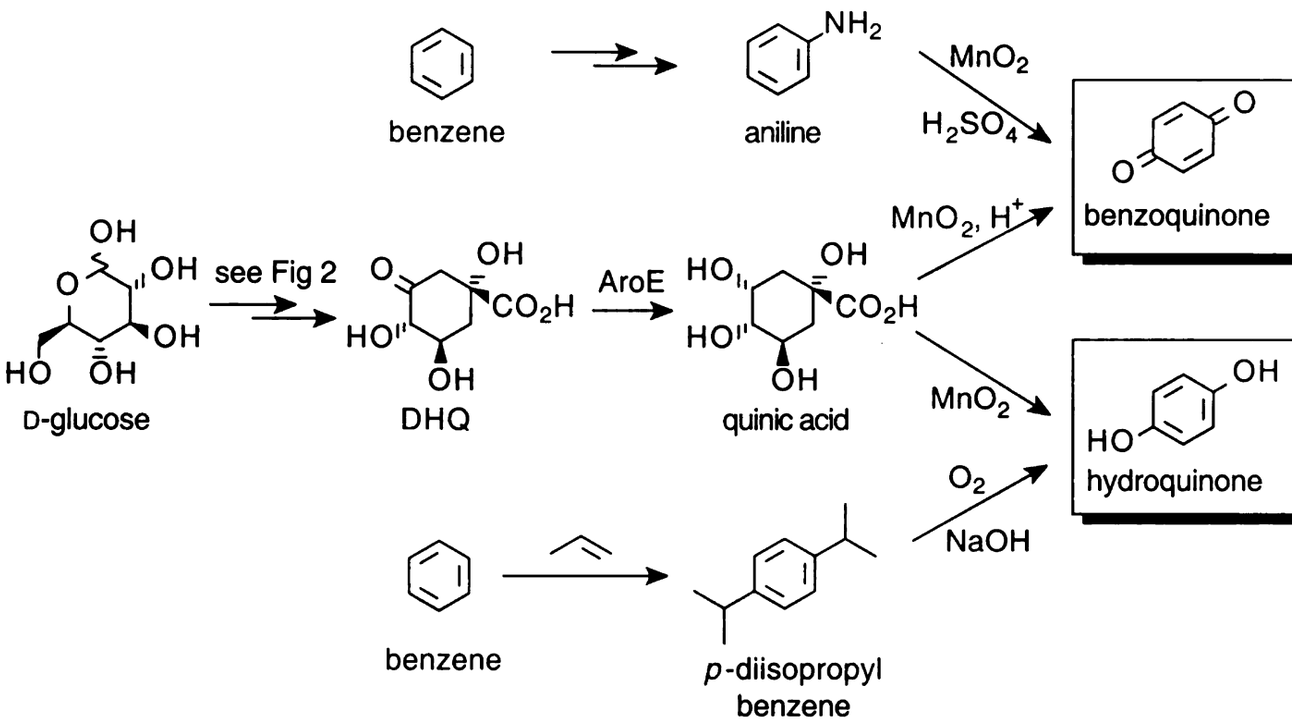
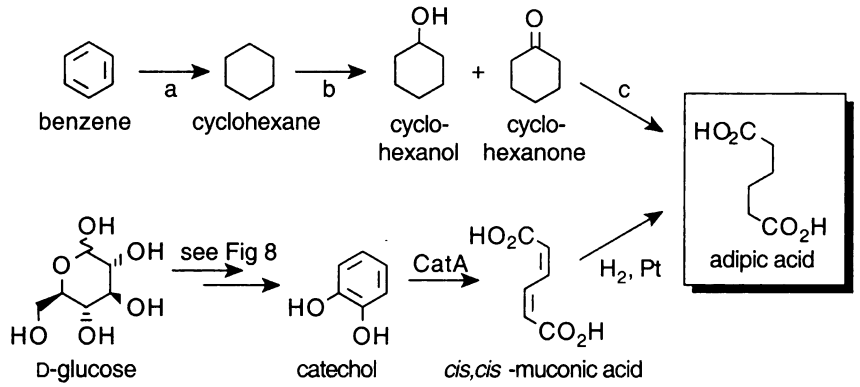


Figure 6. Comparison of benzoquinone and hydroquinone synthesis from glucos and benzene

organic bases along with cobalt or copper salts. Hydroperoxycarbinol and dicarbinol ar produced along with the dihydroperoxide during air oxidation. Treatment with acid ar H_2O_2 converts the hydroperoxycarbinol and dicarbonyl to the dihydroperoxide which cleaved to form acetone and hydroquinone. During the acidic cleavage, explosive organ peroxide can form which presents a safety hazard.[33] Benzoquinone is primari manufactured from oxidation of aniline (Figure 6). The oxidant employed is MnO_2 aqueous H_2SO_4 (Figure 6). Benzoquinone can also be reduced by Fe or hydrogenated t afford hydroquinone. Although accounting for approximately 10% of hydroquinor production,[33] this manufacturing route generates large quantities of $MnSO_4$, $(NH_4)_2SO$ and iron oxide salts.[33]



a = Ni-Al_2O_3, H_2, 370-800 psi, 150-250 °C
b = Co, O_2, 120-140 psi, 150-160 °C
c = Cu, NH_4VO_3, 60% HNO_3, 60-80°C

Figure 7. Comparison of microbial and chemical synthesis of adipic acid.

Inclusion of a *catA* gene encoding catechol 1,2-dioxygenase in a catechol-producing microbe converts catechol into *cis,cis*-muconic acid (Figure 7).[34] The *catA* gene was isolated from *Acinetobacter calcoaceticus*.[35] Catalytic hydrogenation at 50 psi and room temperature using 10% Pt on carbon converts the *cis,cis*-muconic acid into adipic acid.[34] Primarily used in production of nylon-6,6, the annual global demand for adipic acid exceeds 1.9 x 10^9 kg.[36] Benzene is the principal starting material from which adipic acid is currently synthesized (Figure 7). Hydrogenation of benzene to produce cyclohexane is followed by air oxidation to yield a mixture of cyclohexenol and cylohexanone. Nitric acid oxidation then yields adipic acid and nitrous oxide,[37] which is involved in ozone depletion and global warming.[38] Adipic acid production may account for some 10% of the annual increase in atmospheric nitrous oxide levels.[37]

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CHAPTER 2

BIOCATALYTIC CONVERSION OF GLUCOSE TO PROTOCATECHUIC ACID VERSUS ABIOTIC AROMATIZATION OF 3-DEHYDROSHIKIMIC ACID AND 3-DEHYDROQUINIC ACID

I. INTRODUCTION

Protocatechuic acid (PCA) is the common name for 3,4-dihydroxybenzoic acid. Although an industrial large-scale synthesis of this molecule is not available, protocatechuic acid could be a useful starting material for the synthesis of various pharmaceutical and industrial chemicals. For example the PCA-containing structure is part of various drugs used for the treatment of asthma such as Hexoprin[1] (**1**), Rolipram[2] (**2**) and the benzamide[2] (**3**) (Figure 8). In addition to their bronchodialator activity in the treatment of asthma, Epinefrin (**4**) (R: Me) and isoproterenol (**4**) (R: iPr) are two PCA-containing medicines that have also been used in the treatment of glaucoma and cardiovascular disease as cardiostimulants (Figure 8).[3] Mebeverine (**5**), is a PCA-containing molecule that exerts a spasmolytic effect on colonic as well as gastric contraction, and has also been used for the of irritable colon syndrome.[4] Protocatechuic acid and gallic acid have been recently used in the synthesis of catechol-*o*-methyltransferase (COMT) inhibitors of the general structures (**6**), to combat disorders of catecholamine metabolism such as Parkinsons disease.[5] The antitumor agents tomaymycin and chicamycin (**7**) as well as their synthetic analogous are synthesized from protocatechuic acid (Figure 8).[6]

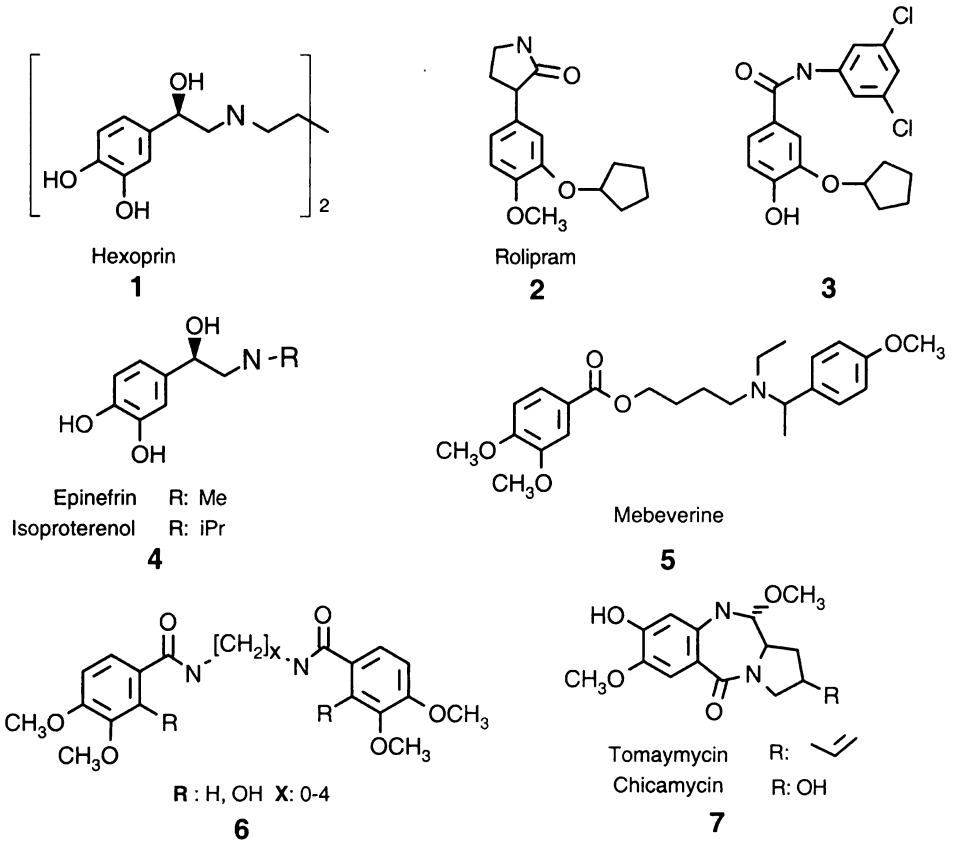


Figure 8. Pharmaceuticals derived from protocatechuic acid

Besides its potential utility as a synthon in the pharmaceutical industry, protocatechuic acid is also a valuable intermediate in the synthesis of catechol, adipic acid, gallic acid, pyrogallol, and vanillin (Figure 9). Decarboxylation of protocatechuic acid using intact *Klebsiella oxytoca M5a1* or recombinant *E. coli* bacteria can produce catechol in high yields and purities as discussed in Chapter 6 of this thesis. In Chapter 4 and Chapter 5, the intermediacy of protocatechuic acid in the biosynthesis of gallic acid is discussed (Figure 9). Protocatechuic acid is also an important intermediate in the biocatalytic synthesis of vanillin,[7] which is an important component of flavors and

fragrances. The commercial synthesis of vanillin, adipic acid and catechol are all derived from petroleum-based non-renewable starting materials.[8] Introducing biocatalytically-synthesized protocatechuic acid as the starting material for the synthesis of the aforementioned industrial chemicals is therefore preferable. Besides the obvious environmental advantages that such a process will provide, the required feedstock consists of abundantly-available, renewable starch, cellulose and hemicellulose.[9] In addition, introducing a cost effective route to protocatechuic acid might increase the industrial utilization of this molecule.

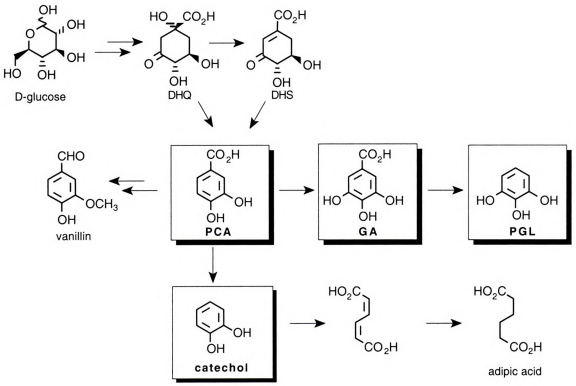


Figure 9. Industrial chemicals derived from protocatechuic acid

In this Chapter, a one-step biocatalytic synthesis of protocatechuic acid from glucose as well as two, two-step syntheses proceeding through intermediacy of microbially-synthesized 3-dehydroshikimic (DHS) acid and 3-dehydroquinic acid (DHQ)

are investigated. In both routes, the common pathway of aromatic amino acid biosynthesis is utilized. In the two-step syntheses, fermentation supernatants containing either 3-dehydroshikimic acid or 3-dehydroquinic acid are thermally dehydrated to protocatechuic acid in high yields. Therefore, the scale of protocatechuic acid production using this hybrid route is dependent on the 3-dehydroshikimic acid and 3-dehydroquinic acid titers that can be achieved using recombinant *E. coli* biocatalysts. In the one-step synthesis, introduction of the *aroZ*-encoded DHS dehydratase protein in the biocatalyst that synthesizes 3-dehydroshikimic acid results in a biocatalyst capable of converting glucose directly into protocatechuic acid. Although the introduction of *aroZ* allows for the most concise synthesis of protocatechuic acid, a key question is whether protocatechuic acid is as nontoxic to the *E. coli* biocatalyst as are 3-dehydroshikimic acid and 3-dehydroquinic acid.

II. SYNTHESIS OF PROTOCATECHUIC ACID

A. Abiotic aromatization of 3-dehydroshikimic acid and 3-dehydroquinic acid

The conversion of bioproducts into valuable industrial and pharmaceutical chemicals is gaining in popularity as a way to replace petroleum with glucose as a source of carbon for chemical synthesis. The common pathway of aromatic amino acid biosynthesis is an excellent source of aromatic acids and hydroaromatics amenable to aromatization. For example, examination of the early pathway intermediates 3-dehydroshikimic acid (DHS) and 3-dehydroquinic acid (DHQ) indicates that these hydroaromatics should be reactive and provide ready access to protocatechuic acid. A simple dehydration of either 3-dehydroshikimic acid or 3-dehydroquinic acid gives the α,β unsaturated intermediates I - III, which would be expected to rapidly tautomerize to afford the more stable aromatic structure of protocatechuic acid (PCA, Figure 10).

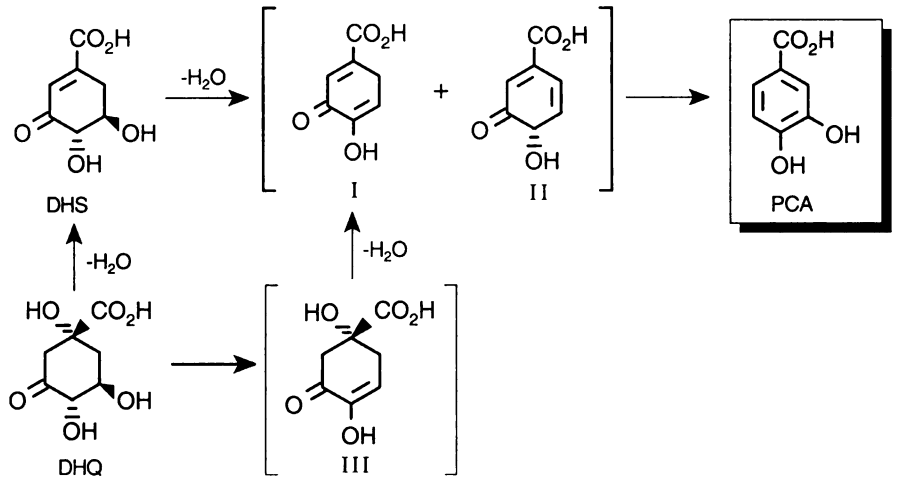


Figure 10. DHS and DHQ dehydration intermediates.

The abiotic aromatization of both 3-dehydroquinic acid and 3-dehydroshikimic acid into protocatechuic acid was investigated, and the reaction conditions were optimized for the dehydration of 3-dehydroshikimic acid (Table 1). Refluxing fermentation broths under various reaction conditions afforded protocatechuic acid in yields varying from 55 to 76% (Table 1). These observed yields were relatively lower than what was expected for a simple dehydration reaction. One possible explanation is that 3-dehydroshikimic acid is oxidative labile. Phosphate-containing solutions (pH~7) of 3-dehydroshikimic acid decompose rapidly in the presence of O_2 and H_2O_2 (Chapter 5).[10] Mechanistic studies showed that phosphate was catalyzing the formation of various enediol intermediates, which rapidly react with O_2 or H_2O_2 (Chapter 3, Figure 23).[10] Enediols of 3-dehydroshikimic acid could also form under dehydration reaction conditions resulting in reaction with O_2 and lowered yields of protocatechuic acid. However, switching to N_2 atmosphere instead of air did not improve the reaction yield (Table 1).

Table 1. Dehydration of DHS under various conditions

			Yield %		
entry	pH	Conditions	DHS	PCA	Time (h)
1.	7	Air	–	72	2
2.	7	N_2	–	76	2
3.	8	Air	–	58	2
4.	8	N_2	–	57	2
5.	6	Air	–	74	3
6.	6	N_2	–	72	3
7.	2	Air	30	55	22

Decreasing the pH of the reaction lowered the dehydration rate and led to incomplete reaction even after 22 h of reflux (Table 1). The same stability to acidic conditions was observed when all the 3-dehydroshikimic acid was recovered after purified

material was stirred in an AcOH/H_2O mixture (v:v, 85:15) at 50 °C for 24 h. However, addition of catalytic amounts of ZnO (0.5 equiv. relative to DHS) catalyzed the formation of protocatechuic acid (80% yield) under identical conditions after 16 h (Chapter 3, Table 8, entry 3).

The best and simplest dehydration conditions appear to entail the refluxing of 3-dehydroshikimic acid at neutral pH under air or N_2 atmospheres. These reaction conditions were then applied to the conversion of 3-dehydroquinic acid into protocatechuic acid. Filtering 500 mL of fermentation supernatant that contained either 3-dehydroshikimic acid or 3-dehydroquinic acid through an Amicon filter to remove all the soluble proteins, and refluxing for 2 h under air, produced protocatechuic acid as the major product. The dehydration rates of both 3-dehydroshikimic acid and 3-dehydroquinic acid were followed by taking aliquots from refluxed supernatants and analyzing them by ^{1}H NMR spectroscopy every 15 min (Figure 11). Although both reactions were completed within 2 h, 3-dehydroshikimic acid gave a lower yield (72%) compared to 3-dehydroquinic acid (85%). During the dehydration of 3-dehydroquinic acid, a steady concentration of 3-dehydroshikimic acid was present throughout the reaction (Figure 11B) indicating that at least part of product protocatechuic acid was coming through 3-dehydroshikimic acid intermediacy in addition to intermediate III (Figure 10). Product purification consisted from an extraction with EtOAc, filtering through charcoal, and concentration of the organic layer until the concentration of protocatechuic acid was approximately 0.5 M. Addition of two volumes of petroleum ether to this solution precipitates protocatechuic acid, which is then recovered by filtration. Typical isolated yields calculated from the starting 3-dehydroshikimic acid (mol/mol) range from 60 to 65%.

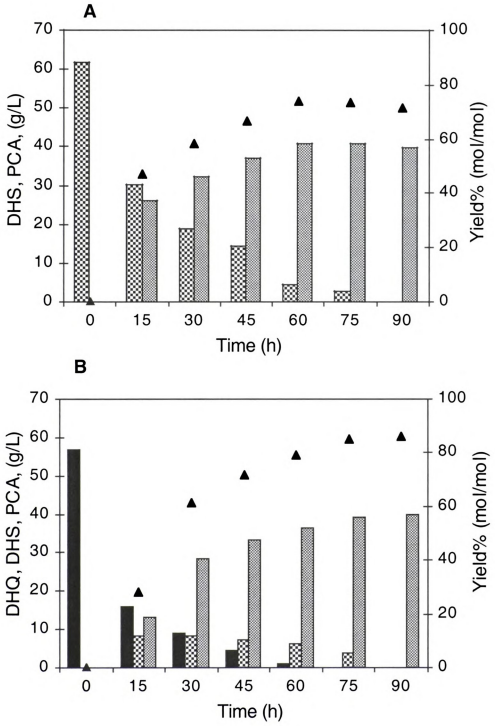


Figure 11. Thermal dehydration of A, DHS; and B, DHQ; DHQ ( ), DHS ( ), PCA ( ) and % yield (▲).

B. Comparison of the biocatalytic synthesis of 3-dehydroquinic acid and 3-dehydroshikimic acid from glucose

The utility of dehydrating either 3-dehydroshikimic acid or 3-dehydroquinic acid as a route to protocatechuic acid ultimately depends on the yield and titers of these hydroaromatics that can be synthesized by microbes. Recombinant *E. coli* cultured under fed-batch fermentor conditions can accumulate 3-dehydroshikimic acid in concentrations as high as 69 g/L.[11] If the same biosynthetic machinery is introduced into host cells that can accumulate 3-dehydroquinic acid, similar titers of this molecule are expected to form.

The host strain used for the construction of the DHQ-synthesizing biocatalyst was *E. coli* QP1.1. The ancestral strain used to construct QP1.1 was *E. coli* AB2848, which contained a mutation in its genomic *aroD* locus.[12] Insertion of an extra *aroB* gene into the *serA* locus of the AB2848 genome created QP1.1.[13] The absence of catalytically-active, *aroD*-encoded DHQ dehydratase, which catalyzes the conversion of 3-dehydroquinic acid into 3-dehydroshikimic acid, resulted in the accumulation of DHQ in culture supernatants of QP1.1 (Figure 2, Chapter 1). Insertion of a second copy of the *aroB*-encoded DHQ synthase in the genome was required for the elimination of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) accumulation.[14] Carbon flow directed into the common pathway was increased upon expression of plasmid-localized $aroF^{FBR}$-encoded DAHP synthase. $AroF^{FBR}$ is a feedback insensitive isozyme of DAHP synthase that was isolated after mutation of native *aroF* followed by selection for more rapid growth in a diffusion gradient chamber against an increasing concentration of *m*-fluorotyrosine.[15] Site-specific insertion of genetic elements in the genomic *serA* locus eliminated 3-phosphoglycerate dehydrogenase activity (Figure 64 Chapter 5), which provided the basis for plasmid maintenance. Due to the *serA* mutation, QP1.1 was unable to synthesize L-serine, and as a result it could only grow if this amino acid was added to the culture medium or upon expression of plasmid-localized *serA*.

Growth of *aroD* mutants of *E. coli*, such as *E. coli* AB2848, 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 acid 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. More details concerning the construction and cultivation under fermentor conditions of such organisms are discussed in Chapter 5.

A biocatalyst very similar to the DHQ-synthesizing constructs was utilized for the accumulation of 3-dehydroshikimic acid. Host strain *E. coli* KL3 contained a mutation in the *aroE* locus. The resulting absence of shikimate dehydrogenase activity results in the accumulation of 3-dehydroshikimic acid in the culture supernatant.[10] For the same purposes discussed above, an extra copy of *aroB* was inserted in the *serA* locus of the genome. The nutritional requirements of QP1.1 also apply to KL3 as a result of the inability of both microbes to synthesize aromatic amino acids and vitamins.

In both DHS- and DHQ-synthesizing constructs, DAHP synthase expression levels as well as the availability of this enzyme's substrates dictate the amount of carbon that is channeled to the common pathway. For this reason, the feedback-insensitive DAHP synthase and transketolase encoded by $aroF^{FBR}$ and *tktA*, respectively, were plasmid-localized to increase expression levels. The role of *tktA*-encoded transketolase overexpression was to increase erythrose 4-phosphate availability. As one of the two substrates of DAHP synthase, the availability of erythrose-4-phosphate has been demonstrated to be limiting in vivo.[11] Depending on the host strain transformed with the $aroF^{FBR}$, *tktA*, serA-encoding plasmid 3-dehydroquinic acid or 3-dehydroshikimic acid was being synthesized. The two most successful DHS-synthesizing biocatalysts contained either one copy of *tktA* along with one copy of $aroF^{FBR}$ under the control of a

strong *tac* promoter (pKL4.124A), or one copy of *tktA* and two copies of *aroF*^{FBR} under the control of their native promoters (pKL4.130A).[11]

Table 2. Comparison of the biocatalytic titers and yields of DHS versus DHQ.

Catalyst	Conc. (g/L) DHS	Conc. (g/L) DHQ	Yield[c] (%)	Relevant Genes
KL3/pKL4.130[a]	69		36	*aroF*^{FBR} ,*aroF*^{FBR}, *tktA, serA*
QP1.1/pKL4.130		(53) 59[b]	40	*aroF*^{FBR} ,*aroF*^{FBR}, *tktA, serA*
KL3/pKL4.124A[a]	52		24	P_{tac}*aroF*^{FBR},*tktA, serA*
QP1.1/pKL4.124A		(43) 47[b]	19	P_{tac}*aroF*^{FBR},*tktA, serA*

a. Ref. 10; b. The maximum value of DHQ is reported. Product titers at the end (48 h) of the fermentation run are in parenthesis c. Yields (mol/mol) of DHQ or DHS based on glucose consumed were calculated at 48 h.

QP1.1/pKL4.124A grew vigorously in a fermentor, reaching the third phase of D.O. control at 10 h and stationary phase at 30 h (Figure 12A). The activity of DAHP synthase was controlled by the amount of IPTG (5 mg) that was added every 6 h starting at 12 h. This concentration of inducer was found to give the highest product titers in the similar DHS-synthesizing biocatalyst (KL3/pKL4.124A).[10] Comparable 3-dehydroshikimic acid (52 g/L) and 3-dehydroquinic acid (47 g/L at 36 h) titers were obtained using this plasmid (Table 2). The synthesis of 3-dehydroquinic acid, however, leveled off after 36 h, and a small product loss (43 g/L at 48 h) was observed during the last 12 h of the fermentation (Figure 12A, Table 2).

Cell growth of QP1.1/pKL4.130 was significantly slower relative to QP1.1/pKL4.124A. QP1.1/pKL4.130 reached the third phase of D.O. control at 18 h and stationary phase of growth at 36-42 h. At 42 h, 3-dehydroquinic acid production terminated. This gave a maximum 3-dehydroquinic acid titer of 59 g/L, whereas at the end of the run (48 h), it decreased to 53 g/L (Table 2, Figure 12B). The yield of

3-dehydroquinic acid synthesized by QP1.1/pKL4.130 even when calculated at 48 h, was slightly higher (40%) compared to DHS-synthesizing KL3/pKL4.130, and approximately double the yield of 3-dehydroquinic acid synthesized by QP1.1/pKL4.124A. The decline in the DHQ concentration after 36 h or 42 h in the fermentation broths of DHQ-synthesizing bacteria might be the result of its oxidation or its further catabolism by the organism. ^{1}H NMR analysis of the supernatant however, indicated formation of only 3-dehydroquinic acid. 3-Deoxy-D-*arabino* heptulosonic acid (DAH) that was present in the fermentation supernatants of DHS-synthesizing biocatalysts was not detectable by 1H NMR in the fermentation broths of all DHQ-synthesizing biocatalysts. Glucose consumption was continued until the end of the fermentation run.

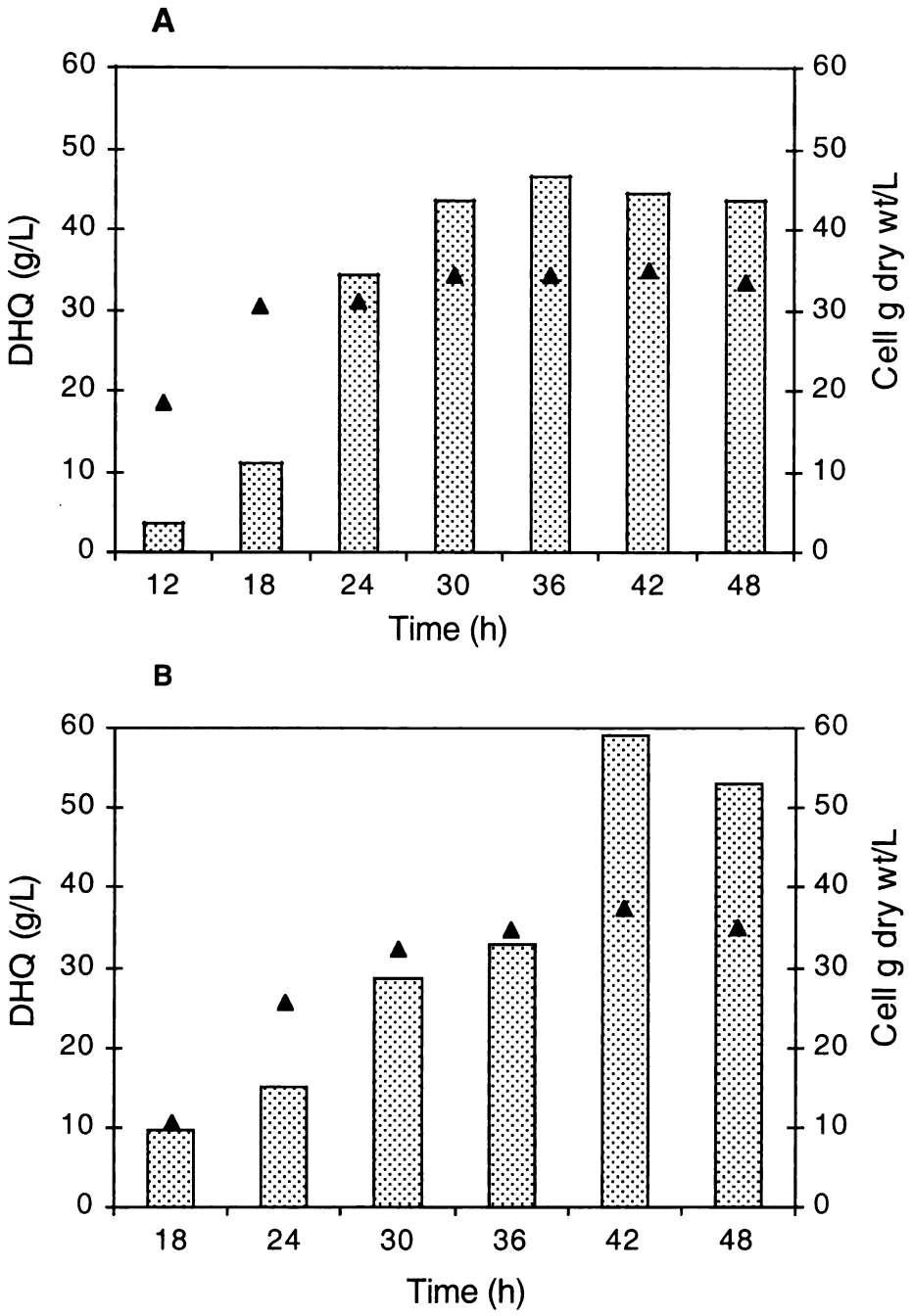


Figure 12. DHQ synthesis under fed-batch fermentor conditions with time. QP1.1/pKL4.124A (A); QP1.1/pKL4.130 (B). DHQ ( ), cell mass (▲)

C. Biocatalytic synthesis of protocatechuic acid from glucose

After exploring the two-step route for the synthesis of protocatechuic acid proceeding via 3-dehydroshikimic acid and 3-dehydroquinic acid intermediacy, the one-step, direct synthesis of protocatechuic acid from glucose was investigated. Introducing the *aroZ* gene encoding DHS dehydratase into a DHS-synthesizing biocatalyst resulted in the synthesis of protocatechuic acid from glucose. The host employed was *E. coli* KL7, which differed from KL3 only in the insertion into the genomic *serA* locus of a cassette containing both *aroB* and *aroZ*.[7] KL7 was also the host utilized for the one-step synthesis of gallic acid from glucose, and is discussed in more detail in Chapter 5 of this thesis.

As a first step, only one plasmid-localized copy of $aroF^{FBR}$ under the control of its native promoter was employed for protocatechuic acid synthesis. The same plasmid also contained an *aroZ* insert to ensure adequate levels of DHS dehydratase expression for complete conversion of 3-dehydroshikimic acid to protocatechuic acid. Plasmid pSK7.54 was constructed after ligation of the *serA* gene in the *Sma*I/CIAP-treated site of pSK4.122 (Figure 13). The 1.9 kb *serA*-containing DNA was isolated as a blunt-end *Dra*I/*Eco*RV digestion of pD2625.

KL7/pSK7.54 cultured under fed-batch fermentor conditions reached the third phase of D.O. control at 13 h and reached stationary phase at 24 h. A steady increase of the protocatechuic acid concentration was observed reaching concentration of 22.5 g/L (17% mol/mol) by the end of the run. Glutamic acid (9.6 g/L) was the only other metabolite synthesized in appreciable amounts (Figure 14).

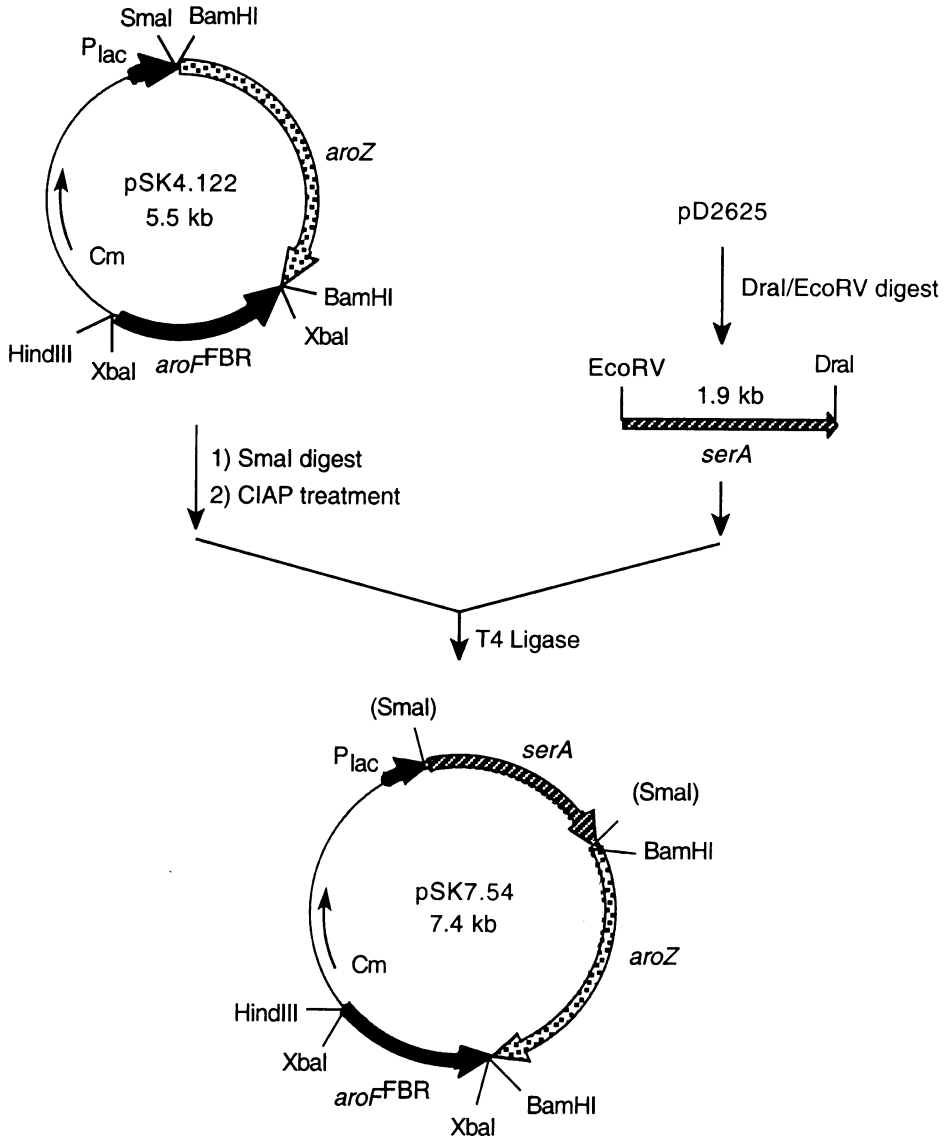


Figure 13. Construction of plasmid pSK7.54

The biosynthesis of glutamic acid as well as possible strategies for preventing its formation are discussed in detail in Chapter 5. When plasmid localization of a single copy of *aroF*^{FBR} was expressed from its native promoter in a DHS-synthesizing biocatalyst, 3-dehydroshikimic acid titers and yields similar to the protocatechuic acid titers and yields synthesized by KL7/pSK7.54 were obtained (Table 3).[11] The purification of protocatechuic acid from the fermentation supernatant consisted of EtOAc extraction followed by partial concentration of the organic layer (500 mM protocatechuic acid). Precipitation of protocatechuic acid as a white powder was achieved after addition of two volumes of petroleum ether relative to the volume of the organic concentrate.

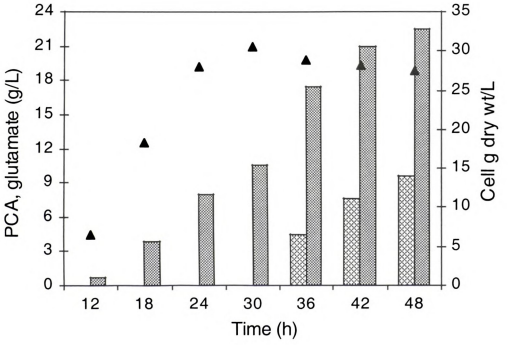


Figure 14. PCA synthesized by KL7/pSK7.54 cultured under fed-batch fermentor conditions. PCA (▨), L-glutamic acid (▩) and cell mass (▲)

Increasing the activity of DAHP synthase by introducing a second plasmid-localized copy of *aroF*^{FBR} resulted in approximately a doubling of the concentration of 3-dehydroshikimic acid synthesized by KL3/pKL4.66A relative to the 3-dehydroshikimic

acid synthesized by KL3/pKL4.33A (Table 3).[10] Since the rate of intracellular protocatechuate formation as well as the final titer did not cause any apparent toxicity problems in KL7/pSK7.54 that expressed a single copy of DHP synthase, a similar strategy was adopted for increasing synthesized protocatechuic acid concentrations. Plasmid pSK7.105 was constructed for this reason. Plasmid pKL4.66B was digested with *Hin*dIII, treated with the Klenow fragment to create blunt ends and dephosphorylated using CIAP. Ligation with the 2.2 kb *aroZ* gene afforded plasmid pSK7.105 (Figure 15). The *aroZ* insert was isolated from pSK4.122 after digestion with *Bam*HI and blunt ends were created using the Klenow fragment.

Table 3. Comparison of DHS and PCA titers and yields when similar biocatalysts grew under fed-batch fermentor conditions.

Catalyst	Conc. (g/L) DHS	Conc. (g/L) PCA	%Yield[b] (mol/mol)	Relevant Genes
KL3/pKL4.33B[a]	20.3		18	*aroF*^{FBR},*serA*
KL7/pSK7.54		22.5	17	*aroF*^{FBR},*aroZ, serA*
KL3/pKL4.66A[a]	38.5		18	*aroF*^{FBR},*aroF*^{FBR}, *serA*
KL7/pSK7.105		10.5	12	*aroF*^{FBR},*aroF*^{FBR}, *aroZ, serA*

a. Ref 11 b. Yield is based in the glucose consumed

KL7/pSK7.105 cultured under fed-batch fermentor conditions gave significantly different results from one run to the next. While in some fermentation experiments KL7/pSK7.105 actually reached the third phase of D.O. control and stayed under D.O. control until 42 h, in other fermentation runs bacteria grew extremely slow with the third phase of D.O. control not reached even after 24 h of cultivation. As a result, these runs were terminated at 24 h. Completed fermentation runs of KL7/pSK7.105 gave after 48 h poor protocatechuic acid titers (10 g/L) and yields (12% mol/mol) (Table 3). DHS-synthesizing biocatalyst KL3/pKL4.66A, which also curried two plasmid-localized

copies of *aroF*^{FBR} expressed from their native promoters, gave 38. 5 g/L of product (Table 3). KL3 and KL7 only differ in the genomic insertion of *aroZ*. Plasmid pSK7.105 is actually constructed from pKL4.66A. Toxicity of protocatechuic acid to the biocatalyst is one possible explanation for the decrease of the protocatechuate synthesis when more carbon flow is directed into the common pathway.

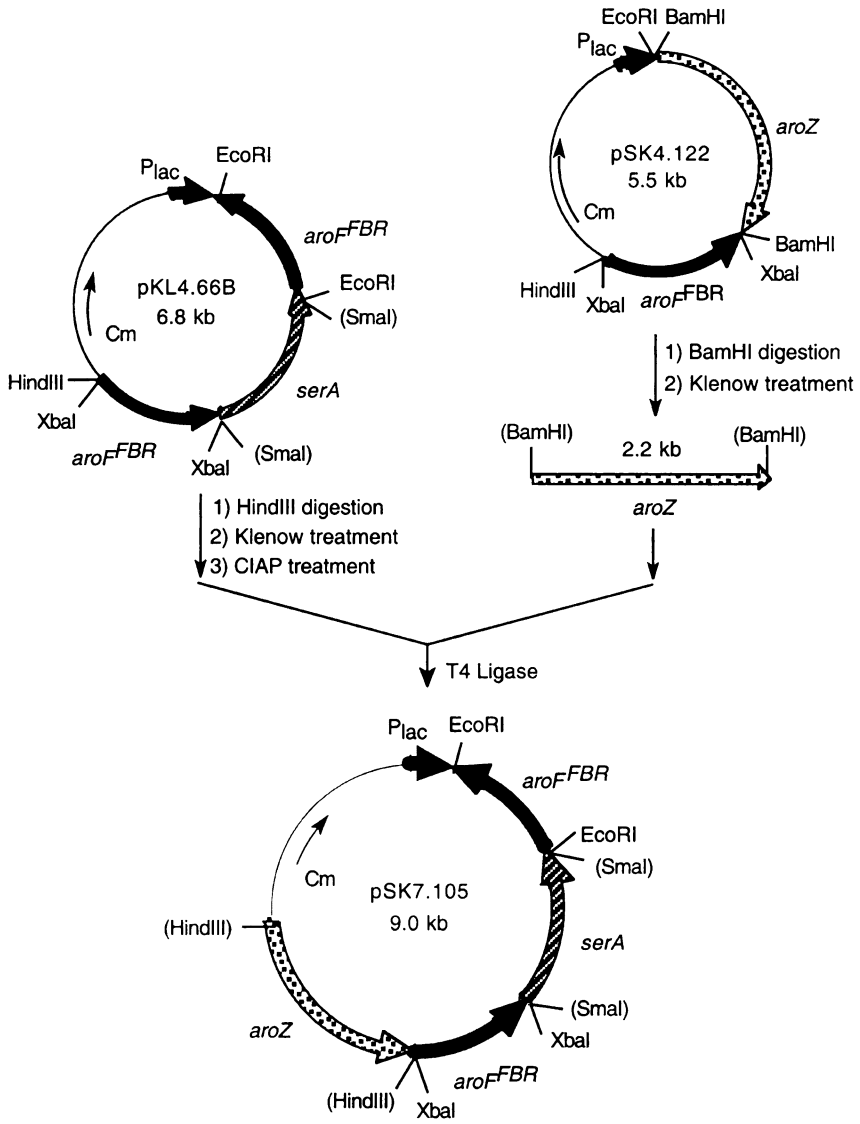


Figure 15. Construction of plasmid pSK7.105

III. DISCUSSION

In this Chapter two basic strategies were investigated for the synthesis of protocatechuic acid from glucose. In the first, microbially synthesized 3-dehydroshikimic acid or 3-dehydroquinic acid were thermally dehydrated to protocatechuic acid. The second route was based on the enzymatic dehydration of 3-dehydroshikimic acid into protocatechuic acid using *aroZ*-encoded DHS-dehydratase. The *aroZ* locus was originally isolated from a *Klebsiella pneumonae* genomic library.[16]

Table 4. Comparison of the PCA titers and yields; one-step versus the two-step synthesis from glucose.

Catalyst	g/L		PCA titer[a] (g/L)	PCA Yield[b] % (mol/mol)
	DHS	DHQ		
KL3/pKL4.130	69		50	26
QP1.1/pKL4.130		59	50	34
KL7/pSK7.54			22.5	17

a. PCA after thermal dehydration of DHS or DHQ or after enzymatic dehydration of DHS.
b. Yield was calculated from the starting glucose.

While the biocatalytic productions of 3-dehydroquinic acid were a bit lower than 3-dehydroshikimic acid when the same biosynthetic genes were utilized (Tables 2 and 4), the one-step protocatechuic acid biosynthesis was a different story. Increasing the intracellular rate of protocatechuate formation by increasing the DAHP synthase activity resulted in cell instability and a general lower titer and yield (Table 3) of synthesized protocatechuic acid. It appears therefore, that the maximum protocatechuic acid concentration that could be tolerated by growing *E. coli* cells during the one-step synthesis from glucose was 22 g/L (Tables 3 and 4). By contrast, bacterial biosynthesis of the non-toxic intermediates 3-dehydroshikimic acid and 3-dehydroquinic acid reached

titers as high as 69 and 59 g/L, respectively. Dehydration of microbially synthesized 3-dehydroshikimic acid and 3-dehydroquinic acid produced 50 g/L of protocatechuic acid (Table 4). The overall yields for synthesis of protocatechuic acid from glucose where microbially synthesized 3-dehydroshikimic acid or 3-dehydroquinic acid were thermally dehydrated were 34% and 26%, respectively (Table 4). An overall yield of protocatechuic acid synthesized from glucose of only 17% was realized for biocatalytic conversion of glucose to 3-dehydroshikimic acid followed by biocatalytic conversion of 3-dehydroshikimic acid into protocatechuic acid. Since no intermediate purification of the 3-dehydroshikimic acid and 3-dehydroquinic acid fermentation broths was required prior to their dehydration and since their concentrations can likely be further improved using better designed biocatalysts,[17] the two-step synthesis of protocatechuic acid appears to be a superior process in both the final product titer obtained as well as the overall yield of protocatechuic acid synthesized from glucose.

Figure 16. ^{1}H NMR of standard protocatechuic acid. δ 7.45 (m, 2H), 6.95 (d, 1H) in D_2O.

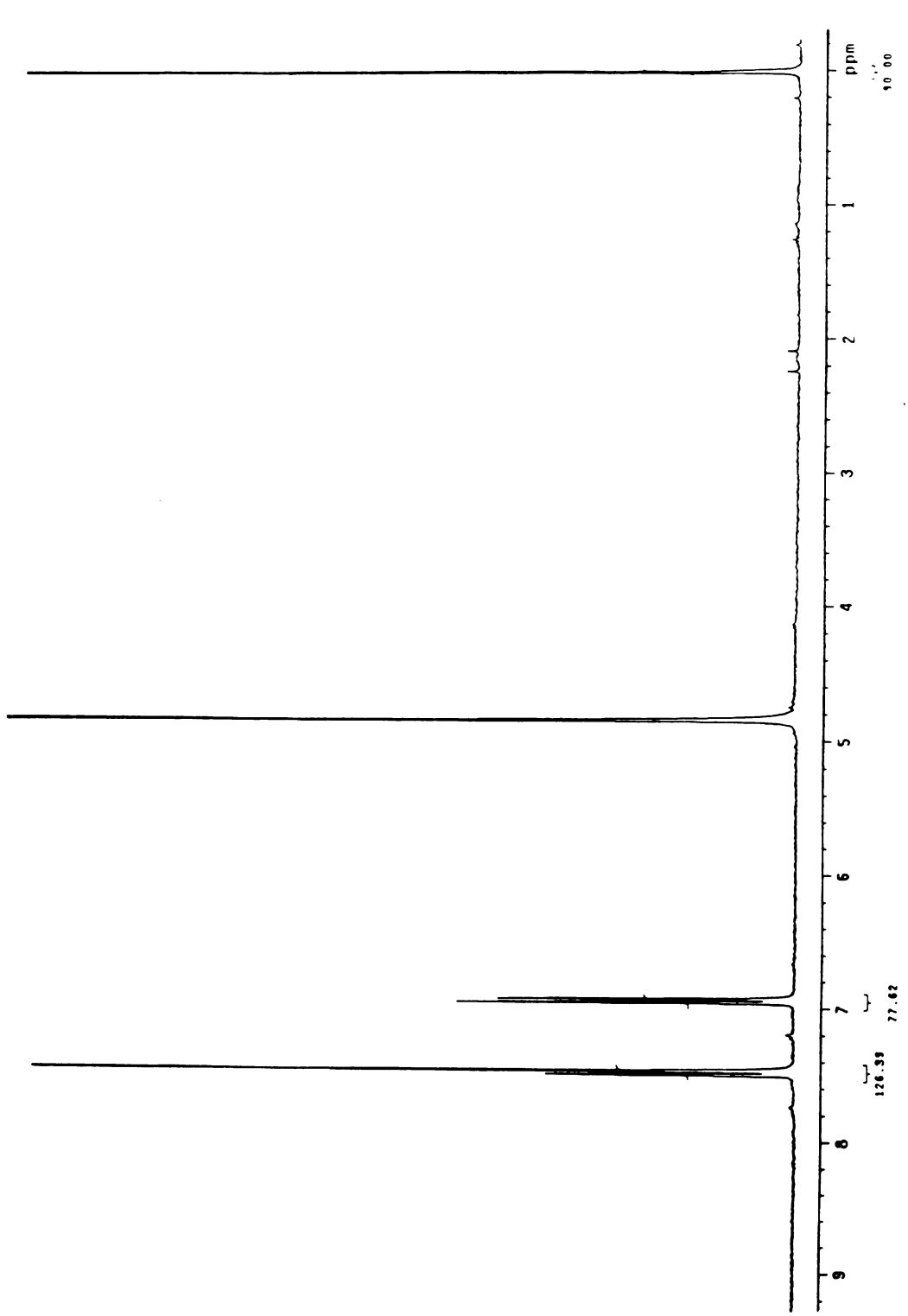

ppm
10.00
9
8
7
6
5
4
3
2
1
126.33
77.62

Figure 17. ^{1}H NMR of the fermentation supernatant of QP1.1/pKL4.130. 3-Dehydroquinic acid resonances: δ 2.35 (d, 1H), 2.39 (d, 1H), 2.55 (dd, 1H), 3.21 (d, 1H), 3.95 (ddd, 1H), 4.36 (d, 1H).

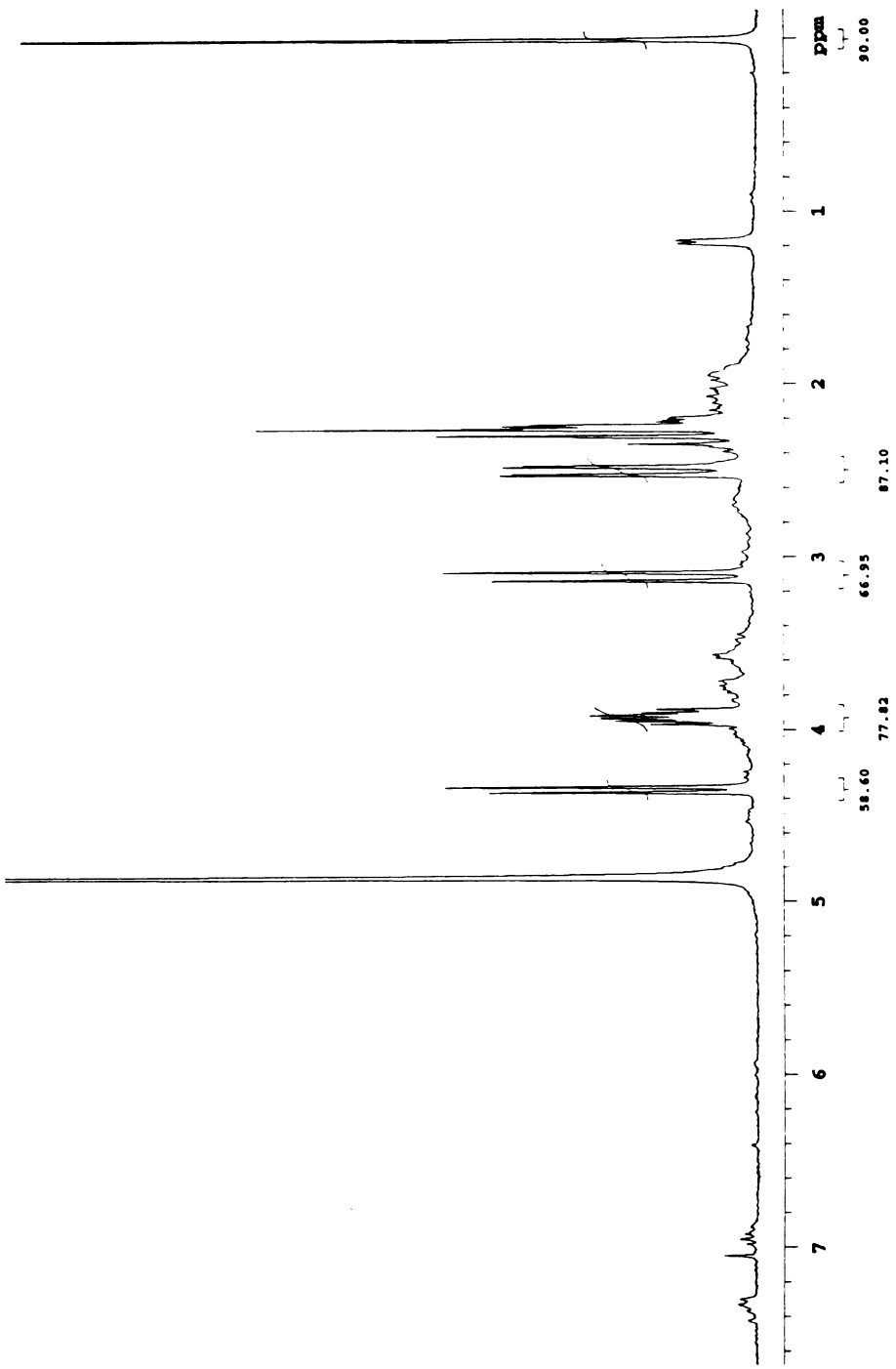
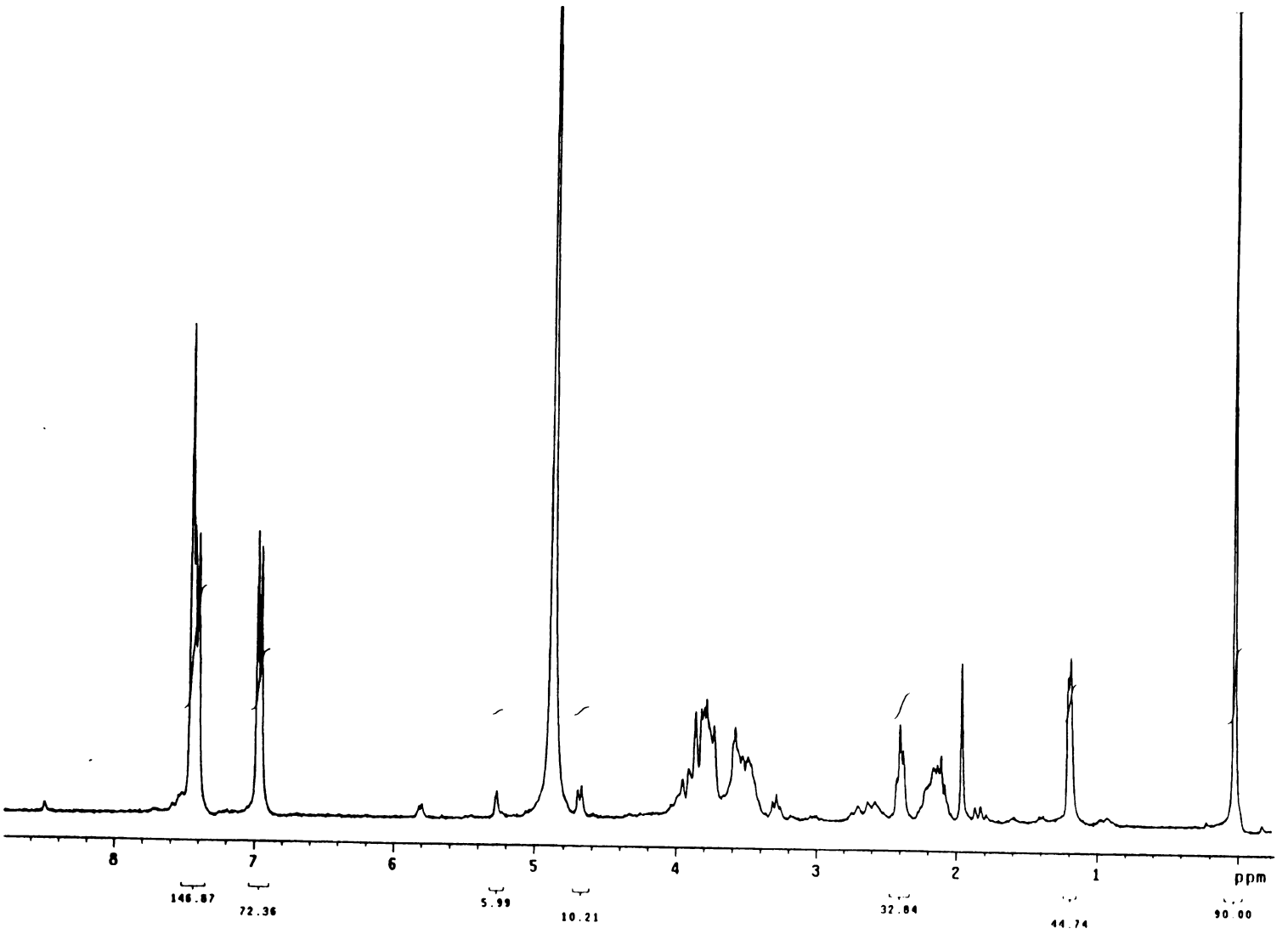

ppm
1
2
3
4
5
6
7
90.00
87.10
66.95
77.82
58.60

Figure 18. ^{1}H NMR of the fermentation supernatant of KL7/pSK7.54. PCA resonances: d; 7.45 (m 2H) and 6.95 (d, 1H). Glutamate and glucose are also present in the supernatant


8
7
6
5
4
3
2
1
ppm
146.87
72.36
5.99
10.21
32.84
44.74
90.00

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CHAPTER 3

CHEMICAL OXIDATION OF 3-DEHYDROSHIKIMIC ACID

I. INTRODUCTION

Gallic acid is the common name for 3,4,5-trihydroxybenzoic acid, a chemical with various industrial applications. It is commercially manufactured by the chemical or enzymatic hydrolysis (Figure 19) of tannin from gallnuts, Aleppo galls, or tara powder, the ground seed pod of the Peruvian tree *Coulteria tinctoria.* About 1.7 x 10^5 kg of gallic acid are globally manufactured annually. Gallic acid has traditionally been used with ferrous sulfate to make various types of inks, particularly the blue-black, permanent-type writing inks. It is used in photothermographic reproduction processes, as a process chemical in engraving and lithography, as a developer in photography and in tanning and fur- and hair-dyeing preparations.[1] Propyl gallate and other gallic acid esters are widely used as commercial antioxidants.

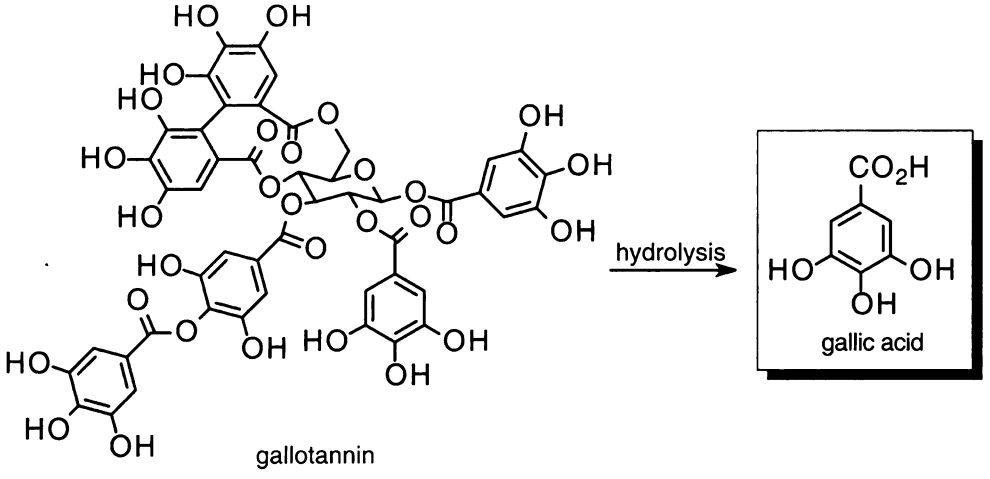


Figure 19: Tara gallotannin and gallic acid structures

As a pharmaceutical, gallic acid has been used as a urinary astringent and internal antihemorrhageant. In veterinary medicine, gallic acid is used for the treatment of diarrhea. Bismuth subgallate (the bismuth salt of gallic acid) is used as a dusting powder in dermatology. Gallic acid is a valuable synthon in the chemical synthesis of various pharmaceuticals. Some examples include trimethoprim[2], a broad-spectrum antimicrobial agent that has been used to treat bacterial infections in humans, mescalin[3], a psychomimetic agent and a starting material in the synthesis of the neuromuscular blocking agent doxacurium chloride[4], and in the synthesis of hexobendin, a coronary vasodilator (Figure 20). In addition, certain gallotannins show potent antitumor activity.[5]

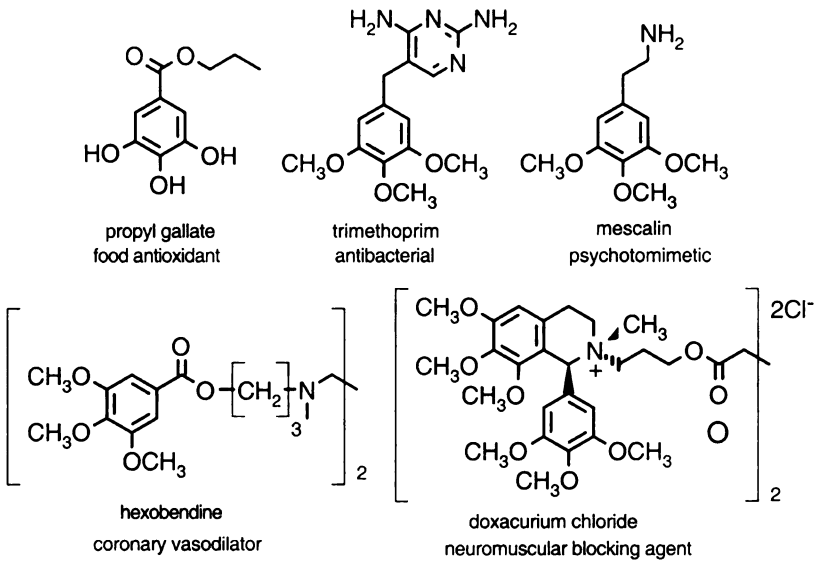


Figure 20: Derivatives of gallic acid

Thermal decarboxylation of gallic acid is the main commercial manufacture of 3,4,5 trihydroxybenzene (pyrogallol). In Chapter 6, the utility of pyrogallol is discussed in more detail. The main industrial manufacture of pyrogallol comes from thermal decarboxylation of gallic acid. Therefore, the availability of this industrially important aromatic molecule is connected to gallic acid production. In Chapters 1 and 2 the

importance of 3-dehydroshikimc acid (DHS) as a synthetic starting material was discussed. Structurally, gallic acid can be viewed as a derivative of 3-dehydroshikimic acid. Chemical or enzymatic oxidation of either hydroxyl group of 3-dehydroshikimic acid, will afford a dicarbonyl intermediate I or II, (Figure 21) which rapidly tautomerizes to the more stable aromatic structure of gallic acid.

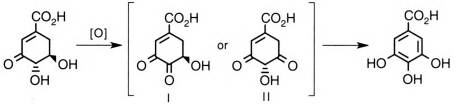


Figure 21. Oxidation of either hydroxy group of DHS will afford GA

In this chapter, various conditions for the chemical oxidation of 3-dehydroshikimic acid are discussed. Catalytic and stoichiometric oxidations using Cu^{+2} salts can afford gallic acid in moderate to high yields. In the stoichiometric oxidations the metals can be recycled and reused, and the reaction solvents consist of water or mixtures of water and acetic acid. 3-Dehydroshikimic acid was found to possess comparable antioxidant activity relative to a variety of commercially available antioxidants and was destroyed under strong oxidation conditions.[6] It reacts with O_2 or H_2O_2 giving a product mixture consisting of gallic acid, protocatechuic acid, tricarballylic acid and pyrogallol (Figure 22) in reactions that are catalyzed by inorganic phosphate.[6] Isolation and purification of the gallic acid product as well as the 3-dehydroshikimic acid from the starting cell supernatant is also discussed.

II. REACTION OF 3-DEHYDROSHIKIMIC ACID WITH O_2 AND H_2O_2: PRODUCTS, MECHANISM AND ASSOCIATED ANTIOXIDANT ACTIVITY

A. Reaction with O_2 and H_2O_2

During ^1H NMR analysis of the fermentation supernatants of 3-dehydroshikimic acid-synthesizing *E. coli* bacteria, a singlet at δ 7.1 ppm was routinely observed. Subsequent characterization of this byproduct revealed that the resonance was associated with the aromatic protons of gallic acid. To determine whether gallic acid formation was due to enzymatic or chemical catalysis, 3-dehydroshikimic acid was stirred under air in 1 M $Na_{1.5}H_{1.5}PO_4$ buffer at pH 6.6. After 50 h at 40 °C, 10% of the 3-dehydroshikimic acid remained and a product mixture was formed consisting of gallic acid (GA, 14%) protocatechuic acid (PCA, 12%), tricarballylic acid (TCBA, 14%), and pyrogallol (PGL, 3%) from which gallic acid was purified in 13% isolated yield (Figure 22, Table 5). The purification of gallic acid began with the extraction of pyrogallol from the reaction solution under neutral pH using EtOAc as the extraction solvent. After acidification to pH 3.5, protocatechuic acid, gallic acid, and some tricarballylic acid were extracted from the reaction with EtOAc. The product mixture was dried, redissolved in H_2O/MeOH (v/v, 9/1) and loaded on to a reverse-phase column consisting of octadecyl-functionalized silica gel. Elution with the same H_2O/MeOH solvent mixture afforded pure gallic acid (13% isolated yield).

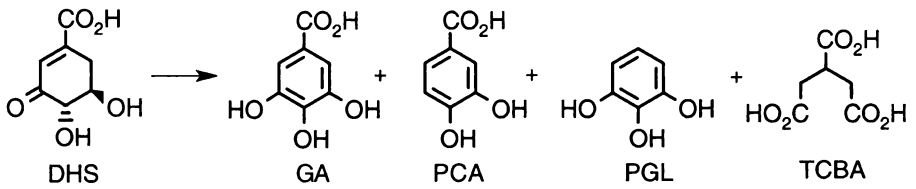


Figure 22. Air and H_2O_2 oxidation products of 3-dehydroshikimic acid in phosphate-catalyzed solutions. DHS, 3-dehydroshikimic acid; GA, gallic acid; PCA, protocatechuic acid; PGL pyrogallol; TCBA, tricarballylic acid.

Reaction of 3-dehydroshikimic acid was a general base-catalyzed process first order in both 3-dehydroshikimic acid and inorganic phosphate with an overall second order rate constant of 1.4×10^{-5} M^{-1} s^{-1}. 3-Dehydroshikimic acid reacted with H_2O_2 in a phosphate buffered solution (1M, pH 6.7) under the same conditions employed for its reaction with O_2. However, oxidation with stoichiometric amounts of H_2O_2 afforded product mixtures that lacked pyrogallol and gave higher yields of gallic acid (22%) relative to protocatechuic acid (10%) and tricarballylic acid (2%) (Table 5).

Table 5. Product distribution of air and H_2O_2 oxidations of DHS

Conditions[b]	DHS (M)	Yield %[a] GA	PCA	PGL	TCBA	DHS	Time
Air, 1M Pi	0.2	14	12	3	14	10	50 h
H_2O_2, 1M Pi	0.2	22	10	–	2	20	36 h
H_2O_2, 1M Pi, 1% Cu^{+2}	0.1	36	3	–	–	–	35 h
H_2O_2, 1M Pi, 1% Fe^{+2}	0.1	36	9	–	–	–	39 h

a. Reaction yield (mol/mol) based on ^1H NMR analysis of the crude reaction mixture at the end of the reaction using 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 as the internal standard.
b. Reaction temperature 40 °C

Addition of catalytic amounts (1% mol/mol relative to DHS) of Cu^{+2} ($CuSO_4$), or Fe^{+2} ($FeSO_4$) to the reactions with H_2O_2 was found to improve the gallic acid to protocatechuic acid ratio, eliminate tricarballylic acid and pyrogallol formation, and increase the reaction yields of gallic acid formation (Table 5). Under these reaction conditions, the gallic acid/protocatechuic acid ratio was 11:1 when Cu^{+2} was added, and 4:1 when Fe^{+2} was added. Purification of gallic acid after reaction with H_2O_2 in the presence of 1% $CuSO_4$, consisted of acidification to pH 2.5 with H_2SO_4 and organic extraction with EtOAc followed by chromatographic separation using octadecyl-functionalized silica gel. The elution solvent was H_2O/MeOH (v/v, 9/1) and pure gallic acid was isolated in 31% (mol/mol) yield based on the starting 3-dehydroshikimic acid. The reaction rates of both the H_2O_2 and air oxidations of 3-dehydroshikimic acid were affected by the inorganic

phosphate concentration. For example a three-fold increase of the phosphate concentration (3 M) under the H_2O_2 oxidation conditions resulted in formation of gallic acid and protocatechuic acid along with complete consumption of 3-dehydroshikimic acid after 5 h of reaction.

A plausible mechanism (Figure 23) for 3-dehydroshikimic acid oxidation involves initial tautomerization of 3-dehydroshikimic acid to enediol A that is catalyzed by inorganic phosphate. Enediol A could then partition between elimination of water to form the protocatechuic acid, which is observed in all 3-dehydroshikimic acid oxidations, and reaction with either O_2 or H_2O_2 to yield gallic acid, tricarballylic acid and pyrogallol. Formation of oxidation products need not be restricted to the reaction of a single enediol given the possibility that enediol A may be in equilibrium with enediol B and dihydrogallic acid. Although more than one mechanism can account for the conversion of enediol A into enediol B (Figure 23), a sigmatotropic 1,5-hydride shift is an intriguing possibility. Subsequent tautomerization of enediol B would afford dihydrogallic acid.

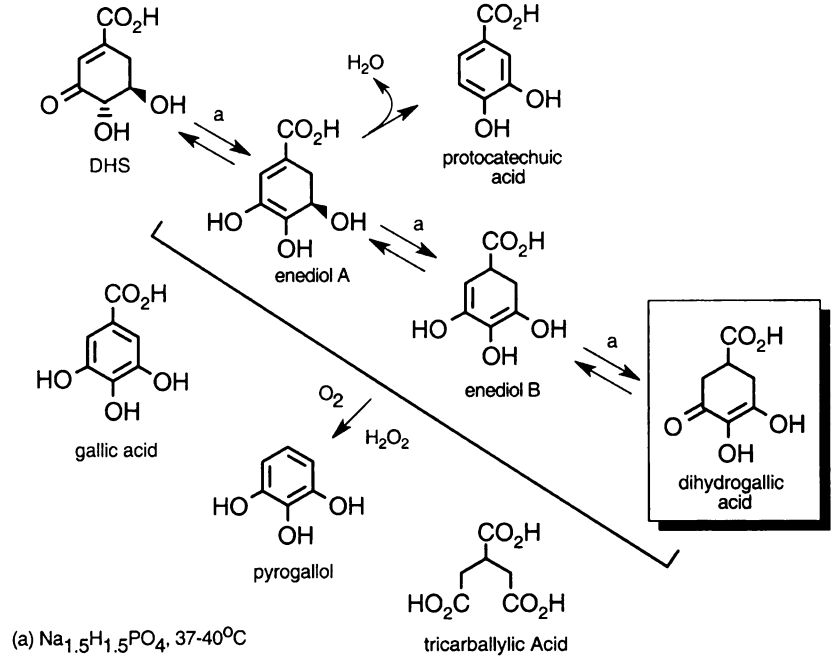


Figure 23. Proposed mechanism for air and H_2O_2 oxidations of DHS

Evidence for the enediol intermediacy followed from the isolation of oxygen-sensitive dihydrogallic acid (Figure 23) along with protocatechuic acid in respective yields of 54% and 36% from an anaerobic, K_2HPO_4 (1 M) solution of 3-dehydroshikimic acid stirred for two days at rt.[6] These reaction conditions differ markedly from the high pressure, Raney nickel hydrogenation of sodium gallate previously used to synthesize dihydrogallic acid.[7] Purification of dihydrogallic acid and subsequent reaction with air in a water solution containing 1 M $Na_{1.5}H_{1.5}PO_4$, produced after 12 h at 37 °C a mixture of gallic acid (3%) pyrogallol (6%), and tricarballylic acid (35%).[6]

The rate dependence of 3-dehydroshikimic acid loss on the concentration of inorganic phosphate is nearly the same in the presence and absence of O_2. Although this indicates that phosphate-catalyzed oxidation of 3-dehydroshikimic acid and conversion of 3-dehydroshikimic acid to dihydrogallic acid share the same rate-determining step, intermediacy of dihydrogallic acid during 3-dehydroshikimic acid oxidation is a different manner. The product ratios determined for dihydrogallic acid oxidation and 3-dehydroshikimic acid oxidation differ markedly. In addition, the dihydrogallic acid/protocatechuic acid product ratio displayed a pronounced dependence on inorganic phosphate concentration in contrast to the gallic acid/protocatechuic acid product ratio that was nearly unaffected by inorganic phosphate concentration during oxidation of 3-dehydroshikimic acid. Therefore, while isolation of dihydrogallic acid establishes that phosphate-catalyzed isomerization of 3-dehydroshikimic acid to enediol tautomers is occurring (Figure 23), the contribution of dihydrogallic acid relative to enediol A and enediol B to the products formed from 3-dehydroshikimic acid reaction with O_2 or H_2O_2 is uncertain.

B. Appraisal of antioxidant activity.

The facile reactivity of 3-dehydroshikimic acid with O_2 or H_2O_2 led to the investigation of its potential antioxidant activity. 3-Dehydroshikimic acid was tested by its ability to inhibit the formation of peroxides and hydroperoxides in lard which are the initial products of lipid oxidation (Figure 24).[6] Its measured antioxidant activity was compared with the measured antioxidant activity of gallic acid and two commercial antioxidants, propyl gallate and *tert*-butylhydroquinone (TBHQ).

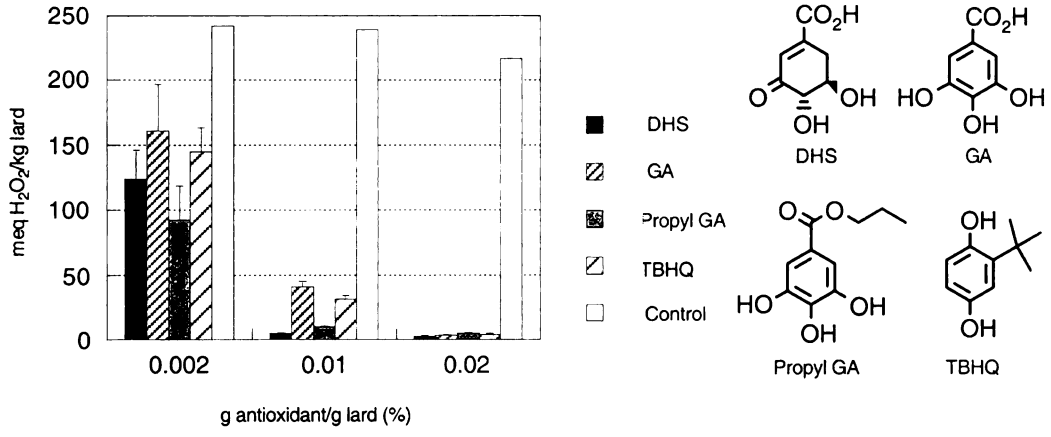


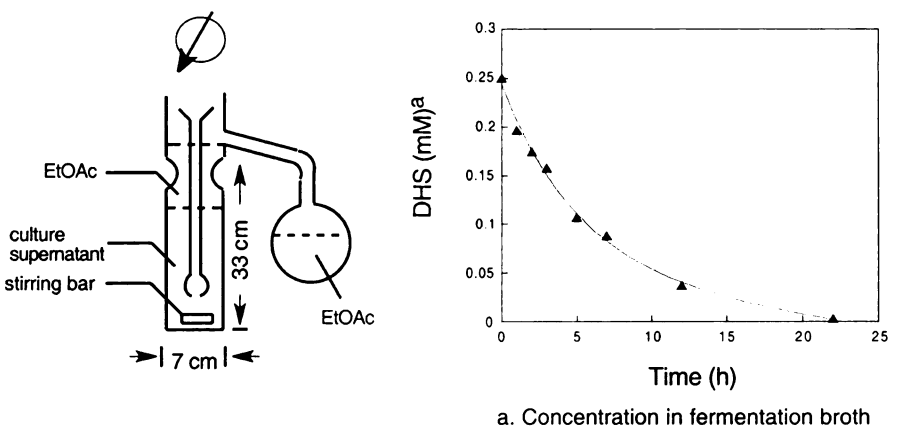
Figure 24. Inhibition of hydroperoxide formation in lard by various commercial antioxidants and DHS

Lard samples, with or without antioxidants, were kept at 60 °C in darkness for 28 days.[8] Peroxide values in units of milliequivalents per kg of lard were determined every 7 days over a 28 day period by standard titration methods.[9] At 0.02 wt%, 3-dehydroshikimic acid provided virtually complete protection for 28 days against oxidative decomposition of the lard (Figure 24). At all concentrations examined, the antioxidant activity of 3-dehydroshikimic acid was equal to or superior to the antioxidant activities of propyl gallate, gallic acid and TBHQ. Even at a concentration as low as 0.002%, 3-dehydroshikimic acid provided significant protection against oxidative decomposition.

III. STOICHIOMETRIC OXIDATIONS OF 3-DEHYDROSHIKIMIC ACID

A. DHS purification

The potential utility of 3-dehydroshikimic acid as a commercial antioxidant or as a starting material in the oxidation to gallic acid required development of methodology appropriate for isolation of 3-dehydroshikimic acid from fermentation broths. Two different methods for the purification of 3-dehydroshikimic acid from fermentation supernatants were investigated. The first consisted of a modified continuous extraction apparatus and the second used anion exchange resins. In either case, a fermentation supernatant containing 3-dehydroshikimic acid was first acidified to pH 2.2 and centrifuged to remove precipitated protein.



a. Concentration in fermentation broth

Figure 25. Modified continuous extractor and extraction profile of DHS from fermentation supernatant

Continuous, liquid-liquid extraction employed vigorous stirring to improve mixing of the organic solvent with the cell supernatant, thereby improving extraction efficiency. Employing a standard continuous extractor in the absence of stirring, about 70% of the 3-dehydroshikimic acid was removed from the water solution after 40 h, whereas stirring at

a rate sufficient to create a colloidal suspension of EtOAc in the culture supernatant enabled the extraction to be complete in 24 h with more than 95% recovery of 3-dehydroshikimic acid (Figure 25). Pure 3-dehydroshikimic acid was obtained after filtration of the EtOAc solution through activated charcoal, drying with $MgSO_4$, and partial concentration of the organic solvent, during which 3-dehydroshikimic acid precipitated as an off-white powder in a 70% isolated yield based on the 3-dehydroshikimic acid present in the starting fermentation broth. Under these extraction conditions, protcatechuic acid, the dehydration product of 3-dehydroshikimic acid, was formed in only trace amounts.

Alternatively, pure 3-dehydroshikimic acid was obtained with the use of a strong anion exchange resin. Fermentation supernatant was adjusted to pH 5.5 and filtered through a column containing AG1x8 (acetate form) anion exchange resin. Both inorganic phosphate and 3-dehydroshikimic acid were adsorbed. After washing the column with water followed by MeOH to remove residual water from the resin, 3-dehydroshikimic acid was selectively eluted (90-95% recovery) with glacial acetic acid. Acetic acid was the solvent employed for stoichiometric or catalytic oxidations of 3-dehydroshikimic acid into gallic acid. 3-Dehydroshikimic acid eluted from the anion exhange column can be oxidized without any further purification. The resin is reusable after washing it with NaOH (0.1 N), and H_2O/AcOH (v/v, 1/1) to regenerate the acetate form of the anion exhange resin.

B. Stoichiometric Cu^{+2} oxidations and metal recycling.

The modest yields of gallic acid and levels of protocatechuic acid byproduct formed when O_2 or H_2O_2 were employed as oxidants led to the examination of other oxidizing conditions. Oxidation of α-hydroxy ketones, such as 3-dehydroshikimic acid, to α-diketones (intermediate I, Figure 21) using Cu^{+2} salts is precedented.[10] Water insoluble oxides such as Fe_2O_3 and CuO were also unsuccessful in oxidizing 3-dehydroshikimic acid. Copper carbonate basic ($CuCO_3 \cdot Cu(OH)_2$) is insoluble in water and has been reported to oxidize α-hydroxy ketones.[11]

Heterogeneous reactions in aqueous phosphate-buffered solutions (1M, pH 6.6) using $CuCO_3 \cdot Cu(OH)_2$ resulted in products consisting of only gallic acid and small amounts of protoctechuic acid (Table 6, entry 1). The product isolation started with a filtration to remove the insoluble copper salts. Extraction of the reaction mixture with EtOAc gave after solvent evaporation a red powder consisting of gallic acid and protocatechuic acid. An off-white powder consisting of the same gallic acid to protocatechuic acid ratio was obtained when the above mixture was dissolved in $MeOH/H_2O$ (v:v 1:9) and filtered through an octadecyl-functionalized silica gel column giving a 51% yield of gallic acid and 2% yield of protocatechuic acid calculated from the starting 3-dehydroshikimic acid. The functionalized silica was acting by negative absorption to remove the color and the recovery of the gallic acid and protocatechuic acid after filtration was 95%. The reaction selectivity towards gallic acid formation was likely higher than 32/1, since some of the protocatechuic acid that contaminated the final product was present prior to the oxidation. Small amounts of protocatechuic acid were present in all fermentation broths of 3-dehydroshikimic acid-synthesizing microbes.[21] The product ratio and yields of the $CuCO_3 \cdot Cu(OH)_2$ reactions were unaffected by whether the reactions were performed under air or nitrogen atmospheres. In any case, complete reaction of 3-dehydroshikimic acid was observed after 4.5 h. However, the product mixture could only

account for about 50% (mol/mol) of the mass of the starting 3-dehydroshikimic acid (Table 6, entries 1, 3).

Table 6. Stoichiometric oxidations of DHS with $CuCO_3 \cdot Cu(OH)_2$, $Cu_X(H_{3-X}PO_4)_2$ and $Cu(OAc)_2$

Entry	Oxidant	Solvent[b]	Pi (M)	Cu(equiv)	Yield %[a] GA	PCA	DHS	GA/PCA	Time (h)	T (°C)
1	$CuCO_3Cu(OH)_2$	H_2O	1.0	4	51	2	–	32/1	4.5	50
2	$CuCO_3Cu(OH)_2$	H_2O	0.25	4	47	4	21	13/1	20	50
3	$CuCO_3Cu(OH)_2$	H_2O	0.25	4	48	3	–	16/1	18	60
4	$Cu_X(H_{3-X}PO_4)_2$	H_2O	1.0	2.2	43	2	–	19/1	12	50
5	$Cu_X(H_{3-X}PO_4)_2$	H_2O	0.25	2.2	31	3	34	10/1	23	50
6	$Cu(OAc)_2$	$AcOH/H_2O$	–	2.2	74	0.7	–	99/1	36	40
7	$Cu(OAc)_2$	$AcOH/H_2O$	–	2.2	53	3.3	–	16/1	12	50

a. (mol/mol) calculated from starting 3-dehydroshikimic acid. b. The concentration of DHS in the reactions in water (entries 1 through 5) was 46 mM, and in the $AcOH/H_2O$ reactions (entries 7 and 8) was 0.79 mM

At the end of the $CuCO_3 \cdot Cu(OH)_2$ reactions, all the copper can be recovered by filtration. However, IR analysis of the recovered copper salts showed absorbances indicating phosphate rather than carbonate functionality. Recycling of copper carbonate from copper phosphate was not attempted given the high affinity of this metal for phosphate (ie $Cu_3(PO4)_2$, $K_{sp} = 1.39x10^{-37}$).[30] Ligand exchange of copper salts from carbonate to phosphate prior to the oxidation of 3-dehydroshikimic acid was therefore a reasonable possibility when $CuCO_3 \cdot Cu(OH)_2$ was utilized for oxidation. Complete reactions of 3-dehydroshikimic acid without significantly changing the product ratios and reaction yields were observed when $Cu_X(H_{3-X}PO_4)_2$ was suspended in phosphate-buffered solutions (pH 6.5) containing 3-dehydroshikimic acid (Table 6). The copper phosphate salts were prepared separately by mixing a solution of $CuSO_4$ with 1.5 equivalents of NaH_2PO_4 (pH 6.5) and recovered by filtration.

The concentration of inorganic phosphate plays an important role in determining the reaction rates associated with 3-dehydroshikimic acid oxidations using stoichiometric quantities of Cu^{+2}. Significantly slower reaction rates and significant concentrations of unreacted 3-dehydroshikimic acid were observed upon reducing the concentration of inorganic phosphate by 4-fold (Table 6, entry 1 versus entry 2 and entry 4 versus entry 5). The reduction in reaction rates upon lowering inorganic phosphate concentrations is consistent with phosphate-catalyzed tautomerization of 3-dehydroshikimic acid to enediol A and enediol B (Figure 23) being a rate-determining step. The lower yields of protocatechuic acid compared to those observed in inorganic phosphate-catalyzed O_2 or H_2O_2 oxidations lacking Cu^{+2} were probably due to the complexation and stabilization of enediol A or B with Cu^{+2} (Figure 23) which impeded dehydration (Figure 26). Subsequent oxidation of the hydroxyl group by electron transfer with the chelated Cu^{+2} would be anticipated to afford gallic acid. Complexation resulting in stabilization of the intermediate enediols with Cu^{+2} could also explain the elimination of tricarballylic acid and pyrogallol. Formation of tricarballylic acid and pyrogallol was previously observed in inorganic phosphate-catalyzed oxidations lacking Cu^{+2}. The overall yield of gallic acid was not affected by the presence or absence of O_2 thereby indicating that loss of gallic acid to reaction with O_2 was not a significant problem.

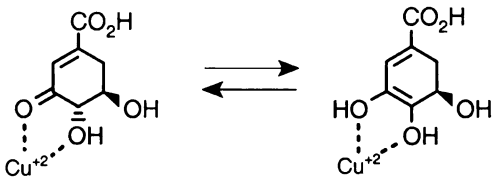


Figure 26. Complex formation of DHS and enediol A with Cu^{+2}

Homogeneous oxidations with Fehling's solution have been reported to give good yields of gallic acid.[12] When 3-dehydroshikimic acid was oxidized under these conditions,

high levels of protocatechuic acid contamination and low yields to gallic acid formaion were observed. Other authors have reported that in $AcOH/H_2O$ mixtures, α-hydroxy ketones were quantitatively oxidized with stoichiometric[10] or catalytic[13] amounts of $Cu(OAc)_2$ under homogeneous conditions.

High yields of gallic acid (74%), and good product ratios (GA/PCA>99/1) were obtained after 3-dehydroshikimic acid was oxidized for 36 h with $Cu(OAc)_2$ (2.2 equiv) dissolved in a mixture of $AcOH/H_2O$ (v/v, 8/2) at 40 °C (Table 6, entry 6). At the end of the reaction, the reaction mixture consisting only of gallic acid and traces of protocatechuic acid was filtered to remove the insoluble copper salts and concentrated almost to dryness. Addition of water produced an acidic solution (pH 3) due to the residual AcOH. Extraction using EtOAc and concentration afforded a brown gallic acid powder, which was redisolved in $MeOH/H_2O$ (v/v, 1/9) and filtered through octadecyl-functionalized silica gel preequilibrated with $MeOH/H_2O$ (v/v, 1/9) adjusted to pH 3 with AcOH addition. This step removed the remaining color, and gallic acid was isolated as an off-white powder that precipitated during solvent concentration (isolated yield 62%). The reaction was complete in 12 h when the temperature was increased to 50 °C, but the overall yield (56%) and product ratio (GA/PCA:16/1) decreased (Table 6, entry 7 verus entry 6).

An additional advantage of this reaction was the relatively easy recycling of $Cu(OAc)_2$. The copper salts filtered in the first step were added to the aqueous solution remaining after the extraction of gallic acid using EtOAc. This mixture was then oxidized with an excess of 30% aqueous H_2O_2. Addition of solid NaOH to give a solution of pH 13 quantitatively precipitated $Cu(OH)_2$, which was recovered by filtration. Copper acetate formed after stirring the $Cu(OH)_2$ in acetic acid for 2 h. The recovery of Cu^{+2} used in the initial oxidation was 93-95%.

Although the heterogeneous oxidations employing $Cu_X(H_{3-X}PO_4)_2$ and $CuCO_3Cu(OH)_2$ offer an advantage in the ease of isolation of the Cu^{+2} oxidant at the end of the reaction, these oxidations afford lower galic acid yields than stoichiometric

homogeneous oxidations employing $Cu(OAc)_2$ at 40 °C. In the case of $CuCO_3 \cdot Cu(OH)_2$, the inability to recover and recycle Cu^{+2} as $CuCO_3 \cdot Cu(OH)_2$ was problematic. The requirement of high phosphate concentrations for stoichiometric, heterogeneous Cu^{+2} oxidations is also not attractive. By contrast, $Cu(OAc)_2$ in $AcOH/H_2O$ gave higher yields of product gallic acid, excellent gallic acid/protocatechuic acid ratios and avoided the need for high salt concenrtations. These advantages combined with the ease of recycling $Cu(OAc)_2$ make this stoichiometric, homogeneous oxidation superioir to the stoichiometric, heterogeneous Cu^{+2} oxidations summarized in Table 6.

IV. CATALYTIC COPPER OXIDATIONS

A. Development of catalytic $Cu(OAc)_2$ oxidations

The high yields and GA/PCA ratios obtained in the stoichiometric oxidations using copper acetate led to a further investigation of its chemistry. Catalytic amounts of $Cu(OAc)_2$ (50% relative to the alcohol) have been reported to oxidize benzoin ($C_6H_5CH(OH)COC_6H_5$) to benzil (diketone) in $AcOH/H_2O$ (v/v, 8/2).[13] Copper was reoxidized in situ with NH_4NO_3 addition.[13b,c] When 3-dehydroshikimic acid was oxidized under the same conditions (Table 7, entry 1) good product ratios were obtained (GA/PCA : 43/1) but the gallic acid yield was low (43%) after 20 h of reaction time compared to the reported yield for benzoin oxidation (100%).[13b,c] Decreasing the amount of Cu^{+2} to 5% relative to 3-dehydroshikimic acid (Table 7, entry 2) did not improve the product yield (38%), or the product ratio (GA/PCA : 19/1). Replacing NH_4NO_3 with H_2O_2 decreased the yield even further after 18 h of reaction time (Table 7, entry 2 versus entry 3).

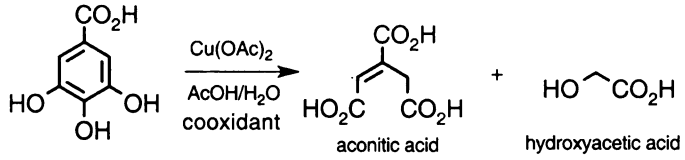
Table 7. Catalytic oxidations of DHS with $Cu(OAc)_2$ and different cooxidants

				Yield %[a]				
Entry	Catalyst	Cu(eguiv)	Oxidant[b]	GA	PCA	DHS	GA/PCA	Time (h)
1	$Cu(OAc)_2$	0.5	NH_4NO_3	43	1	–	43/1	20
2	$Cu(OAc)_2$	0.05	NH_4NO_3	38	2	5	19/1	24
3	$Cu(OAc)_2$	0.1	H_2O_2	21	4.3	34	5/1	18
4	$Cu(OAc)_2$	0.1	Air	48	2.5	30	19/1	23

a. (mol/mol) calculated from the starting 3-dehydroshikimic acid, after product was extracted from the reaction mixture concentrated dryness and weighted b. The reaction solvent was $AcOH/H_2O$ (v/v, 8/2), the temperature was 40 °C and the concentration of DHS was 0.15 M

Atmospheric O_2 could also serve as a cooxidant. Use of atmospheric O_2 as the cooxidant increased the gallic acid yield to 48% from 21% when H_2O_2 was employed as the cooxidant (Table 7, entry 3 versus entry 4). In addition, the products and unreacted

3-dehydroshikimic acid after 23 h of reaction accounted for 81% of the starting 3-dehydroshikimic acid compared to 59% and 45%, respectively, when H_2O_2 and NH_4NO_3 cooxidants were used. Reactions employing catalytic amounts of $Cu(OAc)_2$ were slow and unreacted 3-dehydroshikimic acid was present in the reaction mixtures even after 24 h of reaction time. Inclusion of AcOH in the solvent used for 3-dehydroshikimic acid oxidations was important. No 3-dehydroshikimic acid oxidation was observed in the absence of AcOH.



		Yield %[a]				
Cu(equiv)	cooxidant	GA	aconitic	hydroxyacetic	other	Time (h)
0.1	O_2	>95	–	–	–	16
0.1	H_2O_2	–	20	10	~10	5

a. NMR yields (mol/mol) based on starting gallic acid

Figure 27. Oxidation products of GA using $Cu(OAc)_2$ and air versus H_2O_2 as cooxidants

Improved yields when 3-dehydroshikimic acid was oxidized using catalytic Cu^{+2} and air as the cooxidant versus use of NH_4NO_3 or H_2O_2 as the cooxidant can be attributed to oxidative decomposition of gallic acid. Oxidative gallic acid decomposition was confirmed after reacting it with H_2O_2 in the presence of copper. Under reaction conditions essentially identical to the catalytic oxidation of 3-dehydroshikimic acid, gallic acid was completely consumed after 5 h of reaction yielding hydroxyacetic acid (10%) aconitic acid (20%) and unidentified oxidation products. The total mass of recovered gallic acid oxidation products never accounted for more than 40% (mol/mol) of the beginning gallic

acid. By contrast, when air was employed as the cooxidant, gallic acid was unaffected even after 16 h of reaction time (Figure 27).

Gallic acid overoxidation can explain the product difference in the reactions of 3-dehydroshikimic acid with catalytic amounts of $Cu(OAc)_2$. While the same amounts of 3-dehydroshikimic acid and protocatechuic acid were present at the end of the reactions utilizing either H_2O_2 or O_2 as the cooxidants, the yield of gallic acid was 21% when H_2O_2 was used and 48% when O_2 was used (Table 7, entry 3 versus entry 4). Both NH_4NO_3 and H_2O_2 are strong oxidizing agents ($E_{NO3-/NO}$ =0.96 eV and $E_{H2O2/H2O}$ =1.77 eV), and in addition, H_2O_2 in the presence of Cu^{+2} generates hydroxy radicals under Fenton-like[14,15b] chemistry that can decompose both the starting 3-dehydroshikimic acid and product gallic acid. In support of this argument, product mixtures obtained using Fe^{+2}/H_2O_2 were essentially the same when Cu^{+2} and H_2O_2 were employed in the oxidation of gallic acid. The facile reactivity of gallic acid with superoxide (O_2^-) and alkoxide (RO·) radicals has been reported.[15]

B. Development of $Cu(OAc)_2$/ZnO reactions

From the discussion so far it is apparent that developing reaction conditions for the oxidation of 3-dehydroshikimic acid to gallic acid that are selective and high-yielding is not an easy matter. The reaction must be mild to avoid yield losses caused by gallic acid decomposition and selective to avoid the formation of the dehydration product protocatechuic acid. Cu^{+2} was found to selectively oxidize 3-dehydroshikimic acid, but for the reaction to be high-yielding, stoichiometric amounts are required (Table 6). Catalytic amounts (5-10% mol/mol relative to 3-dehydroshikimic acid) of $Cu(OAc)_2$ in $AcOH/H_2O$ mixtures using O_2 as the cooxidant resulted in good GA/PCA ratios, but slow and incomplete reactions were observed (Table 7).

An intriguing discovery came from the addition of nonoxidative Lewis acids (Zn^{+2}, Mg^{+2}, Mn^{+2}) to the catalytic copper acetate reactions. These metals were found to improve

both the reaction rate and yield when added in catalytic amounts to the reaction mixture. Kinetic experiments were then performed to establish the effects of Cu^{+2} and Lewis acids (ZnO, MgO, MnO) on the reaction rate and product distribution. The reaction progress was followed by measuring the gallic acid production spectroscopically by its absorbance at 298 nm. At certain time points, the oxidation progress and product ratio was confirmed by ^1H NMR analysis. All the reactions were carried out at 50 °C under either air (1 atm), or bubbling O_2 through the reaction mixture. The starting concentration of 3-dehydroshikimic acid was 50 mM.

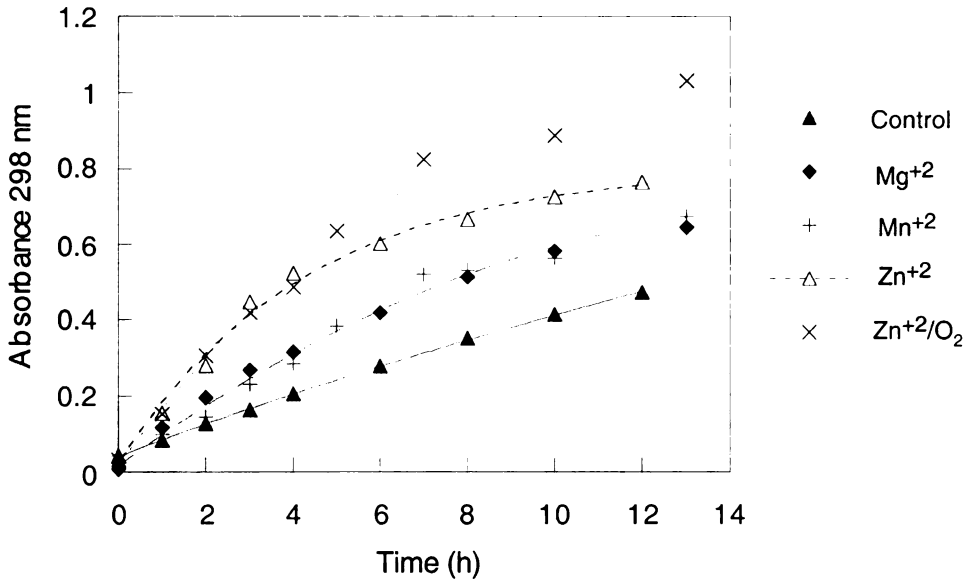


Figure 28. Effect of Lewis acid in the reaction rate of DHS oxidations with catalytic amount of $Cu(OAc)_2$.

The presence of Mn^{+2}, Mg^{+2}, or Zn^{+2} (50% mol/mol relative to 3-dehydroshikimic acid) significantly increased the reaction rate of the 3-dehydroshikimic acid oxidation with $Cu(OAc)_2$ (10% mol/mol relative to 3-dehydroshikimic acid) giving gallic acid and protocatechuic acid as the only products (Figure 28). Their ratio was independent of the

Lewis acid, being 19/1 (mol/mol) in all cases. The reaction rate and yield on the other hand were metal-dependent, with Zn^{+2} giving both the highest yield and reaction rate (Figure 28). In the case of Zn^{+2} catalysis, initial reaction rates were unaffected by switching from air to O_2. However the overall conversion was faster using O_2 probably because of the more efficient reoxidation of Cu^{+1} to Cu^{+2}.

The effect of the concentrations of 3-dehydroshikimic acid, Cu^{+2} and Zn^{+2} on the reaction rate and product ratio was investigated. While no product was obtained after stirring 3-dehydroshikimic acid in $AcOH/H_2O$ mixture for 14 h, slow and incomplete conversion to gallic acid and protocatechuic acid was observed when catalytic amounts of $Cu(OAc)_2$ were added into the mixture (Table 8, entry 1 versus entry 2). In the absence of Cu^{+2}, Zn^{+2} efficiently catalyzed the conversion of 3-dehydroshikimic acid into protocatechuic acid (Table 8, entry 3). Addition of catalytic amounts of Cu^{+2} to this reaction essentially reversed the product ratio giving gallic acid as the major product (Table 8, entry 3 versus entry 4).

Table 8. Catalytic oxidations with $Cu(OAc)_2$ and ZnO.

entry	DHS[b] (M)	cooxidant	Cu (equiv)	Zn (equiv)	Yield %[a] GA	PCA	DHS	GA/PCA	Time (h)
1	0.05	Air	–	–	trace	trace	>90		14
2	0.05	Air	0.1	–	48	2.5	30	19/1	23
3	0.05	Air	–	0.5	1	80	5	1/80	16
4	0.05	Air	0.1	0.5	73	4	–	19/1	23
5	0.05	Air	0.1	0.1	71	5	20	14/1	23
6	0.5	Air	0.05	0.1	70	3	11	24/1	7
7	0.5	O_2	0.05	0.1	64	3.5	14	19/1	7
8	1	Air	0.05	0.1	46	14	8	3/1	7
9	1	O_2	0.05	0.1	64	3.5	9	19/1	6
10	1	O_2	0.05	0.25	67	3	–	19/1	4

a. (mol/mol) based on starting 3-dehydroshikimic acid

b. The reactions were heated at 50 °C in $AcOH/H_2O$ (v/v, 85/15)

A tenfold increase in the concentration of 3-dehydroshikimic acid (0.05 M to 0.5 M) resulted in an increase of the oxidation rate (Table 8, entry 6 versus entry 5). In addition, Cu^{+2} can be reduced form 10% to 5% (mol/mol relative to 3-dehydroshikimic acid) without changing the product ratio or yield (Table 8, entry 5 versus entry 6). Further increases in the 3-dehydroshikimic acid concentration (1 M) required O_2 instead of air for efficient reoxidation of Cu^{+1} to Cu^{+2} and for sustaining a high mol/mol ratio of GA/PCA (Table 8, entry 9 versus entry 8).

The catalytic Cu/Zn reactions could be easily scaled up to oxidize large amounts of 3-dehydroshikimic acid, in solutions containing 3-dehydroshikimic acid in high concentrations. However, bubbling air or O_2, rather than stirring the reaction under air or O_2, was required in oxidations where the 3-dehydroshikimic acid concentration was 0.1 M or 1 M, respectively. Larger-scale reaction (10.5 g) of 3-dehydroshikimic acid (1 M) with $Cu(OAc)_2$ and ZnO (0.05 equiv and 0.25 equiv relative to 3-dehydroshikimic acid, respectively) at 50 °C in AcOH/H_2O (v/v, 6/1) gave a 70% yield of a mixture consisting of gallic acid and protocatechuic acid (GA/PCA :19/1) after 4 h (Table 8, entry 10). A black powder consisting of gallic acid and protocatechuic acid precipitated after concentration of the reaction mixture to one-half of its original volume and cooling in an ice bath. This crude product was redissolved in water (pH 3) and extracted with EtOAc. An off-white powder consisting of gallic acid (>99%) was isolated after fractional precipitation of the concentrated EtOAc with petroleum ether in a 53% (mol/mol) isolated yield based on the starting 3-dehydroshikimic acid. The precipitation step separated contaminating protocatechuic acid from gallic acid. Purifying gallic acid from protocatechuic acid using this precipitation method only worked if the ratio of gallic acid to protocatechuic acid was 15/1 (mol/mol) or higher. Large-scale (11.5 g of 3-dehydroshikimic acid, 50 mM solution) catalytic Cu/Zn reaction (5% Cu^{+2}, 50% Zn^{+2} mol/mol relative to 3-dehydroshikimic acid) can be performed in straight AcOH solvent without changing the GA/PCA ratio (25/1) or

the reaction yield (80%). Pure gallic acid (67% isolated yield) was obtained after purification of the reaction product (Table 10).

The Cu/Zn mixture can be reused at least one more time to oxidize 3-dehydroshikimic acid without intermediate purification and isolation of the metals. After oxidation for 14 h at 50 °C of 1 g of 3-dehydroshikimic acid (0.1 M) in $AcOH/H_2O$ (v/v, 8/2) with $Cu(OAc)_2$ (5% relative to DHS) and ZnO (50% relative to DHS) the reaction mixture containing gallic acid and protocatechuic acid (GA/PCA, 33/1) was concentrated to almost dryness and resuspended in H_2O. After extracting of the gallic acid and protocatechuic acid with EtOAc, the water layer containing the metals was oxidized with an excess of H_2O_2 for 2 days at 50 °C. Addition of the appropriate amount of AcOH to create an $AcOH/H_2O$ of 8/2 (v/v) was followed by addition of 1 g of 3-dehydroshikimic acid and the mixture was heated at 50 °C. After 20 h all the 3-dehydroshikimic acid was consumed giving gallic acid (64% yield) and protocatechuic acid (3% yield) in a ratio of GA/PCA of 19/1. Both reactions were performed under air.

The effect of Zn^{+2} on the reaction rate was evaluated in kinetic experiments where the Cu^{+2} concentration (0.1 equiv relative to to 3-dehydroshikimic acid) remained unchanged while Zn^{+2} varied (Figure 29A). A less dramatic effect in the reaction rate was observed when the concentration of Cu^{+2} varied (Figure 29B) keeping the Zn^{+2} concentration unchanged (0.5 equiv relative to 3-dehydroshikimic acid). When the 3-dehydroshikimic acid was stirred under N_2 in a D_2O/CD_3CO_2D mixture containing ZnO (1 equiv relative to to 3-dehydroshikimic acid), no deuterium exchange was observed at the C-4 or C-5 hydrogens as indicated by ^1H NMR analysis of the crude mixture.

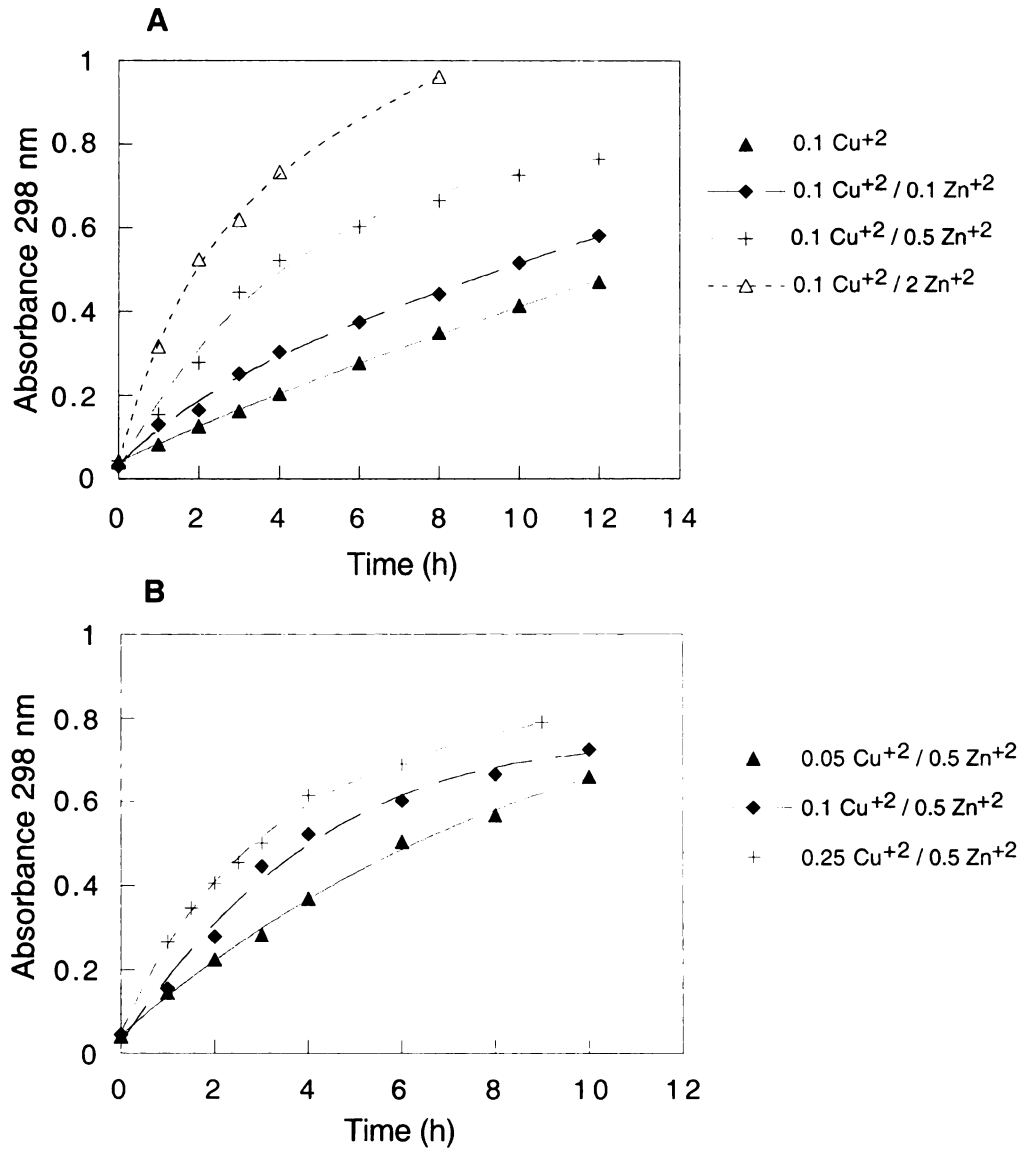


Figure 29. Effect of Zn^{+2} (Graph A) and Cu^{+2} (Graph B) concentrations in the oxidation rates of 3-dehydroshikimic acid with $Cu(OAc)_2$ and ZnO.

C. Selectivity of Cu/Zn reactions

The Cu/Zn reaction conditions were applied to the oxidation of other compounds. No reaction was observed when shikimic acid (SA, Figure 30) was stirred for 24 h at 50 °C in an $AcOH/H_2O$ solution containing $Cu(OAc)_2$ and ZnO (0.2 equiv and 0.5 equiv mol/mol relative to shikimic acid respectively). By contrast, molecules containing an α-hydroxy ketone functionality were found to react efficiently under these conditions. 3-Dehydroshikimic acid (DHS, Figure 30) gave complete conversion to gallic acid and protocatechuic acid (Table 8, entry 4) while 3-dehydroquinic acid (DHQ, Figure 30) gave product mixtures that also consisted of gallic acid and protocatechuic acid with the former being the major product (Table 9). D-Glucose among other sugars has been reported to be oxidized by Cu^{+2} in aqueous basic solutions.[16] However, no reaction was obtained after stirring glucose for 24 h under the Cu/Zn standard (0.2 equiv Cu^{+2} and 0.5 equiv Zn^{+2} relative to glucose) reaction conditions. In the presence of alkali, sugars undergo tautomeric change through an intermediate enediol.

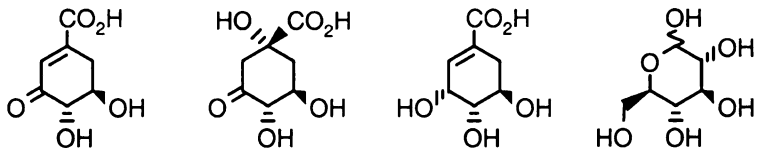


Figure 30. Reaction selectivity of $Cu(OAc)_2/ZnO$ (0.1 equiv, 0.5 equiv) oxidations.

When 3-dehydroquinic acid reacted with $Cu(OAc)_2/ZnO$ (0.1 equiv / 0.5 equiv relative to 3-dehydroquinic acid) in $AcOH/H_2O$, a mixture of gallic acid and protocatechuic acid (9/1, mol/mol) was obtained among other products. The rate of 3-dehydroquinic acid disappearance was similar to that of 3-dehydroshikimic acid under the same Cu/Zn reaction conditions (Figure 31A). However the yield to gallic acid and protocatechuic acid was significantly lower (30%, Table 9, Figure 31B). The reaction was catalyzed by Zn^{+2} , and in

the absence of Cu^{+2} the dehydration product, protocatechuic acid, was the predominant product.

Table 9. Oxidation products of 3-dehydroquinic acid (DHQ) with Cu/Zn

Catalyst	Cu (equiv)	Zn (equiv)	Yield % GA	Yield % PCA	Yield % DHQ	GA/PCA	Time (h)
$Cu(OAc)_2/ZnO$	–	0.5	2	40.5	43	1/24	8
$Cu(OAc)_2/ZnO$	0.1	–	8.5	0.5	53	19/1	23
$Cu(OAc)_2/ZnO$	0.1	0.5	27	3	15	9/1	12

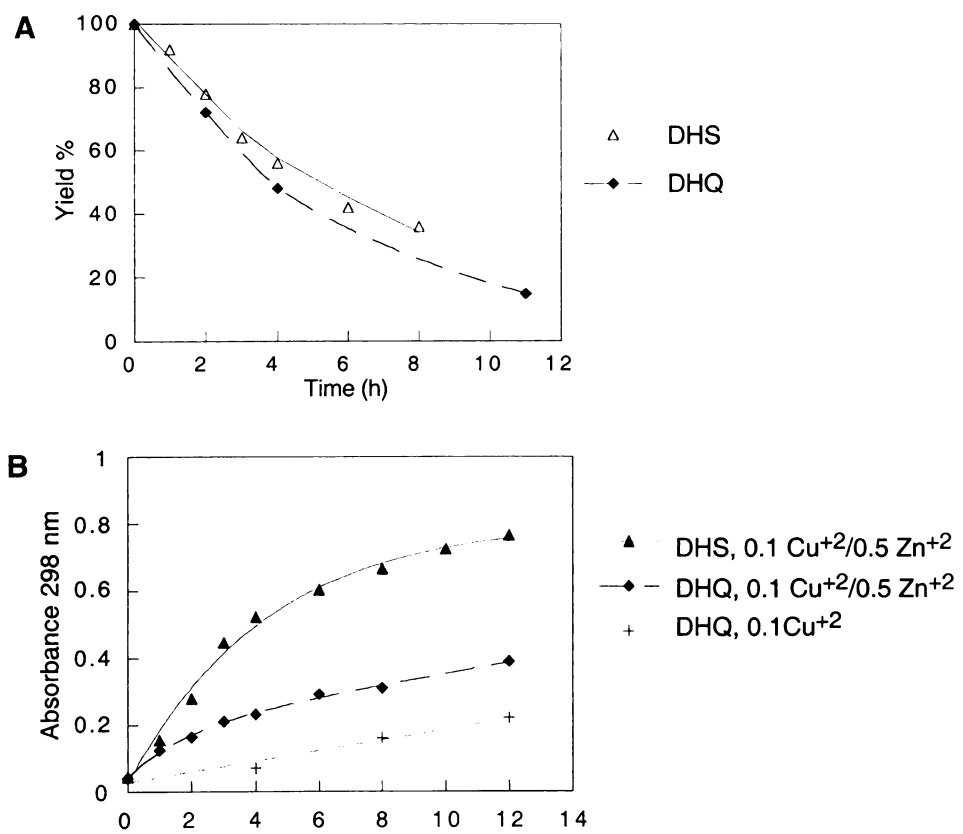


Figure 31. A. Rates of consumption of DHS versus DHQ when both react under the standard Cu/Zn (0.1 equiv and 0.5 equiv) reaction conditions; B. Rates of product formation of DHS versus DHQ.

V. DISCUSSION

The first important observation of this research was the facile reactivity of 3-dehydroshikimic acid with O_2 and H_2O_2. The antioxidant activity of DHS was evaluated by its ability to inhibit the formation of hydroperoxides in lard at 60 °C (Figure 24). This method of appraising antioxidant activity is widely used in the food industry. However, the use of higher temperatures to accelerate antioxidant measurements has been criticized for not providing an accurate parallel of the conditions typically encountered during storage of food and plant oils.[17] Therefore in addition to inhibition of hydroperoxide formation in lard samples, a different assay was examined. The efficacy of test compounds as antioxidants in this assay was measured at 23 °C as the degree of inhibition of fluorescence decay of the fluorophore (3(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid) localized within an artificial lipid membrane.[6] The antioxidant activity of 3-dehydroshikimic acid using this assay was comparable to some commercially employed antioxidant such as α-tocopherol, *tert*-butylhydroquinone (TBHQ) and propyl gallate.[6] Both the peroxide inhibition and fluoresence assays indicate that 3-dehydroshikimic acid possessed significant antioxidant activity.

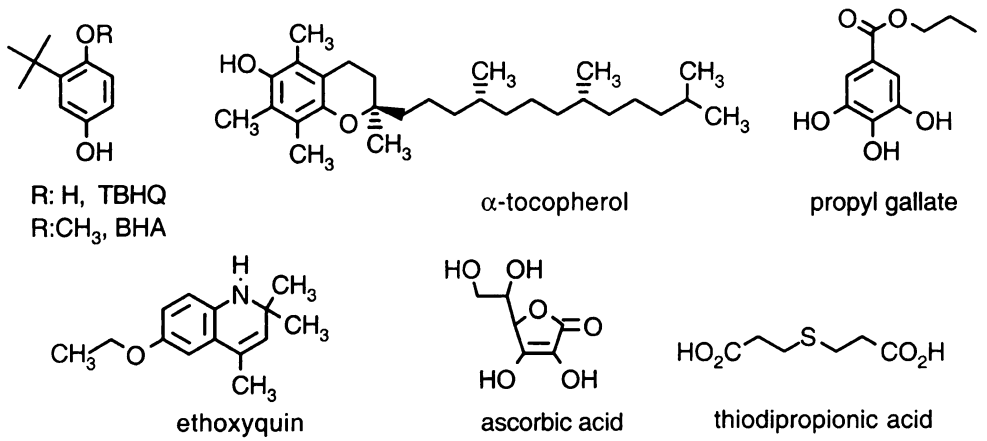


Figure 32. Commercial antioxidants

Commercial antioxidants employed in foods, materials, medicinals, and cosmetics are designed to interfere with either individual or multiple steps in oxidative decomposition processes.[18] One of the larger antioxidant classes quenches radical intermediates formed during initiation and propagation of radical chain reactions involving organic compounds and reactive oxygen species. Radical scavengers include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), TBHQ, Irganox 1010, ethoxyquin, propyl gallate, and the tocopherols (Figure 32). Ascorbic acid is a widely used O_2 scavenger. Thiodipropionic acid functions by degrading peroxides and hydroperoxides by nonradical routes. Ethylenediaminetetraacetic acid (EDTA) and citric acid deactivate metals towards redox reactions with peroxides by complexation and stabilization of the oxidized states of metal ions.

3-Dehydroshikimic acid is unique in that all of the reactivities associated with antioxidant activity are present either in 3-dehydroshikimic acid, 3-dehydroshikimic acid enediol tautomers, or the products formed from 3-dehydroshikimic acid (Figure 23). How much of the observed antioxidant activity can be attributed to 3-dehydroshikimic acid and its enediol tautomers relative to the phenolic products formed from 3-dehydroshikimic acid is unclear. As reductones, 3-dehydroshikimic acid enediols should be able to quench radicals by donation of a hydrogen atom or electron and be capable of metal complexation.[19] Clearly, 3-dehydroshikimic acid and its enediol tautomers are quite reactive with O_2 and H_2O_2. As for the phenolic products formed from 3-dehydroshikimic acid, gallic acid is a phenolic radical scavenger and is known to form complexes with metals.[18c] Protocatechuic acid is also a phenolic radical scavenger precedented to possess antioxidant activity.[14,20]

Besides the potent antioxidant activity that 3-dehydroshikimic acid possesses, the product of 3-dehydroshikimic acid oxidation, gallic acid, is an important starting material in pharmaceutical and flavor applications. High titers and yields[21] were obtained in the biocatalytic production of 3-dehydroshikimic acid from glucose using recombinant *Escherichia coli* bacteria, rendering this molecule a convenient synthon for large scale

synthesis of gallic acid using both microbial and chemical catalysis. Phosphate-catalyzed O_2 or H_2O_2 oxidations of 3-dehydroshikimic acid in water were simple and environmentally benign, but suffered from low yields to gallic acid and accumulation of a variety of byproducts including protocatechuic acid. The latter was the dehydration product of enediol A (Figure 23). Protocatechuic acid formation, besides lowering the gallic acid yield, complicated the purification since a trivial separation technique from gallic acid is not feasible. Addition of catalytic amounts of Cu^{+2} salts under the H_2O_2 oxidation conditions increased the GA/PCA ratio but still gave low product yields due to 3-dehydroshikimic acid and gallic acid decomposition. Clearly, more selective higher yielding oxidation conditions were required for an applicable synthesis of gallic acid from 3-dehydroshikimic acid.

Table 10. Comparison of stoichiometric and catalytic copper oxidations of DHS

Catalyst	Solvent	cooxidant	Cu (equiv)/ Zn (equiv)[c]	Yield % GA	Yield % PCA	Time (h)	% Yield purified GA
$CuCO_3 \cdot Cu(OH)_2$[a]	H_2O,1M Pi	–	4/0	51	2	4.5	50[d]
$Cu(OAc)_2$[a]	AcOH/H_2O	–	2.2/0	74	0.7	36	62
$Cu(OAc)_2/ZnO$[a]	AcOH	Air	0.05/0.5	77	3	11	67
$Cu(OAc)_2/ZnO$[b]	AcOH/H_2O	O_2	0.05/0.25	67	3	4	53

a. DHS concentration 50 mM b. DHS concentration 1M
c. (mol/mol) relative to DHS. d. PCA contamination was present

Oxidation of α-hydroxy ketones is well known to occur with Cu^{+2} salts.[10-13] Haslam first reported the oxidation of 3-dehydroshikimic acid to gallic acid using Fehling's solution.[12] In a phosphate buffered solution, stoichiometric amounts of copper carbonate basic ($CuCO_3 \cdot Cu(OH)_2$) oxidized 3-dehydroshikimic acid, giving after purification a powder consisting of a mixture of gallic acid (51% yield) and protocatechuic acid (2% yield) (GA/PCA:32/1) as the sole products (Table 10). Untreated fermentation supernatant can be oxidized without prior purification of 3-dehydroshikimic acid and the copper can be

recovered from the reaction mixture by a simple filtration. Unfortunately under the high phosphate concentrations required, all the copper carbonate has been transformed to a highly insoluble material, copper phosphate, making the oxidant recycling very difficult.

Higher yields to gallic acid formation (74%) and excellent product ratios (GA/PCA:99/1) were obtained after reaction of 3-dehydroshikimic acid at 40 °C with 2.2 equivalents of $Cu(OAc)_2$ in a $AcOH/H_2O$ mixture (85/15, v/v) from which gallic acid was purified in 62% yield (Table 10). The $Cu(OAc)_2$ was recovered in high yield (93-95%) and re-used in a subsequent oxidation giving reproducible oxidation results. The AcOH reaction solvent is a non-flammable environmentally benign liquid and could be recovered and reused. The microbially produced 3-dehydroshikimic acid prior to its oxidation required a solvent exchange from water to acetic acid and removal of the phosphate salts since inorganic phosphate impurities were shown to inhibit Cu^{+2}-mediated oxidation. Filtration of the fermentation broth through a strong anion exchange resin (AG1x8) resulted in adsorption of both 3-dehydroshikimic acid and inorganic phosphate. Selective elution of 3-dehydroshikimic acid with glacial AcOH provided a solution that was phosphate-free and could be oxidized without any further purification. A one-step removal of inorganic phosphate and exchange of H_2O with AcOH is thus achieved. The resin can be regenerated and reused for at least five times before the anion-binding capacity of the resin declines. Catalytic amounts of $Cu(OAc)_2$ (0.05 mol/mol equiv relative to 3-dehydroshikimic acid) in the presence of ZnO (0.5 or 0.25 mol/mol equiv relative to 3-dehydroshikimic acid) at 50 ^oC and at the same $AcOH/H_2O$ or straight AcOH solvent, catalyzed the formation of gallic acid and protocatechuic acid from which pure gallic acid was purified in 53-67% isolated yield (Table 10). These reactions can be easily scaled up to oxidize large amounts of 3-dehydroshikimic acid in concentrated solutions.

Mechanistic studies on the oxidation of α-hydroxy ketones with Cu^{+2} salts have attracted significant attention.[22] Oxidation of α-hydroxy acetophenone in aqueous pyridine with $Cu(OAc)_2$ showed that two Cu^{+2} ions were consumed for each ketol molecule

oxidized.[22a] Reaction of deuteriated ketol at the methylene position, showed a large isotope effect (k_H/k_D=7.4) indicating that the rate-determining step was the enolization.[22a] In addition, the effect of substituents in the rate of oxidation of α-hydroxyacetophenone was similar to the effect of the substituents on the rate of enolization of the same compound.[22a] At very low concentrations of Cu^{+2}, the rate was independent of the oxidant concentration[22a] whereas at higher concentrations of Cu^{+2} the rate law became dependent on the concentration of Cu^{+2}. In addition, Cu^{+2} is known to catalyze the enolization of α-hydroxy ketones such as acetoacetic ester.[22c] These results were interpreted in terms of the formation of a Cu^{+2}-ketol complex, where the rate-determining step was the removal of the α-methylene proton by the base (hydroxide or pyridine). Kinetic experiments for the oxidation of a variety of sugars in alkaline medium showed first order dependence on the concentration of alkali and carbohydrate, and no dependence on the Cu^{+2} concentration. In these cases, enolization appears to be the rate-determining step.[16]

Even though catalytic amounts of Cu^{+2} have been reported to oxidize ketols in organic solutions, no mechanistic studies have been performed in these systems. In aqueous solutions, we showed that the oxidation rate of 3-dehydroshikimic acid with oxygen, H_2O_2, or insoluble copper salts, was dependent on the rate of enolization catalyzed by inorganic phosphate. In AcOH/H_2O mixtures, Lewis acids and copper (Figure 28, Table 8) catalyzed the reaction. Zn^{+2} was the most effective catalyst relative to Mg^{+2} and Mn^{+2}. In the absence of Cu^{+2}, Zn^{+2} catalyzed the formation of protocatechuic acid. The oxidation rate was dependent on both 3-dehydroshikimic acid and the concentration of the Lewis acid indicating that both are involved in the rate-determining step of the reaction, which is most likely to be the formation of the first 3-dehydroshikimic acid enolate (Figure 33, II). In addition, the lack of H/D exchange in the C4 or C5 carbons that was observed when 3-dehydroshikimic acid reacted with Zn^{+2} in a D_2O/CD_3CO_2D solution, suggests that formation of the enediol is irreversible, or that oxidation by Cu^{+2} is very rapid after the enol has been formed. The presence of a α-hydroxy carbonyl functionality was found to be

essential for the oxidation to occur, since shikimic acid and glucose (Figure 30) were not oxidized. Based on these results and previously reported examinations of copper oxidations, the following mechanism for the Cu/Zn oxidations is being proposed (Figure 33).

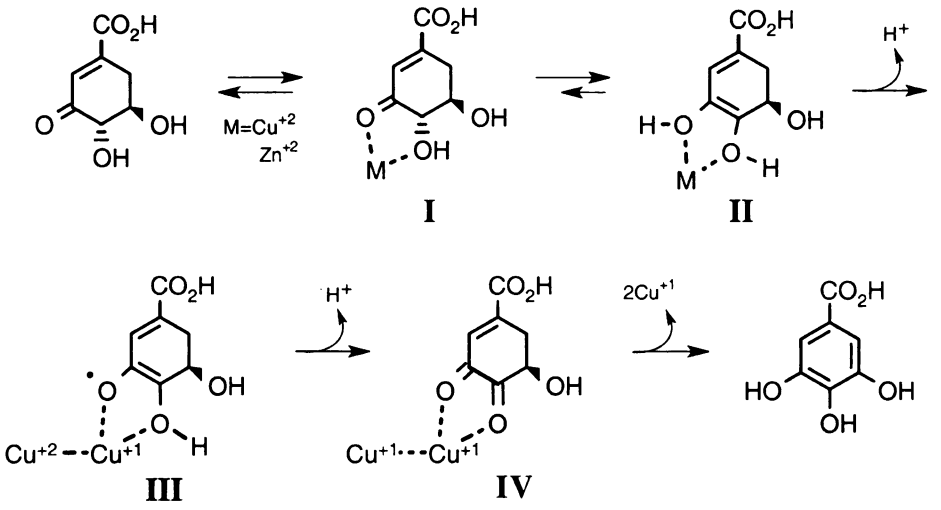


Figure 33. Proposed mechanism for the oxidation of DHS with Cu^{+2} and Cu^{+2}/Zn^{+2} salts

The rate-determining step is the first enolization which is catalyzed by Zn^{+2} and Cu^{+2} with different reaction constants. Zn^{+2} is most likely catalyzing the formation of the enol intermediate by either directly complexing the alcohols of the diol followed by a fast metal exchange with the copper, or by lowering the activation energy of the copper-enol formation probably through an interaction with the diene. Zn-cyclopentadiene complexes are known to form, and both σ- and π- type interactions contribute to the bonding.[23] After the first enolization step (intermediate II), copper oxidizes the substrate by two sequential single electron transfer reactions. Although the enolization step is reversible (II→I), fast oxidation of the alcohol by Cu^{+2} (II→III) prevents this reaction (Figure 33). Reoxidation of the copper by oxygen is a separate reaction. The standard reduction potential for Cu^{+2}/Cu^{+1} is +0.16 eV, while the reduction potential of water formation from oxygen and

protons (O_2 + $4H^+$ +$4e^-$ → $2H_2O$) is +1.23 eV. Reoxidation of Cu^{+1} in AcOH is therefore a spontaneous, thermodynamically-favored process. Other studies[24] have shown that Cu^{+1} has very high affinity for molecular oxygen and forms monomeric and dimeric Cu^{+1}-O_2 structures with simultaneous oxidation to Cu^{+2}.

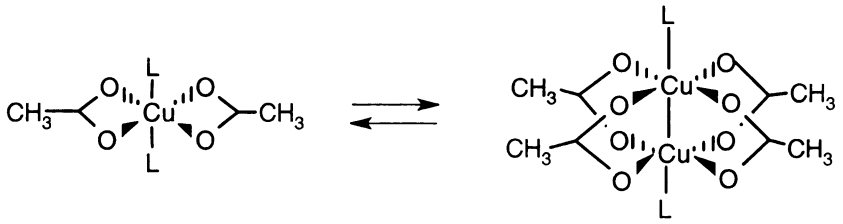


Figure 34. $Cu(OAc)_2$ can form monomeric or dimeric structures in different solvents, L.

The availability of the cupric ion for complexation with the substrate is solvent-dependent. $Cu(OAc)_2$ can form monomeric or dimeric compounds with carboxylic acids (Figure 34).[25] The formation of the dimeric structure is favored in organic solvents. In water, it is destroyed as a result of the formation of monomeric hydrated ions. Other factors that determine the equilibrium are the solvent and the strength of the carboxylic acid. Solvents with low coordination power favor dimeric forms of the complex. The dimeric structure of $Cu(OAc)_2$ was verified by x-ray analysis.[25b,c] The UV-vis absorption spectra of $Cu(OAc)_2$ in crystalline form and in ethanol solution were identical, indicating that the dimeric structure of the complex is retained in ethanol solution.[25a] In acetic acid, the dimeric form predominates.[25] The availability of copper for complexation appears to be important for effective oxidation. It has been reported that catalytic oxidations of steroidal ketols with $Cu(OAC)_2$ in MeOH are inhibited upon addition of water.[13a] In our hands, increasing the water content in the oxidation reactions or using copper salts with strong coordination ligands ($CuSO_4$) under the stoichiometric or the Cu/Zn reaction conditions was found to significantly decrease the reaction rate or did not allow reaction completion. The existence

of the dimeric structure of $Cu(OAc)_2$ can increase the rate of the second electron transfer between the two copper atoms (Figure 33, III→IV) or create a two copper center complex that can perform the oxidation in a single step (Figure 35). The latter is a very likely possibility since a six-membered ring between the $Cu(OAc)_2$ dimer and 3-dehydroshikimic acid is formed, and since acetic acid can be easily exchanged in the coordination sphere.

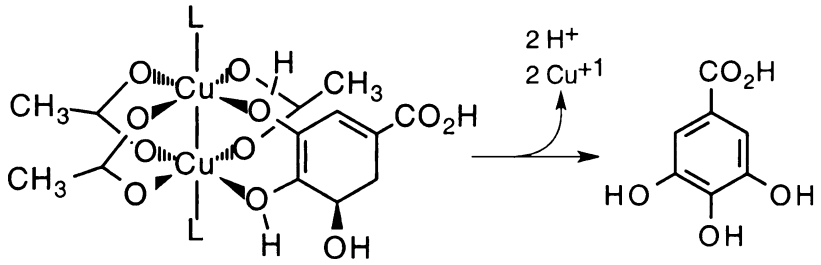


Figure 35. Single step oxidation of DHS using the dimeric $Cu(OAc)_2$ structure.

Catalytic amounts of copper salts have been reported in the literature to catalyze alcohol oxidations under various conditions. Allylic and benzilic alcohols are oxidized at room temperature using cuprous chloride (CuCl, 10% mol/mol relative to the alcohol) and 2,2,6,6,-tertamethylpiperidinyl-1-oxy (Tempo, 10% mol/mol relative to the alcohol) in dimethyl formamide (DMF) using oxygen as the cooxidant.[26] Aliphatic alcohols do not give complete conversions under these conditions. Recently, catalytic oxidations using copper salts in basic media have attracted considerable attention.[27] In toluene, a variety of alcohols are efficiently oxidized using 5 mol %, CuCl·phen (Phen: 1,10 phenanthroline), 5 mol % di-*tert*-butyl-azodicarboxylate hydrazine ($DBADH_2$) and 2 equivalents of K_2CO_3 with oxygen as the ultimate stoichiometric oxidant. The reaction is not selective and oxidizes primary, secondary, allylic or benzylic alcohols at similar rates. Toluene and benzene are the solvents of choice since polar solvents inhibit the reaction.

The catalytic reactions with copper and zinc discovered in this study offer some advantages compared to the above mentioned catalytic oxidations. The solvent system

(AcOH/H_2O, 6/1, v/v) is nonflamable, nontoxic, and environmentally-benign. Low temperatures are used (40-50 °C) and no special precautions are necessary. The reaction can be used in high concentrations (1 M) of 3-dehydroshikimic acid, which allows smaller volumes of solvent to be used. Scaling up is easy and both copper and zinc can be recovered at the end of the reaction. In addition, no other organic ligands or cooxidants are required. Finally, the reaction is high-yielding, mild and selective for α-hydroxy ketone functionality.

3-Dehydroshikimic acid and 3-dehydroquinic acid oxidations employing Cu^{+2} suggest that biosynthesis of gallic acid from either 3-dehydroshikimic acid or 3-dehydroquinic acid may employ an enzyme using Cu^{+2} as a cofactor. Oxidative enzymes which use Cu^{+2} are well known.[28] Ascorbate oxidase, which is catalyzes the oxidation of ascorbic acid to dehydroascorbic acid, contains four copper ions per subunit. One of these copper ions is a center for the electron transfer.[29] The ascorbic acid structure (Figure 32) is very similar to the first enediol intermediate formed in both 3-dehydroshikimic acid and 3-dehydroquinic acid oxidation (Figure 23 and 33).

Figure 36. ^{1}H NMR of 3-dehydroshikimic acid in EtOAc after continuous extraction from the fermentation broth of DHS-synthesizing bacteria cultured under fed-batch fermentor conditions. d 6.42 (d, 1H), 4,28 (d, 1H), 4.00 (ddd, 1H), 3.07 (dd, 1H), 2.66 (ddd, 1H) in D_2O

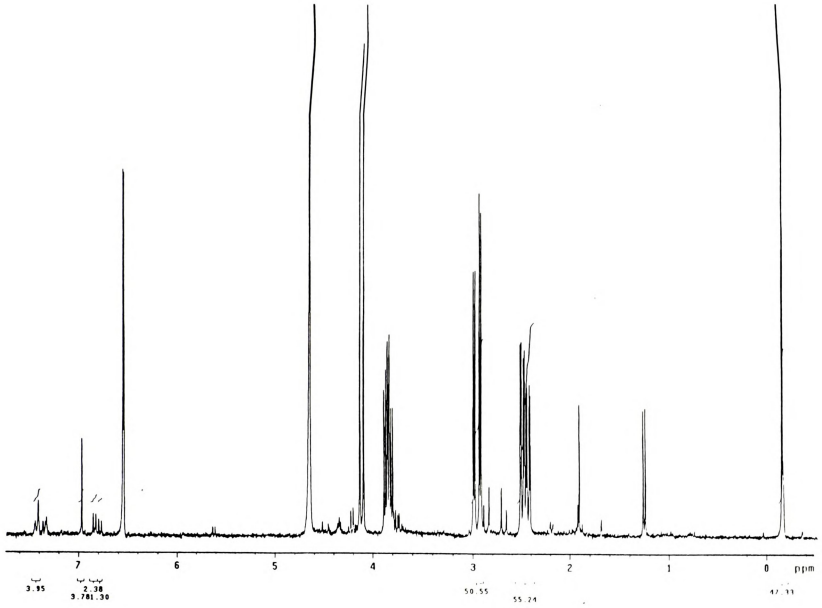

7
6
5
4
3
2
1
0
ppm
3.95
2.38
3.781.30
50.55
55.74

Figure 37. ^{1}H NMR of standard gallic acid: δ 7.1 (s, 2H), in D_2O.

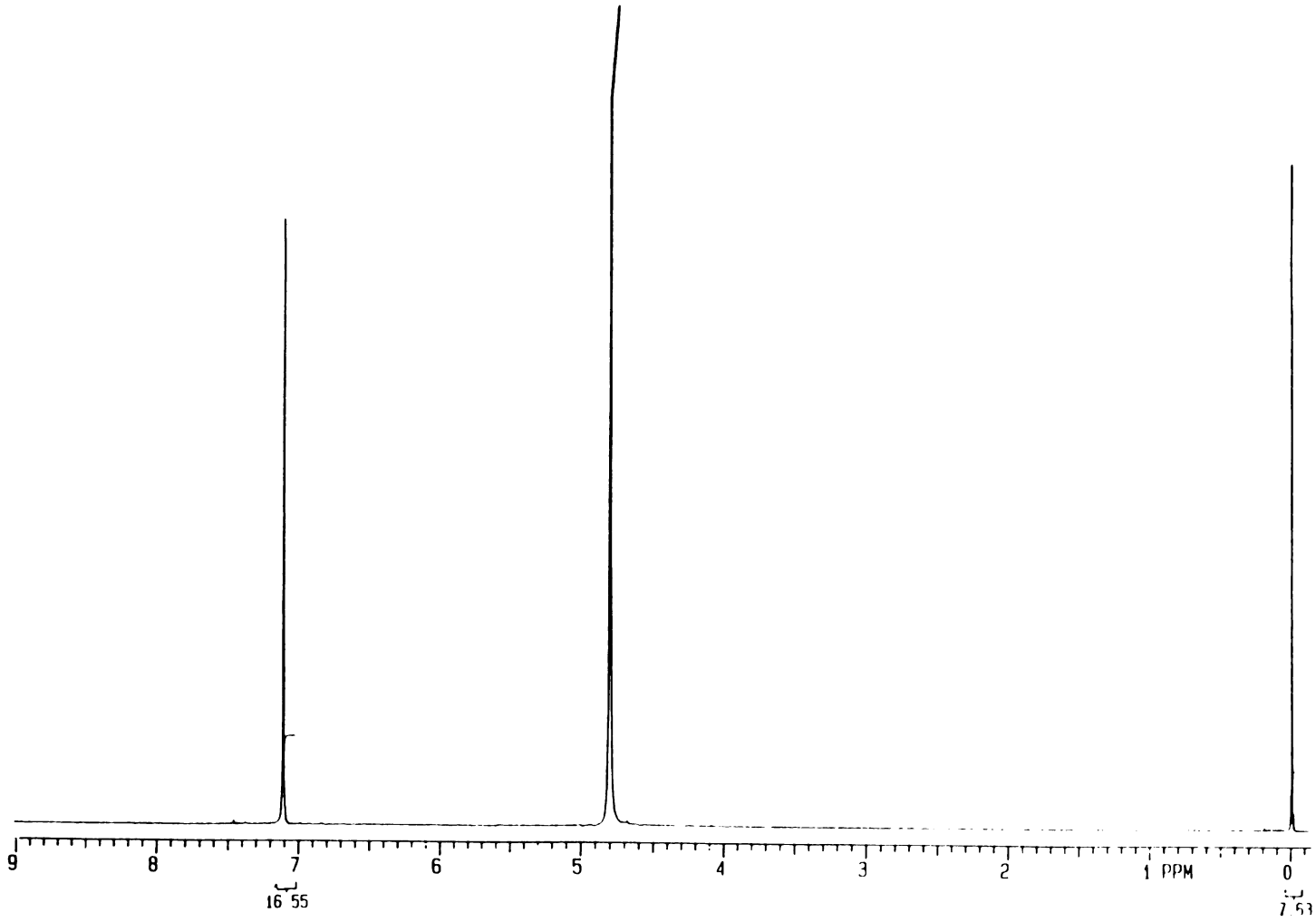

9
8
7
6
5
4
3
2
1 PPM
0
16 55
7 53

Figure 38. ^{1}H NMR of standard pyrogallol : δ 6.55 (d, 2H), 6.75 (t, 1H), in D_2O.

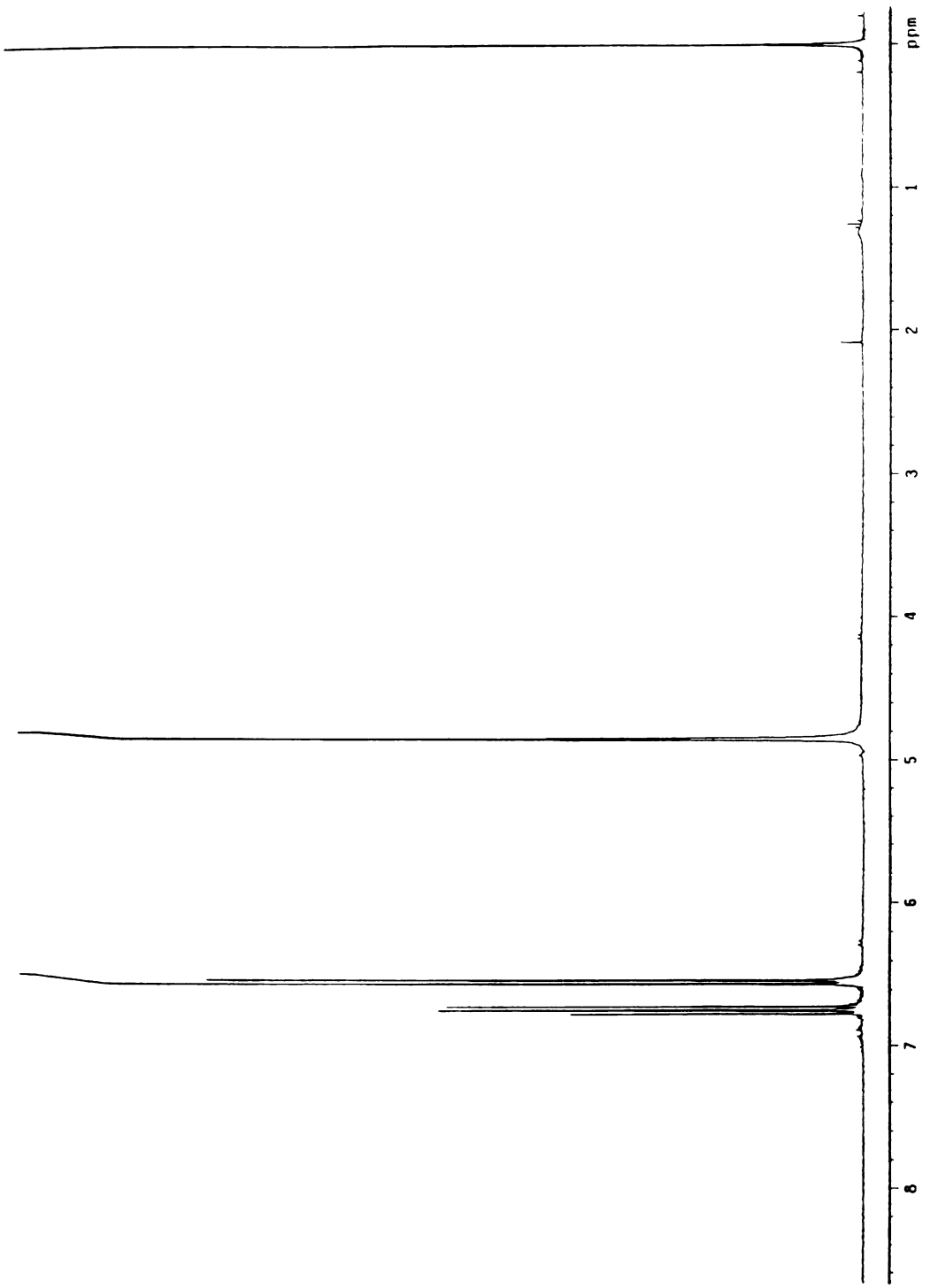

ppm
1
2
3
4
5
6
7
8

Figure 39. ^{1}H NMR standard of tricarballylic acid : δ 3.23 (q, 1H), 2.74 (m, 2H), in D_2O

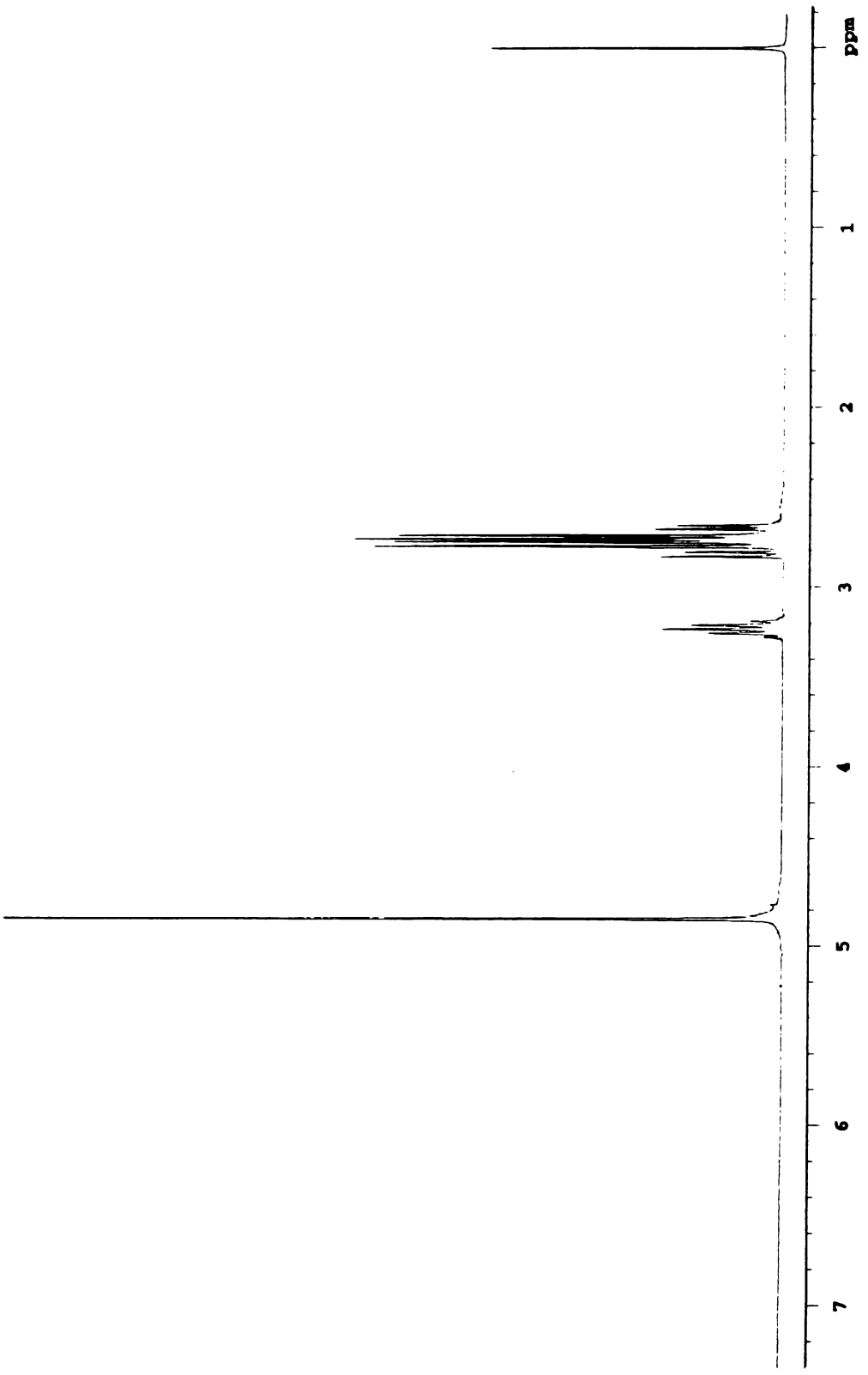

ppm
1
2
3
4
5
6
7

Figure 40. ^{1}H NMR of phosphate (1 M) catalyzed reaction of 3-dehydroshikimic acid with atmospheric O_2 : GA :δ 7.1 (s, 2H); PCA : δ 7.45 (m, 2H) 6.95 (d, 1H); TCBA : δ 3.23 (q, 1H), 2.74 (m, 2H), PGL : δ 6.55 (d, 2H), 6.75 (t, 1H) in D_2O

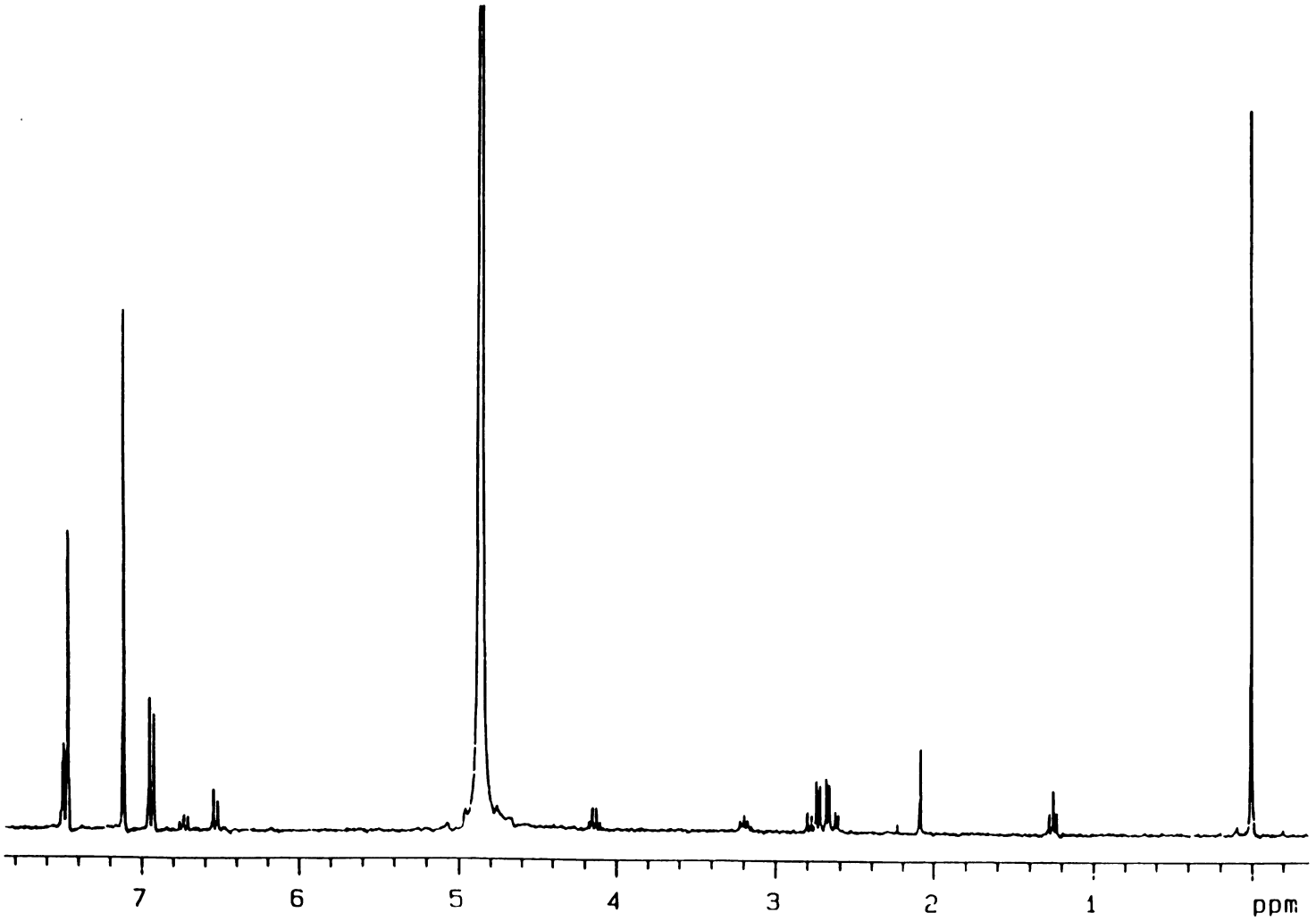

7
6
5
4
3
2
1
ppm

Figure 41. ^{1}H NMR of phosphate-catalyzed reaction of 3-dehydroshikimic acid with H_2O_2 and catalytic (1% mol/mol rel. to DHS) amounts of $Cu(OAc)_2$. A sample from the reaction mixture was filtered through Dowex 50 (H^+) to remove the metals, and the solvent was exchanged with D_2O. GA resonance δ 7.1 (s, 2H), PCA resonances δ 7.45 (m 2H), 6.95 (d, 1H).

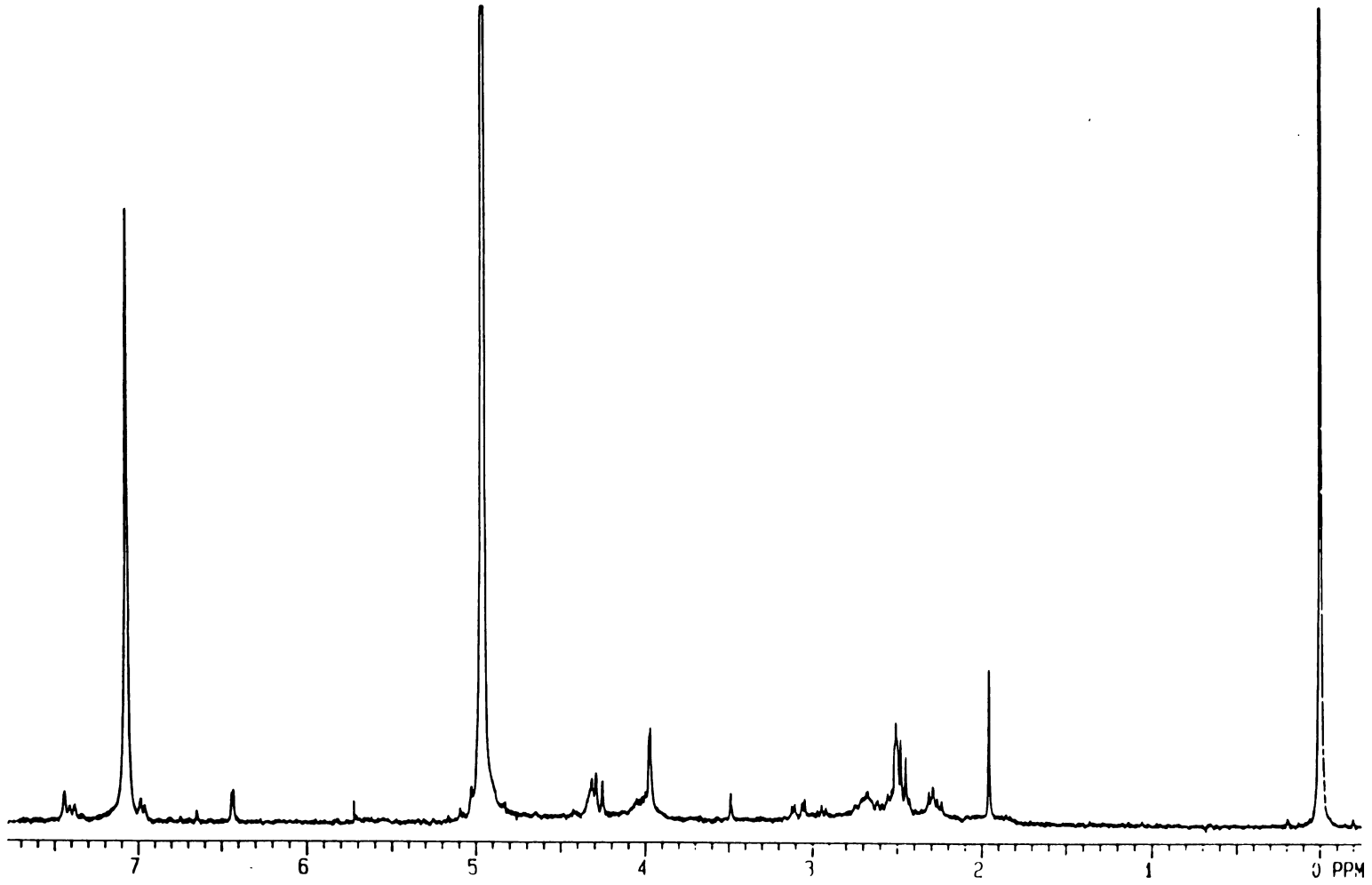

7
6
5
4
3
2
1
0 PPM

Figure 42. ^{1}H NMR of phosphate-catalyzed (1 M) reaction of 3-dehydroshikimic acid with $CuCO_3 \cdot Cu(OH)_2$ (2.2 equiv relative to DHS). A sample from the reaction mixture was filtered through Dowex 50 (H^+) to remove the metals, and the solvent was exchanged with D_2O. GA, DHS, and traces of PCA are present.

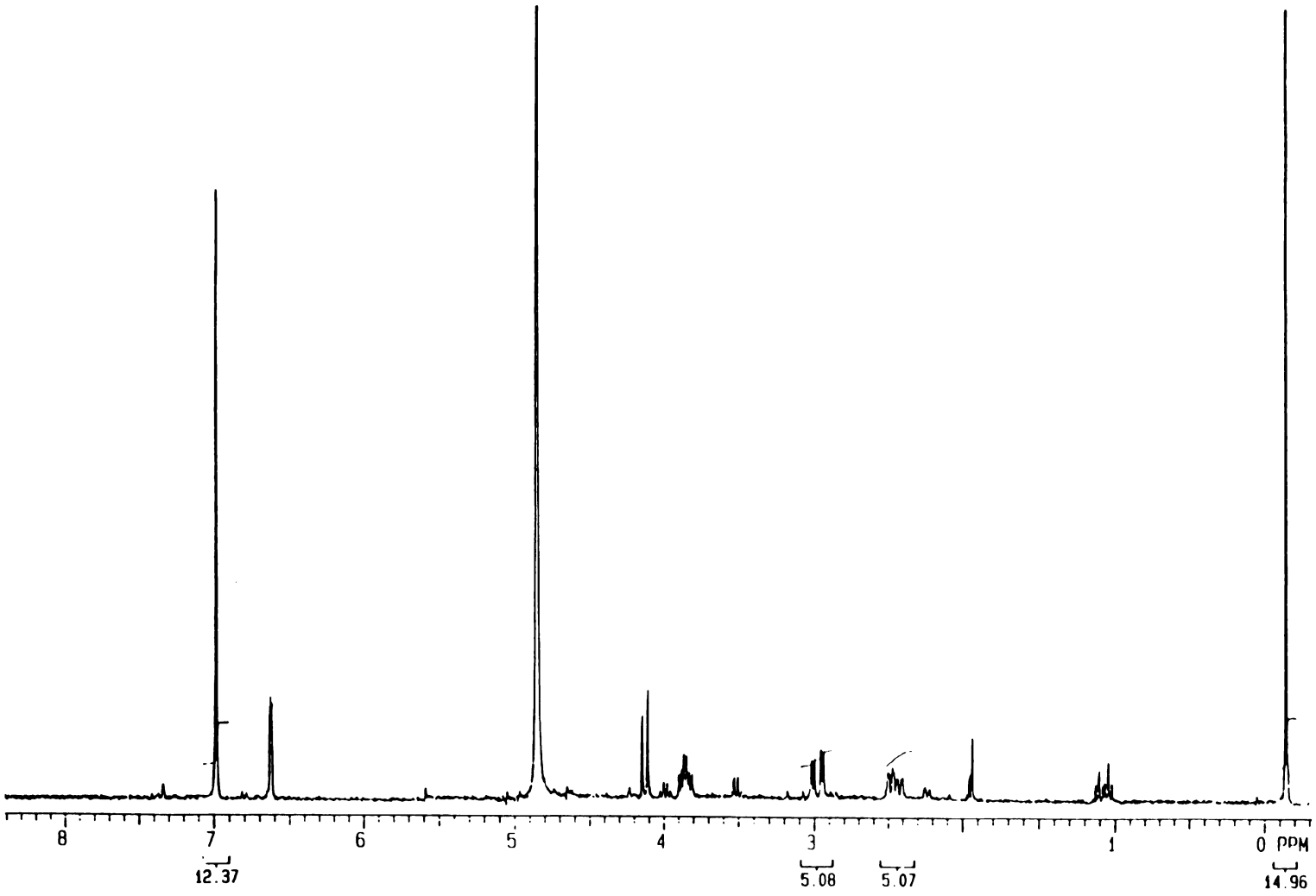

8
7
6
5
4
3
1
0 PPM
12.37
5.08
5.07
14.96

Figure 43. ^{1}H NMR of stoichiometric reaction of 3-dehydroshikimic acid with $Cu(OAc)_2$ in $AcOH/H_2O$ (v/v 8/2). A sample from the reaction mixture was filtered through Dowex 50 (H^+) to remove the metals, and the solvent was exchanged with D_2O. GA and traces of PCA are present.

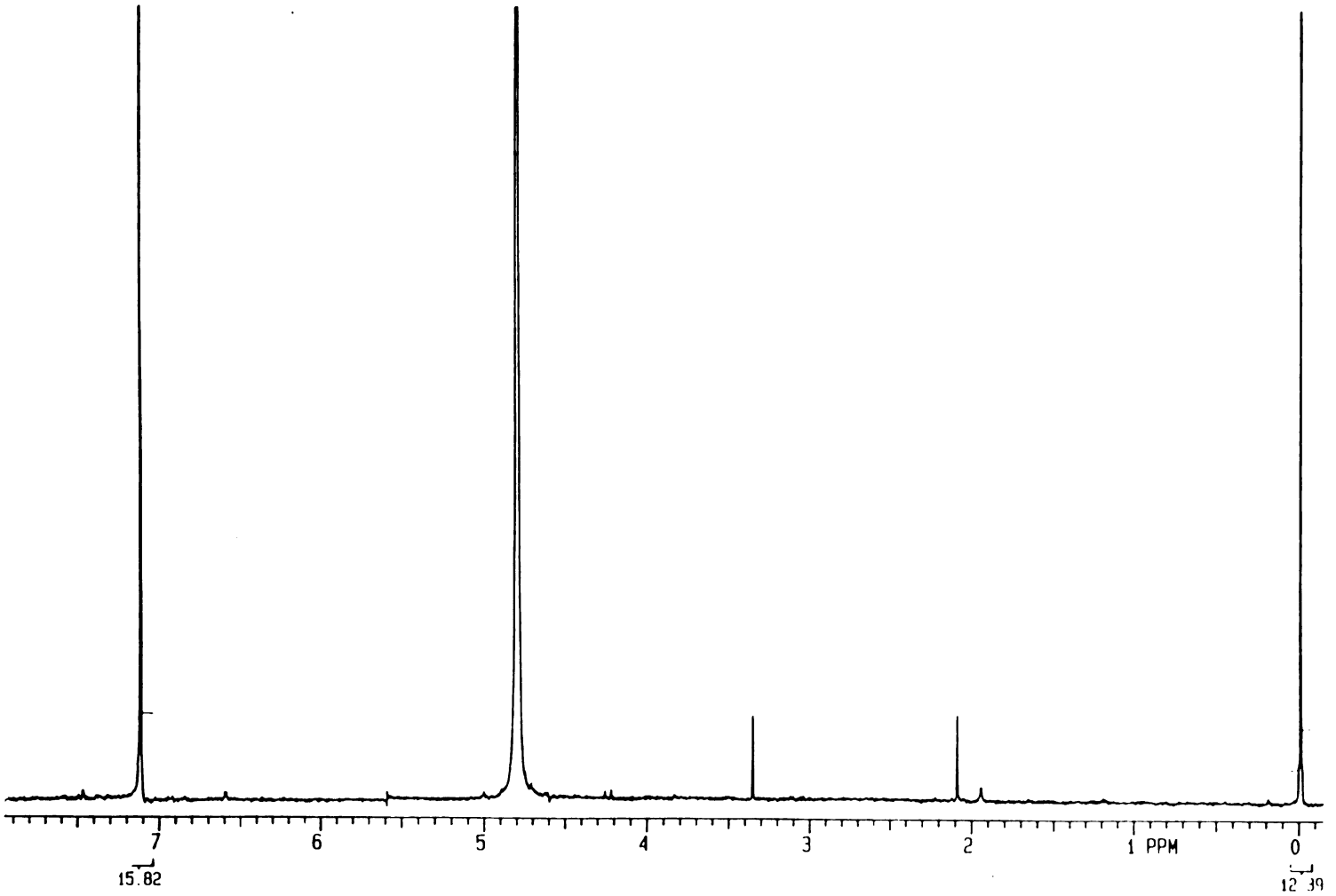

7
6
5
4
3
2
1 PPM
0
15.82
12.39

Figure 44. ^{1}H NMR of reaction of 3-dehydroshikimic acid with catalytic amounts of $Cu(OAc)_2$ (10% rel to DHS) and ZnO (50 rel to DHS) in AcOH/H_2O (v/v, 85/15). A sample from the reaction mixture was filtered through Dowex 50 (H^+) to remove the metals, and the solvent was exchanged with D_2O. GA, PCA and AcOH are present.

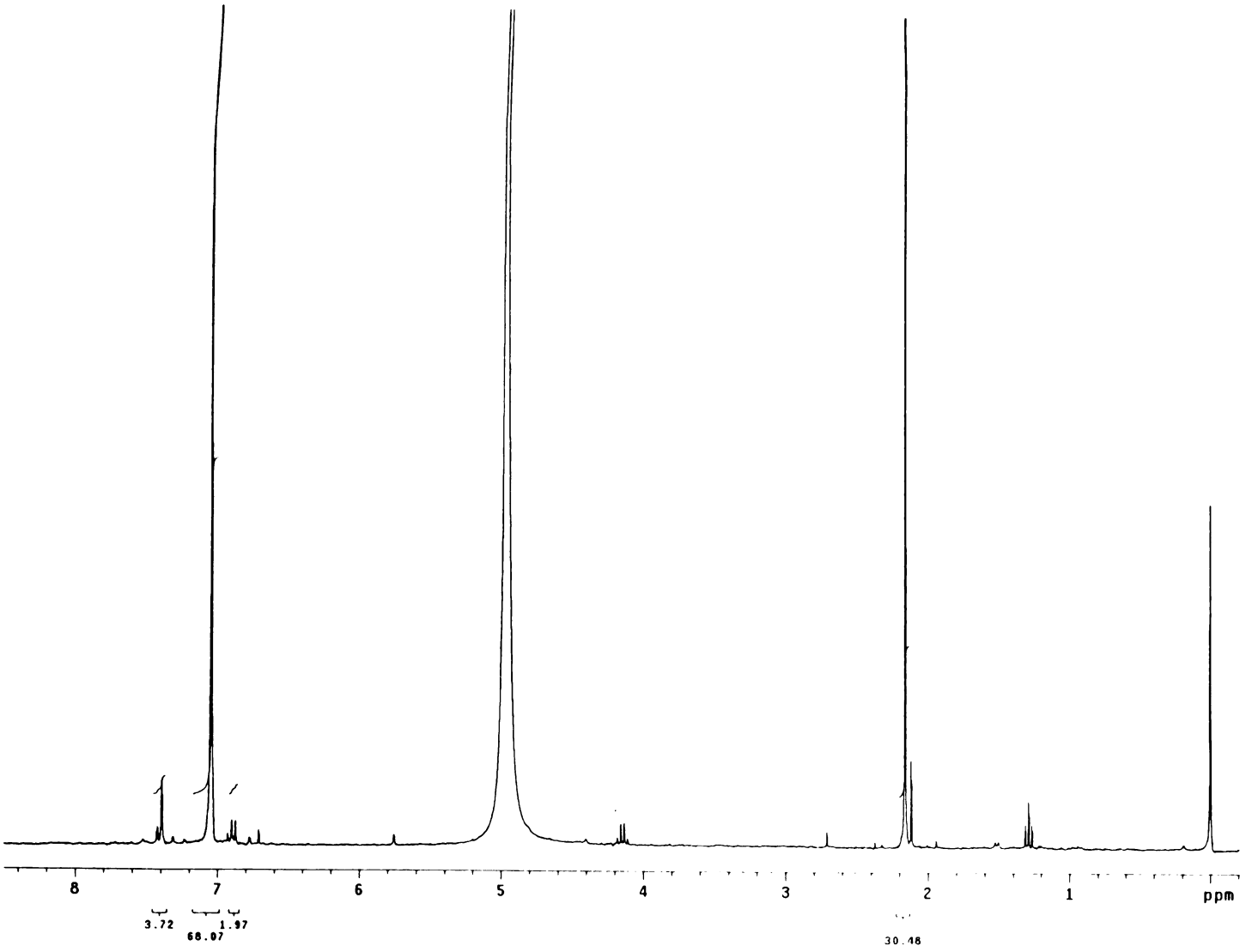
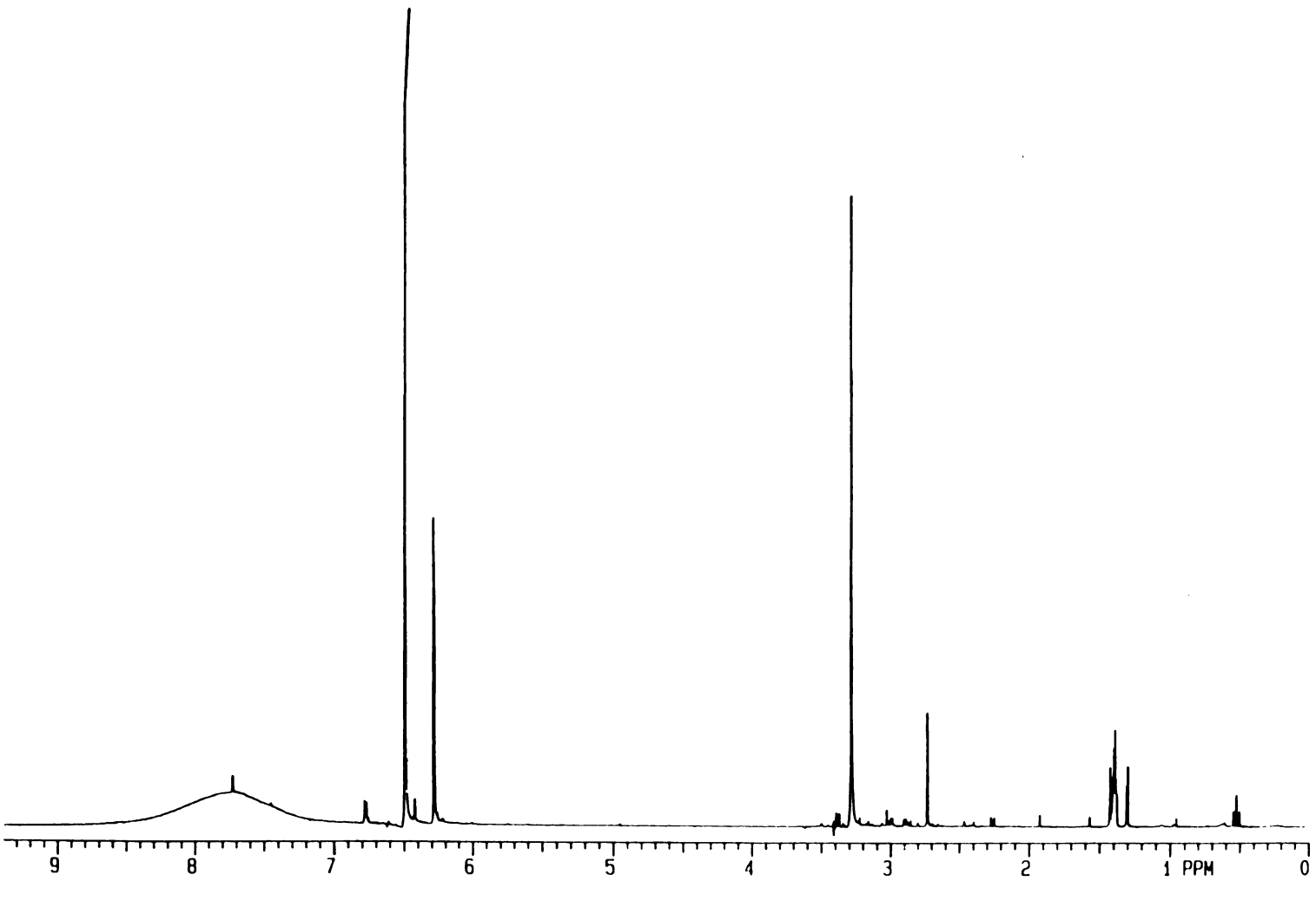

8
7
6
5
4
3
2
1
ppm
3.72
68.07
1.97
30.48

Figure 45. ^{1}H NMR of reaction of gallic acid with H_2O_2 and catalytic amounts (10% mol/mol rel. to GA) of $Cu(OAc)_2$. A sample was filtedred through Dowex 50 (H^+) and the solvent was exchanged with acetone-d_6. GA resonances δ 6.5 (s, 2H), aconitic acid resonances δ 6.3 (s, 1H), 3.3 (s, 2H), gluconic acid resonances, δ 2.7 (s, 2H).


9
8
7
6
5
4
3
2
1 PPM
0

References

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CHAPTER 4

BIOCATALYTIC SYNTHESIS OF GALLIC ACID FROM PROTOCATECHUIC ACID AND 3-DEHYDROSHIKIMIC ACID

I. INTRODUCTION

Gallic acid is widely distributed in plants and fungi as the major component in a variety of biomolecules. In nature, gallic acid is primarily formulated in plant polyphenols[1,2] known as tannins. Gallic acid is obtained by hydrolyis of these polymers. At least 750 metabolites of gallic acid have now been described ranging in molecular weight from 500 to 4000. In Chinese galls (*Rhus semialata*) gallate-containing polymers constitute up to 70% the dry weight of the gullnuts.[1b]

Besides the natural significance of tannins, antitumor activities are displayed by some ellagitannin derivatives.[1c,3] The biosynthesis of these complicated polyphenol metabolites has attracted considerable attention.[1a,b,2] While the complete biosynthetic construction of higher tannins is not fully understood, the initial steps in the biosynthesis of gallotannins have been worked out and consists of a series of esterifications of the glucopyranoside core molecule with gallic acid.[2,1b] Surprisingly, the complete biosynthesis of the most important building block, gallic acid, is still unclear.

It is generally accepted that gallic acid biosynthesis starts from 3-dehydroshikimic acid or phenylalanine which are both metabolites of the common pathway of aromatic amino acid biosynthesis. Two main routes to the biosynthesis of gallic acid have been proposed. In the first L-phenylalanine is processed to 3,4,5-trihydroxycinnamic acid[4] (Figure 46 Route A) or protocatechuic acid[5] (PCA) through caffeic acid intermediacy (Figure 46, Route B). Hydroxylation of protocatechuic acid or oxidative cleavage of the double bond of 3,4,5-trihydroxycinnamic acid will lead to gallic acid formation (Figure 46). Protocatechuic

acid is a widely used metabolite and its biosynthesis is known.[6] 3,4,5-Trihydroxycinnamic acid on the other hand has never been encountered in nature, and as a result, its intermediacy in gallic acid synthesis is questionable. In the second route, gallic acid biosynthesis starts from 3-dehydroshikimic acid (DHS), a much earlier intermediate of the common pathway.[7] Oxidation of 3-dehydroshikimic acid (Figure 46, Route C) or dehydration of 3-dehydroshikimic acid to protocatechuic acid followed by hydroxylation (Figure 46, Route D) constitute a shorter biosynthesis of gallic acid. Recent studies using ^{13}C-labeled metabolites in the fungus *Phycomyces Blakesleanus* indicate 3-dehydroshikimic acid as the precursor of gallic acid biosynthesis.[8] The intermediacy of protocatechuic acid however is still uncertain.[8]

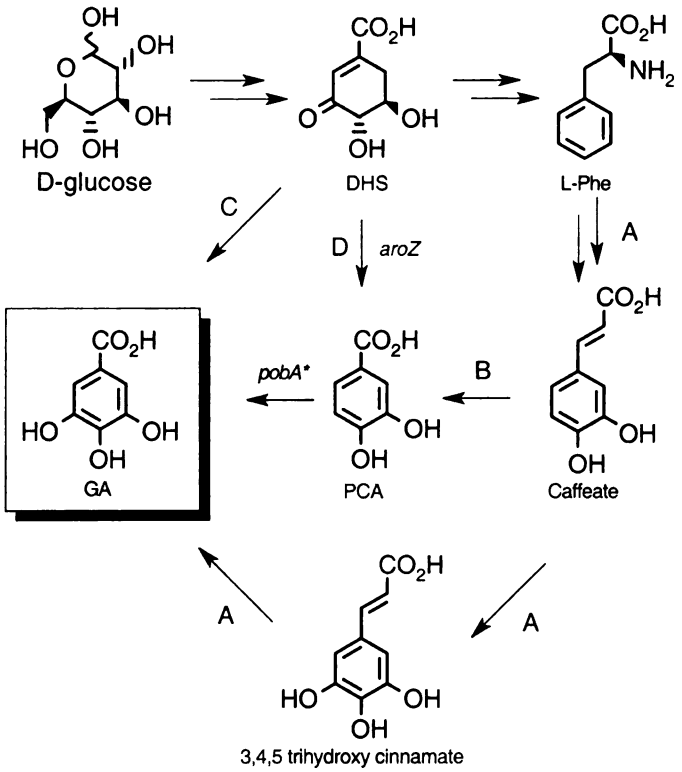


Figure 46. Proposed pathways for the biosynthesis of gallic acid

A naturally-occurring enzyme that can hydroxylate protocatechuic acid resulting in gallic acid formation has not yet been isolated or even detected. However, enzymes that can hydroxylate aromatic rings ortho to hydroxyl substituents are known in nature. The biosynthesis of L-Dopa, for example, requires aromatic *ortho* hydroxylation of L-tyrosine.[9] Many microorganisms can grow on aromatic acids formed as degradation products of plant material that are present in soil. Growth on *p*-hydroxybenzoic acid by these organisms is based in the transformation of this aromatic acid into tricarboxylic acid cycle (TCA) intermediates via the β-ketoadipate pathway.[11,12,20] One step in *p*-hydroxybenzoic acid catabolism involves ortho hydroxylation to yield protocatechuic acid using *pobA*-encoded *p*-hydroxybenzoate hydroxylase (PHB hydroxylase). A variety of organisms including *Pseudomonas*,[10,15,20a] *Acinetobacter*,[11] and *Klebsiella*[12] species contain this enzymatic activity. Other aromatic hydroxylases include a *K. pneumoniae*[13] 3-hydroxybenzoate-6-hydroxylase, which catalyzes the hydroxylation of 3-hydroxybenzoate to 2,5 dihydroxybenzoate (gentisate), and an *E. coli* broad substrate hydroxylase classified as 4-hydroxyphenylacetic acid hydroxylase, which hydroxylates various substituted phenols such as 3-hydroxyphenylacetic acid, phenol, *p*-cresol, resorcinol and hydroquinone.[14]

Considering the variety of natural enzymes that hydroxylate phenolic aromatic ring carbons, the existence of an enzyme capable of hydroxylating protocatechuic acid to form gallic acid seems reasonable. In fact, Ballou and coworkers, while studying the mechanism of *Pseudomonas aeruginosa* PHB hydroxylase encoded by *pobA* found that site-directed mutagenesis resulting in the replacement of tyrosine 385 with a phenylalanine produced an isozyme (PobA*) capable of hydroxylation of protocatechuic acid to form gallic acid.[15] The wild-type enzyme is a dimer of two identical subunits of MW 45,000. Each monomer contains one FAD. The three dimensional x-ray analysis of both the wild-type and the mutant isozymes showed almost identical three dimensional structures.[16,17] In both structures the positions of the flavin and modified residue are not altered.[16b]

Extensive mechanistic studies of the wild-type enzyme revealed that hydroxylation of the aromatic ring occurs in two stages.[15-17] In the first, the enzyme containing the oxidized FAD binds *p*-hydroxybenzoate (K_D=9.5 μM) and NADPH (K_D=210 μM) to form a ternary complex.[15b] In this complex rapid reaction of FAD and NADPH (Step 1, Figure 47) generates the reduced flavin. In the absence of enzyme-bonded *p*-hydroxybenzoate, FAD is reduced by NADPH 100,000 times more slowlly.[15,17] Reduced FAD reacts rapidly with O_2 to form a flavin-hydroperoxide (Step 2, Figure 47) which then hydroxylates the enzyme-bound substrate (Step 3, Figure 47). Elimination of water regenerates the oxidized flavin (Step 4, Figure 47). The decay of the flavin hydroperoxide to oxidized flavin and H_2O_2 (step 5 Figure 47) cannot be measured in the wild-type enzyme due to the small fraction of the enzyme involved. The enzyme hydroxylates *p*-hydroxybenzoic acid with a turnover number of 44 s^{-1} as measured at 25 °C and a pH of 7.9.[15b]

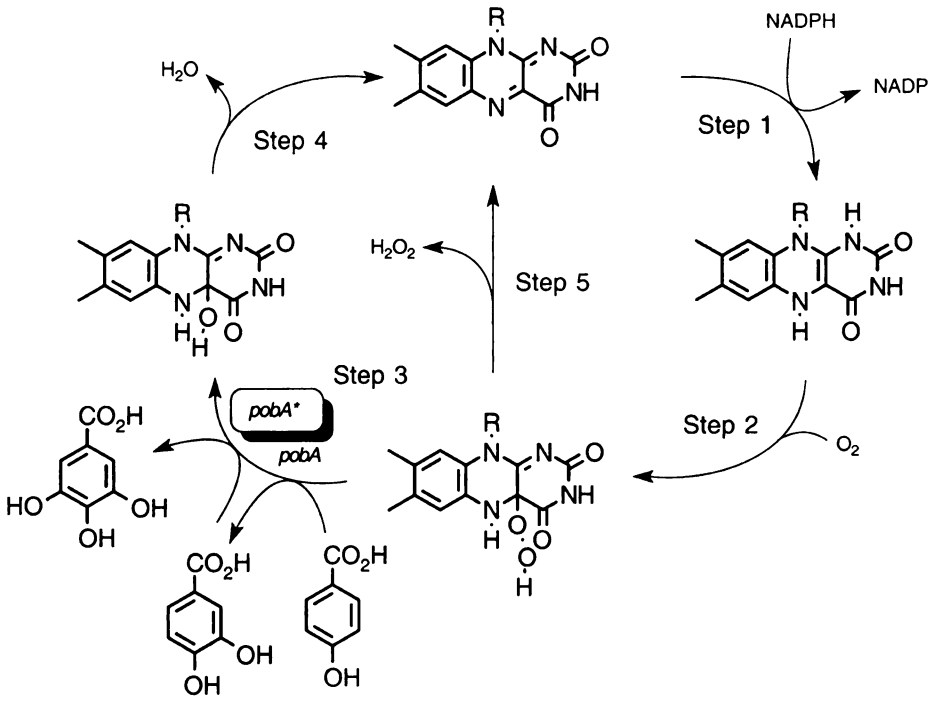


Figure 47. Hydroxylation mechanism of PHB hydroxylase

The mutant (Tyr385→Phe) enzyme utilizes the same general mechanism for the oxidation of protocatechuic acid. The efficiency of gallic acid formation by this enzyme was measured in assays using protocatchuic acid as the substrate. In this case, 20% of the NADPH consumed led to gallic acid formation. The remainder of the reducing equivalents resulted in the formation of H_2O_2 (Step 5, Figure 47).[15b] Although the mutant enzyme has a similar affinity for *p*-hydroxybenzoic acid (K_D=7.9 μM) compared to the wild type (K_D=9.5 μM), its affinity for protocatechuic acid (K_D=66 μM) is higher relative to wild-type (K_D=230 μM). Binding of either protocatechuic acid or *p*-hydroxybenzoic acid in the active site of the mutant isozyme activates reduction of FAD by NADPH with approximately the same rate. However, this reduction is 10 times slower compared to the reduction of FAD by NADPH in the wild-type enzyme using *p*-hydroxybenzoic acid as the substrate. The hydroxylation ability of *p*-hydroxybenzoic acid to protocatechuic acid by the mutant enzyme is 80% as measured by the ratio of product formation versus NADPH oxidation. An overall lower rate of *p*-hydroxybenzoic acid oxidation was also measured (turnover number 0.91 s^{-1}).[15b] No measurements of the turnover number using protocatechuate as the substrate have been reported.

In this Chapter, synthesis of gallic acid utilizing the mutant PHB hydroxylase (PobA*) was examined. The synthesis employed a two-step strategy where 3-dehydroshikimic acid and protocatechuic acid were microbially synthesized independently and then added to the culture supernatant of a biocatalyst that expressed plasmid-localized *pobA**. The efficiency of this mutant enzyme towards gallic acid formation was evaluated in *E. coli* bacteria cultured under fed-batch fermentor conditions. These microbe-catalyzed conversions addressed the fundamental issue of protocatechuic acid and 3-dehydroshikimic acid transport into the *E. coli* cytoplasm and the toxicity issues arising from the intracellular formation of both gallic acid and H_2O_2. These research activities establish the foundation for de novo synthesis of gallic acid from glucose and provide a basis for comparing biocatalytic versus chemical conversion of 3-dehydroshikimic acid into gallic acid.

II. MICROBIAL SYNTHESIS OF GALLIC ACID FROM 3-DEHYDROSHIKIMIC ACID AND PROTOCATECHUIC ACID

A. Construction of the host strain

AB2834, an *E. coli aroE*$^-$ strain[18] was chosen as the parental strain for construction of a gallic acid producing biocatalyst. As a result of the *aroE*-encoded shikimate dehydrogenase mutation, 3-dehydroshikimic acid can not be transformed into shikimic acid and be processed to the biosynthesis of aromatic amino acids through the common pathway enzymes (Figure 48 and Figure 2 Chapter 1). 3-Dehydroshikimic acid is therefore accumulating in the cytosol of AB2834. An *aroBaroZ* cassette was site-specifically inserted into the *serA* locus of *E. coli* AB2834 *aroE*$^-$ via homologous recombination to generate *E. coli* KL7.[19]

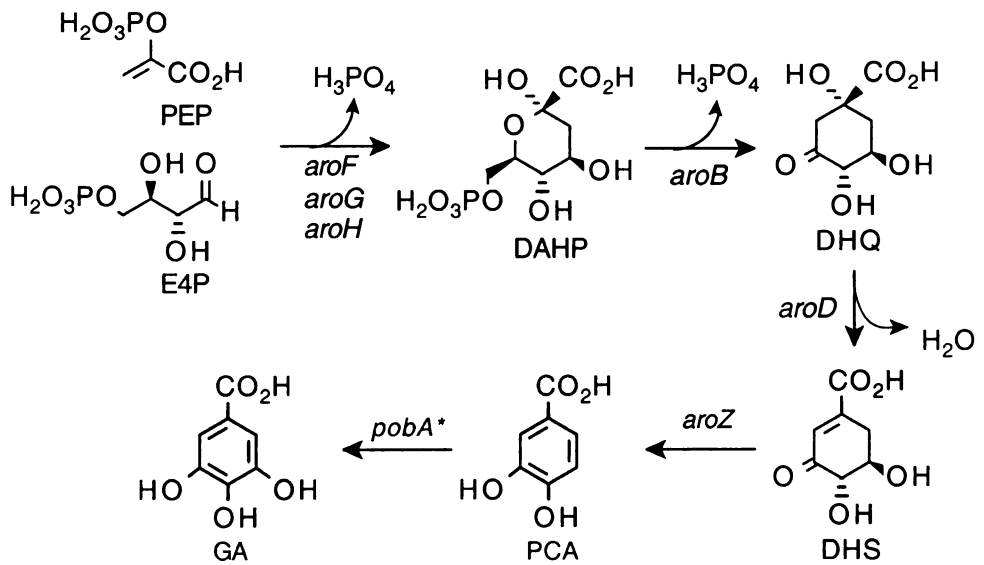


Figure 48. Early steps of the common pathway of aromatic amino acid biosynthesis and the proposed biosynthesis of gallic acid.

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 (Figure 64, Chapter 5). Since *serA*-encoded 3-phosphoglycerate dehydrogenase is an enzyme necessary for the biosynthesis of L-serine, microbial growth in minimal salts medium lacking L-serine supplementation is only possible if plasmids containing *serA* inserts are stablely maintained. This strategy employing nutritional pressure for plasmid maintenance obviates any need for use of antibiotics and plasmid-localized resistance to antibiotics for plasmid maintenance. 3-Dehydroshikimic acid dehydratase, encoded by *aroZ*, catalyzes the conversion of 3-dehydroshikimic acid into protocatechuic acid (Figure 48). AroB-encoded DHQ synthase catalyzes the formation of 3-dehydroquinic acid (DHQ) from 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) (Figure 48). Introduction of a second copy of *aroB* in the genome of *E. coli* was necessary for the elimination of DAH accumulation in biocatalysts that overexpress DAHP synthase (Figure 48).[20]

B. Plasmid Constructions

Overview

The genes that needed to be localized in all plasmids were *pobA** and *serA*. The *pobA** locus was always expressed from a *tac* promoter and encoded the mutant *p*-hydroxybenzoate hydroxylase responsible for oxidation of protocatechuic acid to gallic acid. 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. For synthesis of gallic acid from 3-dehydroshikimic acid, *aroZ* was also plasmid-localized. Expression of *aroZ* localized on a plasmid in addition to the expression of genomic *aroZ* in KL7, ensured ample DHS dehydratase activity for conversion of 3-dehydroshikimic acid into protocatechuic acid when 3-dehydroshikimic acid was added to the culture medium (Figure 48). For microbe-catalyzed synthesis of gallic acid from protocatechuic acid added to the culture medium two different plasmids were tested. One plasmid only carried the *pobA** gene while the other plasmid carried a protocatechuate transport protein encoded by the *pcaK* gene in addition to the *pobA** locus. PcaK is a membrane protein originally isolated from *Psuedomonas putida* and has been shown to facilitate protocatechuate uptake when expressed in *E. coli* bacteria.[21]

Construction of pSK6.76, pSK6.77 and pSK6.118

The open reading frame (ORF) of the mutant (Tyr385Phe) *p*-hydroxybenzoate hydroxylase (PHB hydroxylase, PobA*) was amplified using the polymerase chain reaction (PCR) from the plasmid pNE130.[15b] The primers were designed to include *Eco*RI ends as follows: 5'- GGAATTCCATGAAGACTCAAGTCGCC and 5'- GGAATTCCGCCGTC TTTCGTCGATAT. The PCR-synthesized 1.1 kb fragment of *pobA** was ligated after the *tac* promoter on the *Eco*RI site of the plasmid pKK223-3 giving plasmid pSK4.176 (Figure

49). Plasmid pKK223-3[22] is a cloning vector with a *pMB1* origin of replication. It contains a strong *tac* promoter and a ribosome binding site (RBS) which can be utilized to express gene lacking their native promoters and ribosomal binding sequence. Plasmid pSK4.176 was digested with *Bam*HI and the 1.5 kb $P_{tac}pobA^*$ fragment was ligated in the *Bam*HI site of pKL4.71 making pSK4.232A and pSK4.232B (Figure 50). In pSK4.232B there is one unique *Sma*I site, whereas in pSK4.232A digesion with *Sma*I regenerates the 1.5 kb fragment containing $P_{tac}pobA^*$.

The *aroZ* gene was isolated by PCR amplification using plasmid pSU1-28 as the template.[6a] Primers were designed to contain terminal *Bam*HI sequences as follows: 5-CGGGATCCGCGCATACACATGC and 5-CGGGATCCGGGTACAGAGGGTGTT GT. The amplified *aroZ* (2.2 kb) gene containing its native promoter was ligated in the *Bam*HI site of pSU18 to generate pSK4.99 (Figure 51). Ligation of the $P_{tac}pobA^*$ gene into a *Sma*I site of pSK4.99 created plasmid pSK6.70 (Figure 52). The $P_{tac}pobA^*$ gene was isolated from pSK4.233A as a 1.5 kb *Sma*I fragment. Plasmid pSK6.70 was digested with *Hin*dIII followed by treatment with the Klenow fragment to create blunt ends. Subsequent ligation with *serA* (1.9 kb), isolated from pD2625 after digesting with *Eco*RV/*Dra*I, afforded pSK6.76 (Figure 53).

Plasmid pSK4.122 was created by ligating the $aroF^{FBR}$ gene into an *Xba*I site of pSK4.99 (Figure 54). The 1.1 kb fragment of $aroF^{FBR}$ was isolated from pKL4.66A after digestion with *Xba*I. Ligation of $lacI^Q$ into an *Eco*RI site of pSK4.122 created plasmid pSK4.141 (Figure 55). The 1.1 kb $lacI^Q$ insert was isolated by PCR amplification using pKL4.71A as the template. The primers were designed to contain *Eco*RI ends as follows: 5-CGGAATTCATTTACGTTGACACCATCG and 5-CGGAATTCTTAATTGCGTTGC GCTCAC. Inserting $P_{tac}pobA^*$ into a *Sma*I site of pSK4.141 created pSK4.259. The 1.5 kb fragment of $P_{tac}pobA^*$ was isolated from plasmid pSK4.232A following a *Sma*I digestion (Figure 56).

Digestion of pSK4.259 with *Bam*HI/*Hin*dIII gave a 4.8 kb vector fragment containing P_{tac}*pobA** and $lacI^Q$ along with two other fragments. One fragment contained the 2.2 kb *aroZ* gene and the other fragment contained the 1.1 kb $aroF^{FBR}$ gene. Gell purification of the large fragment and ligation with the 1.8 kb fragment containing P_{tac}*pcaK* afforded plasmid pSK6.68 (Figure 57). The latter gene was isolated from pHJD110 after a *Bam*HI/*Hin*dII digestion. Insertion of the *serA* (*Eco*RV/*Dra*I fragment) gene into a *Sma*I site of pSK6.68 gave pSK6.77 (Figure 58).

Digesting pSK6.68 with *Bam*HI gave a 4.8 kb vector fragment containing P_{tac}*pobA** and $lacI^Q$ along with the 1.8 kb P_{tac}*pcaK* gene. Gel purification of the large fragment, treatment with Klenow fragment, and ligation with *serA* (*Eco*RV/*Dra*I fragment) afforded plasmid pSK6.118 (Figure 59).

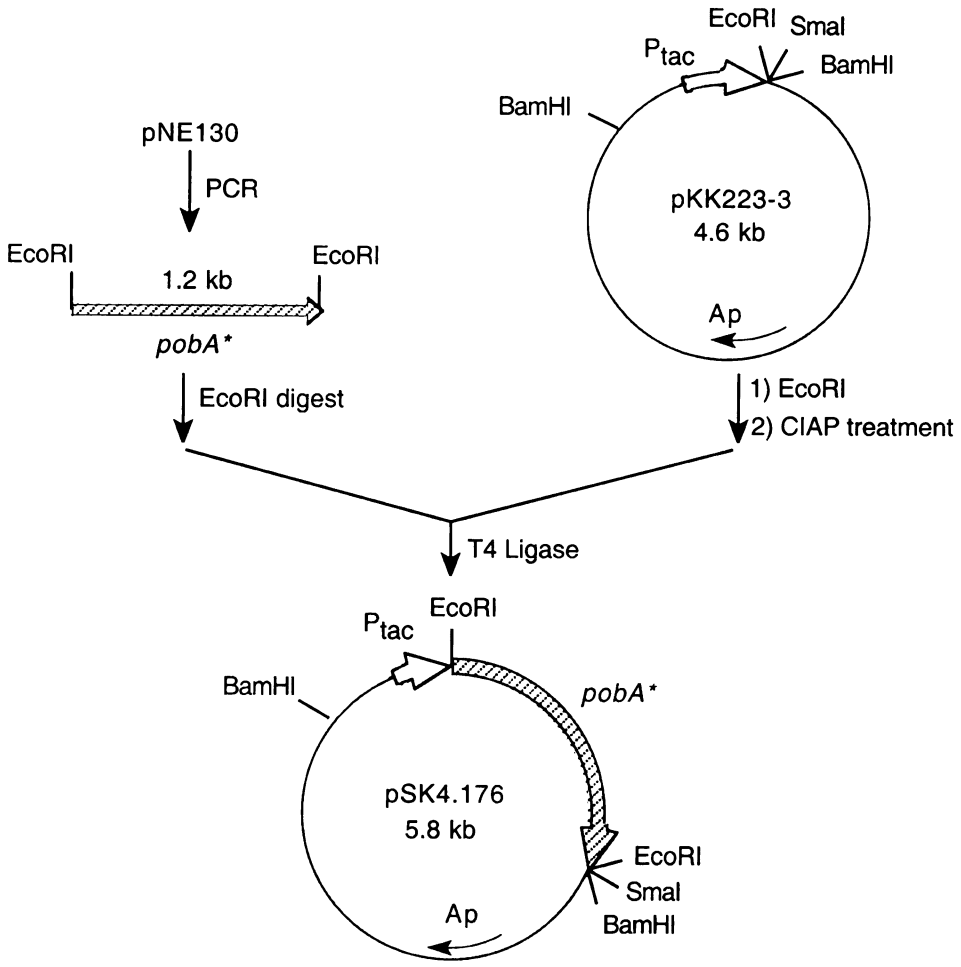


Figure 49. Construction of plasmid pSK4.176

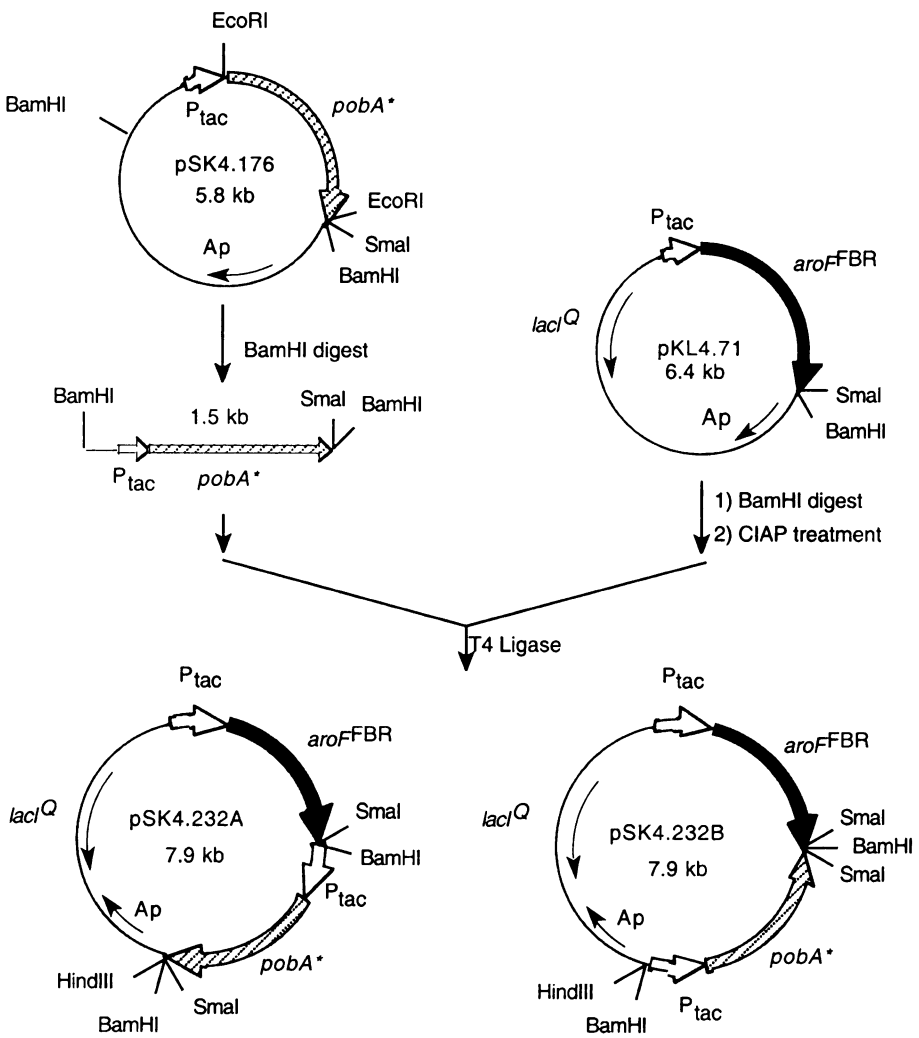


Figure 50. Construction of plasmid pSK4.232A and plasmid pSK4.232B

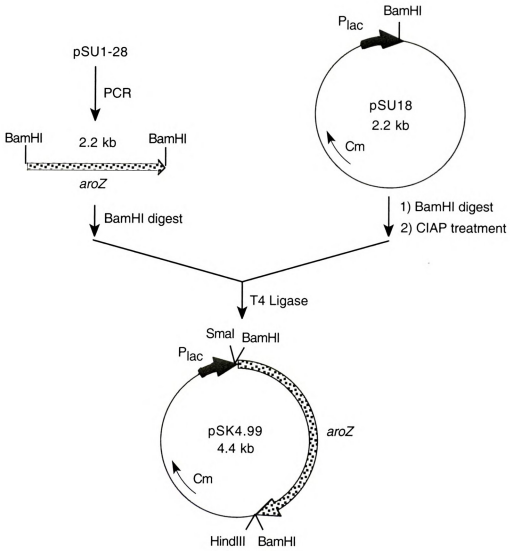


Figure 51. Construction of plasmid pSK4.99

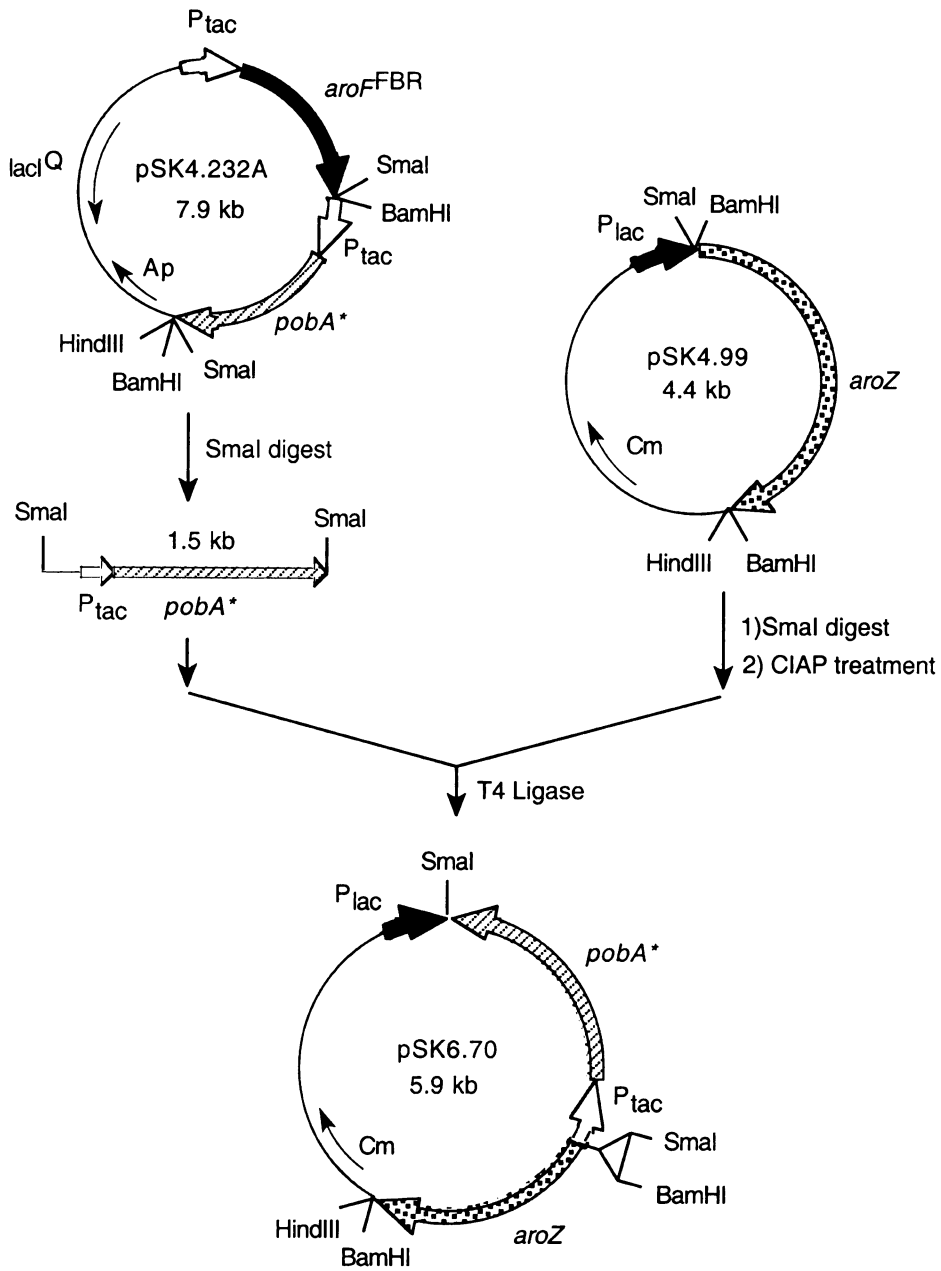


Figure 52. Construction of plasmid pSK6.70

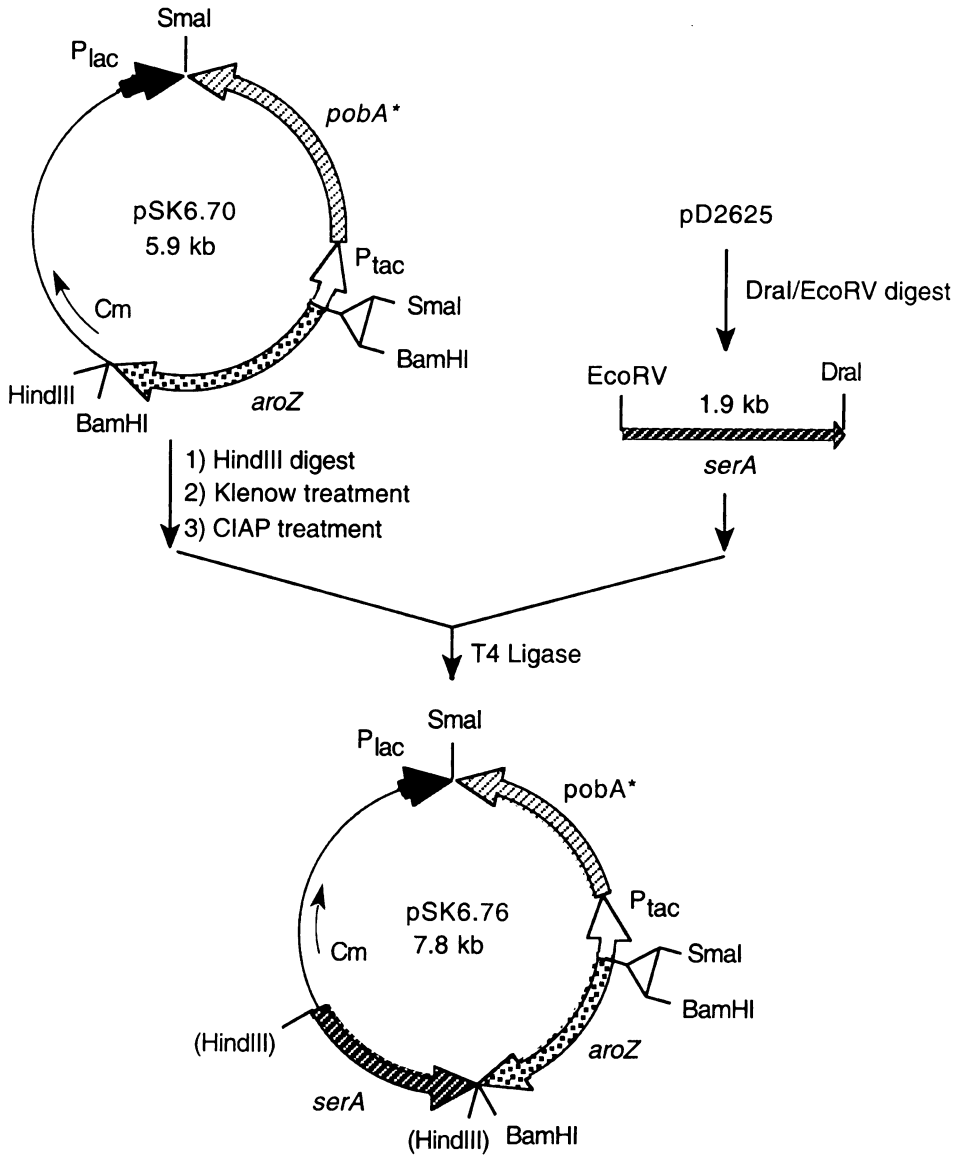


Figure 53. Construction of plasmid pSK6.76

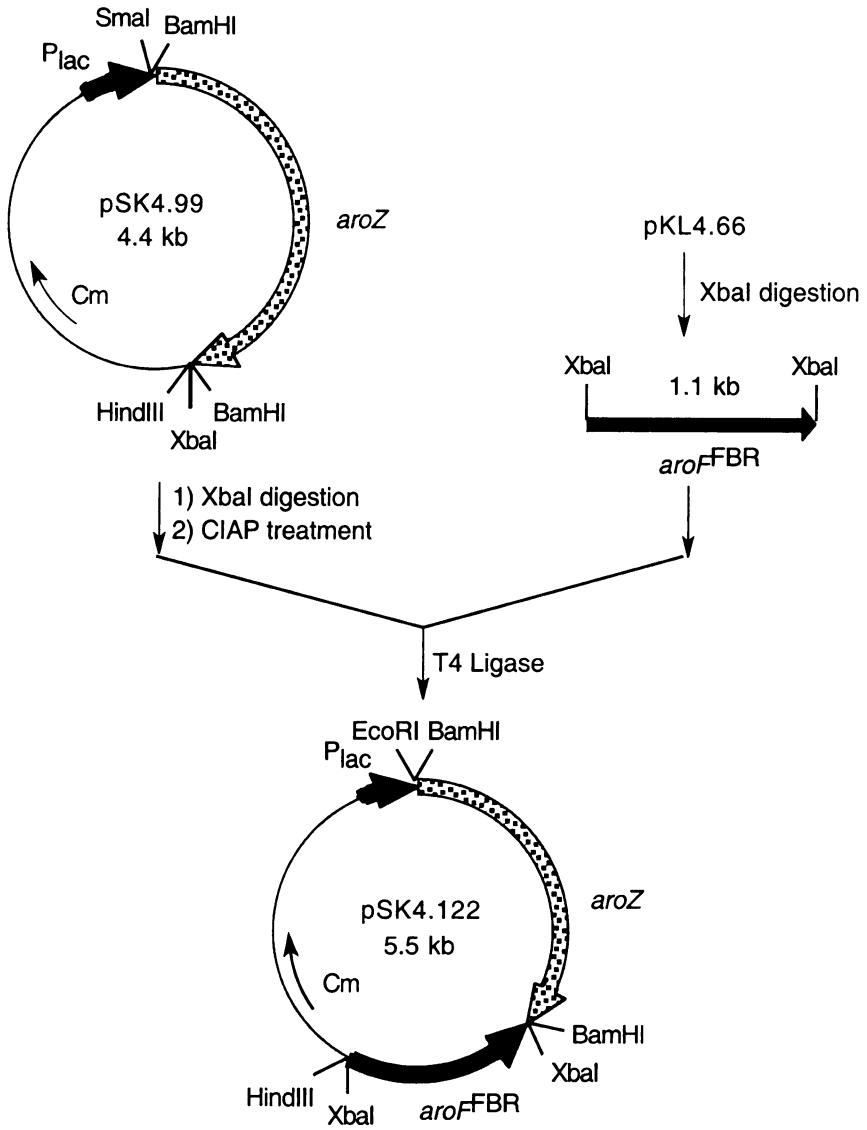


Figure 54. Construction of plasmid pSK4.122

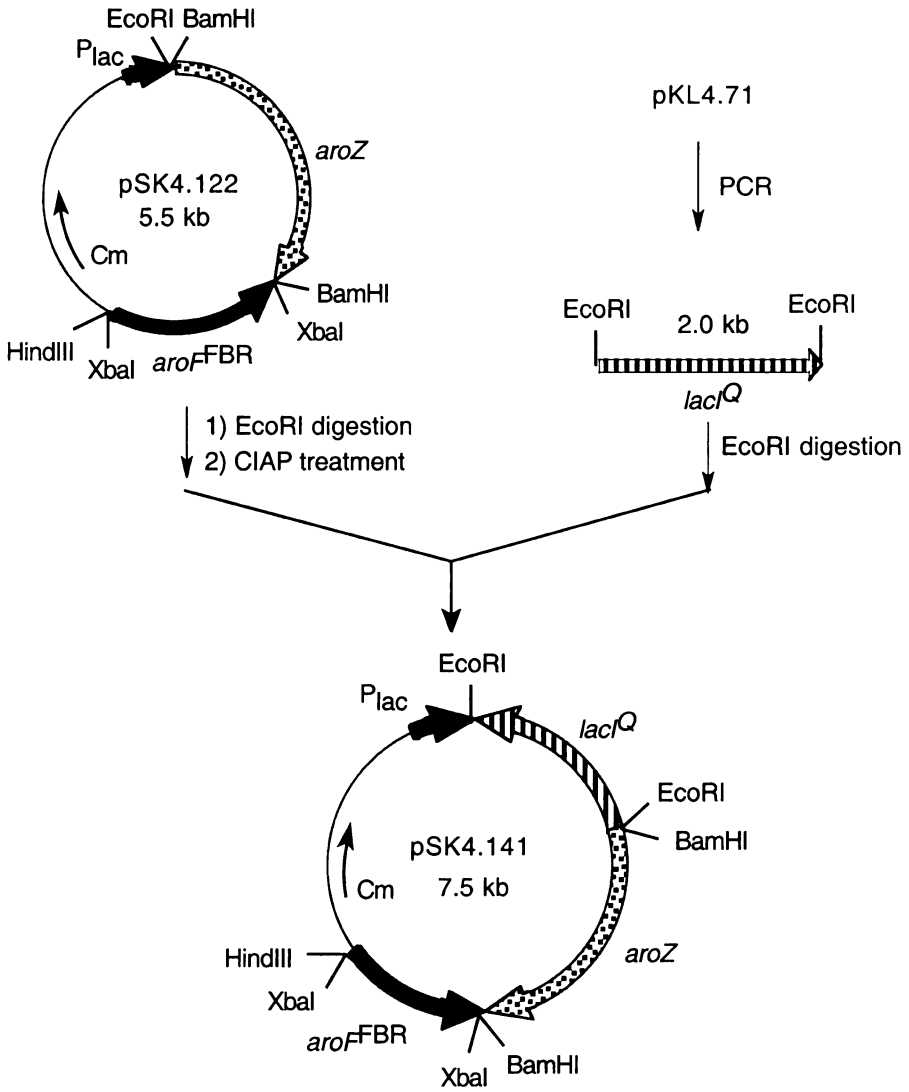


Figure 55. Construction of plasmid pSK4.141

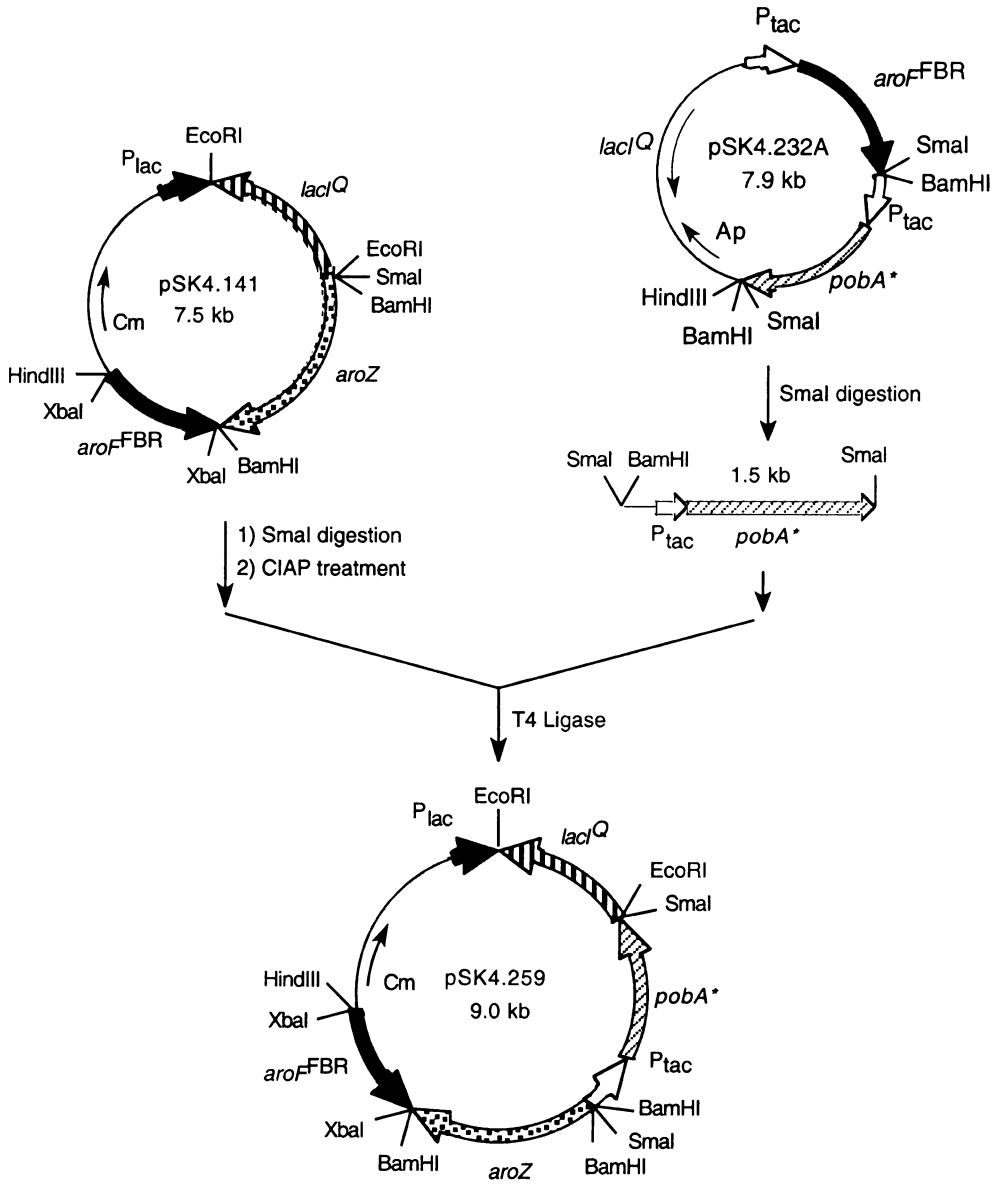


Figure 56 : Construction of plasmid pSK4.259

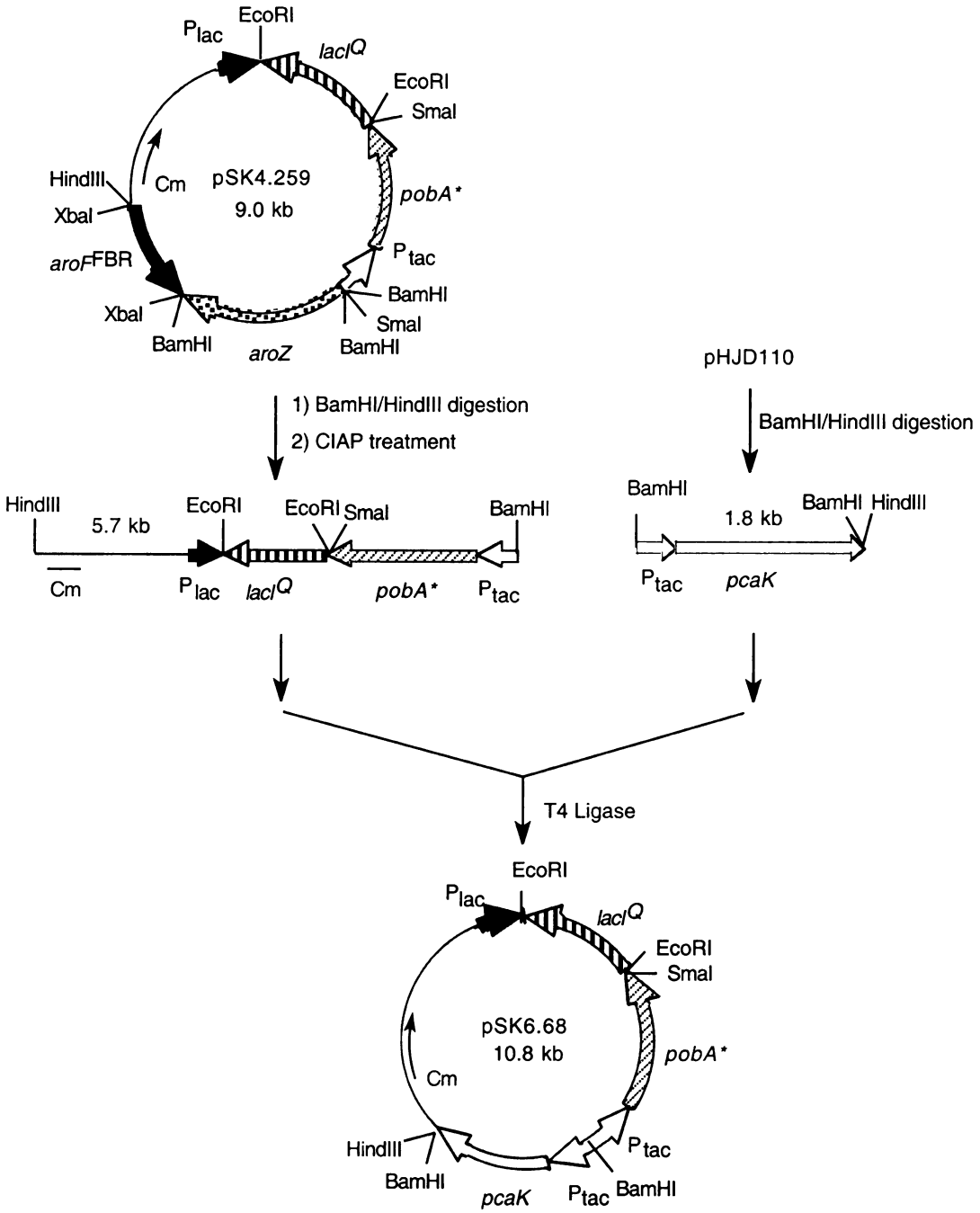


Figure 57: Construction of plasmid pSK6.68

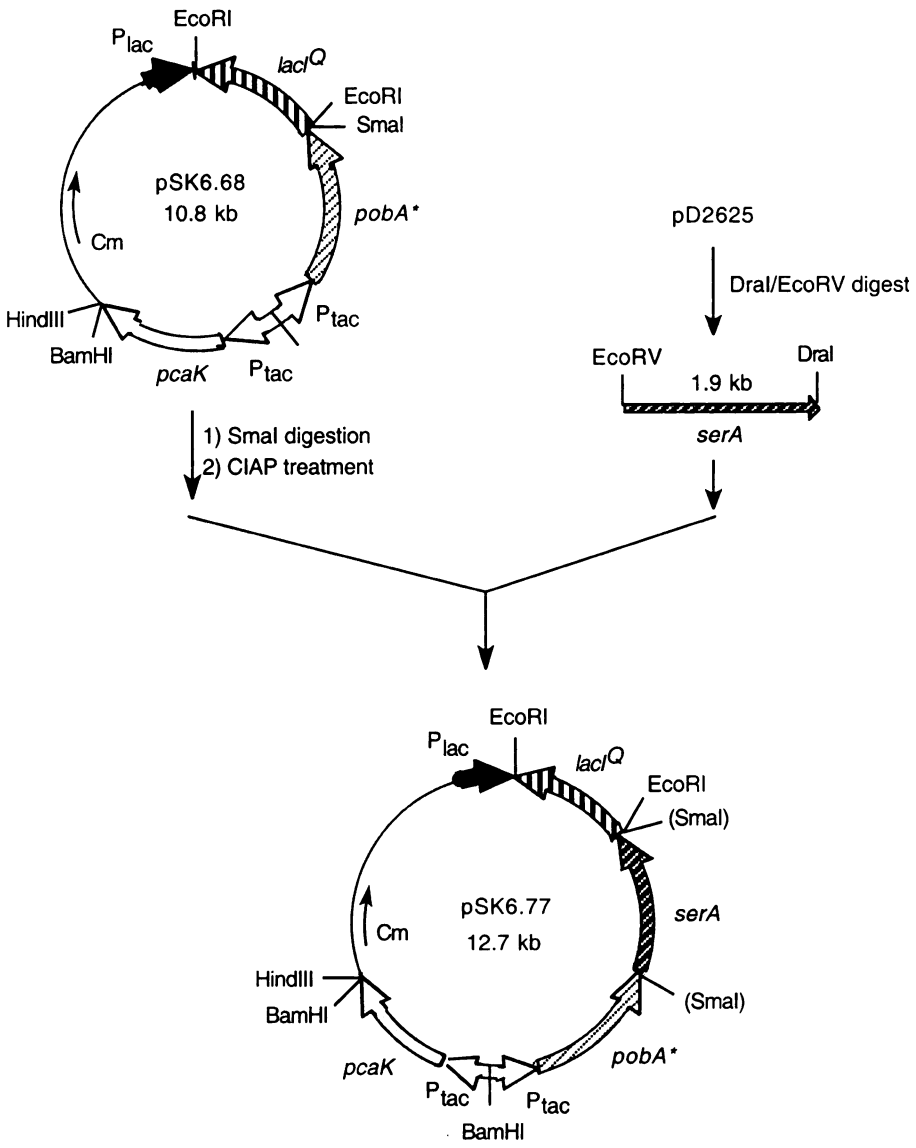


Figure 58: Construction of plasmid pSK6.77

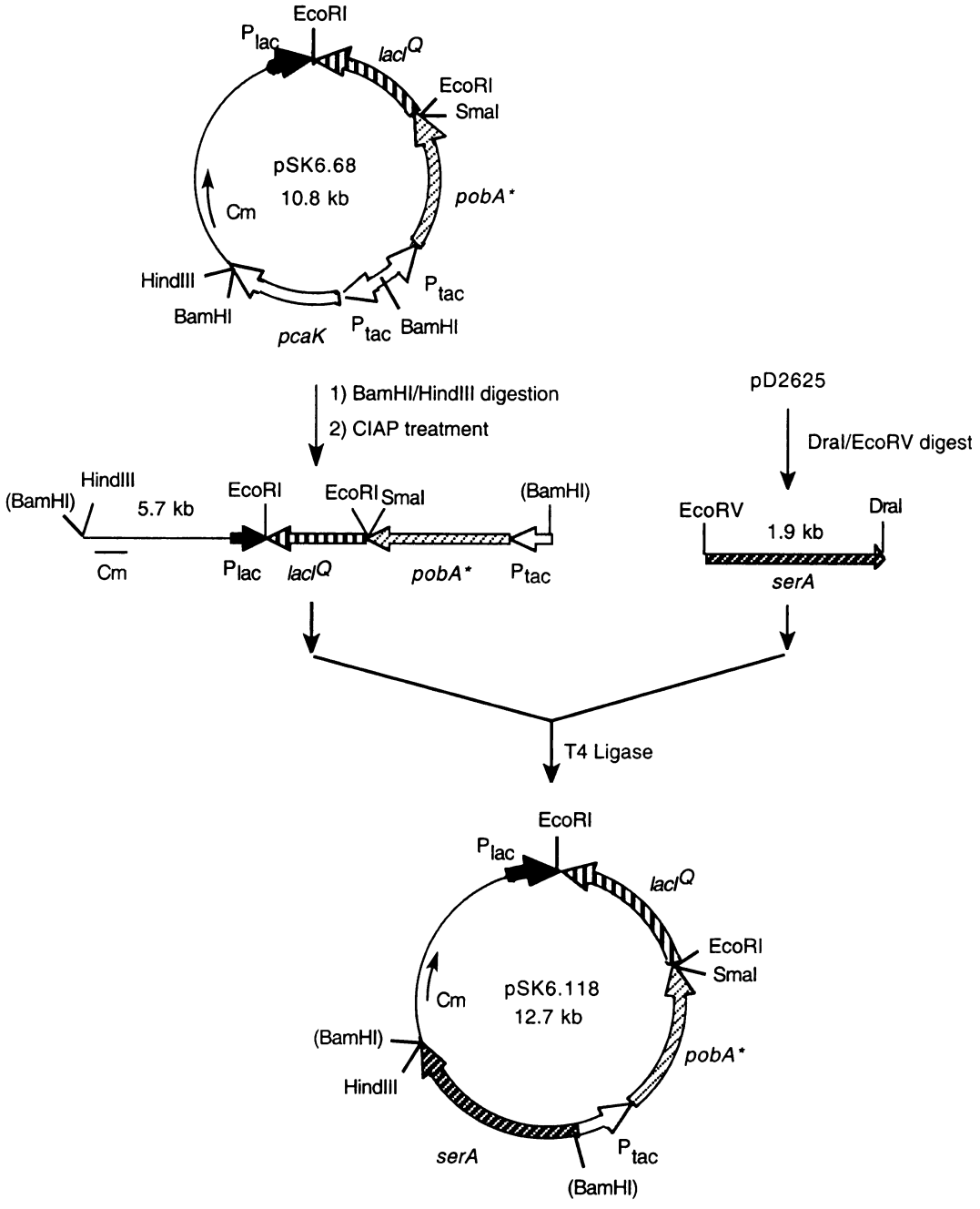


Figure 59. Construction of plasmid pSK6.118

C. Biocatalytic Synthesis of Gallic Acid.

Synthesis of gallic acid from 3-dehydroshikimic acid

Purified 3-dehydroshikimic acid was oxidized utilizing *E. coli* KL7/pSK6.76. Two different methods of 3-dehydroshikimic acid supplementation were tested. Cells grew until the maximum airflow was reached at 11 h, before a sterile-filtered solution (50 mL) of 3-dehydroshikimic acid (10.3 g,) was added in the culture supernatant (60 mM final concentration). Although the Lac repressor protein was not present in this plasmid, IPTG (15 mg) was added every 6 h starting at 12 h (Table 11). Accumulation of protocatechuic acid was observed during the first 12 h following the 3-dehydroshikimic acid addition (24 h, Figure 60A) reaching a maximum value of 4.6 g/L and then slowly decreasing through the next 12 h (36 h, Figure 60A) reaching 3.5 g/L by the end of the fermentation run. At the same time 4.6 g/L of gallic acid were accumulated after 42 h of reaction time resulting in a 48% mol/mol yield based on added 3-dehydroshikimic acid (Figure 60A).

Table 11. PobA* activities (U/mg) and product titers in the synthesis of gallic acid from DHS and PCA.

Construct	24 h	36 h	48 h	GA (g/L)	PCA (g/L)
KL7/pSK6.76[a]	0.040	0.049	0.055	6.3	1.8
KL7/pSK6.118[b]	0.038	0.038	0.036	7.3	6.2

a. DHS added with the glucose feed b. PCA added with the glucose feed

The final gallic acid titer was increased if instead of adding all of the 3-dehydroshikimic acid in one portion, it was added slowly with the glucose feed. The glucose feed solution was prepared by mixing 100 mL of a solution containing 18 g of 3-dehydroshikimic acid, with a 200 mL solution containing 120 g of glucose. An oxygen sensor controlled dissolved oxygen levels via addition of the solution containing glucose

(0.4 g/mL) and 3-dehydroshikimic acid (0.06 g/mL, 0.35 mmol/mL). The rate of addition of the previous glucose feed solution was about 8 mL/h and as a result approximately 0.5 g of 3-dehydroshikimic acid was added to the culture medium every h. By the end of the fermentation run, the total amount of 3-dehydroshikimic acid added to the growing cells was 18 g, producing 6.3 g/L of gallic acid at 48 h in a 45% (mol/mol) yield based on added 3-dehydroshikimic acid. Significant amounts of protocatechuic acid (1.8 g/L) as well as unreacted 3-dehydroshikimic acid (4.2 g/L) were present at the end of the run (Figure 60B). A 20 mL aliquot of fermentation broth was taken at 24 h, 36 h, and 48 h for determination of PHB hydroxylase activity. Although *pobA** activities were stable throughout the fermentation (Table 11), the majority of gallic acid was produced in stationary phase (after 24 h). Interestingly, the concentration of protocatechuic acid showed an initial increase reaching a maximum value of 3.4 g/L at 36 h, after which the concentration of protocatechuic acid steadily decreased to1.8 g/L at 48 h (Figure 60B).

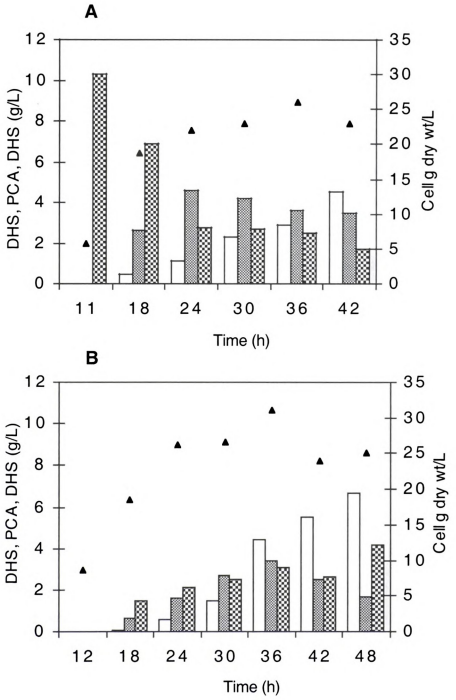


Figure 60. Conversion of DHS into GA using KL7/pSK6.76; A. DHS is all added in one portion at 11 h.; B. DHS is added with the glucose feed. (▭) GA, (▩) PCA, (▩) DHS, (▲) Cell g dry wt/L.

Synthesis of gallic acid from protocatechuic acid

The conversion of 3-dehydroshikimic acid into gallic acid using intact *E. coli* cells demonstrated the ability of this microbe to transport 3-dehydroshikimic acid. More importantly, the intracellular formation of H_2O_2 and gallic acid, both resulting from *pobA** expression, were not sufficient to preclude microbe viability and gallic acid synthesis. Furthermore, the decline in the concentration of protocatechuic acid in the culture supernatant of KL7/pSK6.76 during the final 12 h of continuous 3-dehydroshikimate addition suggested that protocatechuic acid transport into the cytoplasm was occurring. Previous literature precedent indicated that *E. coli* was incapable of transporting protocatechuic acid into its cytoplasm from the culture supernatants.[21b,c] In agreement with the literature, cultivation of KL7/pSK6.118 under shake flask conditions in the presence of protocatechuic acid showed essentially no gallic acid formation, with the concentration of protocatechuic acid remaining unchanged after 24 h of cultivation.

However, when KL7/pSK6.118 was cultured under fed-batch fermentor conditions, 7.7 g/L of gallic acid was synthesized (Figure 61A) after addition of protocatechuic acid (9 g) in one portion when the maximum airflow rate at 10 h was reached. The yield (mol/mol) of the gallic acid formation based on the 3-dehydroshikimic acid added in the fermentation broth was 80%. The decrease in protocatechuate concentration corresponded to an approximately equimolar increase in gallic acid concentration. Apparently, recombinant *E. coli* cultured under fed-batch fermentor conditions is capable of protocatechuate transport into its cytoplasm.

The same gallic acid titer (7.3 g/L) was produced if instead of one portion, protocatechuic acid was added slowly with the glucose feed (Figure 61B). An oxygen sensor was used to control dissolved oxygen levels in the culture medium via glucose addition. The feeding solution contained glucose (0.4 g/mL) and 0.053 g/mL (0.34 mol/mL) of protocatechuic acid, and as a result, the addition of this metabolite was

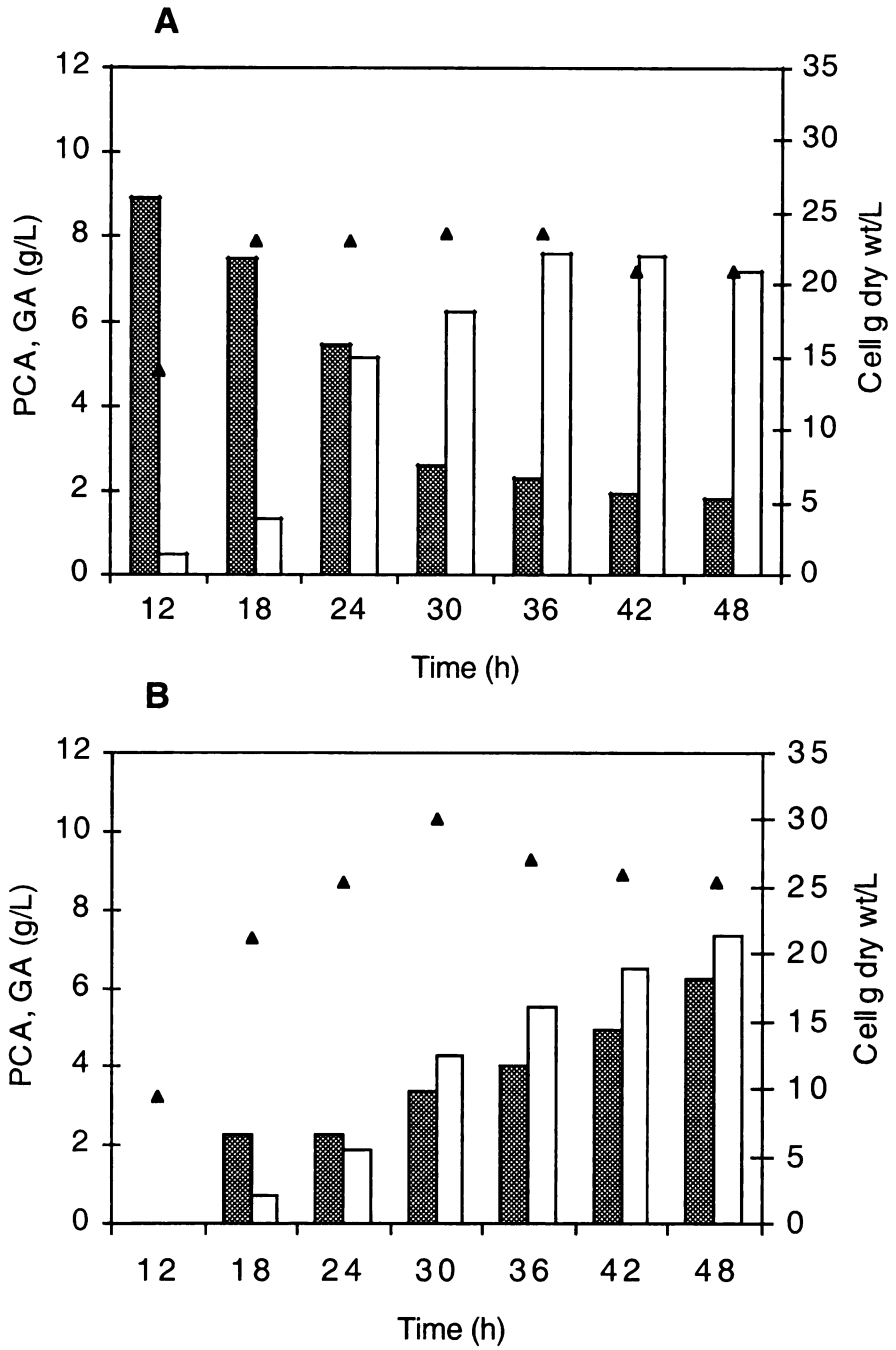


Figure 61. Conversion of PCA into GA using KL7/pSK6.118; A. PCA added all in one portion at 10 h ; B. PCA addition with the glucose feed. (▭) GA, (▩) PCA, (▩) DHS, (▲) Cell g dry wt/L.

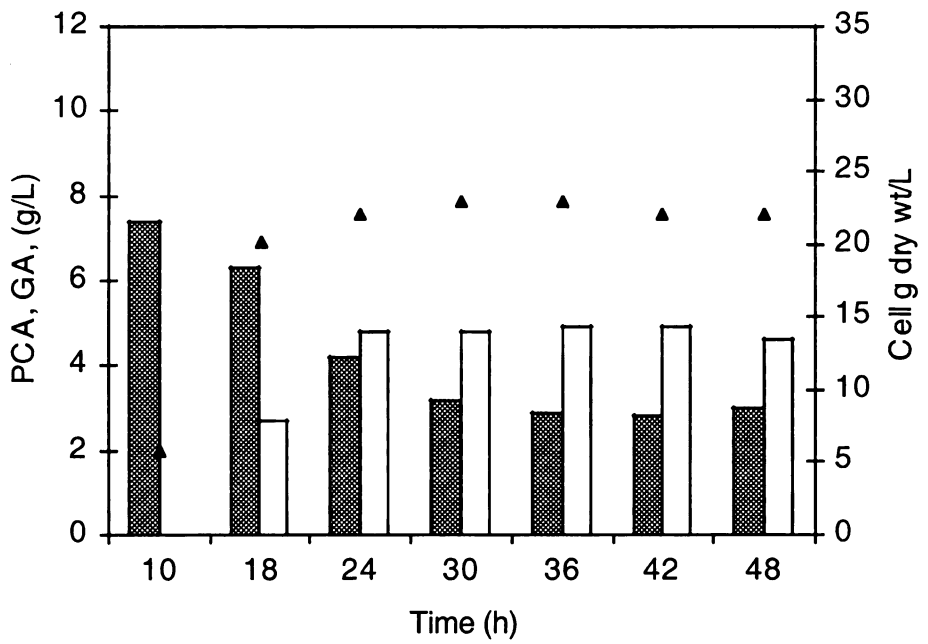
controlled from the rate of glucose consumption by the biocatalyst. The feeding rate was about 7.5 mL/h resulting in addition of around 0.4 g of protocatechuic acid per hour. The yield (mol/mol) of gallic acid synthesized based on protocatechuic acid addition to the fermentation broth was 54%. Addition of IPTG (15 mg) every 6 h starting from the time of maximum airflow was necessary to induce PobA* expression (0.038 U/mg, Table 11). In contrast to pSK6.76, plasmid pSK6.118 carries the repressor Lac protein and IPTG addition was necessary for adequate expression of PobA*. Comparable activities (Table 11) of PobA* were obtained during synthesis of gallic acid from 3-dehydroshikimic acid (pSK6.76) and during synthesis of gallic acid from protocatechuic acid (pSK6.118). However, addition of protocatechuic acid was found to give 1 g/L more gallic acid compared to that obtained with 3-dehydroshikimic acid supplementation.

A continuous increase of the gallic acid concentration with time was observed when either 3-dehydroshikimic acid or protocatechuic acid was added with the glucose feed (Figure 60B and Figure 61B). In contrast, adding all the substrate at one time portion in the mid log phase of cells cultured under fed-batch fermentor conditions, resulted in a more rapid increase of its concentration during the first 12 h following the addition (Figure 60 and Figure 61).

Hartwood et al[21] identified and isolated a gene (*pcaK*) from *Psuedomonas putida* that encoded a membrane protein responsible for protocatechuate and *p*-hydroxybenzoate uptake. They also demonstrated that PcaK was a permease that could be expressed in *E. coli* and facilitate transport of protocatechuic acid into the cell. Plasmid pSK6.77, which carried both *pcaK* and *pobA**, was constructed to test the effect of the permease gene in the synthesis of gallic acid. Transcription of both *pcaK* and *pobA** was under the control of a *tac* promoter. As a result of localizing $lacI^Q$ on the same plasmid, IPTG addition was necessary to induce transcription.

At the mid log phase (10 h after inoculation) of the fermentor culture of KL7/pSK6.77, 9.0 g of protocatechuic acid and the first 15 mg of IPTG were added. The

production of gallic acid during the subsequent 6 h (18 h, Figure 62) was higher (2.5 g/L) relative to the gallic acid (1.4 g/L, Figure 61A) synthesized by KL7/pSK6.118, which lacked permease activity. Furthermore, a significant difference in the cells expressing the *pcaK* gene was their inability to keep the dissolved oxygen (D.O.) at 20% after the second IPTG (15 mg) addition at 18 h. The D.O. increased to values ranging from 40% to 60% by 24 h, and no further gallic acid production or protocatechuate consumption was observed after this time (Figure 62). In experiments where the D.O. was reaching higher values (60%) a decrease of the gallic acid concentration was also observed after 24 h.



	12 h	24 h	36 h
PobA* (U/mg)	0.027	0.101	0.134

Figure 62. KL7/pSK6.77. PCA was added in one portion at 10 h. IPTG (15 mg) was added at 10 h, 18 h, 24 h, 30 h, and 36 h. (▭) GA, (▩) PCA, (▲) Cell g dry wt/L.

Since both *pobA** and *pcaK* genes are controlled by *tac* promoters, the second addition of IPTG provided an incremental increase in their activities. The activity of *pobA**

was 0.027 U/mg two hours after the first IPTG (15 mg) addition (12 h), and was increased to 0.101 U/mg at 24 h before the third addition was due (Figure 62). Although the *pcaK* activities were not assayed due to the complicated assay method for this enzyme requiring ^{14}C-labeled protocatechuic acid,[21c] a similar increase of the activity of *pcaK* was hypothesized to occur. The higher rates of protocatechuate uptake and/or gallic acid and H_2O_2 formation resulting from the increased activities of both *pobA** and *pcaK* after 24 h, was the most likely reason for the inability of these recombinant *E. coli* bacteria to control D.O. levels. A similar loss of the D.O. control was observed when the activity of PHB hydroxylase was increased in biocatalysts carrying a double *pobA** cassette and were synthesizing gallic acid when grown on glucose supplementation (Chapter 5, pg 160-161). Whether the observed inability to control the D.O. levels was due to the elevated intracellular concentration of protocatechuic acid or to its oxidation products gallic acid and H_2O_2, was unclear. The observed toxic effect may have been a combination of both factors.

Although KL7, the host strain used in all the previous gallic acid-synthesizing experiments, carries one copy of the feedback sensitive DAHP synthase on the genome, it can still accumulate protocatechuic acid and gallic acid in the presence of overexpressed *pobA**. To identify the amount of gallic acid and protocatechuic acid coming from glucose using this host, KL7/pSK6118 was grown in a fermentor without any external supplementation of 3-dehydroshikimic acid or protocatechuic acid. (Figure 63). The tyrosine necessary for cell growth that was added to the medium is likely responsible for inhibition of DAHP synthase and the absence of protocatechuic acid and gallic acid synthesis during the first 24 h of fermentor cultivation (Figure 63). ^{1}H NMR analysis of the cell supernatant indicated all the tyrosine added to the fermentor medium was consumed by 24 h. KL7/pSK6.118 ultimatelly synthesized 3.9 g/L of gallic acid and 0.8 g/L of protocatechuic acid. These results provide further evidence favoring protocatechuic acid transport in *E. coli* cultured under fermentor conditions. Almost 5 g/L of gallic acid have been synthesized at 24 h by KL7/pSK6.118 when protocatechuic acid is added at 12 h

(Figure 61A) relative to the 0.07 g/L of gallic acid and 0.5 g/L of protocatechuic acid synthesized de novo from glucose by KL7/pSK6.118 at 24 h.

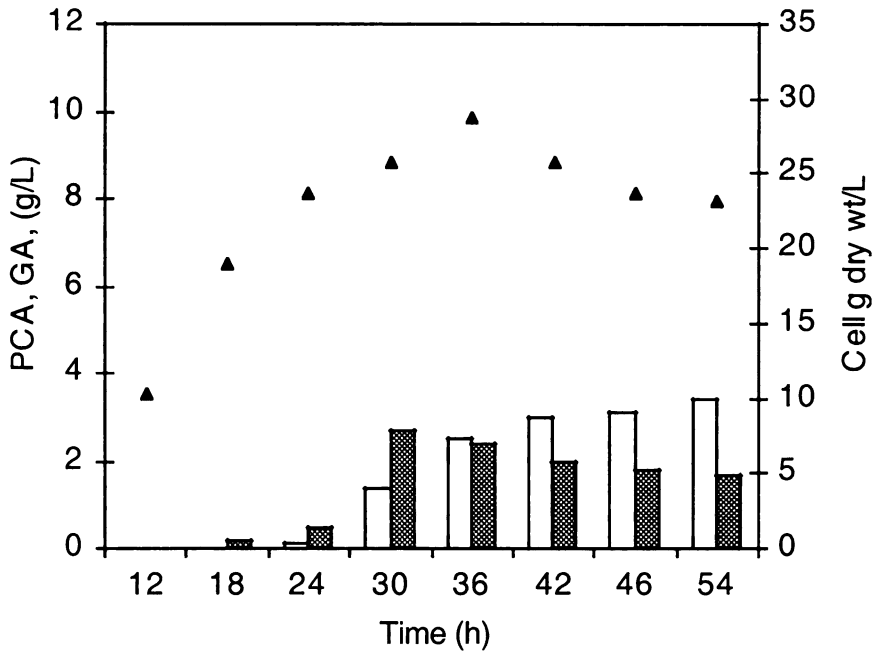


Figure 63: KL7/pSK6.118 cultured in glucose. No external PCA was added. (▭) GA, (▩) PCA, (▲) Cell g dry wt/L.

III. DISCUSSION

In this Chapter, biocatalytic synthesis of gallic acid (GA) from 3-dehydroshikimic acid (DHS) and biocatalytic synthesis of gallic acid from protocatechuic acid (PCA) were examined. Microbe-catalyzed conversion of 3-dehydroshikimic acid into gallic acid can be viewed as the biocatalytic alternative to chemical oxidation of 3-dehydroshikimic acid examined in Chapter 3. Microbe-catalyzed synthesis of gallic acid from 3-dehydroshikimic acid and protocatechuic acid also serve as incremental steps towards achieving a de novo synthesis of gallic acid from glucose using a single microbial biocatalyst. Microbial synthesis of gallic acid from protocatechuic acid allows attention to be focused on heterologous expression in *E. coli* of *Pseudomonas aeruginosa pobA**-encoded *p*-hydroxybenzoate hydroxylase.[21] The H_2O_2 byproduct of PobA* activity could overwhelm the microbe's ability to reduce this oxidant and thus be lethal towards the microbial biocatalyst. Microbial synthesis of gallic acid from 3-dehydroshikimic acid allows *aroZ*-encoded DHS dehydratase activity and conversion of 3-dehydroshikimic acid into protocatechuic acid to be separated from challenges associated with direction of carbon flow down the common pathway of aromatic amino acid biosynthesis. Whether *E. coli* can transport 3-dehydroshikimic acid and protocatechuic acid from the culture medium is central to whether these intermediates are lost to de novo gallic acid synthesis as a consequence of inadequate AroZ and PobA* activities and resulting export out of the cytoplasm. Potential toxicity of gallic acid towards *E. coli* can also be appraised by examining its synthesis from 3-dehydroshikimic acid and protocatechuic acid.

PobA* is derived from *pobA*-encoded *p*-hydroxybenzoate hydroxylase, which catalyzes the formation of protocatechuic acid from *p*-hydroxybenzoate. This is an essential step in the β-ketoadipate pathway, which enables *P. aeruginosa* to use *p*-hydroxybenzoate as a sole source of carbon during growth. PobA is a dimeric protein that contains one FAD molecule per monomer. FAD reduction by NADPH and subsequent reaction of $FADH_2$

with O_2 generates a reactive flavin-hydroperoxide intermediate which hydroxylates *p*-hydroxybenzoate (Figure 47).[16,17] For PobA, product protocatechuic acid is not a substrate for the enzyme. By contrast, protocatechuic acid is further hydroxylated to gallic acid by mutant isozyme *pobA**.

Whole cell synthesis of gallic acid using a cytosolic protein such as PobA* required sufficient intracellular accumulation of protocatechuic acid. For this reason, protocatechuic acid or its biosynthetic precursor 3-dehydroshikimic acid must enter the cell. In fact, supplementation of *E. coli* cells cultured under fermentor conditions with either 3-dehydroshikimic acid or protocatechuic acid resulted in gallic acid formation. A significant difference in the rate of gallic acid production was observed as a result of the protocatechuic acid or 3-dehydroshikimic acid supplementation method. For example, when protocatechuic acid was added in one portion at the mid log phase of bacterial growth, a rapid decrease of the concentration of protocatechuic acid followed by an equivalent increase in the concentration of gallic acid was observed. Gallic acid and protocatechuic acid concentrations remained approximately constant 12 h from the time of protocatechuate addition (Figure 61A). In the case of protocatechuic acid addition with the glucose feed, gallic acid formation as well as protocatechuate accumulation followed a constant rate of increase, which lasted until the end of the fermentation at 48 h (Figure 61B). A similar pattern of gallic acid production versus precursor utilization was also observed when 3-dehydroshikimic acid was added in one portion (Figure 60A) versus 3-dehydroshikimic acid addition with the glucose feed.

Two *E. coli* host strains RB791 and KL7, carrying the *aroZ* locus encoding DHS dehydratase localized on pKS4.99, produced protocatechuic acid when cultured in minimal medium containing 3-dehydroshikimic acid under shake flask conditions. Cell-free minimal solutions containing 3-dehydroshikimic acid treated under the same shake flask conditions produced trace amounts of protocatechuic acid as a result of chemical dehydration of 3-dehydroshikimic acid, while the concentration of 3-dehydroshikimic acid

in the medium was basically unchanged. Transport of 3-dehydroshikimic acid into the *E. coli* cytoplasm may be mediated by protein(s) encoded by the *shiA* locus, which catalyzes the transport of the structurally similar shikimic acid.[22]

A protocatechuate transport system has not been identified in *E. coli*. However, a variety of organisms that grow in soil utilize transport systems for aromatic acids as an evolutionary advantage for growth in natural environments where these molecules are at very low concentrations. A permease protein, designated PcaK, was identified in *Pseudomonas putida* as a transporter of both *p*-hydroxybenzoate and protocatechuate.[21b,c] When plasmid-localized *pcaK* was expressed in *E. coli* cells cultured under shake flask conditions, protocatechuate transport was observed. In the same study, *E. coli* cultured under shake flask conditions, showed no detectable protocatechuic acid transport in the absence of *pcaK* expression.[21c]

No gallic acid was detected when *E. coli* KL7/pSK6.118 was cultured in minimal salts medium containing protocatechuic acid under shake flask conditions. However, the same *E. coli* construct produced significant amounts of gallic acid when cultured in minimal salts medium containing protocatechuic acid under fed-batch fermentor conditions. Inclusion in the biocatalyst of the *pcaK*-encoded protocatechuate permease enzyme (KL7/pSK6.77) did not change the final concentration of the produced gallic acid, but instead, it increased the rate of protocatechuate transport during the first 6 h after its addition as measured by the rate of gallic acid formation. When *pcaK* was present (KL7/pSK6.77) 2.5 g/L of gallic acid were synthesized at 18 h whereas cells lacking this activity (KL7/pSK6.118) produced 1.4 g/L of gallic acid at the same time point (Figure 61A and Figure 62). In these experiments, protocatechuic acid was added in one portion at the time of maximum airflow rate at 10-12 h. In the absence of external addition of protocatechuic acid, KL7/pSK6.118 cultured in minimal salts medium under fed-batch fermentor conditions produced trace amounts of gallic acid (0.07 g/L) and protocatechuic acid (0.5 g/L) even after 24 h of culturing time (Figure 63).

These results suggest that protocatechuic acid is making its way from the culture supernatant into the cytoplasm under fed-batch fermentor conditions. Although a protocatechuate permease protein has not been identified in *E. coli*, it is possible that under the fermentor conditions of growth a membrane enzyme responsible for protocatechuate uptake is expressed. Alternatively, protocatechuate might be able to utilize an uptake system that is designed for other structurally similar compounds and expressed in *E. coli* under fermentor conditions. A diffusion-controlled uptake of protocatechuic acid through the membrane into the cytoplasm can not be excluded. Uncharged aromatic acids can diffuse across biological membranes in a concentration dependent rate.[21c,24] Maybe this is the reason for the difference in rate of gallic acid synthesis in cells that grow under continuous versus one time protocatechuate addition. In the one time addition, the high concentration of protocatechuate in the medium can increase the rate of protocatechuate diffusion through the membrane. A small diffusion-controlled uptake of protocatechuic acid will be amplified under the high cell density conditions that exist in fermentor-cultured bacteria. Whatever the reason for protocatechuate transport by *E. coli* bacteria, it is only occurring when cells are cultured under fed-batch fermentor conditions. No protocatechuate uptake was observed when cells grew under shake-flask conditions.

Chemical oxidation of 3-dehydroshikimic acid to gallic acid using copper and zinc salts was discussed extensively in Chapter 3. Reacting 3-dehydroshikimic acid (1 M solution) with $Cu(OAc)_2$ and ZnO (0.05 egiuv and 0.25 equiv mol/mol relative to 3-dehydroshikimic acid) in $AcOH/H_2O$ mixture, gave after 4 h of reaction time complete conversion to gallic acid and protocatechuic acid in 67% and 3% respective yields (Table 12). After purification, gallic acid was isolated in 53% isolated yield based on the starting 3-dehydroshikimic acid. Biocatalytic oxidation of 3-dehydroshikimic acid using recombinant *E. coli* bacteria (KL7/pSK6.76) on the other hand, gave gallic acid in 48% yield when added in one portion, and 45% when added with the glucose feed (Table 12). *E.*

coli KL7/pSK6.118 oxidized protocatechuic acid in 80% yield when added in a single portion, and 54% yield when added with the glucose feed (Table 12).

Table 12. Comparison of GA yields and titers using chemical and biocatalytic oxidation of DHS and PCA.

Catalyst	Substrate	GA (g/L)	Yield[d] %	GA/PCA	Time (h)
KL7/pSK6.76[a]	DHS	3.6	48	1/1	42
KL7/pSK6.76[b]	DHS	6.3	45	2/1	48
KL7/pSK6.118[a]	PCA	7.7	80	4/1	48
KL7/pSK6.118[b]	PCA	7.3	54	1/1	48
$Cu(OAc)_2/ZnO$[c]	DHS	114	67	19/1	4

a. One time substrate addition b. Continuous feed
c. Table 10, Chapter 3 d. (mol/mol) based on the added substrate

The chemical oxidation offered some advantages compared to the biocatalytic synthesis. The product mixture consisted of very small percentage of protocatechuic acid impurities and for this reason, purification of gallic acid was achieved by petroleum ether precipitation. Scale-up of the chemical oxidations was not a problem. The reaction can be performed in high concentrations of 3-dehydroshikimic acid (1 M) and 14-17 g of 3-dehydroshikimic acid were oxidized giving reproducible reaction product mixtures and high yields. For the biocatalytic oxidations adding more substrate to the bacterial culture did not increase the gallic acid titer, as shown in the protocatechuic acid oxidation when one-time addition of substrate was replaced with continuous feeding of this substrate.

Biocatalytic conversion of 3-dehydroshikimic acid and protocatechuic acid required longer reaction times than chemical oxidation of 3-dehydroshikimic acid. The presence of significant amounts of protocatechuic acid contaminant in the synthesized gallic acid is also problematic for the biocatalytic synthesis of gallic acid from 3-dehydroshikimic acid and protocatechuic acid. However, no decomposition of 3-dehydroshikimic acid or gallic acid was encountered and as a result, the remaining 3-dehydroshikimic acid can be recovered at

the end of the reaction. Although all the solvents and metals can be recycled at the end of the chemical reaction, the Cu^{+2}/Zn^{+2} regeneration is problematic and their handling increases the reaction cost and add extra steps to the whole process. Catalyst recovery and recycling is avoided in the biocatalytic conversion of 3-dehydroshikimic acid and protocatechuic acid into gallic acid.

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CHAPTER 5

BIOCATALYTIC SYNTHESIS OF GALLIC ACID FROM D-GLUCOSE

I. INTRODUCTION

Microbe-catalyzed conversion of D-glucose into bioproducts such as L-phenylalanine and L-tryptophan is intimately related to microbe-catalyzed synthesis of industrial chemicals such as adipic acid and catechol from D-glucose (Fig).[1] 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. Numerous intermediate common pathway metabolites such as 3-dehydroshikimic acid (DHS), are shared in the biosynthesis of aromatic amino acids and the biocatalytic synthesis of adipic acid, catechol, and vanillin.[2,3] Carbon flow directed into this common pathway can be conveniently measured by the accumulation of 3-dehydroshikimic acid in the culture supernatants of microbial mutants lacking shikimate dehydrogenase activity. Under fed-batch fermentor conditions, 3-dehydroshikimic acid titers up to 69 g/L are synthesized using recombinant *E. coli* bacteria that overexpress certain key enzymes of the common pathway.[4]

In Chapter 4, the enzymatic conversion of 3-dehydroshikimic acid into gallic acid using recombinant *E. coli* was discussed. Synthesis of gallic acid followed the external addition of pure 3-dehydroshikimic acid to the culture medium of bacteria grown under fed-batch fermentor conditions that overexpressed plasmid-localized, *aroZ*-encoded DHS

dehydratase and the mutant PHB hydroxylase (*pobA**). Modest yields of gallic acid formation were obtained from 3-dehydroshikimic acid (45-48%). When protocatechuic acid was added to the fermentation broth, the yields of gallic acid formation were dependent on the supplementation method and reached values up to 80% when protocatechuic acid was added in one portion in the mid log phase of bacteria cultured under fed-batch fermentor conditions (Table 12, Chapter 4). A drawback in the synthesis of gallic acid using both these conditions resulted from the substantial amounts of protocatechuate impurities that were accumulating in the cell supernatant giving ratios of GA/PCA anywhere between 1/1 to 4/1 (mol/mol) (Table 12, Chapter 4). The need to employ a separate fermentation/isolation to obtain 3-dehydroshikimic acid or protocatechuic acid and the difficulty in removing protocatechuic acid from gallic acid reduces the attractiveness of these syntheses as strategies for obtaining gallic acid. However, the ability of intact *E. coli* to synthesize gallic acid in substantial amounts under fermentor growing conditions was clearly demonstrated by these experiments.

As discussed in this Chapter, introduction of *aroZ* and *pobA** loci into an *E. coli* construct that accumulated 3-dehydroshikimic acid resulted in a microbe capable of de novo synthesis of gallic acid from glucose. No additional modification of the host microbe was required since gallic acid is not a natural metabolite of *E. coli* and can not be further metabolized. The activities of PobA*, $AroF^{FBR}$, and common pathway enzymes that limit carbon flow were manipulated to optimize the product yields, titers, and GA/PCA ratios. The inability to control the dissolved oxygen as well as a growth inhibition was encountered when recombinant bacteria carrying a double *pobA** loci grew under fed-batch fermentor conditions. Introduction in this biocatalyst of a H_2O_2-degrading catalase

enzyme encoded by *katG* did not solve the problem. Gallic acid titers up to 20 g/L accompanied with low protocatechuate contamination (GA/PCA: 19/1 mol/mol) were synthesized in 12% yield (mol/mol based in glucose consumption), after optimizing the activities of PobA*, $AroF^{FBR}$ as well as the fermentation conditions.

II. BIOCATALYST CONSTRUCTION AND FED-BATCH FERMENTOR CONDITIONS FOR GA SYNTHESIS FROM GLUCOSE

A. Shared genomic and plasmid elements

All of the gallic acid-synthesizing biocatalysts shared several genetic and recombinant elements including a mutation in the genomic *aroE* locus, an *aroBaroZ* cassette inserted into the genomic *serA* locus, plasmid-localized *serA*, and plasmid-localized *pobA** and $aroF^{FBR}$. *E. coli* AB2834,[5] an *aroE* mutant lacking shikimate dehydrogenase activity, was the ancestral strain used to construct the KL7 host used in all of the gallic acid syntheses. The absence of catalytically-active, *aroE*-encoded shikimate dehydrogenase, which catalyzed the conversion of 3-dehydroshikimic acid into shikimic acid, in addition to the genome-localized, *aroZ*-encoded DHS dehydratase, which catalyzes the conversion of 3-dehydroshikimic acid into protocatechuic acid (PCA), resulted in the accumulation of protocatechuic acid in the culture supernatants of *E. coli* KL7.[3] Growth of *E. coli* KL7 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 expression of plasmid-localized, $aroF^{FBR}$-encoded of DAHP synthase, wild-type expression levels of *aroB*-encoded DHQ synthase are inadequate in *E. coli* AB2834 for

conversion of substrate DAHP into product 3-dehydroquinic acid (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 gallic acid. An approximately two-fold increase in DHQ synthase activity, resulting from introduction of a second copy of *aroB* into the genome of *E. coli* AB2834, has been previously employed to eliminate DAH accumulation.[6] Expression levels of DHS dehydratase associated with genome-localized *aroZ* are adequate for conversion of 3-dehydroshikimic acid into protocatechuic acid even when carbon flow into the common pathway is increased.[3] Introduction of a second genomic copy of *aroB* and a genomic copy of *aroZ* resulted from site-specific insertion of a cassette consisting of *aroB aroZ* with flanking *serA* nucleotide sequences into the *serA* locus of *E. coli* AB2834 through homologous recombination.[3]

Disruption of the genomic *serA* locus also provided the basis for plasmid maintenance. The *serA* locus encodes D-3-phosphoglycerate dehydrogenase, which is the first of the three enzymes responsible for serine biosynthesis (Figure 64).[7] Since KL7 is a serine auxotroph, growth in medium lacking L-serine supplementation required expression of plasmid-localized *serA* carried by all KL7 biocatalysts. Using this nutritional pressure for plasmid maintenance avoided the necessity of using antibiotics in fermentation medium. Nonetheless, all of the gallic acid-synthesizing plasmids carried markers encoding antibiotic resistance. This enabled antibiotic pressure in addition to nutritional pressure to eliminate microbial contamination and ensured plasmid maintanance in inoculants.

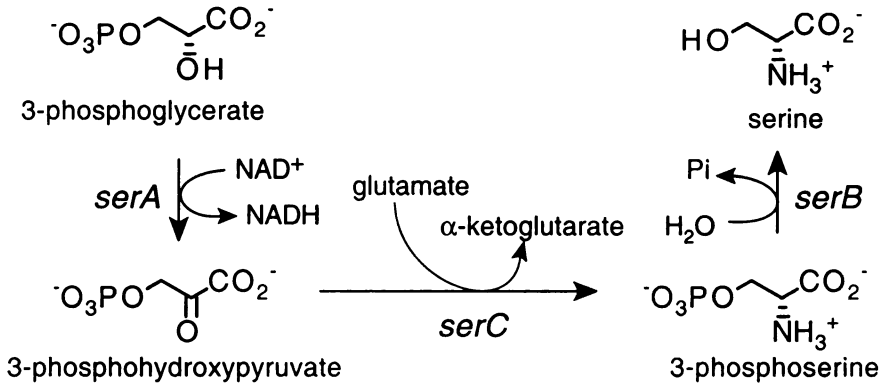


Figure 64. Serine biosynthesis. *SerA*: 3 phosphoglycerate dehydrogenase; *serC*: 3-phosphoserine aminotransferase; *serB*: 3-phosphoserine phosphatase.

In *E. coli*, the most important regulation of DAHP synthase is feedback inhibition of the enzymes by aromatic amino acids.[8] All 3-dehydroshikimic acid-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 *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*-flourotyrosine.[9] 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.[10]

B. Fed-batch fermentor conditions

Fed-batch fermentations were 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 65). The temperature, pH, and glucose feeding were controlled with a PID controller. The temperature was maintained at 36 °C, and pH was maintained at 7.0 by addition of concentrated NH_4OH or 2 N H_2SO_4. Dissolved oxygen was maintained at 10% of air saturation or in some experiments 20% of air saturation throughout fermentation runs using a Braun polarographic probe. Antifoam (Sigma 204) was added manually as needed.

Inoculants were grown in 100 mL M9 minimal containing the appropriate antibiotic for 10 h to 12 h at 36 °C with agitation at 250 rpm. The inoculants were then transferred to the fermentor. The initial glucose concentration in the fermentation varied from 20 g/L to 22 g/L according to the growth rates of different constructs. Three different methods were used to maintain dissolved oxygen (D.O.) level at 10% 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 10-12 h were required before the impeller reached its maximum stirring rate. The mass flow controller then maintained D.O. levels at 10% saturation at the constant impeller stirring rate by increasing the airflow rate until a preset maximum value (1.0 L/L/min) was reached. Approximately 1.5 h were needed for the airflow to increase to its maximum rate. At a

constant impeller stirring rate (940 rpm) and constant airflow (1 L/L/min), D.O. levels were then maintained at 10% saturation by oxygen-sensor-controlled glucose feeding for the rest of the fermentation. At the beginning of this stage, dissolved oxygen levels fell below 10% 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.

Another method for culturing cells under fed-batch fermentor conditions was also employed. This method differed in how D.O. levels were controlled during the third phase. As with the previous method D.O. during the first two phases of growth were maintained by increasing the impeller rate and airflow. After the impeller rate and airflow reached their preset maximum levels (750 rpm and 1 L/L/min respectively) the D.O. was maintained at the 10% set value by changing the impeller rate (750 to 1350 rpm). The impeller in the first phase reached the maximum value (750 rpm) after 10 h and 1.5 h more was required for the airflow to reach the preset value of 1 L/L/min in the second phase. The airflow was then increased to 1.65 L/L/min for the first 12 h of the third phase and was subsequently set to a value of 1.35 L/L/min for the rest of the fermentation run. At the same time, a continuous glucose feed ensured that 15 to 25 g/L of glucose was always present in the fermentation supernatant. The glucose feed (60% w/v) was manually set to values ranging from 0.15 mL/min for the first 30 h, to 0.05 mL/min for the rest of the fermentation run, as required from the glucose concentrations in the broth. Measurements of the glucose concentrations were performed every 2 h during the first 12 h of phase three, and every 6 h for the rest of the run, using an enzymatic coupled assay kit purchased by Sigma diagnostics. This assay is based on the measurement at 340 nm of

the absorption of NADH that is produced as a result of the enzymatic oxidation of glucose that is present in an aliquot drown from the fermentation broth. Baffles were introduced in the fermentation vessel to ensure rapid O_2 transfer and maintenance of D.O. levels. Baffles are vertical strips of stainless steel that are installed perpedincular to the wall of the tank and prevent swirling and vortex formation of the stirred liquid inside the fermentor vessel (Figure 65). Oxygen mass transfer experiments identified an impeller rate of 750 rpm in baffled Braun fermetors gives the same mass transfer of O_2 as the impeller rate of 940 rpm in fermentors without baffles.[11]

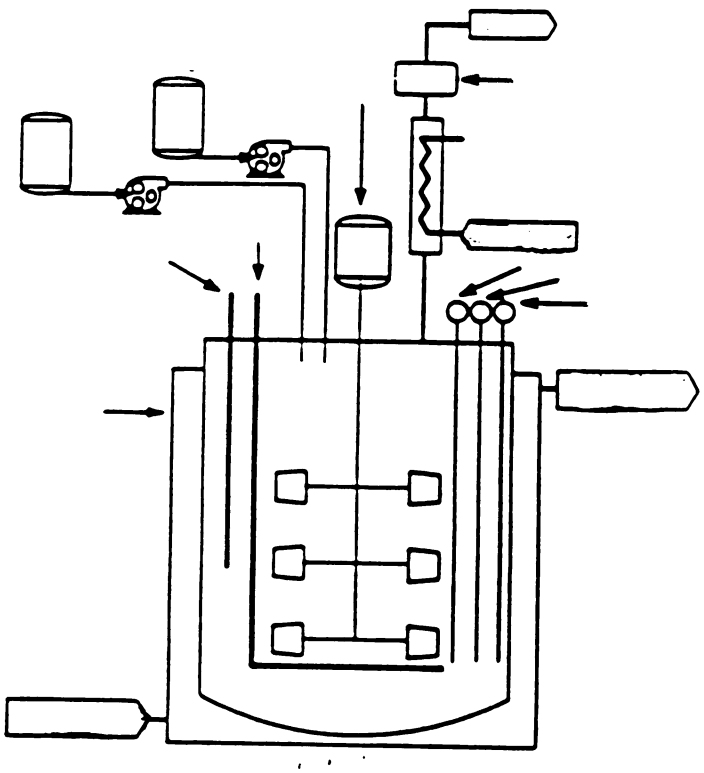


Figure 65. Braun Fermentor

For both fed-batch fermentation conditions, the concentration of the glucose feed was 60% (w/v). The PID control parameters were 0.0 (off) for the derivative control (τ_D), 999.9 s (maximum control action) for the integral control (τ_I), and 950.0% for the proportional band (X_p). When glucose addition was used for controlling D.O. levels, unregulated additions were sometimes problematic and depended on the recombinant *E. coli* construct being cultured.

All gallic acid-producing biocatalysts were evaluated using the glucose-limited fermentor conditions. The best gallic acid producer (KL7/pSK6.161) was then tested using glucose-rich fermentation conditions. Fermentor runs in both glucose-limited and glucose-rich conditions typically entered logarithmic growth 6 h after inoculation. Depending on the strain approximately 24 h or 30 h was required for fermentor cultures to move 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. Small amounts of acetic acid (5-10 mM) sometimes accumulated during the first 12 h after inoculation of the fermentation broth. Concentrations of acetic acid declined or were absent for most of the logarithmic and early stationary microbial growth phases. However, a biocatalyst that carried the *pck* gene (KL7/pSK7.104) accumulated substantial amounts of acetate resulting in the loss of D.O. control after 42 h. Significant amounts of glutamate (up to 110 mM) were accumulating in all gallic acid-producing fermentations starting from 30 to 36 h. Maximum productivity in gallic acid synthesis generally started at 24 h and continued until 48 h. Gallic acid synthesis typically did not continue beyond 48 h.

III. OPTIMIZATION OF THE BIOCATALYST AND FERMENTOR CONDITIONS FOR GALLIC ACID BIOSYNTHESIS

A. Exploring the relationship between DAHP synthase and PHB hydroxylase activities and the titers and yields of gallic acid and other metabolites.

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 3-dehydroshikimic acid-synthesizing strains by use of $aroF^{FBR}$ which is obtained via photochemical mutagenesis of *aroF*.[9] 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.[12]

The importance of DAHP synthase activity in the amount of carbon that is channeled to the aromatic pathway has been evaluated.[4] A correlation between the final product titers and yields of the aromatic pathway, and the activity levels of DAHP synthase was obtained in biocatalysts that grew under fed-batch fermentor conditions and accumulated 3-dehydroshikimic acid.[4] Maximum 3-dehydroshikimic acid titers and yields (52 g/L, 24% respectively) were obtained when $AroF^{FBR}$ was expressed from a strong *tac*

promoter. 3-Dehydroshikimic acid titers up to 20 g/L were synthesized in 18% (mol/mol) yield from glucose when AroF^{FBR} was expressed from its native promoter. 3-Dehydroshikimic acid titers of 41 g/L synthesized in 21% (mol/mol) yield from glucose were realized when an extra *aroF* promoter in addition to the *aroF*^{FBR} gene was plasmid-localized.[4] The purpose of adding an extra *aroF*^{FBR} promoter was to titrate away the cellular supply of TyR protein, which binds to the operator region of *aroF*^{FBR}. Some percentage of the *aroF*^{FBR} under transcriptional control of its native promoter thus evaded *tyrR* binding and were derepressed. In view of these strategies used to regulate DAHP synthase activity for optimal 3-dehydroshikimic acid synthesis, it was therefore desirable to explore the role of DAHP synthase activity in determining the titers and yields of microbe-synthesized gallic acid. Various biocatalysts were thus constructed that contained *aroF*^{FBR} expression systems similar to those previously exploited in the 3-dehydroshikimic acid-synthesizing constructs.

Plasmid pKD11.291A[4], which carried a copy of *aroF*^{FBR} with its native promoter and an extra copy of the *aroF* promoter, was the vector utilized for the construction of the first gallic acid-synthesizing biocatalyst. Digestion of pKD11.291A with *Hin*dIII, treatment with Klenow fragment to create blunt ends, and dephosphorylation with CIAP, gave plasmid pSK4.282 after ligation with the P_{tac}*pobA** gene (Figure 66). The latter was isolated as a 1.5 kb fragment after *Sma*I digestion of pSK4.232A (Figure 50, Chapter 4). The original vector from which pKD11.291A was constructed was plasmid pSU18. This plasmid carries a *p15* origin of replication with a copy number of approximately 12 per cell.[14b] It also contains a *lac* promoter and a genetic marker encoding for Cm resistance.

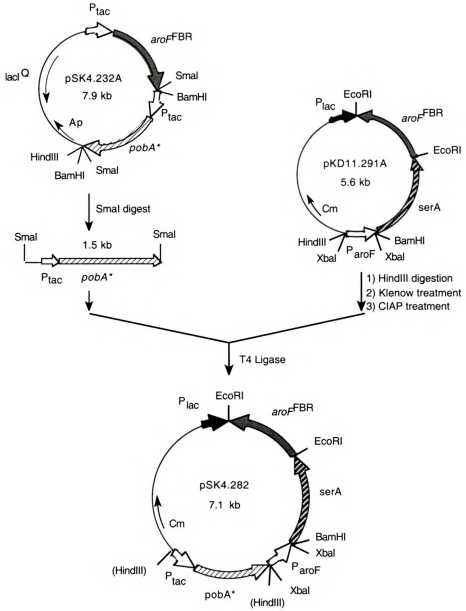


Figure 66. Construction of plasmid pSK4.282

KL7/pSK4.282 cultured under fed-batch fermentor conditions reached stationary phase at around 30 h under the glucose-limited conditions. The production of gallic acid started after 18 h and increased steadily throughout the whole run reaching a maximum of 8.3 g/L at the end of the run at 48 h (Figure 67). Using pKD11.291A that was used to construct pSK4.282, 41 g/L of 3-dehydroshikimic acid were synthesized when KL3/pKD11.291A was cultivated under fermentor conditions.

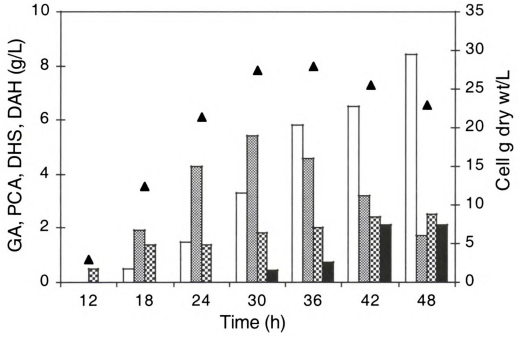


Figure 67. KL7/pSK4.282. Cell growth (▲) and GA (▭); PCA (▩); DHS (▩) and DAH (■) production.

Protocatecuic acid (PCA) reached a maximum concentration of 5.5 g/L at 30 h and then declined to 1.7 g/L after 48 h. 3-Dehydroshikimic acid (DHS, 2.5 g/L) and 3-deoxy-D-*arabino*-heptulosonic acid (DAH, 2.1 g/L) also accumulated at 48 h (Figure 67). The significant decrease of protocatechuate concentration can be explained by its transport into the *E. coli* cytoplasm and its subsequent oxidation to gallic acid. In Chapter 4,

protocatechuate uptake by *E. coli* cells was shown to occur in recombinant *E. coli* cultured under fermentor conditions. The total yield (mol/mol) based on the glucose added into the medium, including DHS, DAH, GA, and PCA, was 14%. DAHP synthase and PHB hydroxylase activities were monitored at three different time points (Table 13). PHB hydroxylase was expressed from the beginning of the fermentation and its activity was stable throughout the whole run (0.055 U/mg). In contrast, the activity of DAHP synthase showed a significant decrease after 24 h. Loss of AroF activity during the stationary phase of *E. coli* growth has been attributed to protease activity[13] and has been shown to occur in DHS-synthesizing bacteria cultured under fed-batch fermentor conditions.[4]

Table 13. KL7/pSK4.282. Product titers, total yields and specific activities of $AroF^{FBR}$ and PobA*.

PobA* (U/mg)[a]			$AroF^{FBR}$ (U/mg)[a]			g/L				Total[b]	GA[c]
24 h	36 h	48 h	24 h	36 h	48 h	GA	PCA	DHS	DAH	Yield %	Yield%
0.09	0.12	0.11	1.0	0.013	0.04	8.4	1.7	2.5	2.1	14	8

a. μmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.

One approach taken to increase the levels of DAHP synthase expression entailed replacement of the native promoter of $aroF^{FBR}$ with a strong *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 ß-D-thiogalactopyranoside (IPTG). AroF specific activity was thus controlled by the amount and frequency of IPTG added to the fermentor medium. Varying the DAHP synthase activity in 3-dehydroshikimic acid-synthesizing biocatalysts by changing the amount of added IPTG was found to be very important in determining the amount of carbon flow channeled into the common pathway. An optimum addition of 4.8 mg IPTG every 6 h starting from the beginning of phase three at 12 h was found to give the highest 3-dehydroshikimic acid titers (52 g/L).[4]

Plasmid pSK4.272 was created for the inducible, controlled expression of $aroF^{FBR}$. The low efficiency of protocatechuic acid hydroxylation using the mutant PHB hydroxylase enzyme required high expression levels that were only achieved with the use of a strong promoter. Consequently, in addition to $aroF^{FBR}$, PHB hydroxylase was expressed from a P_{tac} promoter. Plasmid pSK4.272 was constructed after ligation of a *serA* gene into a *Sma*I site of pSK4.232B (Figure 68). The *serA* gene was isolated as a blunt end fragment (1.9 kb) after *Dra*I/*Eco*RV digestion of pD2625. The cloning vector

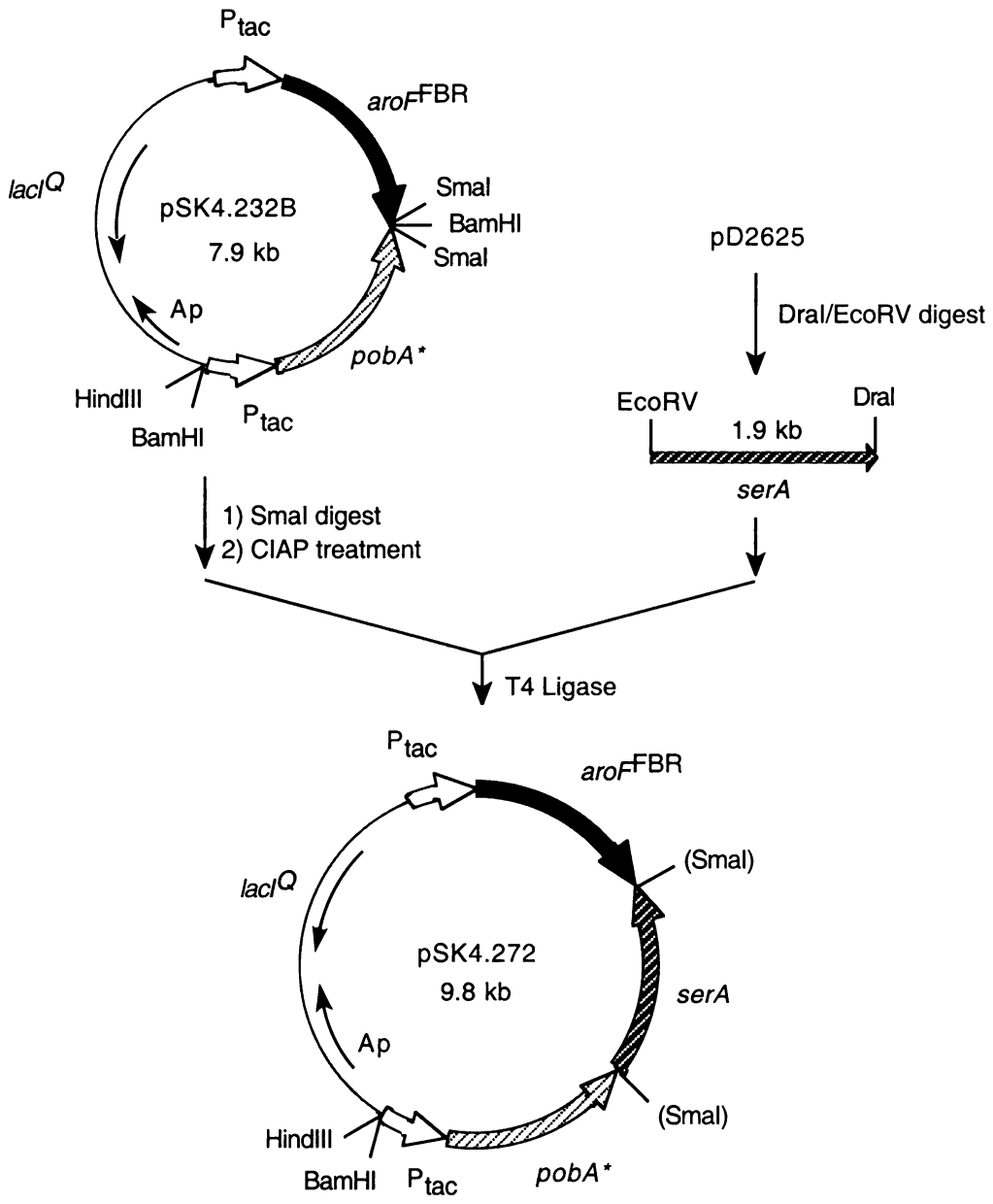


Figure 68. Construction of plasmid pSK4.272

that was originally used for the construction of pSK4.232 was pJF118EH. This plasmid contains a *tac* promoter and a $lacI^Q$ gene.[14a] With a copy number of about 15 per cell, it also contains a *pMB1* origin of replication and an ampicilin resistant marker.

Two different IPTG concentrations (5 and 30 mg) were added every 6 h starting from the beginning of phase three at 12 h (mid log phase) during cultivation of KL7/pSK4.272 under fed-batch fermentor conditions. Cells reached stationary phase at 24 h, which was 6 h earlier than KL7/pSK4.282 cultured under identical fed-batch fermentor conditions. Minimal expression of *pobA** and $aroF^{FBR}$ genes (0.014 U/mg and 0.037 U/mg, respectively before the first IPTG addition at 12 h) during the first 12 h, due to the expression of the plasmid-localized $lacI^Q$ repressor gene, was the reason for this significant growth difference.

Table 14. KL7/pSK4.272. Product titers, total yields and specific activities of $AroF^{FBR}$ and PobA*.

IPTG	PobA* (U/mg)[a]			$AroF^{FBR}$ (U/mg)[a]			g/L				Total[b]	GA[c]
(mg)	24 h	36 h	48 h	24 h	36 h	48 h	GA	PCA	DHS	DAH	Yield %	Yield%
5	0.017	0.022	0.037	0.27	0.25	0.086	3.2	20.8	3.4		19	3
30	0.035	0.055	0.055	4.9	4.0	1.6	14	1.7	4.1	5	19	11

a. μmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.

Slower growth rates of recombinant *E. coli* cultivated under fed-batch fermentor conditions have been reported in 3-dehydroquinic acid (Chapter 2) and 3-dehydroshikimic acid-synthesizing[4] biocatalysts when two plasmid-localized $aroF^{FBR}$ genes were introduced in the host strain. Addition of 5 mg IPTG every 6 h starting at 12 h after inoculation resulted in predominant formation of protocatechuic acid (20.8 g/L) relative to

gallic acid (3.2 g/L) and 3-dehydroshikimic acid (3.4 g/L) (Figure 69A). The total yield for gallic acid, protocatechuic acid and 3-dehydroshikimic acid was 19% (mol/mol) relative to the consumed glucose (Table 14).

The GA/PCA ratio was reversed from 1/8 (mol/mol) in favor of protocatechuic acid to 8/1 (mol/mol) in favor of gallic acid when instead of 5 mg of IPTG, 30 mg IPTG were added every 6 h starting from the beginning of phase three at 12 h after inoculation (Figure 69B, Table 14). Under these conditions 14 g/L of gallic acid was synthesized by 48 h along with protocatechuic acid (PCA, 1.7 g/L), 3-dehydroshikimic acid (DHS, 4.1 g/L) and 3-deoxy-D-*arabino*-heptulosonic acid (DAH, 5 g/L) (Table 14). An increase in both DAHP synthase and PHB hydroxylase activities reflected the higher IPTG concentrations in the fermentation supernatant. Although the GA/PCA ratio was directly linked to the *in vivo* expression levels of *pobA**, the significant difference in $aroF^{FBR}$ activity did not affect the total yield of aromatic pathway products (Table 14).

These results clearly demonstrated the significance of *pobA** activity for obtaining good ratios of gallic acid to protocatechuic acid. Further improvement of this ratio, or even better, complete elimination of protocatechuic acid, can potentially be achieved by a further increase of the PHB hydroxylase activity. Besides the higher titers of gallic acid that such a construct might provide, elimination of the protocatechuate contamination offers the additional advantage of simplifying the purification process of gallic acid. In contrast to *pobA**, whose increased expression was important for improving the GA/PCA ratio, the activity of $aroF^{FBR}$, which is responsible for increasing the carbon flow into the

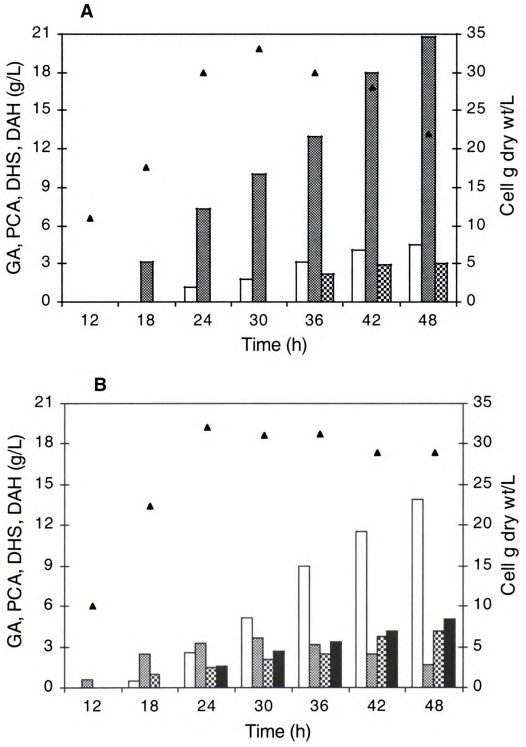


Figure 69. KL7/pSK4.272. Cell growth (▲) and GA (□); PCA (▨); DHS (▩) and DAH (■) production. A, 5 mg IPTG and B, 30 mg IPTG added every 6 h starting at 12 h.

common pathway, did not appear to play a significant role on the total yield of the common pathway products (Table 14).

Plasmid pSK6.233 was designed to satisfy both the above requirements. Increased activities of PHB hydroxylase were to be achieved after adding a second copy of *pobA** expressed from a *tac* promoter. The $lacI^Q$ repressor gene was again included in the construct to afford selective and controlled expression of the *pobA** genes. Plasmid pSK6.225 was first created after ligating the second P_{tac}*pobA** into the *Sma*I site of pSK4.232B (Figure 71). This 1.5 kb DNA insert was isolated as a *Sma*I fragment from pSK4.232A (Figure 50, Chapter 4). Digestion of pSK6.225 with *Hin*dIII, treatment with Klenow fragment to create blunt ends and dephosphorylation using CIAP gave pSK6.233 after ligation with the 1.9 kb *serA*-containing fragment (Figure 72). The later was isolated from pKD2625 as a *Dra*I/*Eco*RV fragment.

The first 12 h of KL7/pSK6.233 cultured under fed-batch fermentor conditions showed a very similar growth profile compared to the single *pobA** construct (KL7/pSK4.272) reaching phase two at 11 h where the first addition of IPTG (30 mg) was performed. Just before the second IPTG addition (30 mg) at 18 h, the cells started loosing the ability to control the D.O., which steadily increased from the 10% set point at 17 h to 75% at 24 h. Growth inhibition was also observed as indicated by the stabilization of the cell density measurements from the time when the D.O. start increasing (17 h) until the 24 h time point where the experiment was terminated.

The introduction of a second copy of the *pobA** gene rendered the cells incapable of completing a fermentation run due to an inability to control D.O. levels during their

cultivation and because of growth cessation 17 h after inoculation. Although various reasons such as plasmid instability can be responsible for this behaviour, increased rates of intracellular formation of gallic acid and/or H_2O_2 in growing bacteria might play an additional role in the creation of the observed toxic effect.

The toxicity of H_2O_2 in growing cells is well established.[15] A number of catalase-defecient mutants of *E. coli* which exhibit no assayable catalase activity have been studied. The only physiological difference between the catalase mutants and their parent strains was found to be a 50- to 60- fold greater sensitivity to killing with H_2O_2.[15] To our knowledge, toxicity studies in *E. coli* using gallic acid have not been reported. In contrast, the well known antioxidant activity of free gallic acid or various esters (Chapter 3) has been utilized to protect cell damage from singlet oxygen and free radicals.[16] In our hands, gallic acid inhibited cell growth of RB791 and KL7 *E. coli* in minimal plates containing gallic acid in concentrations as low as 5 mM. Inhibition of growth of RB791 and KL7 in liquid minimal salts medium containinig gallic acid was also established in shake flasks. Under these conditions when 5 mL minimal-salt solutions containing 0.0, 0.1, 0.5, 1 and 5 mM of gallic acid were inoculated with 0.1 mL of an overnight culture of *E. coli* cells (RB791 and KL7), growth inhibition was observed at concentrations of 1 mM gallic acid for RB791 and 5 mM gallic acid for KL7.

In bacteria grown under fed-batch fermentor conditions, the effect of gallic toxicity in the biosynthesis of 3-dehydroshikimic acid was investigated. KL3/pKL4.130B,[4] which carries a plasmid-localized cassette containing two $aroF^{FBR}$ genes expressed from their native promoter as well as a transketolase gene (*tkt*), was utilized for this reason. In the absence of gallic acid addition, KL3/pKL4.130B, produced 69 g/L of 3-dehydroshikimic

acid after 48 h of cultivation (Figure 70A).[4] In the presence of 15 g/L (88mM) of gallic acid, the same biocatalyst when cultured under identical conditions in a fed-batch fermentor, produced 33 g/L of 3-dehydroshikimic acid. Gallic acid (8 g) was added to the fermentation broth at 16 h when phase three of growth was reached creating a concentration of 43 mM of gallic acid in the broth. When late log phase was reached at 24 h, another portion of gallic acid (8.5 g/L) was added in the cell supernatant and a total concentration of 88 mM of gallic acid was obtained in the broth. No growth problems were encountered when KL3/pKL4.130 was growing in the presence of gallic acid and the D.O. was kept under good control until the end of the fermentation run. However in the presence of gallic acid KL3/pKL4.130 grew into lower cell densities (Figure 70A) and produced lower concentrations of both 3-dehydroshikimic acid and 3-dehydroquinic acid (Figure 70). Curiously, large amounts of glutamic acid were synthesized reaching 16 g/L (110 mM) by the end of the run (Figure 70B).

Although both gallic acid and H_2O_2 are toxic metabolites when formed in substantial amounts, the high rates of intracellular formation of H_2O_2 was thought to be the most likely reason for the observed toxicity. As part of its adaptive response to oxidative stress, *E. coli* produces two inducible hydroperoxidases called HPI and HPII.[17] Both these enzymes catalyze the conversion of H_2O_2 into water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). Upon exposure to sublethal levels of H_2O_2 during the exponential growth phase, HPI expression is induced.[18] The reason of the observed toxicity may due to the low native activity levels of HPI, which might not be sufficient to detoxify the cytocolic solution when *pobA** was overexpressed.

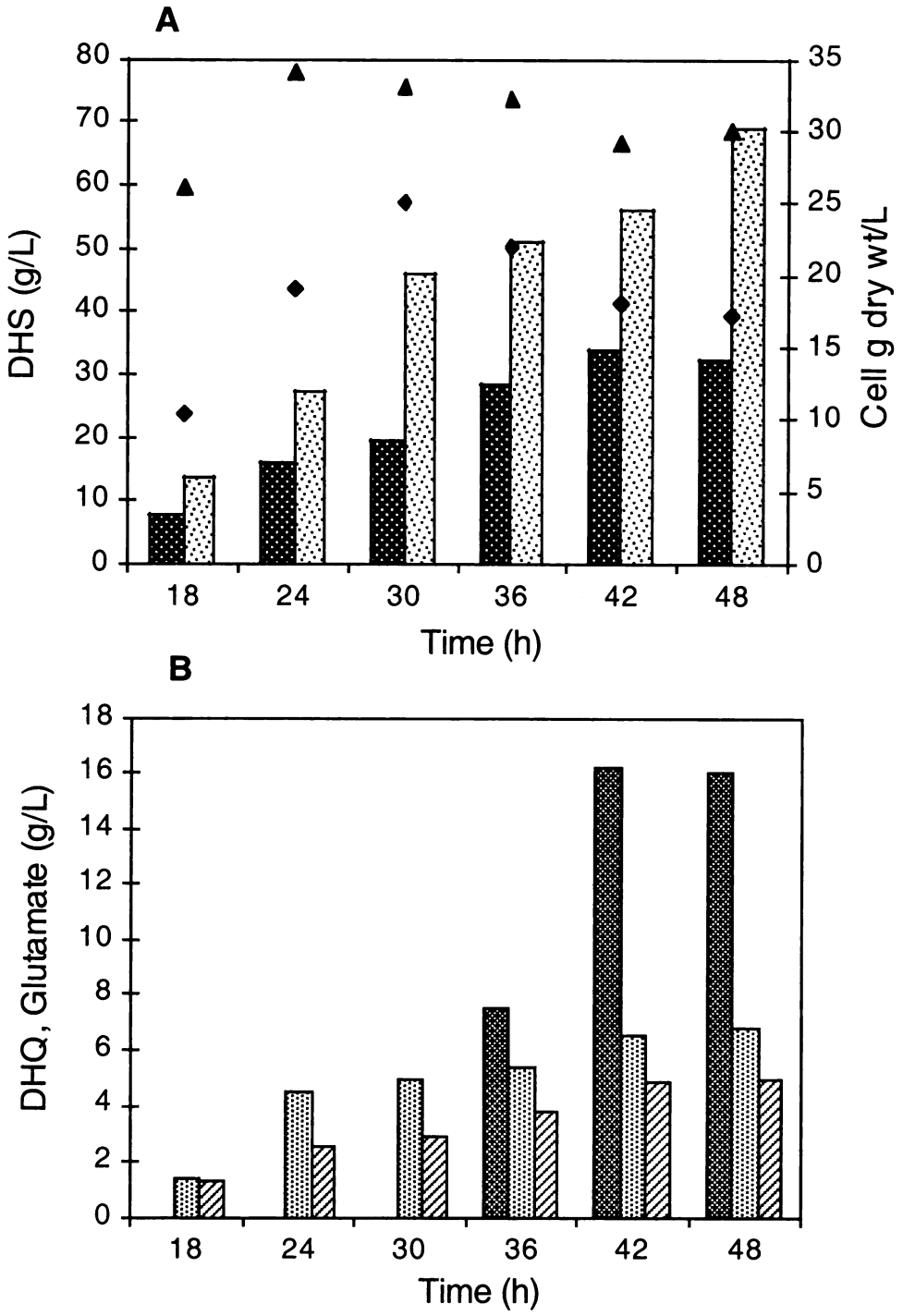


Figure 70. KL3/pKL4.130B. Production of DHS with and without addition of GA in the supernatant; A, Cell mass (▲) and DHS production (▭) of bacteria cultured without GA addition,[4] and cell mass (◆) and DHS production (▬) of bacteria cultured in the presence of GA; **B**, DHQ (▨) and glutamate (▬) production in bacteria cultured in the presence of GA, and DHQ production (▭) of bacteria cultured without GA addition.[4]

Localizing HPI on a plasmid was intended to increase the activity of this enzyme. Plasmid pSK6.270 was designed for this reason. The *katG*-encoded[19] gene that expresses HPI was isolated from the genome of *E. coli* RB791 using the polymerase chain reaction (PCR). Primers that contain *Xba*I terminal sites were designed as follows: 5-GCT CTAGATCTCAACTATCGCATCCGTG and 5-GCTCTAGACTTGCTTCATGAAA TCCAGC. The 2.4 kb *katG* PCR fragment containing its native promoter was digested with *Xba*I and ligated at the same site of pSU18 creating plasmid pSK5.119 (Figure 73). Subsequent insertion of the *serA* gene into pSK5.119 gave plasmid pSK6.261 (Figure 74). The *serA* gene was isolated as a *Dra*I/*Eco*RV fragment from pD2625 and was ligated to a salI site of pSK5.119 that has previously been blunt-ended with Klenow fragment and dephosphorelated with CIAP. A 4.3 kb fragment containing the *katG-serA* cassette was isolated from pSK6.261 after *Hin*dIII/*Sma*I double digestion. Treatment of this DNA cassette with Klenow fragment and ligating to a Klenow/CIAP treated *Hin*dIII site of pSK6.225 created plasmid pSK6.270 (Figure 75).

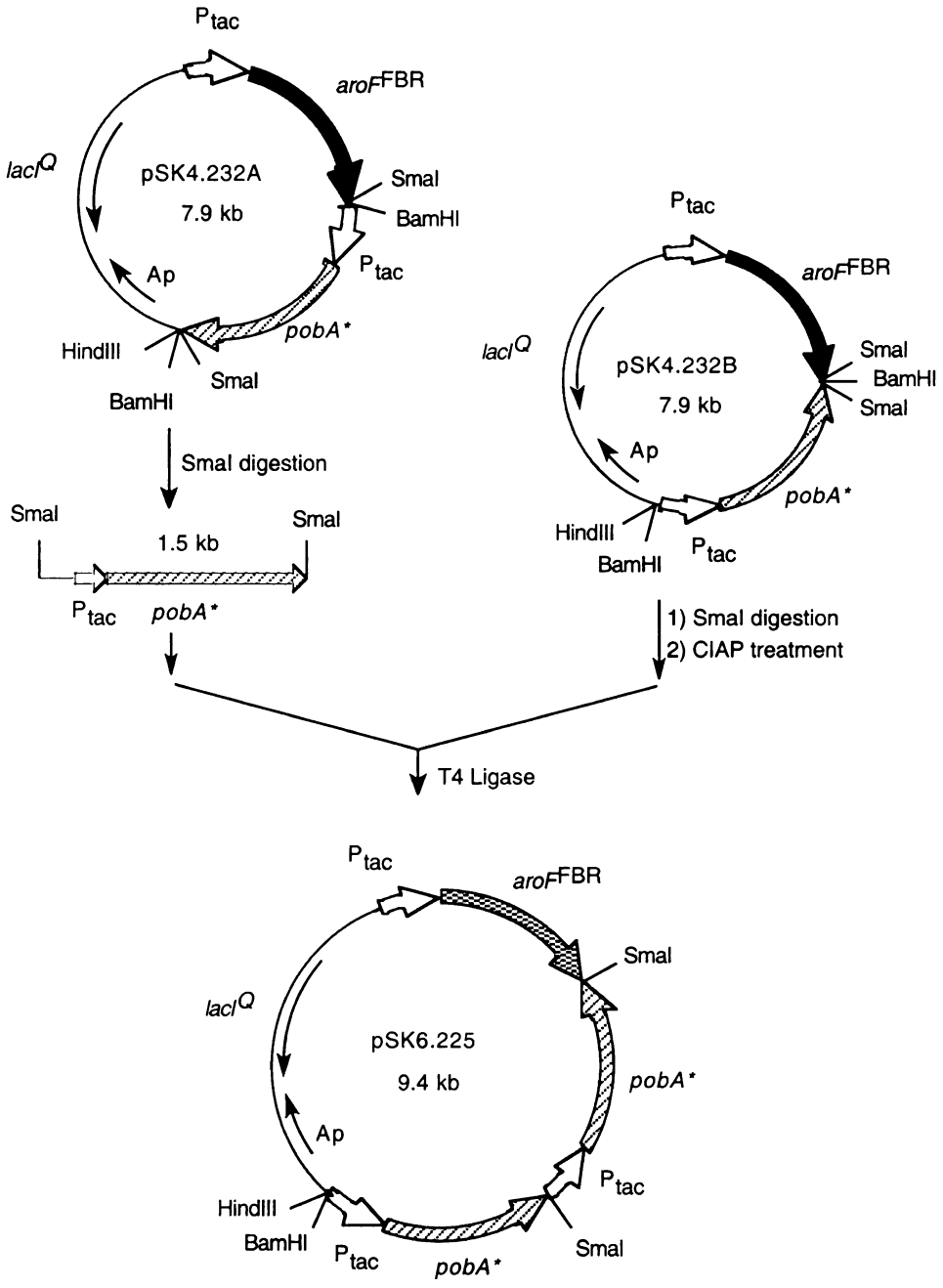


Figure 71. Construction of plasmid pSK6.225

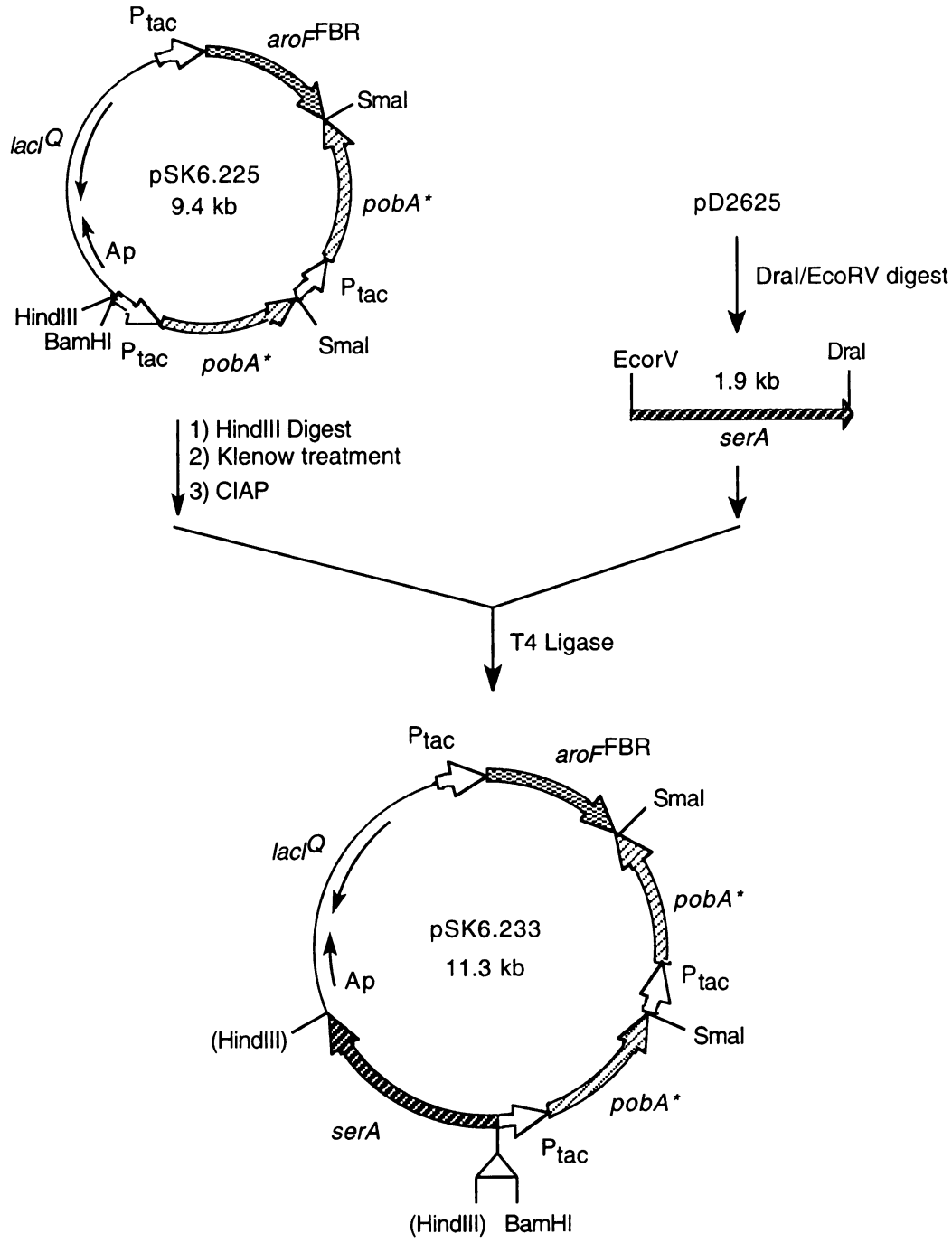


Figure 72. Construction of plasmid pSK6.233

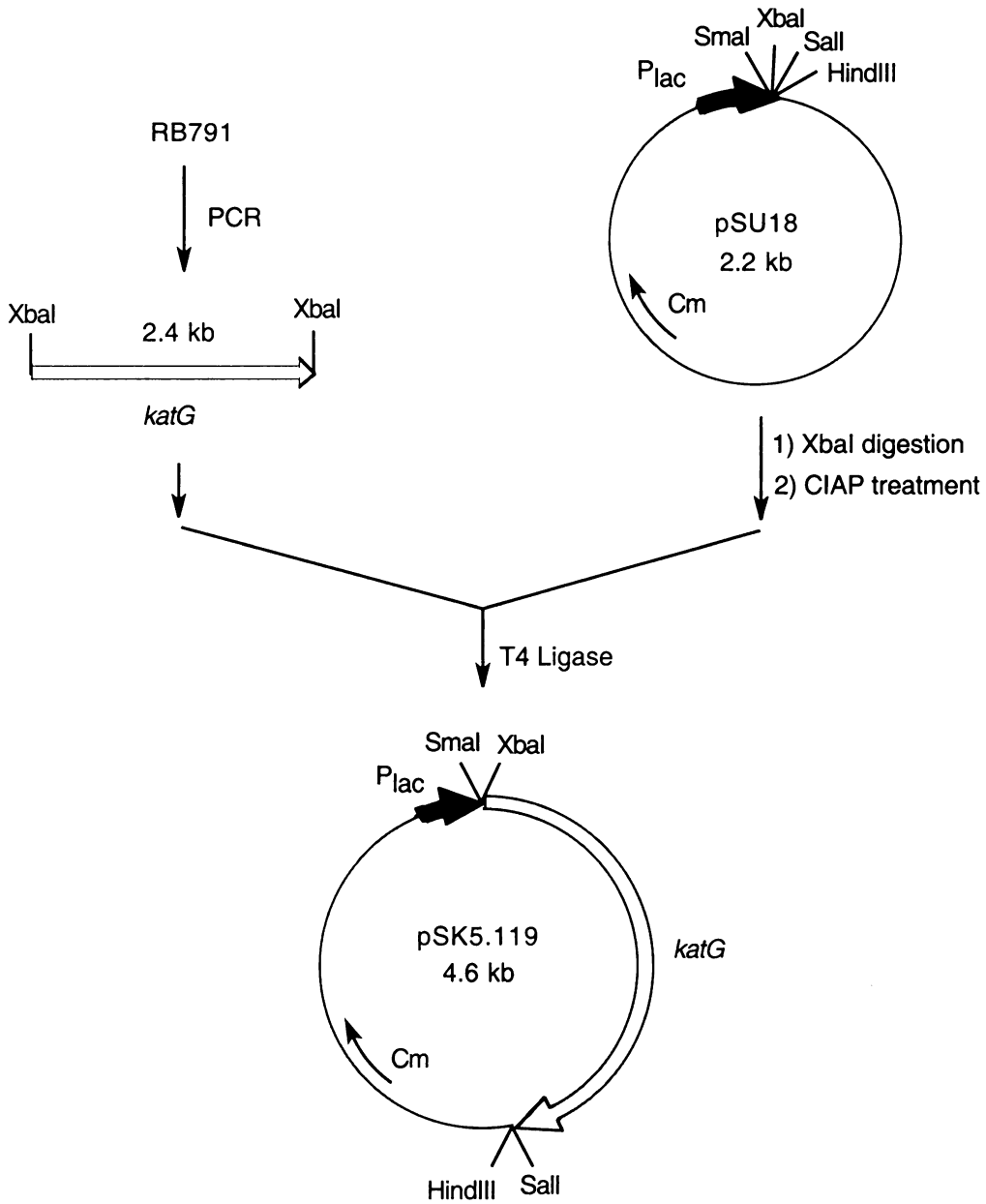


Figure 73. Construction of plasmid pSK5.119

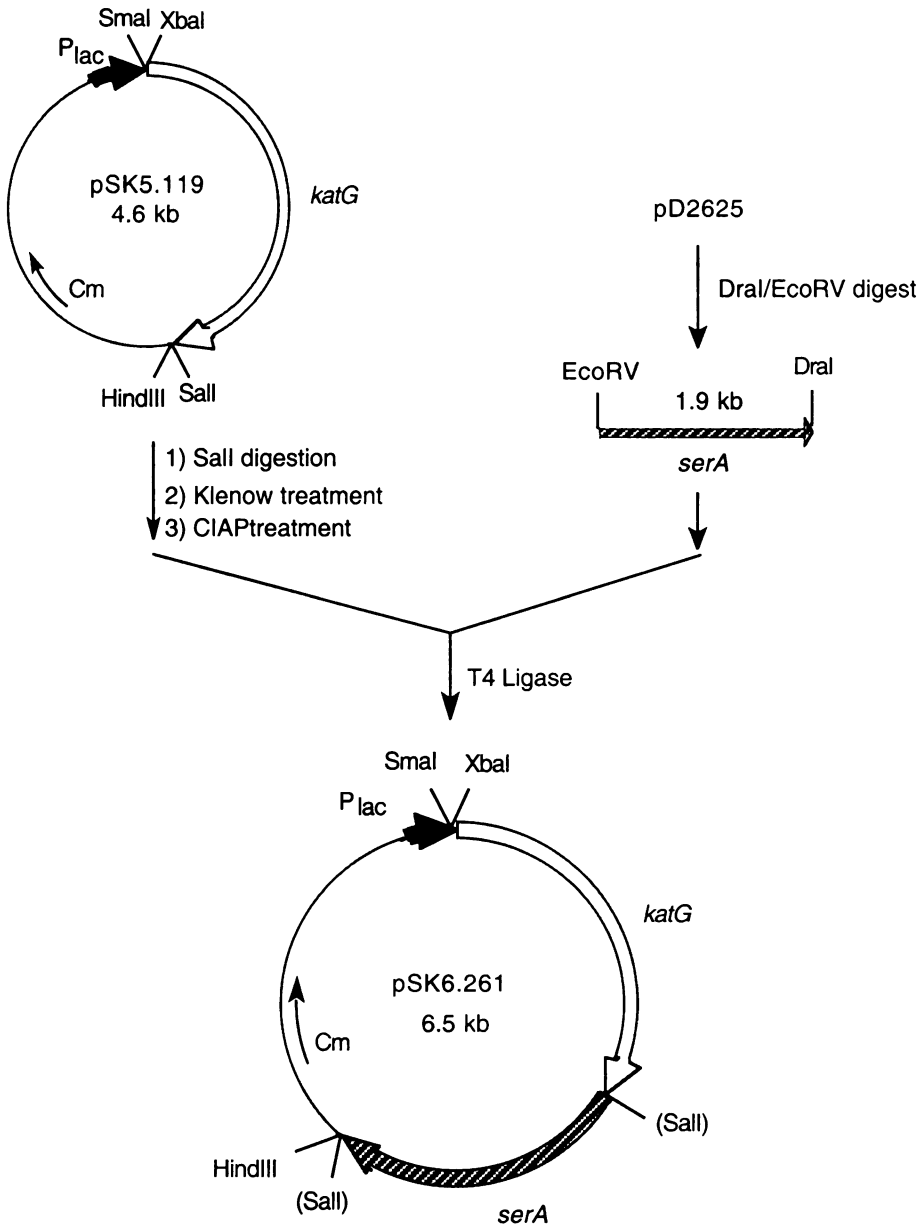


Figure 74. Construction of plasmid pSK6.261

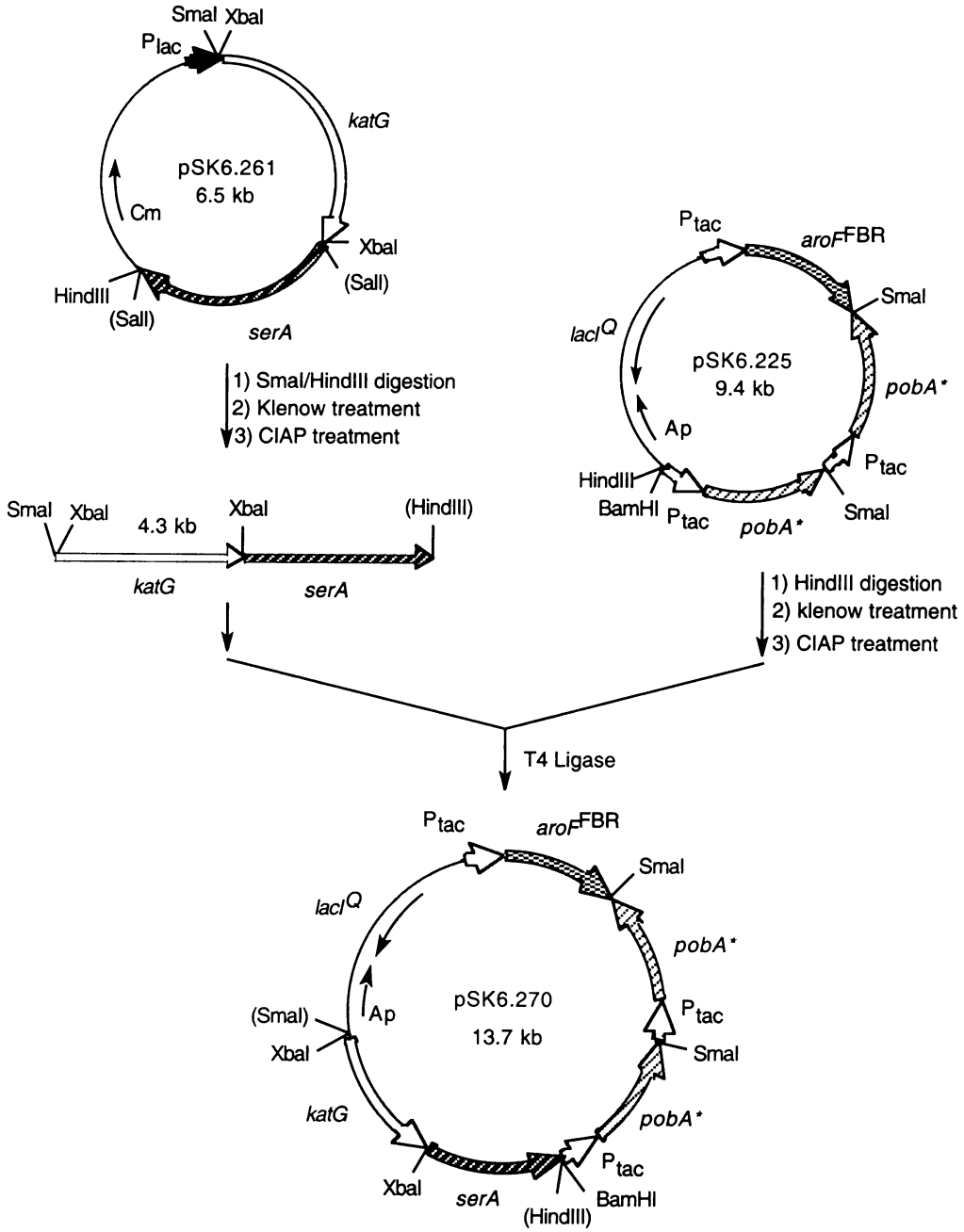


Figure 75. Construction of plasmid pSK6.270

Culturing KL7/pSK6.270 under fed-batch fermentor conditions showed an identical growth profile to the biocatalyst that was carrying two copies of *pobA** but lacking KatG overexpression (KL7/pSK6.233). Airflow reached its maximum rate at 11 h, but the same loss of ability to control the D.O. as well as the same cessation of cell growth, was observed at 17 h before the second IPTG addition (at 18 h) was due. However, the activity levels of KatG were high as indicated by enzyme assays that were performed at 12 and 24 h (0.24 and 0.12 U/mg respectively). When similar enzyme assays were performed in cells that lacked the plasmid-localized KatG (KL7/pSK4.272) no measurable peroxidase activity was detected. Therefore, although HPI was successfully overexpressed, this did not help the bacteria that carried two *pobA** loci grow normaly under fed-batch fermentor conditions. This result suggests that H_2O_2 accumulation could not be blamed for the observed loss of D.O. control and the cessation of cell growth, at least in the biocatalysts that contain two P_{tac}*pobA** genes.

Removing the second copy of *pobA** was then designed to eliminate the observed toxic effect caused by the double *pobA** cassette. At the same time $aroF^{FBR}$ expression from its native promoter would ensure enough carbon flow to the aromatic pathway. Plasmid pSK6.161 was constructed (Figure 76) to fulfill these requirements. Digestion of pSK4.259 (Figure 56, Chapter 4) with *Bam*HI gave a 5.9 kb fragment containing $lacI^{Q}$, $aroF^{FBR}$, and P_{tac}*pobA** and the 2.2 kb *aroZ* gene. Gel purification of the large fragment, treatment with Klenow fragment to create blunt ends, and ligation with *serA* gave pSK6.161 (Figure 76). Plasmid pSK4.259 was constructed from vector pSU18.

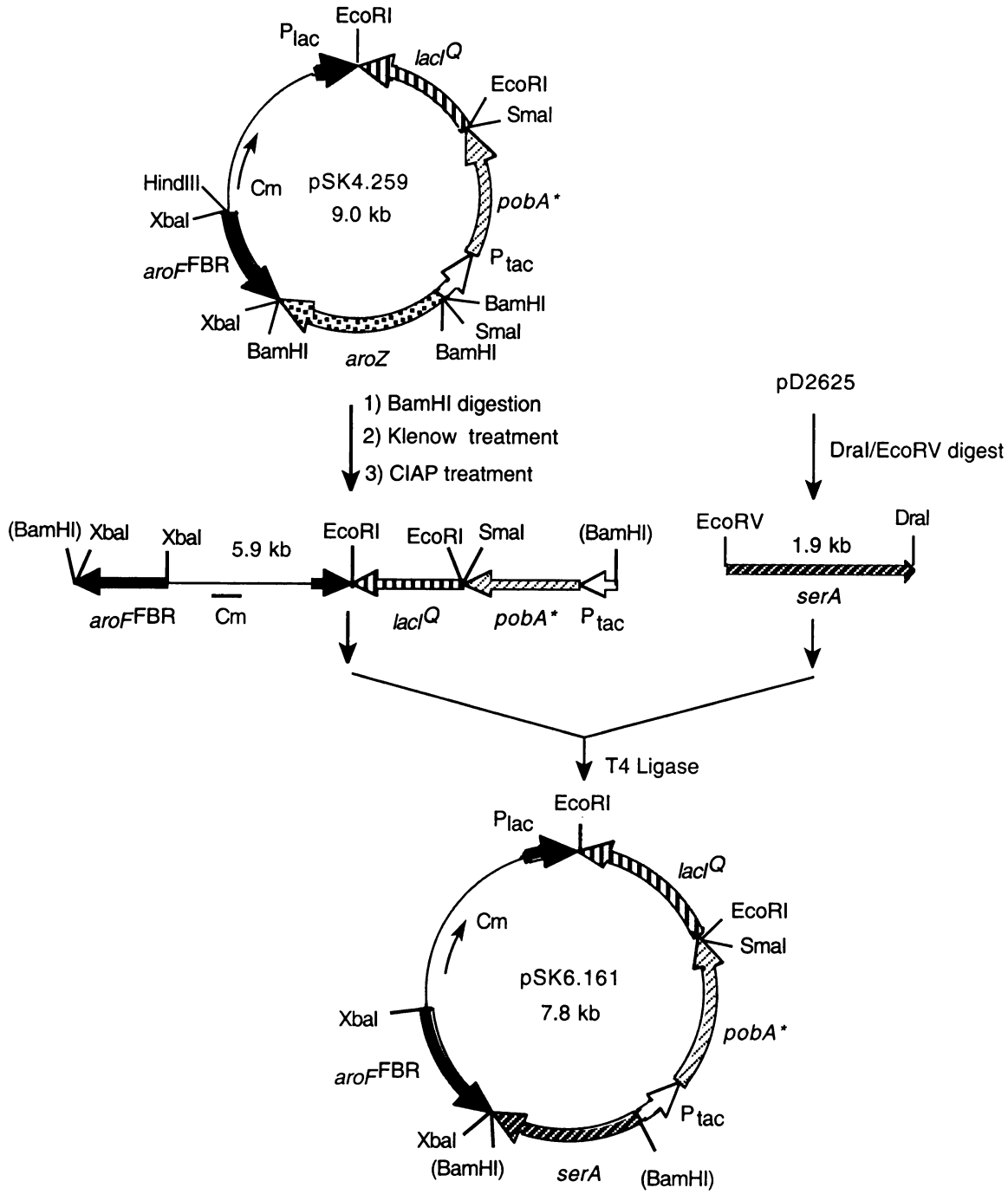


Figure 76. Construction of plasmid pSK6.161

Culturing KL7/pSK6.161 under the standard glucose-limited fermentation conditions resulted in 16.3 g/L of gallic acid along with 0.7 g/L of protocatechuic acid, 2.2 g/L of 3-dehydroshikimic acid, and 4.0 g/L of 3-deoxy-D-*arabino*-heptulosonic acid after 48 h giving a total yield of 18% (mol/mol) of products synthesized from the consumed glucose (Table 15). The cells reached stationary phase at 24 h after which the majority of gallic acid was synthesized (Figure 77). IPTG (30 mg) was added every 6 h starting at the end of phase two (12-13 h). Although high levels of *pobA** activity were produced, the formation of protocatechuic acid was not completely eliminated. Utilizing its native promoter, DAHP synthase activities were lower relative to the construct where DAHP synthase was expressed from a *tac* promoter (KL7/pSK4.272), however, the total yield of products synthesized was almost the same (Table 14, 30 mg IPTG, versus Table 15).

Table 15. KL7/pSK6.161. Product titers, total yields and specific activities of $AroF^{FBR}$ and PobA*.

PobA* (U/mg)[a]			$AroF^{FBR}$ (U/mg)[a]			g/L				Total[b]	GA[c]
24 h	36 h	48 h	24 h	36 h	48 h	GA	PCA	DHS	DAH	Yield %	Yield %
0.12	0.14	0.17	2.3	1.2	0.6	16.3	0.7	2.2	4.0	18	12

a. µmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.

In contrast to the 3-dehydroshikimate-synthesizing biocatalysts, a clear correlation between DAHP synthase activity and the product titers and yields can not be drawn in the case of gallic acid-synthesizing biocatalysts. The product yields in every biocatalyst tested so far were within the range of 14-19% mol/mol based on consumed glucose. The activity of PHB hydroxylase on the other hand, had to be very carefully controlled. A certain level was required for obtaining good gallic acid to protocatechuic

acid ratios while introduction of a double $P_{tac}pobA^*$ cassette resulted in culture instability as observed experimentally from cessation of growth and loss of the ability to control the D.O. leves in bacteria cultured under fed-batch fermentor conditions.

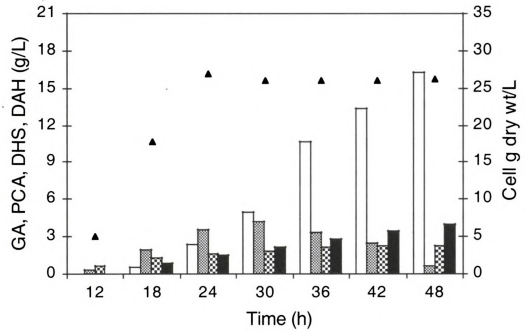


Figure 77. KL7/pSK6.161. Cell growth (▲) and GA (☐); PCA (▨); DHS (▩) and DAH (■) production. IPTG (30 mg) was added every 6 h starting at 12 h.

B. Elimination of DHS and DAH accumulation and the attendant impact on GA titers and yields.

All biocatalysts that have been constructed so far accumulated significant amounts of the common pathway intermediates 3-dehydroshikimic acid (DHS) and 3-deoxy-D-*arabino*-heptulosonic acid (DAH). Accumulation of these molecules reflected the limiting activities of DHQ synthase (AroB) and DHS dehydratase (AroZ). 3-Dehydroquinic acid (DHQ) rather than DAH had been reported to form in significant concentrations in the cell supernatant of 3-dehydroshikimic acid-synthesizing constructs cultured under fed-batch fermentor conditions.[4] These constructs utilized *E. coli* KL3, a host strain that was identical to KL7 with the exception that only *aroB* instead of *aroBaroZ* was inserted in the *serA* locus of AB2834 genome.[4] DAH accumulation was also observed in the protocatechuic acid-synthesizing biocatalysts as discussed in Chapter 2. The formation of DAH was therefore an inherent property of the gallic acid- and protocatechuic acid-producing bacteria. In fact, inhibition studies of DHQ synthase, which catalyzes the formation DAHP to DHQ (Figure 86), revealed that protocatechuic acid was a relatively potent competitive inhibitor with a K_i of 65 μM.[20] The K_m for DAHP for the same enzyme was 17 μM. Accumulation of DAH may thus reflect inhibition of DHQ synthase by protocatchuic acid.

Increasing the activity levels of DHS dehydratase (*aroZ*) was the first strategy examined for elimination of the accumulation of common pathway intermediates. Introducing an extra copy of the *aroZ* gene into the biocatalyst was anticipated to eliminate 3-dehydroshikimiate formation and accordingly increase the final gallic acid and

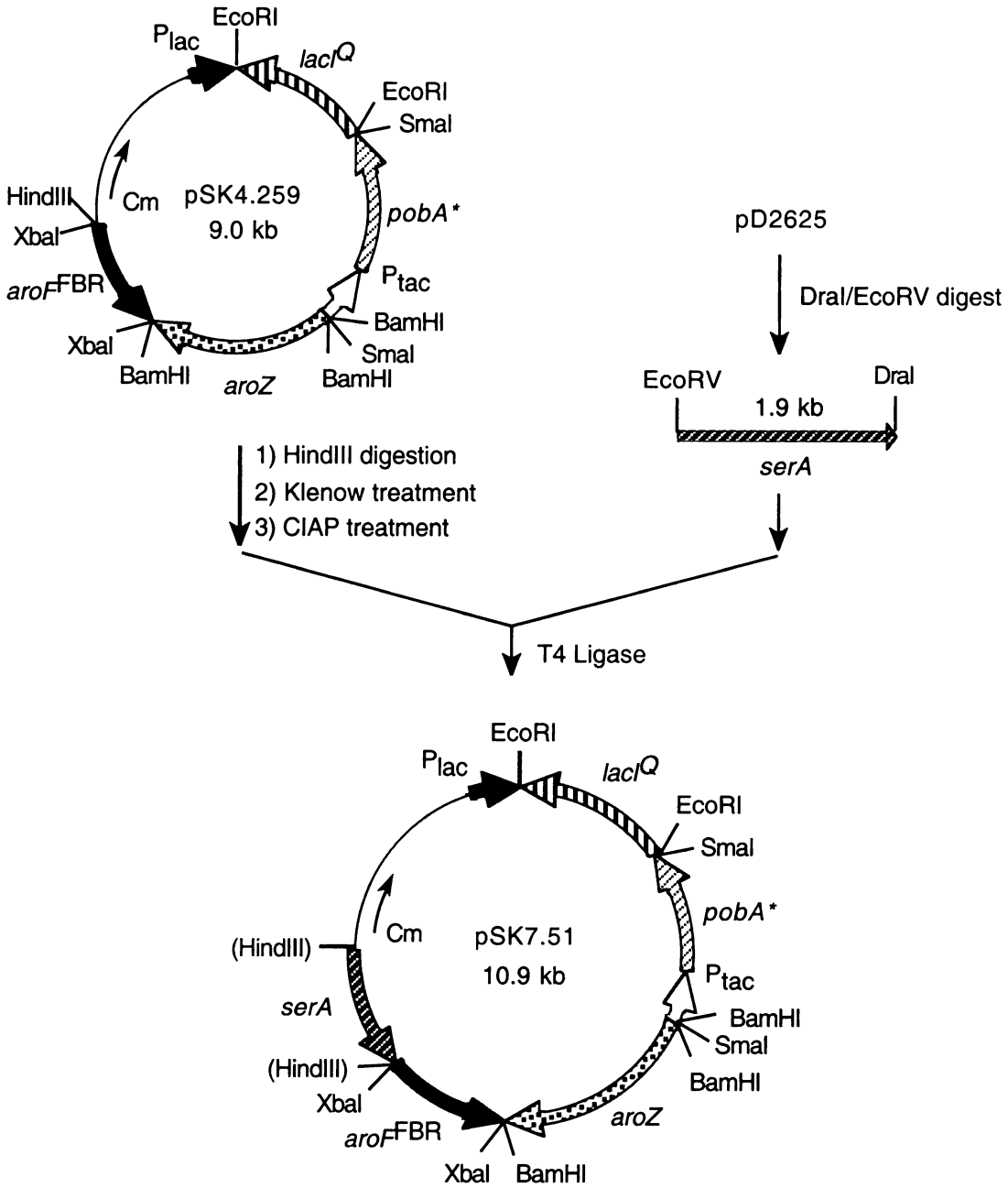


Figure 78. Construction of plasmid pSK7.51

protocatechic acid titers. Plasmid pSK7.51 was constructed (Figure 78) for this reason. Digestion of plasmid pSK4.259 (Figure 56 Chapter 4) with *Hin*dIII, treatment with Klenow fragment and dephosphorylation with CIAP gave after ligation with *serA*, plasmid pSK7.51. The *serA* gene was isolated as a 1.9 kb *DraI*/*Eco*RV fragment from pD2625. Note that both pSK7.51 and pSK6.161 were derivatives of the same pSK4.259 plasmid, which was originally constructed from a pSU18 vector.

Cultivation of KL7/pSK7.51 under fed-batch glucose-limited fermentor conditions, showed the usual profile reaching phase two and stationary phase after 11 h and 24 h respectively (Figure 79). However, the amount and frequency of IPTG addition was very critical for the outcome of these fermentations. Good control of the D.O. was kept until 42 h when 10 mg of IPTG was added at 12, 18 and 24 h (30 mg total). In contrast, loss of the ability to control the D.O. levels was observed after the second addition of IPTG (30 mg) at 24 h, at which time the experiment was terminated.

Table 16. KL7/pSK7.51. Product titers, total yields, and specific activities of AroF^{FBR} and PobA*.

IPTG	PobA* (U/mg)[a]			AroF^{FBR} (U/mg)[a]			g/L				Total[b]	GA[c]
(mg)	24h	36h	48h	24h	36h	48h	GA	PCA	DHS	DAH	Yield %	Yield %
10[d]	0.036	0.048	0.041	0.48	0.36	0.19	11.6[e]	1.1	–	4.3	14	9
30[f]	0.086			0.34								

a. µmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.
d. IPTG added at 12, 18, 24 h; e.This is the final concentration at 48h. The maximum at 36 h was 13g/L
f. IPTG added at 12 h and 18 h.

A similar toxicity effect was observed in the biocatalyst carrying a double *pobA** locus (KL7/pSK6.233). Successful expression of the *katG*-encoded hydroperoxidase I that degrades H_2O_2 did not restore bacterial growth or help control the D.O. levels.

Combining this result with our gallic acid toxicity studies, it is reasonable to assume that the rate of intracellular gallic acid formation was part of, or the only reason, for the observed toxicity. A similar argument can be made in this case of *aroZ* overexpression. Introducing an extra copy of DHS dehydratase resulted in a higher rate of protocatechuate formation. Under these conditions of increased protocatechuic acid production, tuning the activity levels of *pobA** was critical for the survival of the organism (Table 16). PHB hydroxylase activities of about 0.04 U/mg resulting from the low IPTG additions, gave 11.6 g/L of gallic acid (GA), 1.1 g/L protocatechuic acid (PCA) and 4.3 g/L 3-deoxy-D-*arabino*-heptulosonic acid (DAH) without any 3-dehydroshikimic acid (DHS) at the end of the run (Figure 79, Table 16). Increasing the activity of PHB hydroxylase to 0.086 U/mg resulted in loss of the D.O. levels. However, the higher rate of protocatechuate formation can not be excluded as another reason of the observed toxicity.

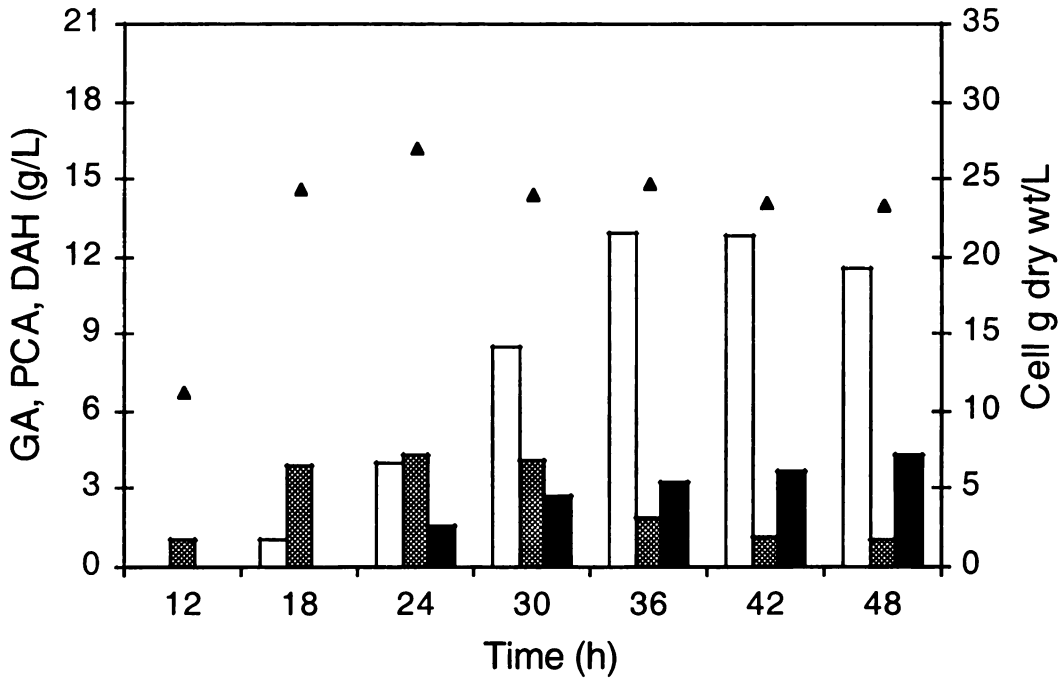


Figure 79. KL7/pSK7.51 Cell growth (▲) and GA (▭); PCA (▩) and DAH (■) production. IPTG (10 mg) were added at 12 h, 18 h and 24 h.

Removal of the impediment to carbon flow at DHS dehydratase after plasmid-localization of *aroZ* was expected to lead to an increase of the final protocatechuic acid and gallic acid titer. Close examination of the gallic acid synthesized during the first 36 h of KL7/pSK7.51, showed values that closely match the sum of the gallic acid and 3-dehydroshikimic acid concentrations synthesized by KL7/pSK6.161 during the same time period (Table 17). The amounts of protocatechuic and 3-deoxy-D-*arabino*-heptulosonic acid synthesized by both biocatalysts were very similar. Clearly, 3-dehydroshikimic acid was being channeled into the synthesis of additional gallic acid. In addition, when *aroZ* was overexpressed, the sum of all the common pathway products was increased until 42 h after inoculation (Table 17). The significant difference in the final product concentrations came during the last 12 h of cultivation. The gallic acid production leveled off and somewhat decreased in KL7/pSK7.51,which carried the extra *aroZ*. By contrast, a steady increase in the synthesis of gallic acid was observed for KL7/pSK6.161 lacking the extra *aroZ* gene (Figure 77 versus Figure 79).

Table 17. Comparison of product formation with time between KL7/pSK6.161 and KL7/pSK7.51

	pSK6.161 (mM)					pSK7.51 (mM)				
Time (h)	GA	PCA	DHS	DAH	Total[a]	GA	PCA	DHS	DAH	Total[a]
24	15	23	9	8	55	23	25	0	9	57
30	29	27	11	11	78	50	28	0	13	91
36	54	17	13	14	101	76	27	0	16	119
42	78	10	14	17	119	76	12	0	18	106

a. mol DHS+DAH+GA+PCA.

Removal of both 3-deoxy-D-*arabino*-heptulosonic acid (DAH) and 3-dehydroshikimic acid (DHS) from the fermentation medium was achieved with a biocatalyst that carried both *aroZ* and *aroB* encoding, respectively, DHS dehydratase and DHQ synthase. Plasmid pSK7.80 was constructed for this purpose. Double digestion of pSK4.141 with *Sal*I/*Hin*dIII gave after ligation with *aroB,* plasmid pSK7.52 (Figure 80). The 1.6 kb *aroB* gene was isolated from pKD14.136B after *Sal*I/*Hin*dIII digestion. Cloning of P_{tac}*pobA** (1.5 kb *Sma*I fragment from pSK4.232A) into a *Sma*I site of pSK7.52 gave plasmid pSK7.61 (Figure 81). Subsequent ligation of *serA* (1.9 kb *Eco*RV/*Dra*I fragment from pD2625) into a Klenow fragment, CIAP *Hin*dIII site of pSK7.61, gave plasmid pSK7.80 (Figure 82). The vector (pSK4.141) that was used for pSK7.80 construction was the same pSU18-based plasmid that was also utilized for the construction of both pSK6.161 and pSK7.51.

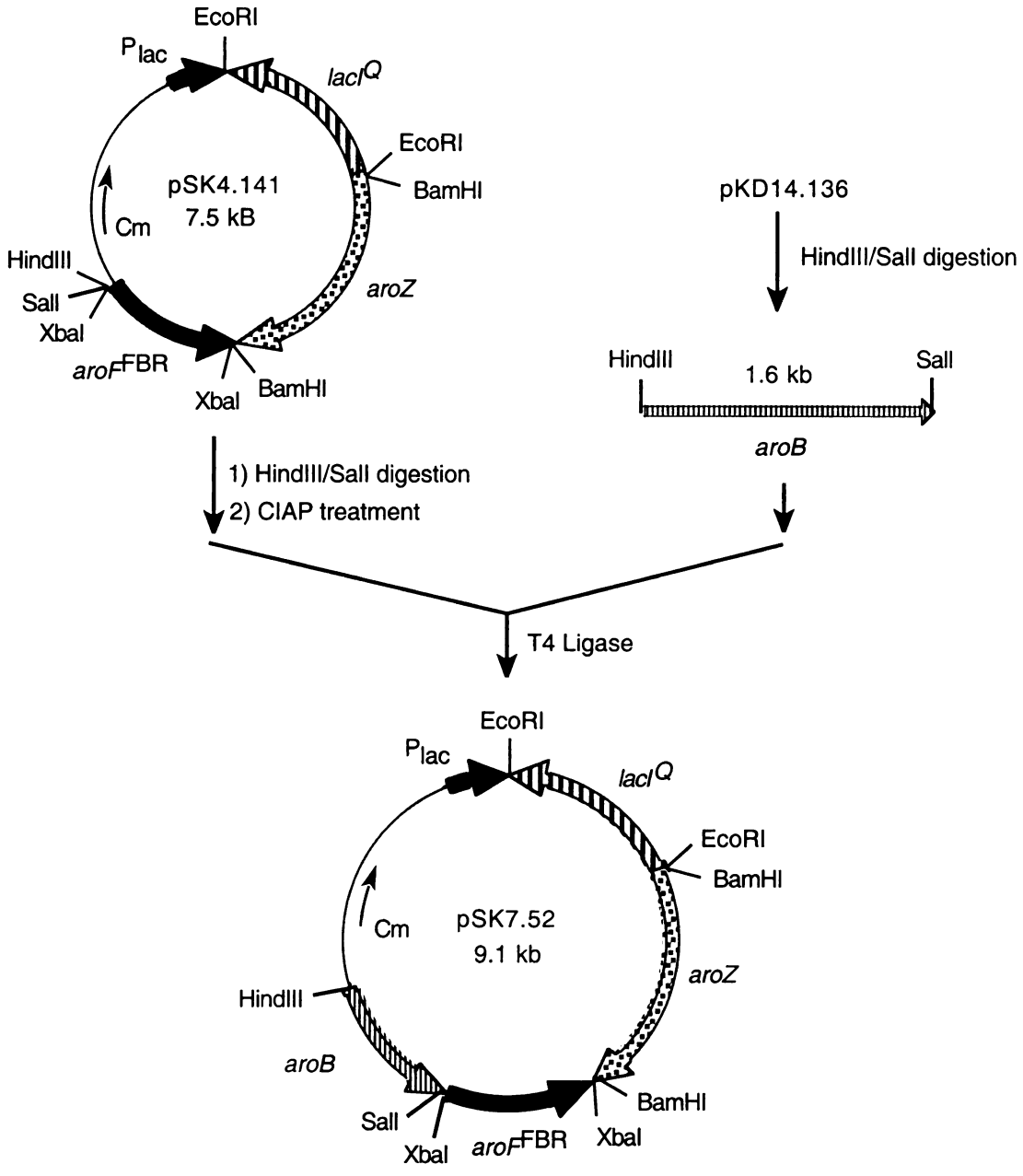


Figure 80. Construction of plasmid pSK7.52

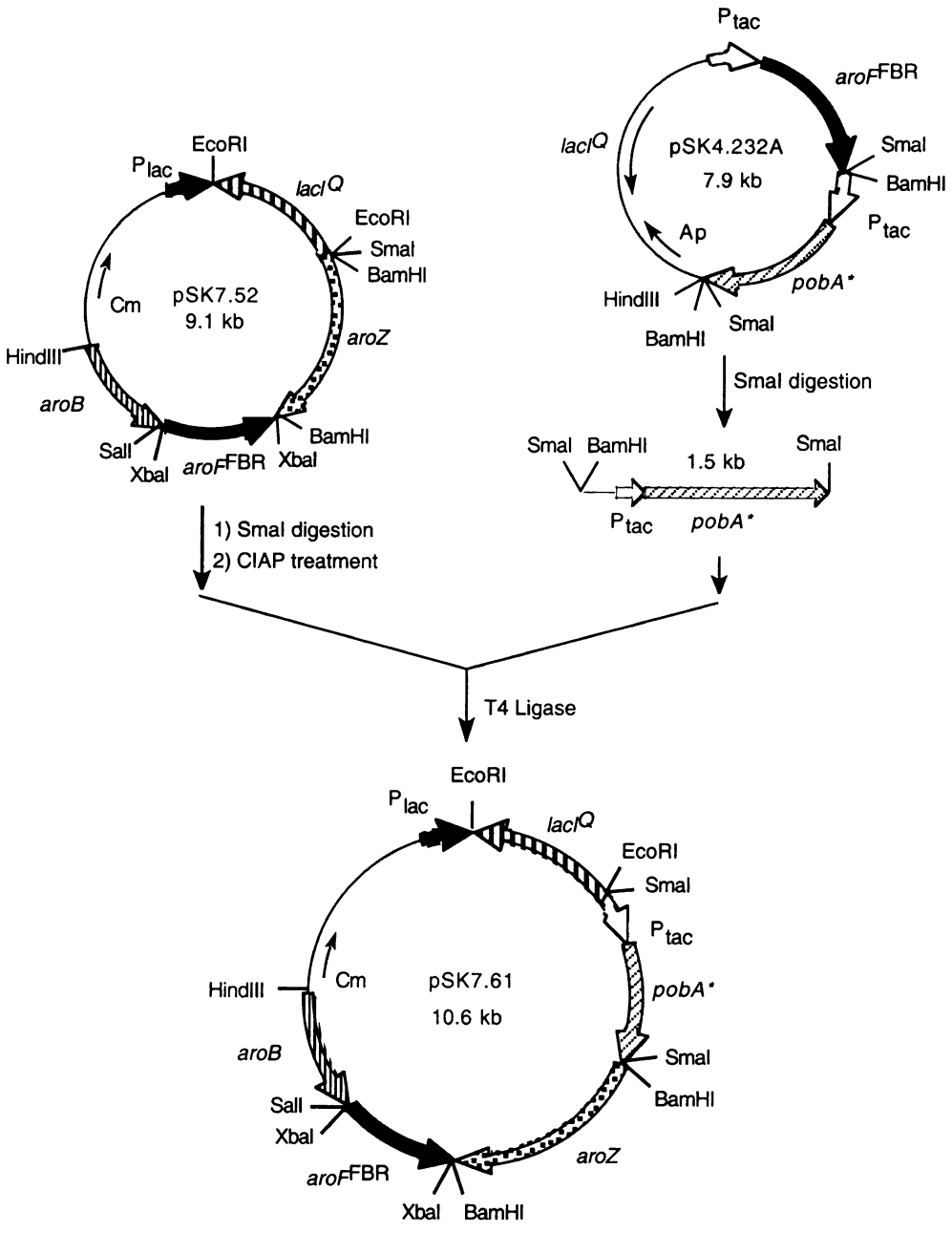


Figure 81. Construction of plasmid pSK7.61

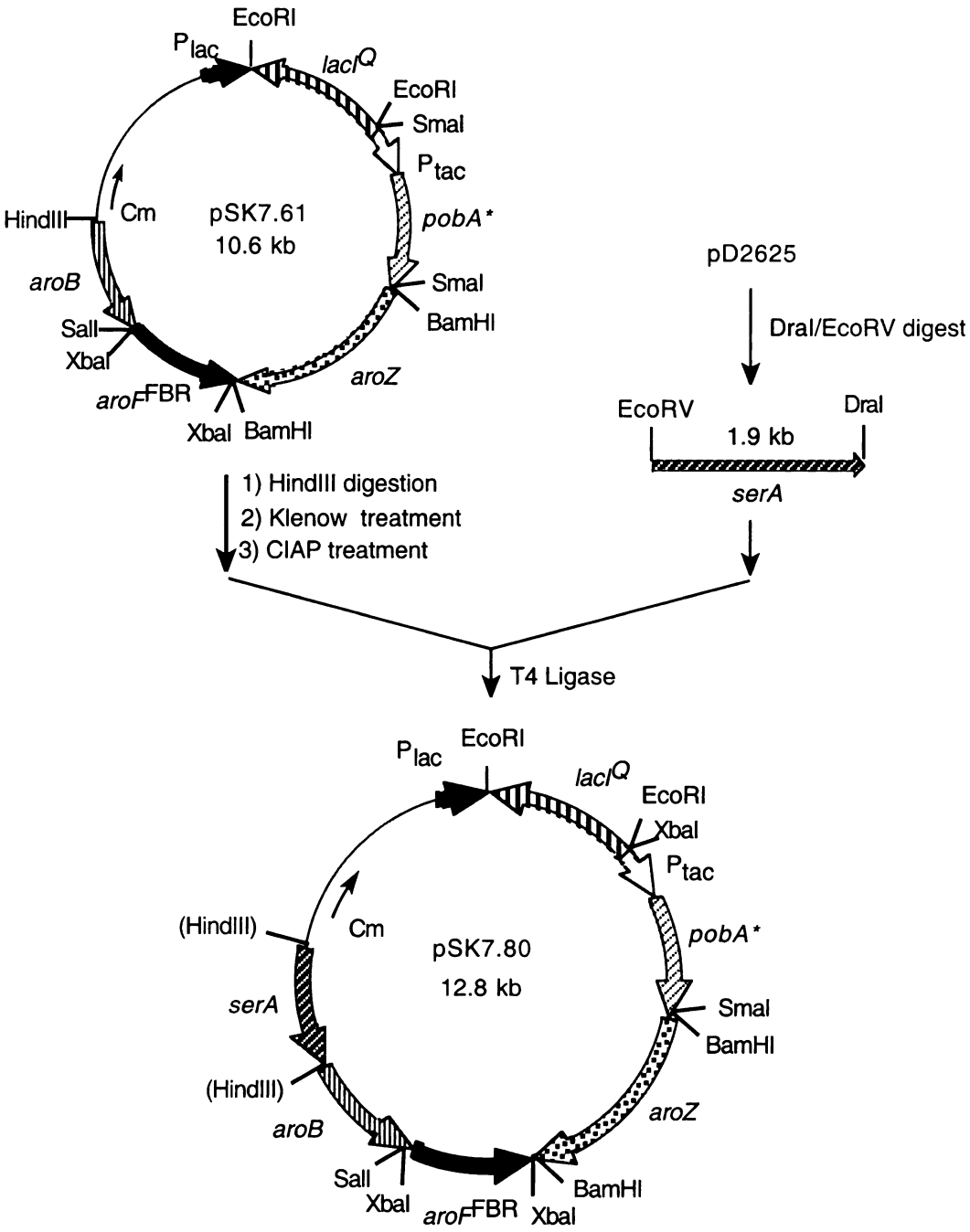


Figure 82. Construction of plasmid pSK7.80

It came as no surprise to find that KL7/pSK7.80 cultured under fed-batch fermentor conditions followed a similar profile compared to its previously discussed biocatalytic ancestors (KL7/pSK6.161 and KL7/pSK7.51). More specifically, cells reached phase two after 11 h, and entered stationary phase at 24 h. Control of D.O. levels was maintained even after 48 h, which in contrast to KL7/pSK7.51, was unaffected by the amounts of added IPTG. No loss of D.O. control or cessation of cell growth were encountered with this biocatalyst, even when 30 mg of IPTG were added every 6 h. The concentration of the added IPTG (10 or 30 mg added every 6h) however, had a profound effect on the product ratio. A relatively small increase of the PHB hydroxylase activity (from ~0.025 to ~0.036 U/mg) basically reversed the GA/PCA ratio from 2/3 (mol/mol) in favor of protocatechuic acid into 4.2/1 (mol/mol) in favor of gallic acid (Figure 83, Table 18). No intermediate accumulation of either 3-deoxy-D-*arabino*-heptulosonic acid or 3-dehydroshikimic acid was observed, however, the final gallic acid titer and the product yield was lower, than the parent biocatalyst KL7/pSK6.161. The lack of toxic side effects in the cell culture of KL7/pSK7.80 was probably due to the lower maximum specific activities of *pobA**.

Table 18. KL7/pSK7.80. Product titers, total yields and specific activities of AroF^{FBR} and PobA*.

IPTG (mg)	PobA* (U/mg)[a]			AroF^{FBR} (U/mg)[a]			g/L				Total[b] Yield %	GA[c] Yield %
	24h	36h	48h	24h	36h	48h	GA	PCA	DHS	DAH		
10	0.020	0.028	0.031	0.44	0.13	0.16	5.1	12.3	–	–	14	4
30	0.032	0.039	0.038	0.40	0.22	0.19	14.3	2.5	–	–	14	11

a. µmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.

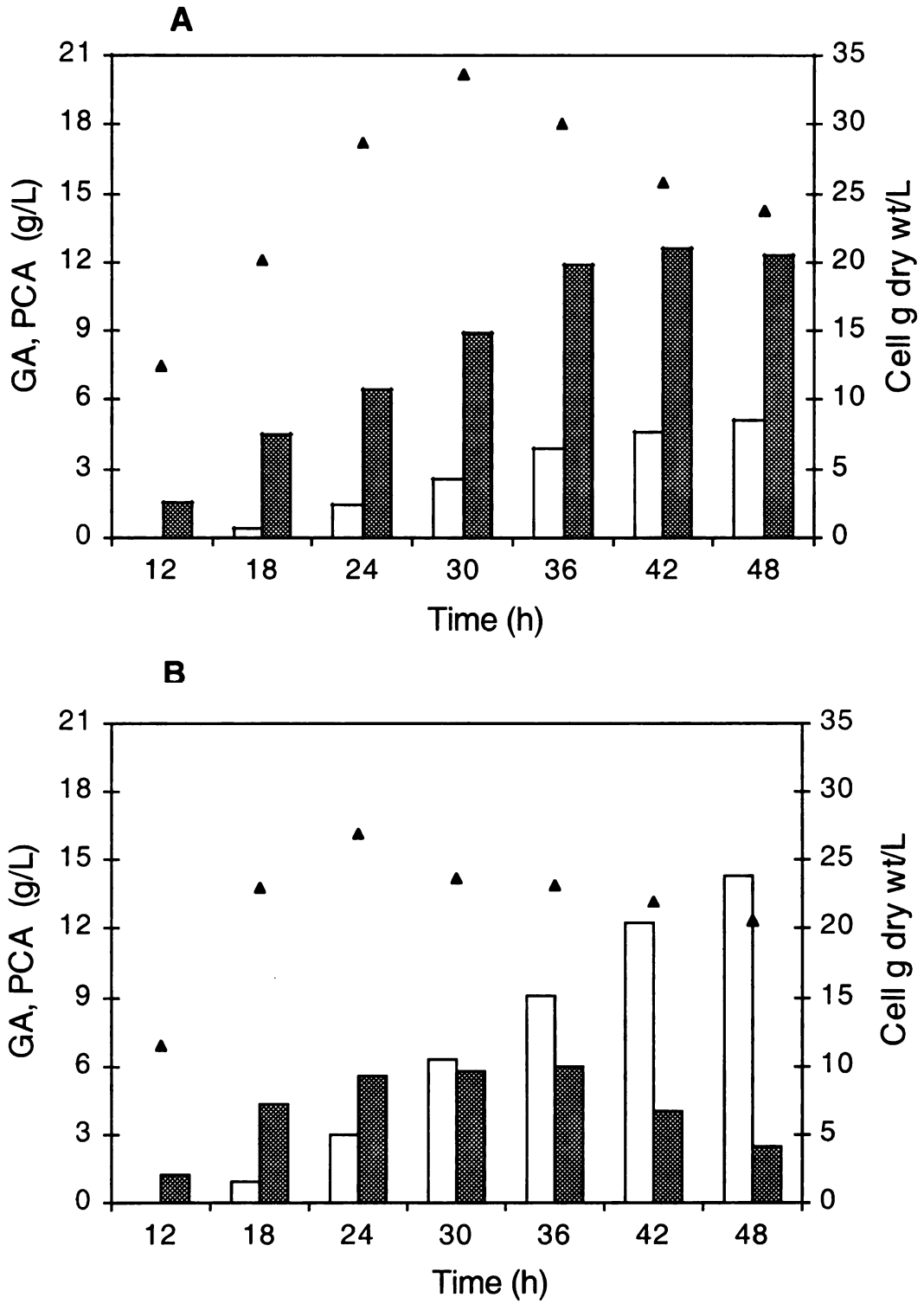


Figure 83. KL7/pSK7.80 Cell growth (▲) and GA (▭) and PCA (▩) production. IPTG was added every 6 h starting at 12; A. 10 mg IPTG added every time point; B. 30 mg IPTG added every time point.

C. The role of α-ketoglutarate and fermentation conditions in the product titers and yields

As part of the ^{1}H NMR spectra of gallic acid-producing biocatalysts, a set of peaks in the aliphatic region was routinely obtained. Although absent in the early time points of the fermentation, their intensity increased after 36 h. Purification of the corresponding metabolite began with refluxing a gallic acid-containing fermentation supernatant for 2 h in basic pH (8.0). The non-aromatic 3-dehydroshikimic acid intermediate was dehydrated into protocatechuic acid, and extracted (pH 2.2) with EtOAc along with all the other aromatic molecules present in the fermentation broth. The cell supernatant, which after extraction still contained the unknown metabolite, was filtered through activated charcoal, neutralized with NaOH (pH 7) and passed through an AG1x8 anion exchange resin (OH form), which absorbed the unknown metabolite. Washing the column with 15 % (v/v) AcOH in H_2O eluted the unknown molecule as a single product. The ^{1}H NMR and ^{13}C NMR of this purified metabolite were identical to the spectrum of L-glutamic acid. In a typical fermentation, L-glutamate was first observed at 30 h to 36 h, and depending on the catalyst, accumulated to concentrations reaching 110 mM by the end of the fermentation run (Figure 84).

In *E. coli,* glutamic acid is synthesized from α-ketoglutarate. Two separate pathways are responsible for its biosynthesis.[21] In the first, ammonia can be directly assimilated into glutamate via a reaction catalyzed by the *gdh*-encoded enzyme, glutamate dehydrogenase (GDH). This reaction involves an NADPH-specific reductive amination of α-ketoglutarate, to produce glutamate (Figure 85).[22] The K_m value for ammonia for

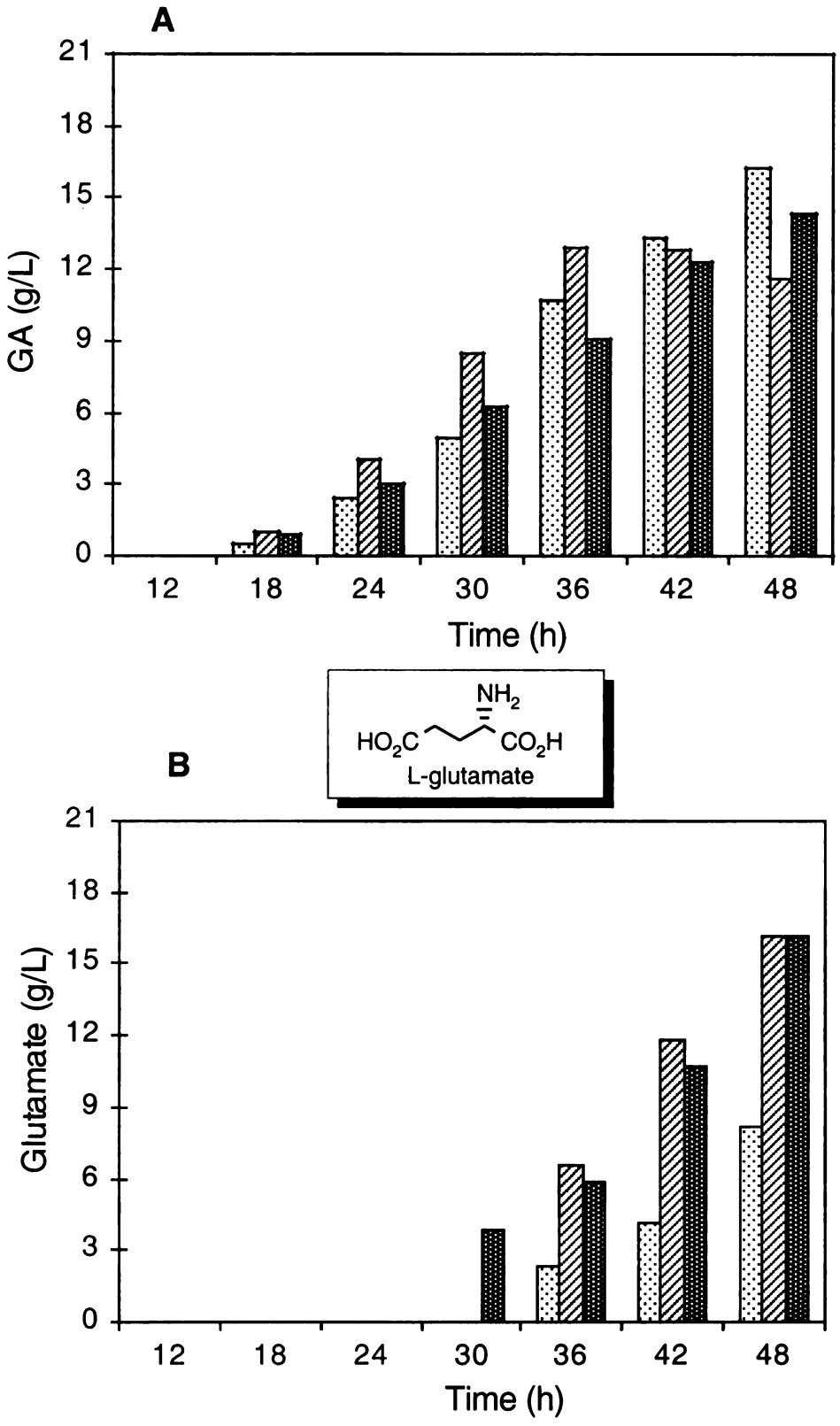


Figure 84. GA production (A) versus L-glutamate production (B) in KL7/pSK6.161 (▭), KL7/pSK7.51 (▨) and KL7/pSK7.80 (▬).

GDH is ~10^{-1} and the enzyme cannot function efficiently under low ammonia concentrations. Therefore, when ammonia concentration is limited (<1 mM) or absent, other nitrogen sources for the synthesis of L-glutamate are utilized. Under these circumstances, glutamate is synthesized indirectly from α-ketoglutarate with the action of two enzymes glutamine synthase and glutamate synthase.[21]

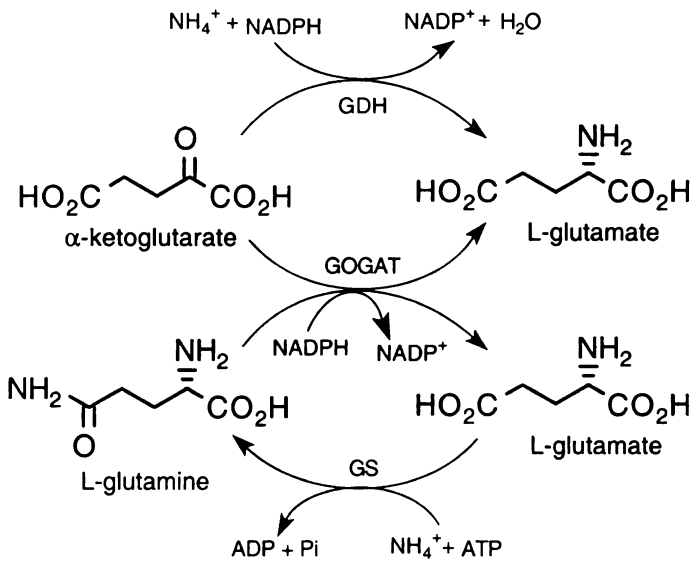


Figure 85. Biosynthesis of L-glutamate from α-ketoglutarate. GDH: glutamate dehydrogenase; GS: glutamine synthase; GOGAT: glutamate synthase.

Condensation of L-glutamate with ammonia to form glutamine with the action of glutamine synthase (GS) is followed by the transfer of the glutamine amide group into the α-carbon of α-ketoglutarate to finally generate two molecules of glutamine (Figure 85). The latter reaction is catalyzed by glutamate synthase (GOGAT) encoded by the *gltBDF* operon and utilizes FAD and NADPH as cofactors.[23] During growth in glucose-rich media, *E. coli* expresses high levels of both enzymes. Glutamine synthase (GS) has a considerably higher affinity for NH_4^+ than glutamine dehydrogenase (GDH), and catalyzes the reaction in the expense of ATP. Therefore, GDH will be the primary

enzyme for glutamate biosynthesis when cells are growing in low ATP and rich ammonia-containing medium (>1 mM), whereas GOGAT will be utilized when nitrogen availability is limited or if other sources of nitrogen are present.[21]

Glutamate accumulation is derived from the high intracellular formation of α-ketoglutarate. The latter is an intermediate of the tricarboxylic acid (TCA) cycle (Figure 86). Aerobic growth of *E. coli* bacteria in glucose-containing medium utilize only part of the TCA cycle, mainly for the biosynthesis of the important precursor metabolites oxaloacetate, α-ketoglutarate and succinyl-CoA (Figure 23).[24] Under these conditions, oxaloacetate is synthesized from phosphoenolpyruvate (PEP) through an anaplerotic reaction catalyzed by the *ppc*-encoded PEP carboxylase (Figure 86).[25a] The reverse reaction (oxaloacetate to PEP) is catalyzed by the *pck*-encoded PEP carboxykinase.[25b] Oxaloacetate is condensed with pyruvate to form citric acid in the first reaction of the TCA cycle catalyzed by *gltA*-encoded citrate synthase. Besides its use in the synthesis of oxaloacetate, PEP is also enzymaticaly produced from pyruvate with the action of the *pps*-encoded PEP synthase. Therefore, all the carbon entering the TCA cycle under growth of *E. coli* in aerobic conditions on glucose is coming from pyruvate (Figure 86).

From the discussion so far it appears that glutamate production is due to the high intracellular pyruvate accumulations. With the action of *pps*-encoded PEP synthase, pyruvate is transformed into PEP, which then partitions between reaction with erythrose-4-phosphate (E4P) catalyzed by DAHP synthase ($AroF^{FBR}$) to form 3-deoxy-D-*arabino*-heptulosonic 7-phosphate acid (DAHP) and reaction with

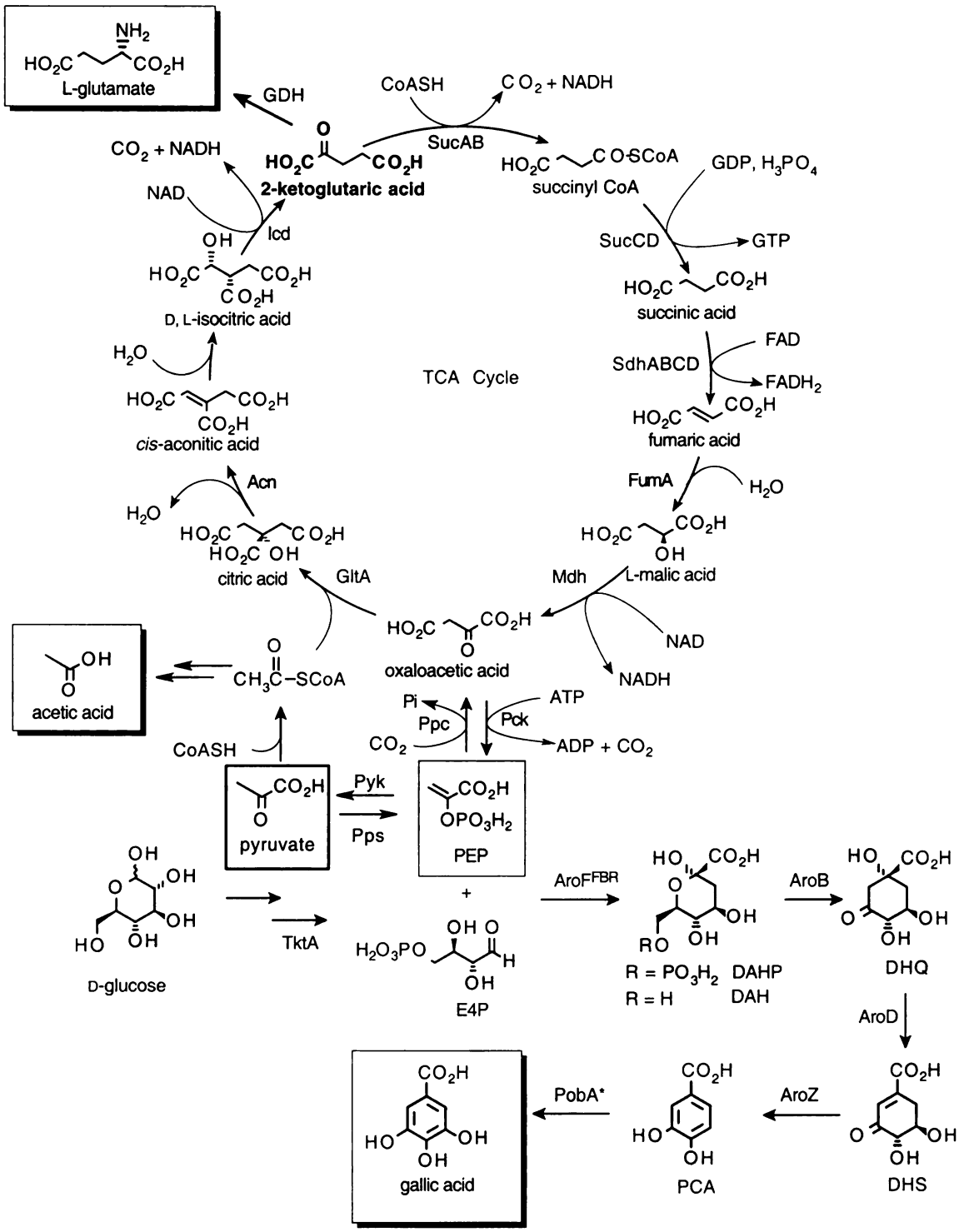


Figure 86. Glutamate biosynthesis and GA production. TktA: transketolase; $AroF^{FBR}$: feedback-insensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; AroB: 3-dehydroquinate synthase; AroD: 3-dehydroquinate dehydratase; AroE: shikimate dehydrogenase; Pck: PEP carboxykinase; GltA: citrate synthase; Acn: aconitase; Icd: isocitrate dehydrogenase; SucAB: 2-ketoglutrarate dehydrogenase; SucCD: succinyl-CoA synthetase; SdhABCD: succinate dehydrogenase; FumA: fumarase; Mdh: malate dehydrogenase; AroZ: DHS dehydratase; PobA*: PHB hydroxylase; Ppc: PEP carboxylase; Pps: PEP synthase; Pyk: pyruvate kinase.

CO_2 catalyzed by *ppc*-encoded PEP carboxylase to form oxaloacetate (Figure 23). While the first reaction drives carbon to the aromatic pathway and ultimately to gallic acid formation, the second route removes PEP with accompanying formation of oxaloacetate and α-ketoglutarate (Figure 86). Subsequent GDH-catalyzed reaction of α-ketoglutarate with NH_4^+ to form L-glutamate (Figure 85) and export into the supernatant, is the way the cells may be utilizing to remove the excess carbon from the TCA cycle. NH_4^+ is abundant during the fermentation experiments due to the use of NH_4OH to control the pH of the cell supernatant. Large amounts (10-20 mL) of concentrated NH_4OH are added during a typical fermentation run.

Is it possible to channel the carbon currently lost to L-glutamate formation towards gallic acid synthesis? Enhancing the activity of Pck was expected to increase intracellular PEP concentrations, and as a result, increase the amount of carbon directed into the common pathway. Plasmid pSK7.104 (Figure 87) was constructed for this reason. Pck was isolated from pKL2.222. The latter consisted of the pSU18 vector (2.2 kb) and the 2.3 kb *pcK* gene cloned in the *Eco*RI/*Bam*HI sites. Since pSU18 and the *pck* gene were very similar in size, pKL2.222 was digested with *Eco*RI /*Bam*HI followed by *Nco*I. While the first double digestion released the 2.2 kb *pck* gene, *Nco*I cut the remaining pSU18 vector in to two smaller pieces (1.0 and 1.2 kb). After this triple digestion, *pcK* was easily gel-purified, treated with Klenow fragment and ligated into the CIAP treated *Sma*I site of pSK6.161 giving plasmid pSK7.104 (Figure 87).

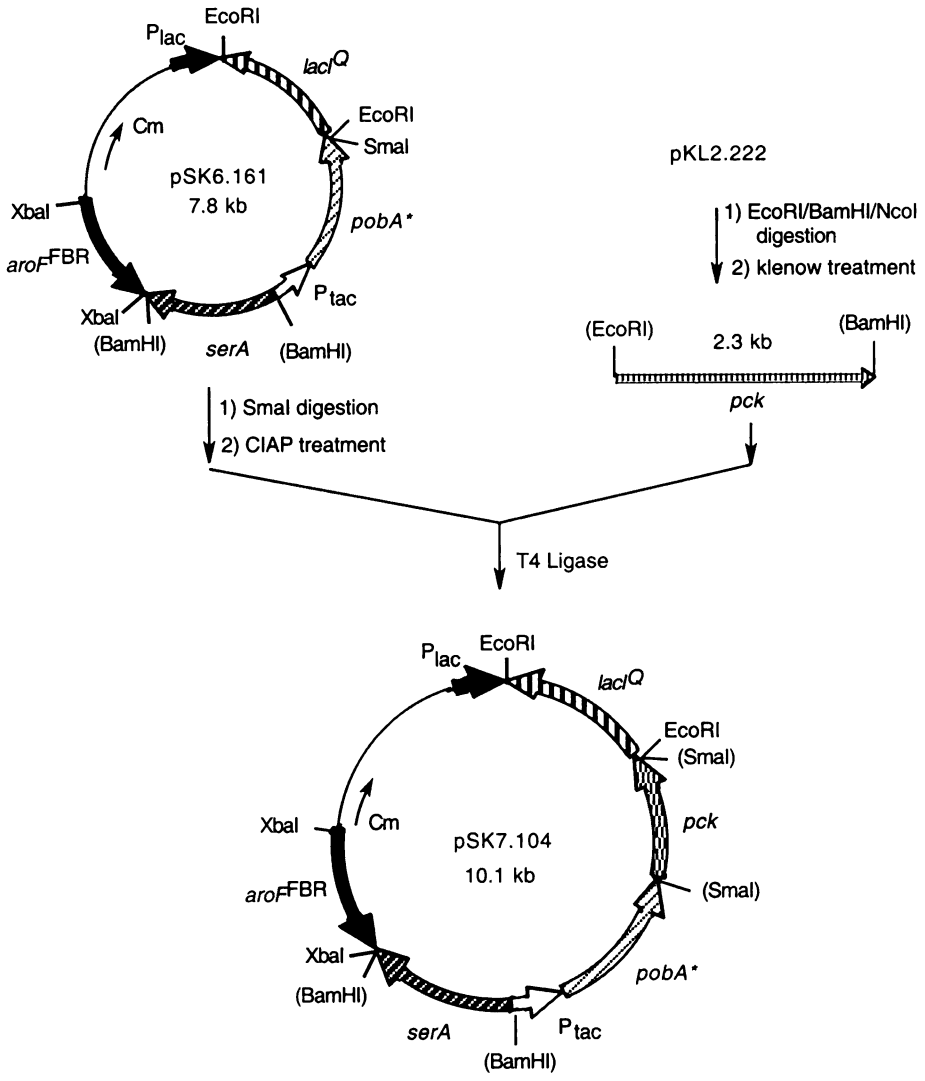


Figure 87. Construction of plasmid pSK7.104

KL7/pSK7.104 cultivated under fed-batch fermentor conditions reached stationary phase at 30 h. IPTG (30 mg) was added every 6 h starting at 12 h. After 36 h however, D.O. levels could not be maintained at 10% of air saturation. The fermentator run was therefore terminated at 42 h. Poor gallic acid titers (5.3 g/L) were obtained and were accompanied by the formation of 2.6 g/L of protocatechuic acid, 1.4 g/L 3-dehydroshikimic acid and 1.8 g/L 3-deoxy-D-*arabino*-heptulosonic acid (Table 19). As expected, no glutamate formation was observed but instead, acetic acid accumulated reaching a concentration of 70 mM (4.2 g/L) by the end of the fermentation run at 42 h (Figure 88).

Acetic acid has never been observed to form in significant amounts in the gallic acid-synthesizing microbes, which have been tested so far. This difference can be attributed to the overexpression of PEP carboxykinase, which removes oxaloacetate from the TCA cycle to form PEP. As a result, pyruvate cannot enter the TCA cycle and be removed by the cell in the form of ketoglutarate and glutamate (Figure 86). This pyruvate excess is now disposed from the cell in the form of acetate, utilizing a different enzymatic pathway (Figure 86).

Table 19. KL7/pSK7.104. Product titers, total yields, and acetate formation.

g/L					Total[a,b]	GA[a]
GA	PCA	DHS	DAH	acetate	Yield %	Yield %
5.3	2.6	1.4	1.8	4.2	12	5

a. At 42 h b. mol GA+PCA+DHS+DAH/mol glucose

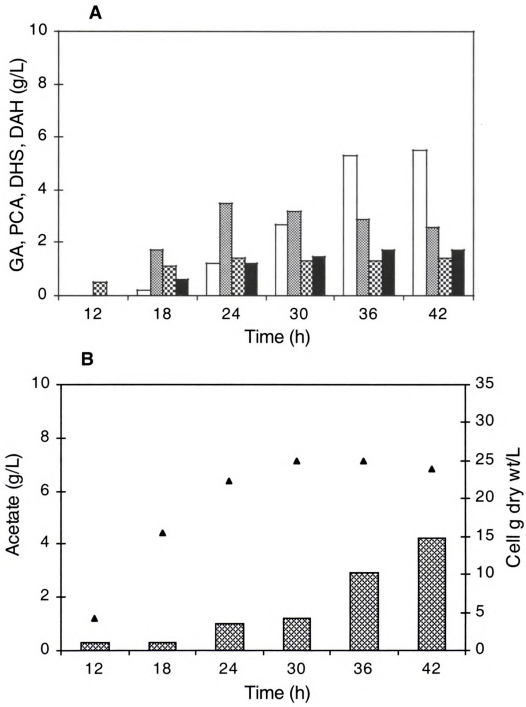


Figure 88. KL7/pSK7.104. Fermentation products (A); and acetic acid formation (B). Graph A: GA ( ); PCA ( ); DHS ( ); DAH ( ). Graph B: Cell mass (▲) and acetate ( ) formation

The extra PEP that is produced in KL7/pSK7.104 after overexpression of PEP carboxykinase was not channeled to the common pathway and ultimately to the formation of gallic acid by the action of DAHP synthase as shown from the poor yields and titers of gallic acid formation. In fact, the production of gallic acid was much lower compared to the construct lacking *pck* overexpression (KL7/pSK6.161). Fermentations that produced large amounts of acetic acid are often accompanied by synthesis of low amounts of the desired product. Besides the gallic acid-synthesizing biocatalysts this effect was also observed in the shikimic acid as well as the 3-dehydroshikimic acid fermentations.[11] In addition, literature reports indicate that acetic acid formation can slow the bacterial growth and/or inhibit recombinant-protein biosynthesis.[26]

All the fermentations so far described use glucose to control the D.O. levels during phase three of growth. Phase three occupies the major part of the fermentation time (30-36 h) and it is during this time that the majority of gallic acid is being synthesized. Keeping the D.O. levels at a set value (20% or 10%) is the way to control the rate of glucose addition. In response to an increase in D.O. levels, glucose is pumped into the supernatant. Immediate consumption of the added glucose triggers more vigorous respiration with an attendant drop in D.O. levels. With the use of D.O. levels to control glucose addition, glucose never accumulates in the culture supernatant thereby making the cells operate under glucose-limited conditions. Periodic ^{1}H NMR analysis of the cell broth reveals no detectable concentrations of glucose. These fermentation conditions were also utilized in all the 3-dehydroshikimic acid-producing experiments[4] as well as in the testing and optimizing of all the gallic acid-producing biocatalysts.

A significant improvement in the synthesis of gallic acid was observed when more aggressive glucose feeding was utilized as a result of decreasing the D.O. set point from 20% to 10%. While all the previously discussed fermentations were performed in conditions where the D.O. was kept at 10%, KL7/pSK6.161 cultured under 20% D.O. synthesized 9.2 g/L of gallic acid (GA), 1.0 g/L protocatechuic acid (PCA), 3.2 g/L of 3-dehydroshikimic acid (DHS) and 3.8 g/L of 3-deoxy-D-*arabino*-heptulosonic acid (DAH) and 14 g/L of glutamate in 48 h (Figure 89). The total yield (mol/mol) of all the aromatic pathway products was 15% based on the glucose consumed. These product titers and yields were considerably different from the 16.3 g/L of GA, 0.7 g/L of PCA, 2.2 g/L DHS, 4.0 g/L of DAH and 5 g/L of glutamate that accumulated when the same biocatalyst was cultured under 10% D.O. (Table 23, entry 1 versus entry 2).

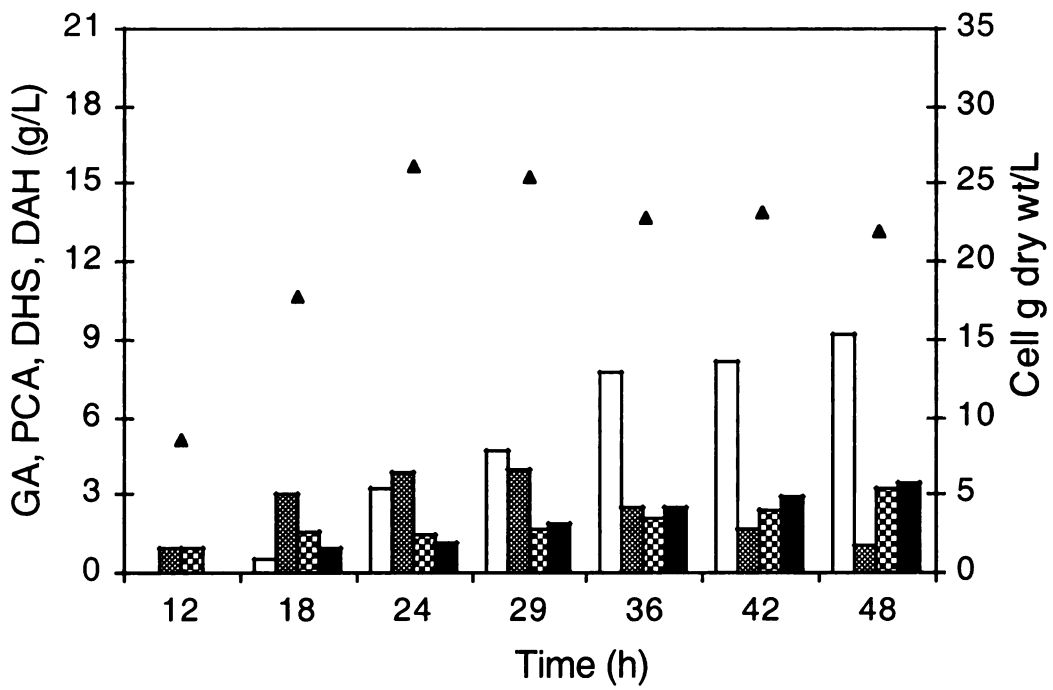


Figure 89. KL7/pSK6.161. Cell growth (▲) and GA (▭); PCA (▩); DHS (▩) and DAH (■) production in fermentations run under 20% D.O. conditions.

To maintain lower D.O. levels (i.e 10% D.O. versus 20% D.O.) a higher concentration of glucose needs to be maintained in the culture medium by the fermentor. If the improvement in titers and yields of gallic acid reflects increased glucose concentrations, switching from glucose-limited to glucose-rich culture conditions warranted investigation. Along these lines, a constant rate of addition of glucose was used to maintain glucose concentrations between 15-25 g/L during phase three of growth. The D.O. set point was the same (10%) and was now controlled by changing the impeller rate. Growing the best gallic acid-producer, KL7/pSK6.161, under these conditions significantly increased the product titer and yield (Figure 90).

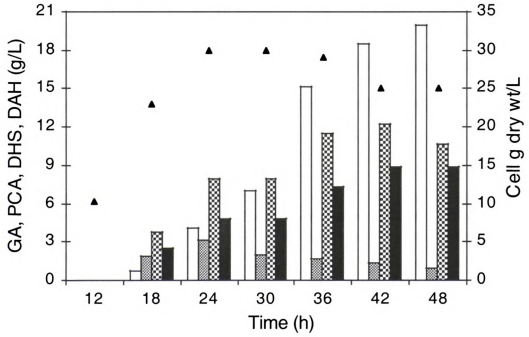


Figure 90. KL7/pSK6.161. Cell growth (▲) and GA (▭); PCA (▨); DHS (▩) and DAH (■) production. Fermentations run under glucose-rich conditions and D.O. concentration of 10%.

The growth characteristics of KL7/pSK6.161 under the glucose-rich conditions were the same as those in the glucose-limited fermentation conditions, reaching stationary phase at 24 h and achieving the same cell densities (25-30 g/L). However, product titers reached 20 g/L of gallic acid (GA), 0.9 g/L of protocatechuic acid (PCA), 10.7 g/L of 3-dehydroshikimic acid (DHS) and 8.9 g/L of 3-deoxy-D-*arabinose*-heptulosonic acid (DAH) with significant amounts of glutamate (14 g/L) accumulating after 48 h (Table 20, and Table 23 entry 3). Although the total yield (mol/mol) accounting for all the aromatic pathway intermediates was higher, the yield for gallic acid synthesis was exactly the same (12%, Table 20). The increased carbon that is channeled into the common pathway under these conditions is apparently expended in increased accumulation of the pathway intermediates 3-deoxy-D-*arabinose*-heptulosonic acid and 3-dehydroshikimic acid rather than the end product gallic acid. Accumulation of 3-dehydroshikimic acid and of 3-deoxy-D-*arabinose*-heptulosonic acid, resulting from the rate-limiting activities of *aroB,* was also encountered in the glucose-limited conditions.

Table 20. KL7/pSK6.161. Product titers, total yields, and specific activities of AroF^{FBR} and PobA* when cultured under glucose-rich fermentation conditions.

PobA* (U/mg)[a]			AroF^{FBR} (U/mg)[a]			g/L				Total[b]	GA[c]
24 h	36 h	48 h	24 h	36 h	48 h	GA	PCA	DHS	DAH	Yield %	Yield %
0.054	0.066	0.083	1.40	0.80	0.75	20	0.9	10.7	8.9	25	12

a. mmol/min b. mol DHS+DAH+GA+PCA/mol glucose c. (mol/mol) based on the glucose consumed.

During the toxicity experiments utilizing gallic acid, it was observed that after incubating a neutral phosphate-buffered solution containing gallic acid at 37 °C in a shake oven, the solution from colorless gradually became green and after 24 h it turned to a dark

brown solution. Since gallic acid is a known antioxidant that was shown to decompose under Fehling conditions (Chapter 3), its sensitivity towards air oxidation in shake flask and fermentation conditions was investigated. After shaking a minimal salt solution containing 25 mM gallic acid at 37 °C under air for 24 h, a gradual loss of its concentration was observed (Figure 91). When the same solution was degassed and shaked in a sealed flask under argon, no decomposition was observed. ^{1}H NMR analysis of the supernatant shaken under air for 24 h showed the presence of the residual gallic acid and small concentrations of aconitic acid as the only products. Aconitic acid was the major product of the oxidation of gallic acid in a neutral phosphate-buffered solution in the presence of H_2O_2 and catalytic amounts of Cu^{+2} (Chapter 3). The stability of gallic acid was also tested under fermentation conditions where the levels of D.O. (20%) were closely monitored during the whole process. Gallic acid was added to KL3/pSK6.118 at the beginning of phase three of growth in a fermentor under 20% D.O.. Again, decomposition of gallic was observed after 24 h (Figure 91).

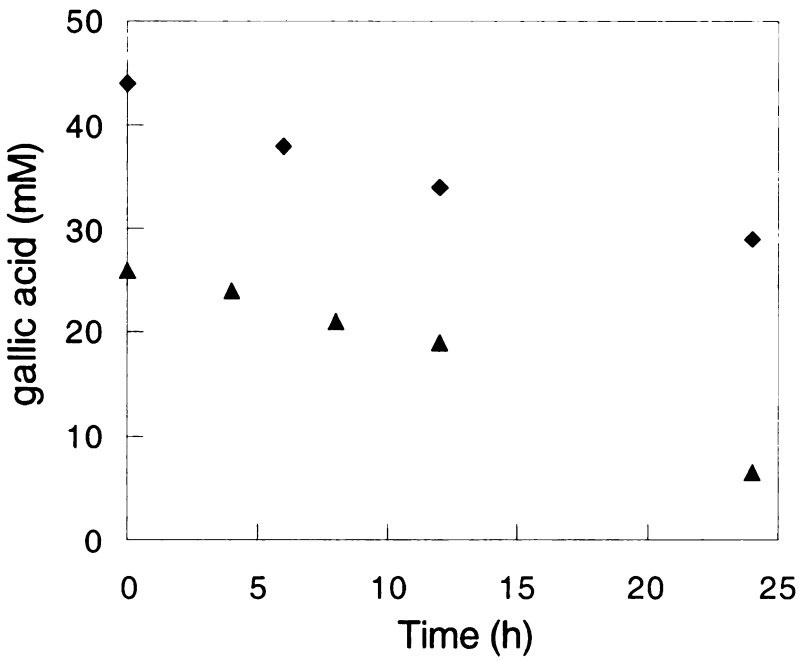


Figure 91. Decomposition of gallic acid under shake flask conditions (▲) and when supplemented in a fermentor where KL3/pKL6.118 was cultured under 20% D.O. (◆).

IV. DISCUSSION

In Chapter 4, the synthesis of gallic acid following the addition of protocatechuic acid or 3-dehydroshikimic acid to the broth of recombinant *E. coli* cultured under fermentor conditions was discussed. In addition to the important elucidation of 3-dehydroshikimic acid and protocatechuic acid transport into the cytoplasm of *E. coli* bacteria, it was demonstrated that the mutant PHB hydroxylase (*pobA**) originally isolated from *P. aeruginosa* was expressed in *E. coli* at levels suitable for synthesis of gallic acid. In this Chapter, recombinant *E. coli* capable of de novo synthesis of gallic acid from glucose were constructed. The biocatalytic pathway to gallic acid was constructed in 3-dehydroshikimic acid-accumulating *aroE*$^-$ hosts. Dehydration of 3-dehydroshikimic acid into protocatechuic acid was catalyzed by *aroZ*-encoded DHS dehydratase, which was localized on the genome of *E. coli* KL7. Subsequent hydroxylation of protocatechuic acid to form gallic acid was catalyzed by plasmid-localized *pobA**-encoded PHB hydroxylase, which was expressed from a strong *tac* promoter (Figure 86).

The amount of carbon that was directed into product is controlled by the activity of the first enzyme of the common pathway of aromatic amino acid biosynthesis, DAHP synthase. For this reason, a feedback-insensitive isozyme of *aroF* designated as *aroF*^{FBR} was localized on the same plasmid that also contained *pobA**. Tuning the activities of both *pobA** and *aroF*^{FBR}, as well as optimizing the fermentation conditions, led to the synthesis of 20 g/L of gallic acid in 12% (mol/mol) yield from glucose along with various other pathway intermediates. Increasing the activities of *aroZ*-encoded DHS dehydratase and *aroB*-encoded DHQ synthase, expression of the *katG*-encoded HPI enzyme that decomposes H_2O_2, and elimination of L-glutamate formation were some of the routes that were investigated to increase gallic acid titers and yields.

A detailed discussion of the PHB hydroxylase mechanism was presented in Chapter 4. As a reminder, it has been previously established that only 20% of the

NADPH consumed by the mutant enzyme leads to the formation of gallic acid. The rest of the reducing equivalents are released as H_2O_2. It was therefore surprising that with such an inefficient enzyme and with the potential toxicity of both gallic acid and H_2O_2, significant amounts of gallic acid could be synthesized. In fact, introducing a second copy of *pobA** into the biocatalyst (KL7/pSK6.233) caused growth inhibition and an inability to control the D.O. levels in biocatalysts cultured under fed-batch fermentor conditions. A similar impact on the cells cultivated under fed-batch fermentor conditions was observed when the rate of intracellular protocatechuic acid formation was increased after increasing DHS dehydratase activity as a consequence of plasmid-localization of *aroZ* (KL7/pSK7.51).

Plasmid loss resulting from genetic instability associated with cells carrying two plasmid-localized *pobA** genes might be one of the reasons responsible for the observed toxicity in KL7/pSK6.233 when cultivated under fermentor conditions. However, the increased rate of intracellular gallic acid and/or H_2O_2 formation can not be excluded from the reasons causing the observed inhibition of cell growth and loss of the D.O.. Gallic acid toxicity was established in shake flask experiments where concentrations of gallic acid as low as 5 mM were able to completely inhibit growth of KL7 and RB791 in minimal salts media containing glucose. Under fed-batch fermentor conditions, 88 mM of gallic acid added to the broth of a DHS-synthesizing biocatalyst (KL3/pKL4.130B) reduced the final DHS synthesis by 50% (Figure 70). The toxicity of H_2O_2 has been well established by other researchers.[15]

The growth inhibition and loss of D.O. control in bacteria that carry a plasmid-localized *aroZ* gene (KL7/pSK7.51) also point to gallic acid and/or H_2O_2 toxicity. While low activities of *pobA**, as controlled by the amount of IPTG added to KL7/pSK7.51 cultivated under fermentor conditions, did not cause growth problems or loss of D.O. control until 42 h of growth, increasing the activity of *pobA** as a result of higher amounts of IPTG addition resulted in growth inhibition and loss of D.O. control in

KL7/pSK6.233. The rate of protocatechuic acid formation is assumed to be the same in both cases (low and high IPTG addition) since *aroZ,* which catalyzes protocatechuic acid formation from 3-dehydroshikimic acid, is expressed from its native promoter and should not be affected by the additions of IPTG. In addition, no accumulation of 3-dehydroshikimic acid is observed in KL7/pSK7.51 cultivated under both low and high IPTG conditions indicating that the activity of DHS dehydratase under low and high IPTG concentrations, is adequate to convert 3-dehydroshikimic acid into protocatechuic acid at a rate that matches the rate that 3-dehydroshikimic acid is biosynthesized. Therefore, in the catalyst that carries the plasmid-localized *aroZ* locus (KL7/pSK7.51), the rate of protocatechuate formation is dependent on the rate of 3-dehydroshikimic acid biosynthesis, which is controlled by the activity of the first enzyme of the common pathway, DAHP synthase. Since the activities of DAHP synthase are very similar in KL7/pSK7.51 cultivated under fermentor conditions utilizing both the high and low IPTG concentrations during the first 24 h (Table 16), the rate of 3-dehydroshikimic acid production and as a result that of protocatechuic acid, must be the same. It appears therefore, that the only difference between growth inhibition, loosing control of the D.O. and normal growth, is the activity of *pobA** in KL7/pSK7.51 cultivated under fed-batch fermerntor conditions. Since in other biocatalysts higher specific activities of *pobA** have been obtained without causing any toxicity problem (Table 21), it is reasonable to conclude that the products of *pobA** rather than the actual enzyme expression were the reasons for the observed toxicity in KL7/pSK7.51 when higher IPTG concentrations were utilized.

Distinguishing between gallic acid and H_2O_2 toxicity relied on the expression of a gene that could degrade H_2O_2 to H_2O and O_2. *E. coli* carries two catalase enzymes for H_2O_2 protection, hydroperoxidase I (KatG) and hydroperoxidase II (KatF).[17] KatG is both a catalase and a peroxidase enzyme that is expressed in the exponential growth phase. Its expression is controlled by another protein indicated as OxyR.[18] Although the

exact regulation of both these proteins is under investigation, it is known that H_2O_2 rapidly induces *katG* expression during cell growth.[18] Carrying its native promoter, *katG* was was amplified by PCR from the genome of RB791 and cloned into the plasmid that also contained the double *pobA** cassette. Despite the high activities of catalase that were obtained (0.24 U/mg at 18 h, 0.12 U/mg at 24 h and 0.04 U/mg at 36 h), cell growth inhibition and loss of D.O. were unaffected in cells cultivated under fed-batch fermentor conditions. The previously discussed plasmid instability present in the catalyst that carries the double *pobA** cassette can still cause part of or be the only reason of the observed growth inhibition and D.O. loss of control in the biocatalyst that overexpresses *katG* in addition to the double *pobA**. However, if the observed effect is arising from the products of *pobA** expression, gallic acid and H_2O_2, plasmid-localization of *katG* and successful expression in the biocatalyst (KL7/pSK6.270) suggests that gallic acid and not H_2O_2 is the toxic metabolite. The rate of gallic acid export might not be fast enough to prevent reaching toxic concentration levels in the cytoplasm. After export of gallic acid into the culture supernatant, its toxicity may be substantially decreased, since fermentor cells cultured under fermentor conditions can tolerate gallic acid concentrations up to at least of 20 g/L.

A different reason for the observed toxicity caused by *pobA** overexpression could be argued. The mutant hydroxylase still possessed its native activity, which consisted of the hydroxylation of *p*-hydroxybenzoate (PHB) to protocatechuic acid. *p*-Hydroxybenzoate, which is essential for the biosynthesis of coenzyme Q, is synthesized from chorismic acid, an intermediate downstream from 3-dehydroxyshikimic acid in the common pathway. Complementation of this essential metabolite in the *aroE*$^-$ KL7 biocatalysts, was achieved by external addition of 10 mg PHB in every fermentation. Possible removal of *p*-hydroxybenzoate from the cytosol by the action of *pobA**, may deplete the cellular biosynthesis of coenzyme Q. Although this might have been an additional reason for the observed toxicity in the construct carrying the double *pobA**

cassette, it was unlikely that it was causing any problems in the single *pobA** biocatalysts. PobA* activities as high as 0.17 U/mg did not cause any problems in cells (KL7/pSK6.161) carrying a single copy of this enzyme (Table 21, entry 5).

Although some strains can not handle very high hydroxylase activities, a certain activity level of this enzyme is required to produce good gallic acid to protocatechuic acid ratios. Activity levels of *pobA**, which are toxic, are also dependent on the biocatalyst under investigation. Since a delicate balance between toxicity and good product formation was required, various biocatalysts that express PHB hydroxylase in different levels were constructed. PobA* was always being expressed from a strong inducable *tac* promoter, in the presence of the $lacI^Q$ repressor gene. Besides the amount of IPTG inducer, the maximum activities of *pobA** were also dependent on the number of genes expressed from the same plasmid. For example, when 30 mg of IPTG were utilized to induce *pobA** expression, the maximum activity of PobA* was higher in biocatalysts that cerryed less genes in the plasmid (Table 21, entry 5 versus entries 2 and 4).

Table 21. Comparison of the GA to PCA product ratio as a function of the PobA* activities.

		g/L		PobA* (U/mg)			GA[a]	
entry	Biocatalyst	GA	PCA	24 h	36 h	48 h	Yield %	IPTG[b]
1.	KL7/pSK4.272	3.2	20.8	0.017	0.022	0.037	3	5
2.	KL7/pSK4.272	14	1.7	0.035	0.055	0.055	11	30
3.	KL7/pSK7.80	5.1	12.3	0.020	0.028	0.031	4	10[c]
4.	KL7/pSK7.80	14.3	2.5	0.032	0.039	0.038	11	30
5.	KL7/pSK6.161	16.3	0.7	0.120	0.138	0.170	12	30

a. (mol/mol) based on the glucose consumed b. IPTG additions at 12, 18, 24, 30, 36, 42 h;
c. IPTG additions at 12, 18, 24 h.

The gallic acid to protocatechuic acid product ratio was sensitive to the hydroxylase expression levels. However, a specific activity of 0.035-0.05 U/mg was found to be enough to produce gallic acid as the major product (Table 21). The activity

measurements of *pobA** that are presented in these thesis, represented the equivalents of NADPH (μmol/min) consumption (loss of OD_{340}) as a result of protocatechuate addition to cell lysate, and not the μmol/min formation of the gallic acid. The wild-type enzyme oxidizes one molecule of *p*-hydroxybenzoic acid into protocatechuic acid for every molecule of NADPH that is consumed. The mutant PHB hydroxylase however, incorporated only 20% of the consumed NADPH into product. A simple calculation revealed that the actual activity towards gallic acid that was necessary for adequate product formation using this mutant hydroxylase was only 0.007-0.01 U/mg. Biocatalysts that expressed almost three times this basic activity (Table 21, entry 5) still did not completely eliminate the formation of protocatechuic acid. Under these conditions, protocatechuate uptake limitations, rather than *pobA** activities was the reason of protocatechuic acid presence at the end of the fermentation run. In Chapter 4 the diffusion-controlled protocatechuate uptake in recombinant *E. coli* cultured under fermentor conditions was discussed. It is possible, that the low concentrations of protocatechuic acid towards the end of the fermentation experiments were limiting its uptake rate and as a result, its oxidation to gallic acid and complete elimination from the fermentation broth.

While PHB hydroxylase activities were responsible for the gallic acid to protocatechuic acid ratios, the activities of DAHP synthase controlled the amount of carbon that was channeled into the aromatic pathway. A 3-dehydroshikimic acid-synthesizing *E. coli* construct similar to the gallic acid-synthesizing construct discussed in this Chapter, has been evaluated for the amount of carbon that was directed into the aromatic pathway as a function of the DAHP synthase activity.[4] The theoretical maximum yield of 3-dehydroshikimic acid synthesis in cells growing on glucose is 43% (mol/mol). In a biocatalyst carrying a plasmid-localized copy of $aroF^{FBR}$ expressed from a *tac* promoter, the highest reported total yield including 3-dehydroshikimic acid,

3-dehydroquinic acid and gallic acid was 24% (mol/mol based on glucose consumed) while the total product titer reached concentrations as high as 0.36 M (Table 22, entry 6).[4]

When the same control of DAHP synthase was utilized in the gallic acid-producing constructs, the total titer including gallic acid (GA), protocatechuic acid (PCA), 3-dehydroshikimic acid (DHS) and 3-deoxy-D-*arabino*-heptulosonic acid (DAH) was significantly lower compared to that of the 3-dehydroshimikc acid-synthesizing biocatalysts while the total yield was only 5% lower (Table 22, entries 1,2 versus 6). However, the activity range of DAHP synthase in both gallic acid- and 3-dehydroshikimic acid-synthesizing biocatalysts was very similar when the same amount of IPTG was utilized. Increasing the activity of DAHP synthase by an order of magnitute in the gallic acid-synthesizing bacteria did not change either the total yield or the total product titer (Table 22, entry 1 versus entry 2).

Table 22. Comparison of the total titers and yields as a function of the AroF^{FBR} activities.

#	Biocatalyst	Total[a] Yield %	Total[a] Titer (M)	AroF^{FBR} (U/mg) 24 h	36 h	48 h	IPTG
1.	KL7/pSK4.272	19	0.16	0.27	0.25	0.086	5
2.	KL7/pSK4.272	19	0.12	4.9	4.0	1.6	30
3.	KL7/pSK6.161	18	0.13	2.25	1.23	0.58	30
4.	KL7/pSK7.80	14	0.10	0.48	0.36	0.19	30
5.	KL3/pKL4.33B[b]	18	0.13	0.23	0.014	0.018	NA
6.	KL3/pKL4.79B[b]	24	0.36	0.30	0.27	0.42	5

a. For the DHS-synthesizing biocatalysts (entries 5 and 6) titer includes DHS, DHQ and GA. For the GA-synthesizing biocatalysts (entries 1-4) titer includes GA, PCA, DHS, DAH; b. Ref. 4

Switching DAHP synthase expression from a *tac* promoter to the native P_{aroF} promoter in the 3-dehydroshikimic acid-synthesizing bacteria, significantly decreased the total product titer accompanied by a smaller decrease in total yield (Table 22, entry 5

versus entry 6). Both the titers and yields of the gallic acid-synthesizing bacteria remained unchanged when the expression of DAHP synthase was switched from a *tac* to a P_{aroF} promoter (Table 22, entry 2 versus entry 3). The yields were further decreased when the rate limiting enzymes *aroB* and *aroZ* were expressed in the same plasmid (Table 22, entry 4). These experimental results suggest that the gallic acid-synthesizing biocatalysts are relatively insensitive to the expression levels of DAHP synthase, in contrast to very similar biocatalysts that synthesize 3-dehydroshikimic acid. As a result, a simple correlation between gallic acid titers and $aroF^{FBR}$ specific activities is not valid in the gallic acid-synthesizing biocatalysts.

A different route that was taken to increase the total gallic acid titers and yields entailed elimination of L-glutamate accumulation. The large amounts of glutamate that were synthesized from all the gallic acid-synthesizing biocatalysts were the result of the accumulating pyruvate into the cytosol. Pyruvate is the end product of the glycolysis pathway that *E. coli* are utilizing to produce energy and assemble biomass when grown on glucose.[27] Another major source of intracellular pyruvate formation comes from the PTS-catalyzed uptake of glucose.[28] Glucose transport into the cytoplasm of *E. coli* is catalyzed by a group translocation mechanism consisting of three cytosolic and two membrane proteins known as the phosphotransferase system (PTS).[28] Utilizing this system, glucose transported into the cell and at the same time, phosphorylated into glucose-6-phosphate. Phosphorylation of glucose with this mechanism provides the energy required to drive glucose into the cytoplasm and at the same time generates glucose-6-phosphate, which is the first intermediate of glycolysis.[27,28] The phosphate group that is utilized by the PTS system to phosphorylate glucose is originaly provided

by phosphoenolpyruvate (PEP). After transferring the phosphate group to the first enzyme of the PTS system, PEP is transformed into pyruvate.[28] The net result of this process is that for every glucose molecule that enters the cytoplasm a PEP molecule is dephosphorylated into pyruvate.

Excess pyruvate that is generated by PTS-catalyzed glucose uptake and glycolysis has to be disposed of by the cell. For this purpose, the TCA cycle is utilized. In the first step of this cycle, one molecule of oxaloacetate is condensed with pyruvate to form citric acid (Figure 86). After a sequence of reactions in the TCA cycle, citric acid is transformed into α-ketoglutarate, which after amination to L-glutamate, is exported out from the cell (Figure 86). The net result of this process is to export from the cell pyruvate in the form of L-glutamic acid. When *E. coli* grows aerobically on glucose, oxaloacetate is synthesized from PEP through an anaplerotic reaction catalyzed by the *ppc*-encoded PEP carboxylase enzyme. A different route to the synthesis of oxaloacetate starts from the TCA cycle intermediate isocitrate with the action of isocitrate lyase (AceA), which catalyzes the formation of glyoxylate from isocitric acid, and malate synthase (aceB), which catalyze the condensation of glyoxylate with acetyl-CoA to form malic acid.[29] This anaplerotic pathway, formally called the glyoxylate bypass, is only induced in bacteria (including *E. coli*) that are growing on acetate or fatty acids.[29]

According to the discussion so far, the need for the cell to channel excess pyruvate through the TCA cycle requires large amounts of PEP to be transformed into oxaloacetate. PEP is condensed with erythrose-4-phosphate to form DAHP with the action of DAHP synthase, which is the first enzyme of the common pathway (Figure 86). DAHP synthase's activity and substrate availability are important parameters for the amount of

carbon that is channeled to the common pathway.[4] Removal of PEP towards the TCA cycle was anticipated to limit the amount of carbon that can be channeled to the common pathway thereby lowering the titers and yields of gallic acid formation.

For this reason, overexpression from a plasmid of the *pck*-encoded PEP carboxykinase, which catalyzes the formation of PEP from oxaloacetate, was intended to recycle oxaloacetate back to PEP and increase the carbon that can be driven to the common pathway with the action of DAHP synthase (Figure 86). Although *pcK* expression eliminated the formation of glutamate, large amounts of acetate were synthesized resulting in lower yields and titers of gallic acid. The eliminaion of glutamate formation was indicative that Pck was functioning. However, in the absence of adequate amounts of oxaloacetate, pyruvate was exported from the cell in the form of acetic acid utilizing a different sequence of enzymatic reactions (Figure 86). The presence of large concentrations of acetic acid in the fermentation broths of *E. coli* bacteria has been reported to limit product titers and yields.[26]

Glutamate accumulation was also observed in the protocatechuate-synthesizing biocatalysts (Chapter 2). In addition, when gallic acid was added in the supernatant of 3-dehydroshikimic acid-synthesizing bacteria (KL3/pKL4.130B) cultured under fermentor conditions, large amounts of glutamate (16 g/L) accumulated in the supernatant along with 32 g/L of microbially-synthesized 3-dehydroshikimic acid. In the absence of gallic acid supplementation, KL3/pKL4.130B synthesized 69 g/L of 3-dehydroshikimic acid and only traces of glutamate. It appears, therefore, that gallic acid and/or protocatechuic acid accumulation in the culture supernatants of *E. coli* KL3 and KL7 is triggering intracellular accumulation of pyruvate, which is exported from the from the cell

in the fermentation broth in the form of glutamate or as acetate when *pcK* is overexpressed. This accumulation of pyruvate might be the result of gallic acid's and protocatechuic acid's inhibition of enzyme(s) of the TCA cycle preventing complete oxidation of pyruvate to CO_2 leading to accumulation of glutamate, or enzymes that are involved in the direct decomposition of pyruvate. Since direct evidence for such inhibition is not available and since other more complicated effects might result from the accumulation of gallic acid and protocatechuic acid, their effect on the intracellular accumulation of pyruvate is unclear.

The fermentation conditions were the last parameter that was optimized before maximum gallic acid titers (20 g/L) were synthesized. The first improvement in the gallic acid titer and yield was observed when the D.O. of KL7/pSK6.161 grown under fed-batch fermentor conditions, was decreased from 20% to 10%. Gallic acid decomposition was shown to occur in shake flasks as well as in the fermentation supernatants of biocatalysts cultured under 20% D.O. conditions (Figure 91). The decomposition rate of gallic acid in the fermentation broth of biocatalysts grown under 10% D.O was not measured, but it is unlikely that it can be much different than the 20% D.O. conditions and explain the large difference of gallic acid titers that were obtained between the 20% D.O. (9.2 g/L GA) and the 10% D.O. (16.3 g/L GA) growth conditions (Table 23). The observed increase of gallic acid titers can rather be explained by the higher glucose consumption and fermentation yield that accompanied the 10% D.O. fermentation conditions compared to the 20% D.O. conditions (Table 20). The consumed glucose was increased even more, when at the same D.O. set point (10%), the fermentation was performed under glucose-rich instead of glucose-limiting conditions. The yield (mol/mol) of gallic acid biosynthesis

Table 23. KL7/pSK6.161. Product titers, total yields, glucose consumed and specific activities of PobA* and AroF^{FBR} in fermentations run under different glucose feeding conditions and D.O. set points.

	PobA* (U/mg)[a]			AroF^{FBR} (U/mg)[a]		
entry	24 h	36 h	48 h	24 h	36 h	48 h
1.	0.040	0.047	0.052	3.2	1.49	0.58
2.	0.12	0.14	0.17	2.30	1.20	0.50
3.	0.054	0.066	0.083	1.40	0.80	0.75

a.μmol/min

			g/L							
entry	D.O. %	excess glucose	GA	PCA	DHS	DAH	glutamate	Total[a] Yield %	GA[b] Yield %	consumed glucose (g)
1.	20	No	9.2	1.0	3.2	3.8	14	15	8	152
2.	10	No	16.3	0.7	2.2	4.0	5	18	12	180
3.	10	Yes	20	0.9	10.7	8.9	14	25	12	225

a. mol DHS+DAH+PCA+GA/mol glucose consumed b. (mol/mol) based on glucose consumed

based on the consumed glucose was the same in both the glucose-rich and glucose-limited conditions. However, a large increase in the concentration of the common pathway intermediates, 3-dehydroshikimic acid and 3-deoxy-D-*arabino*-heptulosonic acid, was observed. As a result, accounting for all the common pathway products (GA, PCA, DHS, and DAH), a higher yield (mol/mol based on glucose consumed) was obtained in the bacteria cultured under the glucose-rich conditions compared to the total yield of the bacteria cultured under glucose-limited conditions (Table 23, enrty 2 versus entry 3). Clearly, as the amount of glucose that was entering the cell during the course of a complete fermentation was increased, the gallic acid titers and total production yields (accounting for DHS, DAH, GA and PCA) also increased.

In *E. coli* cells, glucose is transported into the cytoplasm through the PTS transport system with concomitant phosphorylation using PEP as the phosphorus donor, yielding glucose-6-phosphate and pyruvate.[28] The high requirements of PEP for the transport and phosphorylation of glucose using the PTS system, reduces the availability of this metabolite for DAHP synthase, and as a result lowers the yields and titers of the metabolites that are synthesized through the common pathway (Figure 86). The importance of PEP availability in both the titers and yields of metabolites that are synthesized from the common pathway was shown in the case of 3-dehydroshikimic acid-synthesizing biocatalysts. Plasmid-localization of the *pps*-encoded PEP synthase, which catalyzes the formation of PEP from pyruvate, significantly increased the titers and yields of 3-dehydroshikimic acid in biocatalysts that were grown under fed-batch fermentor conditions.[30] When a glucose facilitator gene (*glf*), which can transport glucose in the expence of ATP and not PEP was introduced to the same biocatalyst, increased amounts of carbon were channeled to the common pathway and increased the titers and yields of the 3-dehydroshikimic acid biosynthesis.[30] It seems therefore that as the PEP concentration in the cytoplasm of 3-dehydroshikimic acid-synthesizing bacteria is increased as a result of the recycling of PEP from pyruvate, or as a result of the use of a non-PEP requiring glucose facilitator gene, higher yields and titers of 3-dehydroshikimic acid are obtained.

The experimental results obtained from the gallic acid-synthesizing fermentations show that the amount of carbon that is channeled to the common pathway, as well as the glucose consumed, are increased as glucose availability increases. This is achieved either in the form of more aggressive glucose feeding of the fermenting bacteria in the glucose-

limited conditions, or in the form of cultivation under glucose-rich fermentation conditions. A non-PEP requiring transport system of glucose into the cytoplasm in addition to PTS is possible when *E. coli* is cultured under fed-batch fermentor conditions and the glucose concentration in the broth becomes more abundant. If this is indeed the case, considering the results previously discussed in the 3-dehydroshikimic acid-synthesizing biocatalysts, both the total yields of all the common pathway products, as well as the total amount of consumed glucose will be increased. That was observed experimentally in the gallic acid-synthesizing *E. coli* cultured under glucose-limited versus glucose-rich fermentation conditions supporting the additional non-PTS transport of glucose under these conditions.

In the literature, other mechanisms of glucose uptake that do not rely on PEP phosphorylation have been described in *E. coli* bacteria.[31] An *E. coli* strain that was incapable of transporting glucose utilizing the PTS system due to a mutation, was able to grow on glucose supplementation. In this case cells are utilizing the galactose proton symport system for glucose uptake without the need to dephosphorylate PEP.[32] The intracellular phosphorylation of glucose to glucose-6-phosphate, which is essential for glucose metabolism, was performed by a non-PTS glucokinase enzyme encoded by *glk*.[33]

Figure 92. ^{1}H NMR of L-glutamate standard. δ 3.78 (t, 1H), 2.51 (t, 2H), 2.15 (m, 2H) in D_2O

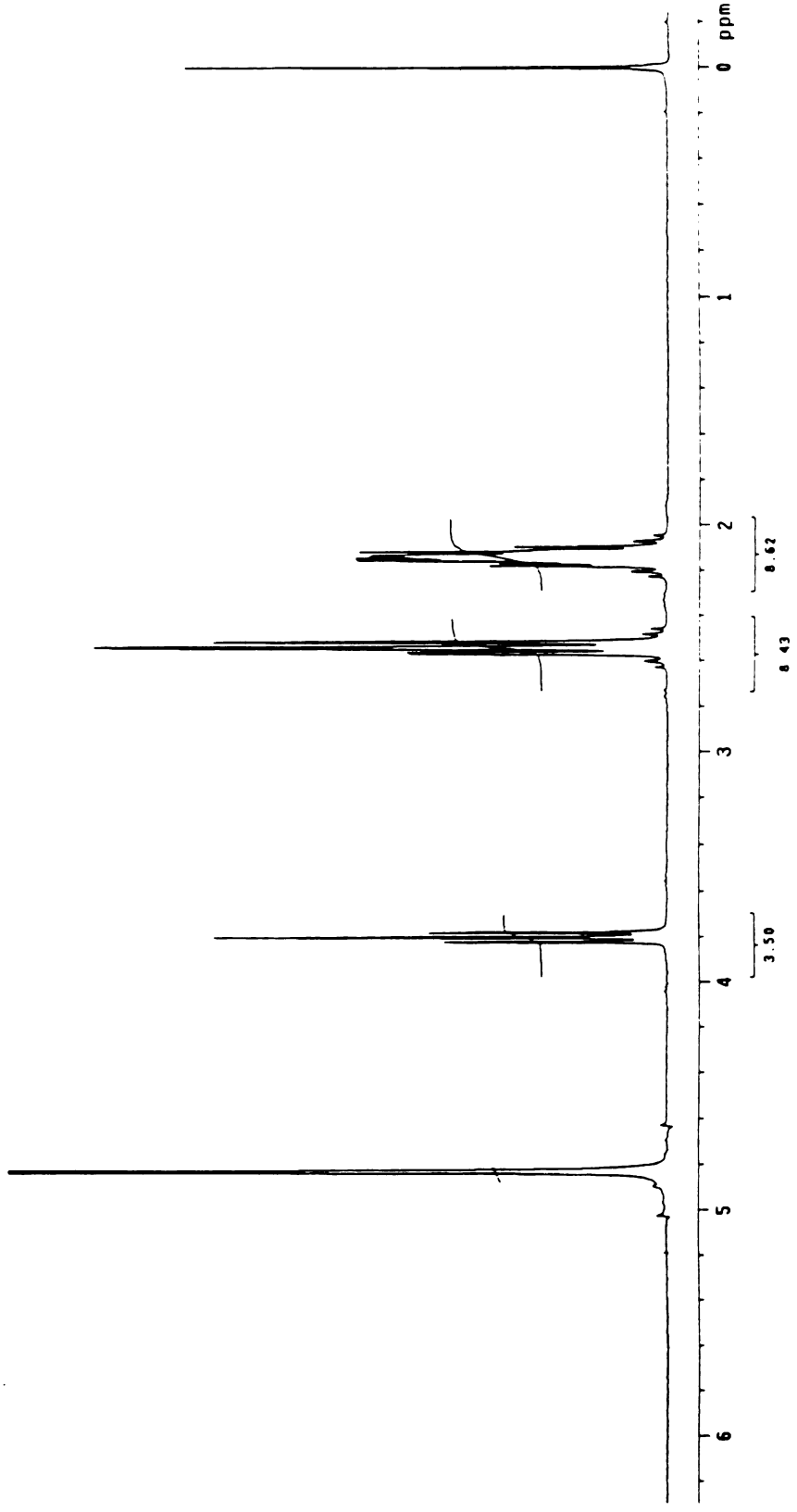

0 ppm
1
2
3
4
5
6
8.62
8.43
3.50

Figure 93. ^{1}H NMR of the fermentation supernatant of KL7/pSK6.161 cultured under glusose-limited conditions at 10% D.O. GA resonance δ 7.1 (s, 2H), PCA resonance δ 7.45 (m, 2H), 6.95 (d, 1H), DHS resonances δ 6.42 (d, 1H), 4.28 (d, 1H), 4.00 (ddd, 1H), 3.07 (dd, 1H), 2.66 (ddd, 1H). DAH resonances δ 1.80 (dd, 1H), 2.20 (dd, 1H) covered by glutamate, 3.45 (dd, 1H), 3.83 (m, 2H), 3.95 (ddd, 1H). Glutamate resonances δ 3.78 (t, 1H), 2.51 (t, 2H), 2.15 (m, 2H), in D_2O

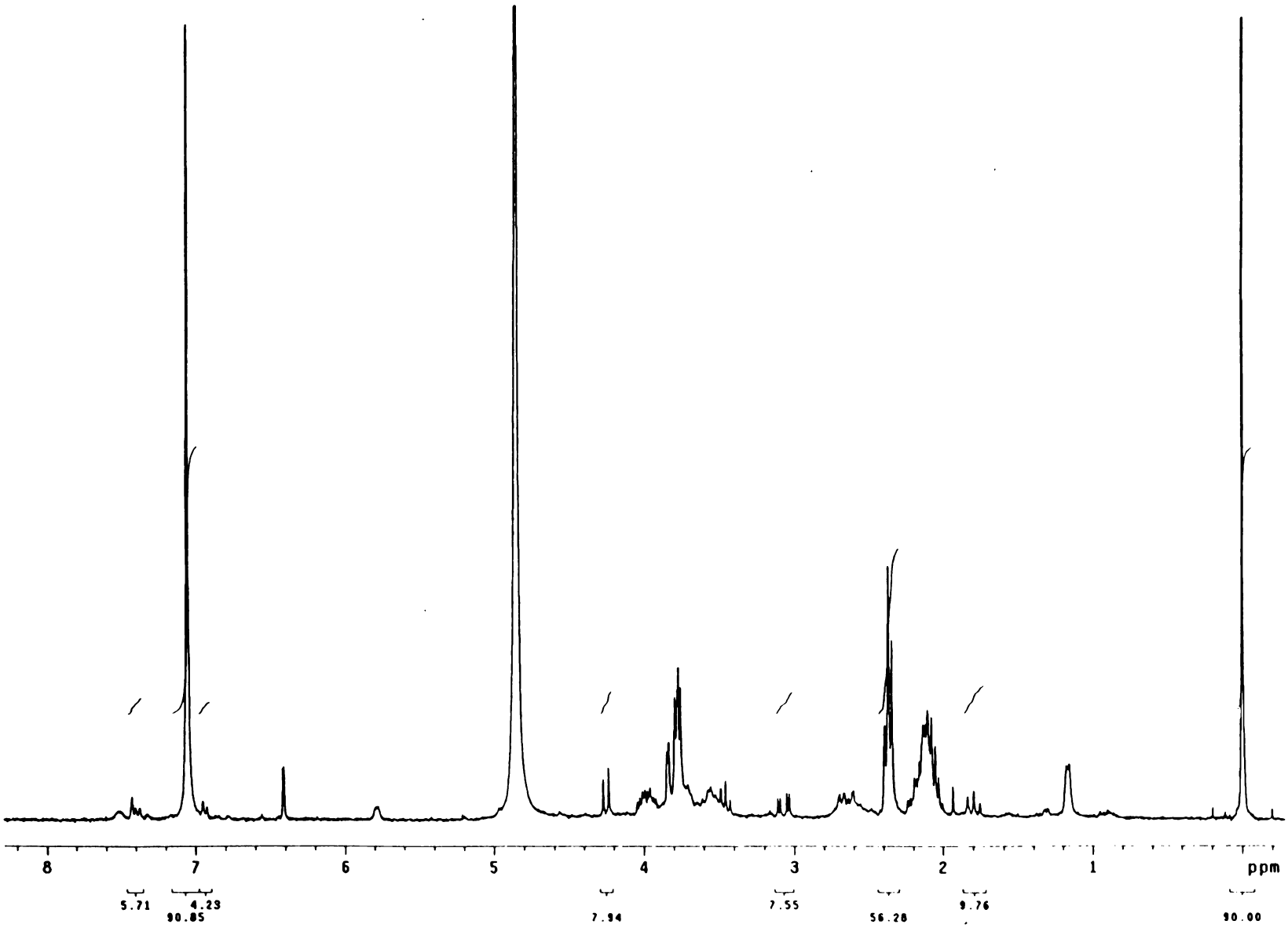

8
7
6
5
4
3
2
1
ppm
5.71
4.23
90.85
7.94
7.55
56.28
9.76
90.00

Figure 94. ^{1}H NMR of the fermentation supernatant of KL7/pSK6.161 cultured under glucose-rich conditions at 10% D.O.. GA, PCA, DHS, DAH glucose and glutamate are present (D_2O).

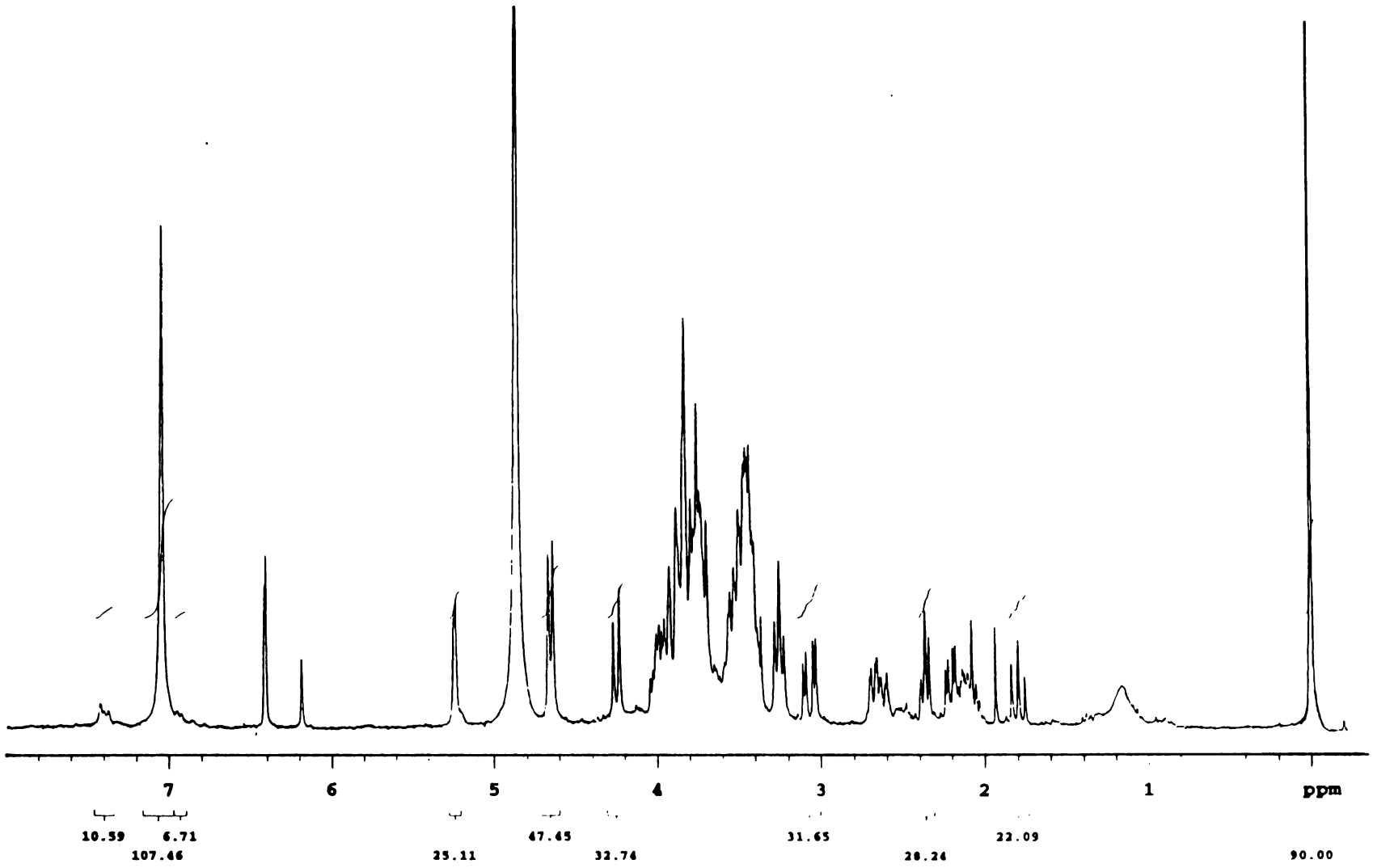

7
6
5
4
3
2
1
ppm
10.59
6.71
107.46
25.11
47.45
32.74
31.65
28.24
22.09
90.00

Figure 95. ^{1}H NMR of the fermentation supernatant of KL7/pSK7.51 cultured under glucose-limited conditions at 10% D.O. GA, PCA, DAH, glutamate and glucose are present (D_2O).

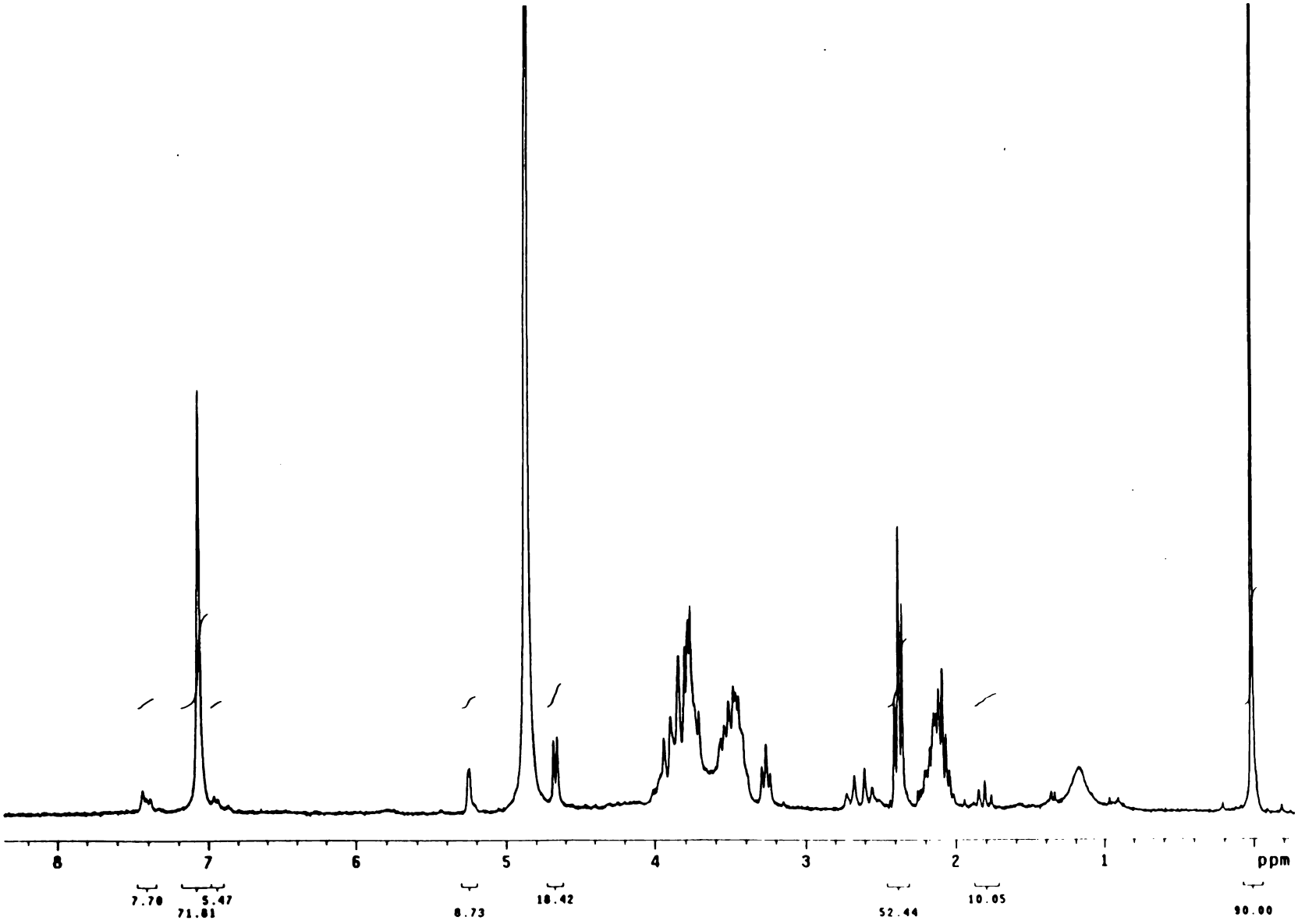

8
7
6
5
4
3
2
1
ppm
7.70
5.47
71.81
8.73
18.42
52.44
10.05
90.00

Figure 96. ^{1}H NMR of the fermentation supernatant of KL7/pSK7.80 cultured under glucose-limited conditions at 10% D.O. . GA, PCA and glutamate are present (D_2O).

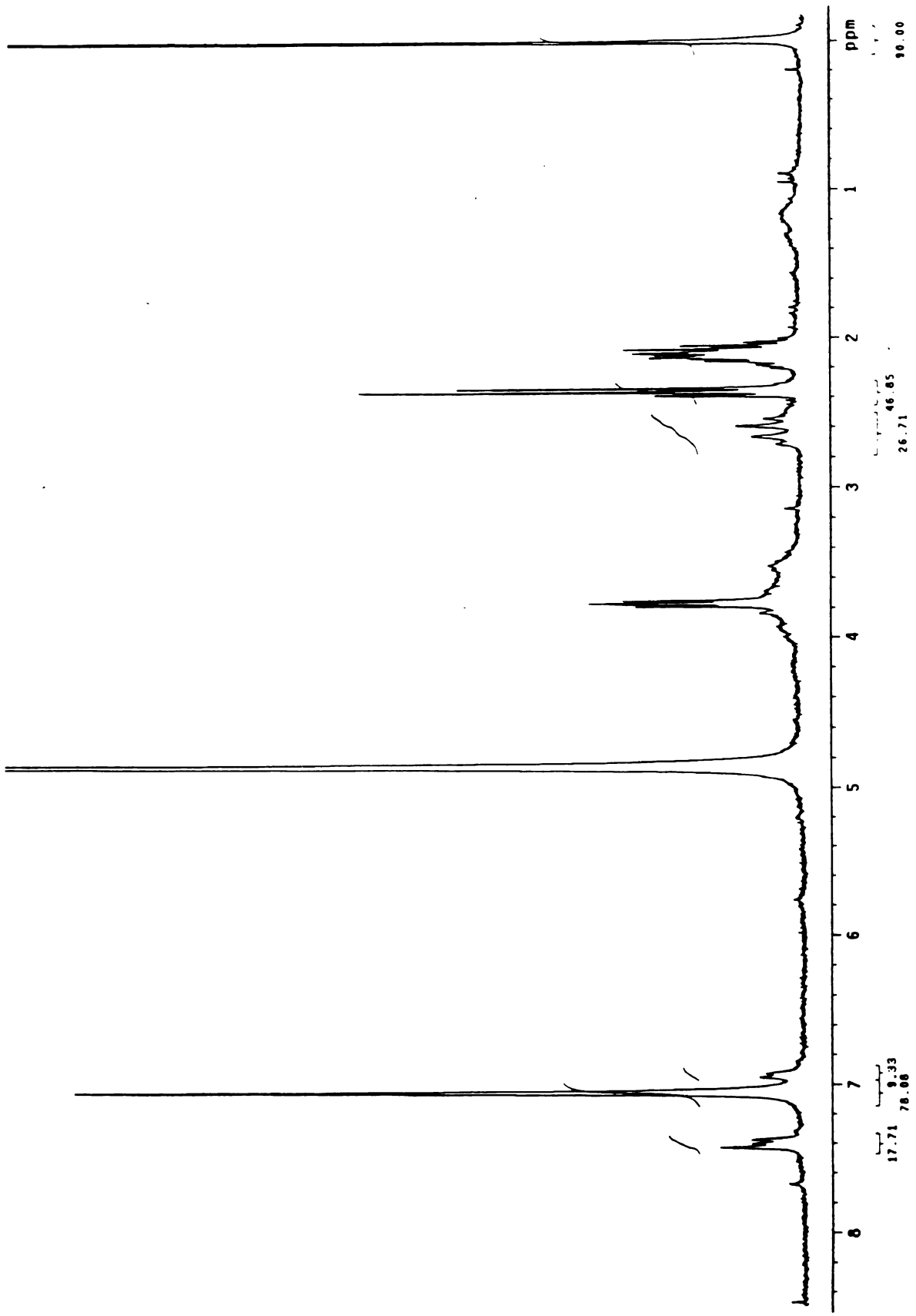

ppm
8
7
6
5
4
3
2
1
17.71
9.33
78.08
46.85
26.71
90.00

Figure 97. ^{1}H NMR of the fermentation supernatant of KL7/pSK7.104 cultured under glucose-limited conditions at 10% D.O. . GA, PCA, DHS, DAH, acetate and glucose are present (D_2O).

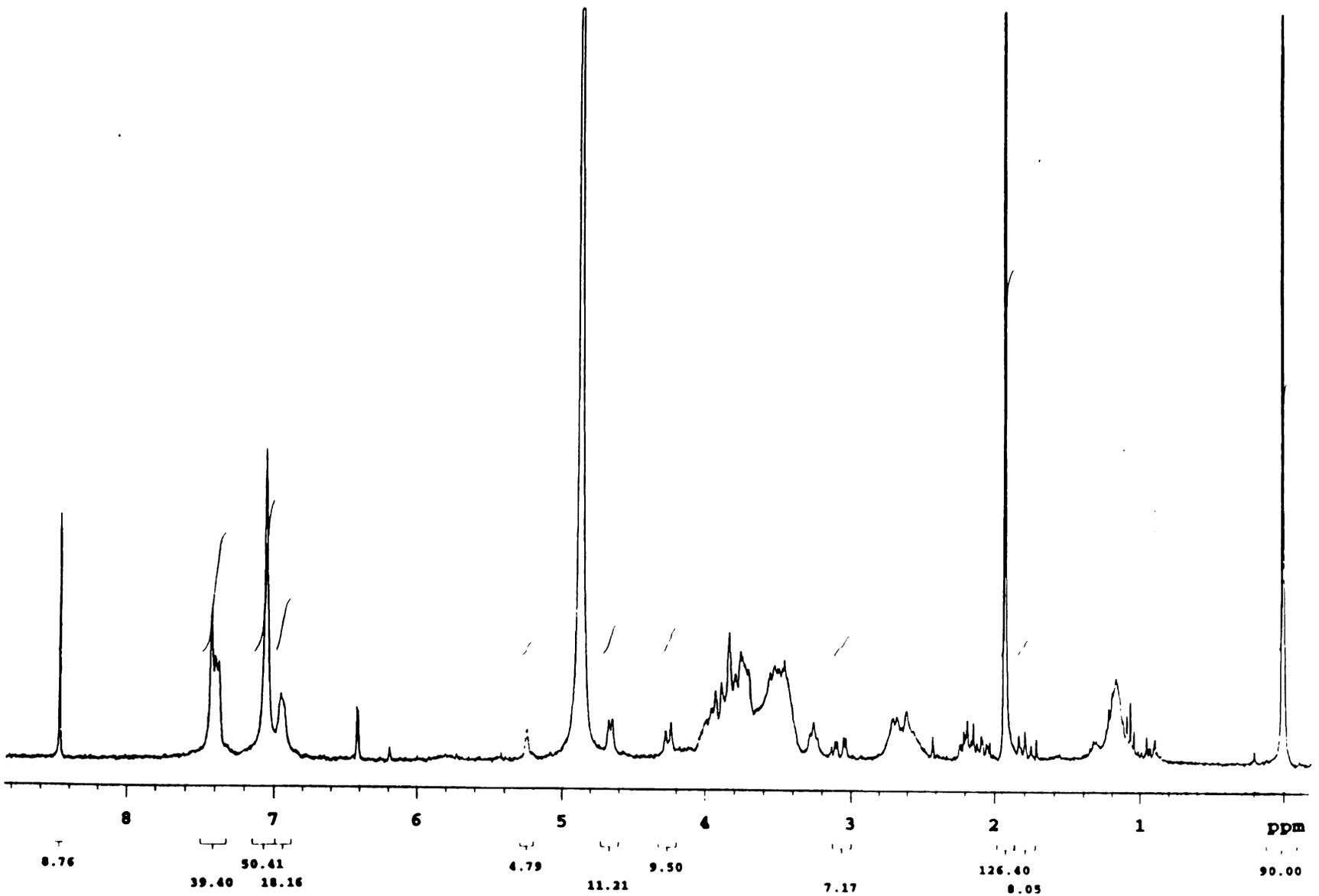
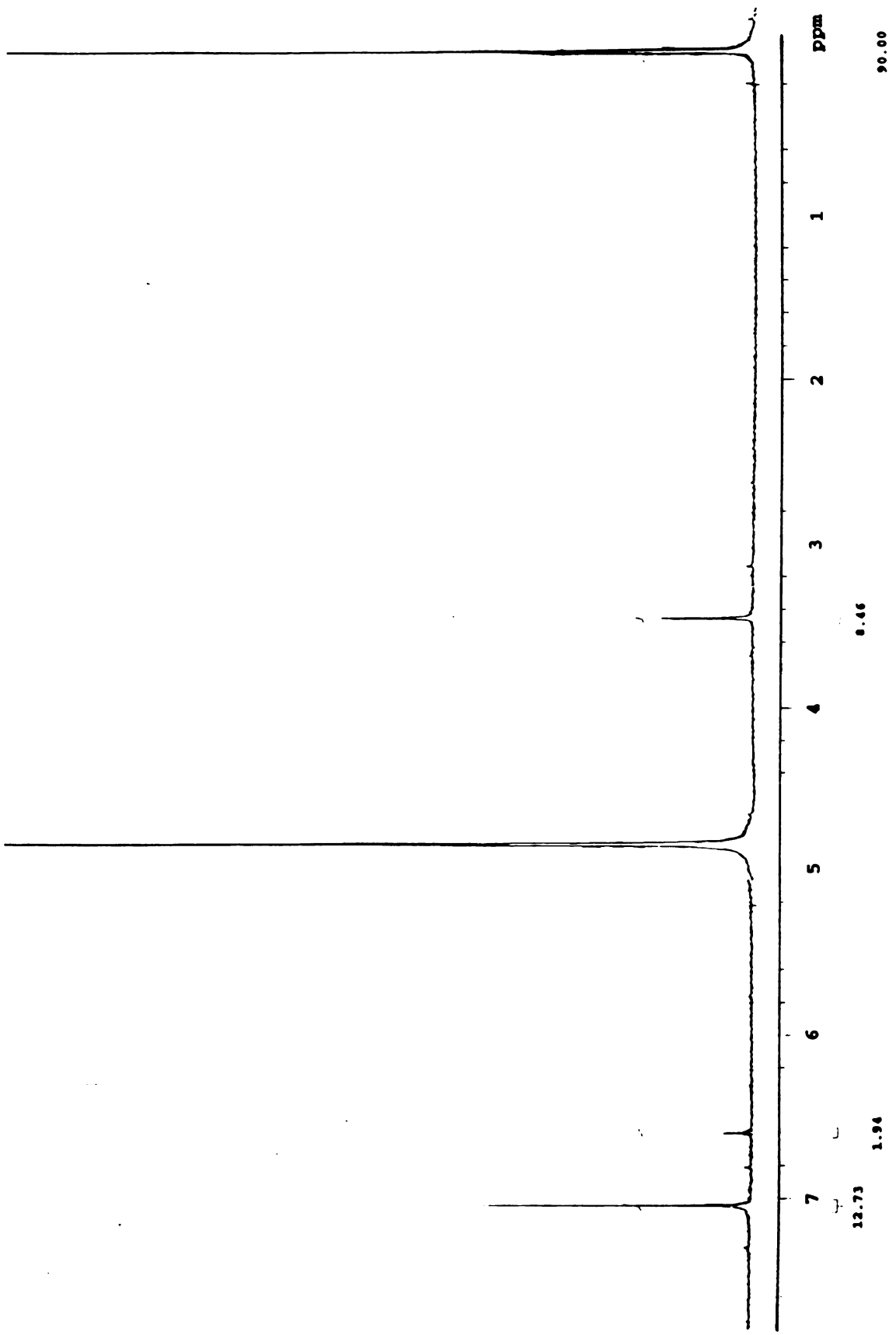

8
7
6
5
4
3
2
1
ppm
8.76
39.40
50.41
18.16
4.79
11.21
9.50
7.17
126.40
8.05
90.00

Figure 98. ^{1}H NMR of the supernatant of a gallic acid solution after stirring for 24 h on a shake-fkask at 37 °C under air. GA resonances δ 7.1 (s, 2H), Aconitic acid resonances δ 6.6 (s, 1H), 3.45 (s, 2H).


ppm
1
2
3
4
5
6
7
90.00
8.46
12.73
1.94

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CHAPTER 6

BIOCATALYTIC CONVERSION OF GALLIC ACID AND PROTOCATECHUIC ACID INTO PYROGALLOL AND CATECHOL

I. INTRODUCTION

Pyrogallol (PGL) and catechol are the common names for 1,2,3-trihydroxy-benzene and 1,2-dihydroxybenzene respectively. Pyrogallol is widespread in nature as a component of tannins, anthocyanins, flavones, and alkaloids,[1] while catechol and its derivatives are found in lignin, wood, and other higher plants. Both molecules are valuable industrial chemicals with a global annual production of around $2x10^5$ kg/yr for pyrogallol and $2.5x10^7$ kg/yr for catechol. Estimating catechol's world production is difficult due to captive markets where catechol is produced and then converted into higher, value-added products before ever seeing the open market as catechol. The main commercial use of pyrogallol is as a building block for synthesis of pharmaceuticals and pesticides while catechol is used in fur dyeing, leather tanning, and photographic development. Catechol is also a building block for synthesis of polymerization inhibitors, perfumes, pharmaceuticals and pesticides.

Pyrogallol is the oldest and one of the more versatile of the photographic developing agents in use. The ease of oxidation of this molecule is the basis for its use in fur and hair dying and as a chemical reagent for the estimation of oxygen.[2,3] Purpurogallin is a red brown to black mordant dye formed by oxidation of pyrogallol. A rubberized pyrogallol-formaldehyde adhesive improves the adhesion of rubber to nylon.[2] A

chemiluminescence pyrogallol system[3] is used to determine traces of chromium(III). Glass fiber for reinforcement of cementitious products is protected from corrosion by a pyrogallol dip.[4] Phosphite esters of 4,6 dialkylpyrogallol are effective heat and light-stabilizers for plastics.[5]

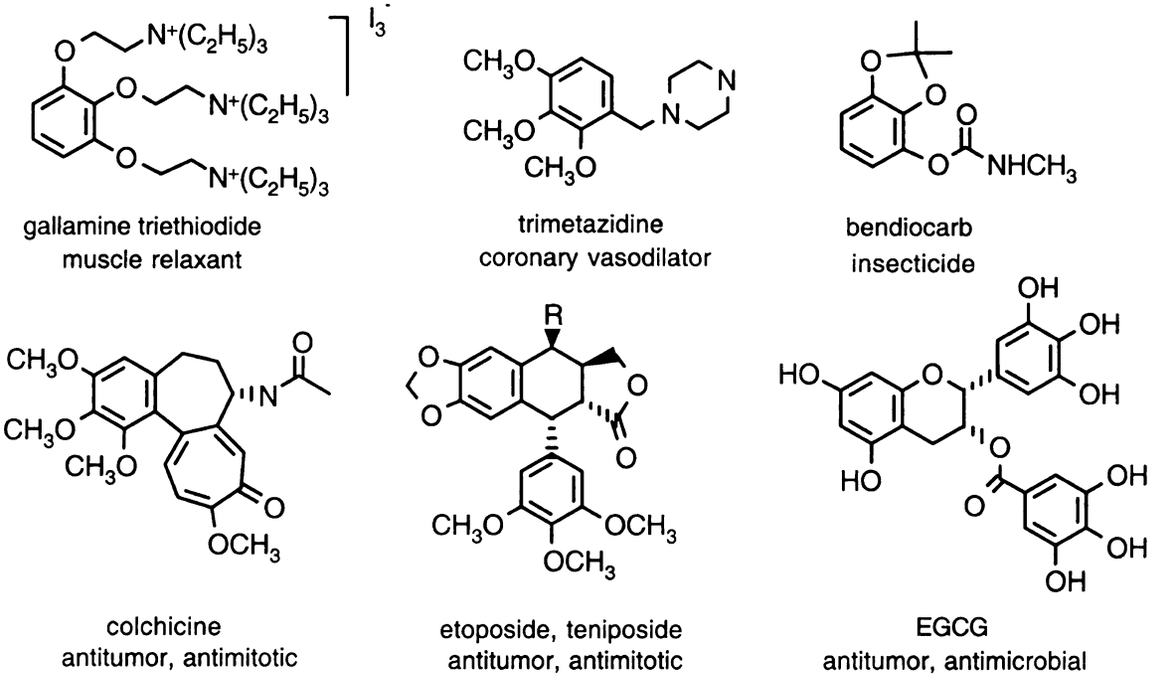


Figure 99. Derivatives of pyrogallol.

Many derivatives of pyrogallol have been used as pharmaceuticals. Gallamine triethiodide is an important muscle relaxant in surgery, and it is also used in convulsive-shock therapy (Figure 99). Trimetazidine is used as a coronary vasodilator, and bendiocarb is a widely used insecticide for the control of cockroaches, crickets, ants, fleas and other insects (Figure 99). The structure of pyrogallol is part of a variety of natural products with various pharmaceutical activities. Colchicine (Figure 99), the major component of autumn crocus, *Colchicum automnale L.*, is a potent drug in cancer

chemotherapy.[6] Due to its severe toxicity colchicine has limited clinical use, but various other structural analogues have been synthesized.[7] Other less-toxic, broad-spectrum antitumor drugs that contain the pyrogallol core structure include podophylotoxin and its semisynthetic derivatives etoposide and teniposide (Figure 99).[8] Both the gallic acid and pyrogallol structures are present in EGCG, a compound originally isolated from green tea, that possesses wide biological activity.[9] The synthetic value of pyrogallol and its close analogue, gallic acid, in the chemical synthesis of the previous pharmaceuticals becomes apparent.

The main commercial synthesis of pyrogallol comes from thermal decarboxylation of gallic acid. Gallic acid is heated with half its weight of water in a copper autoclave until the pressure reaches 12 atm and the temperature is 175 °C. Steam and carbon dioxide are released but sufficient water is retained to maintain the pyrogallol as a liquid.[10] The cooled solution is decolorized with animal charcoal and is then evaporated until the volatile pyrogallol distills into iron receivers. The solidified material is purified by repeated distillation, sublimation, or vacuum distillation at 200 °C in the presence of dialkyl phthalates.[10]

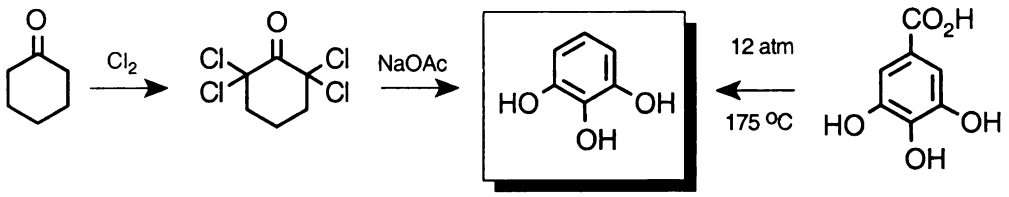


Figure 100: Chemical synthesis of pyrogallol.

Because of the continuing uncertainties of supplies of gall nuts and tara powder, which are used for gallic acid and pyrogallol manufacture, there is interest in the development of synthetic processes. Various approaches were investigated in the synthesis of pyrogallol from readily available petrochemicals. The first synthetic pyrogallol that was industrially manufactured used basic hydrolysis of 2,2,6,6-tetrachlorocyclohexanone (Figure 100).[11] The preparation of the starting material by chlorination of cyclohexanone in the presence of collidine as the catalyst has been patented.[12] Note that cyclohexanone's main commercial synthesis begins with benzene, a petroleum-derived chemical with known toxic and carcinogenic activity. Other synthetic approaches involve hydroxylation of resorcinol with 50% H_2O_2 in the presence of hexafluoroacetone at 60 °C to give a mixture of pyrogallol and 1,2,4-trihydroxybenzene,[13] or hydrolysis of 2,6-dimethoxyphenol using 48% hydrobromic acid.[14] The 2,6-dimethoxyphenol is produced by reaction of 2,6-dibromophenol with sodium methoxide.

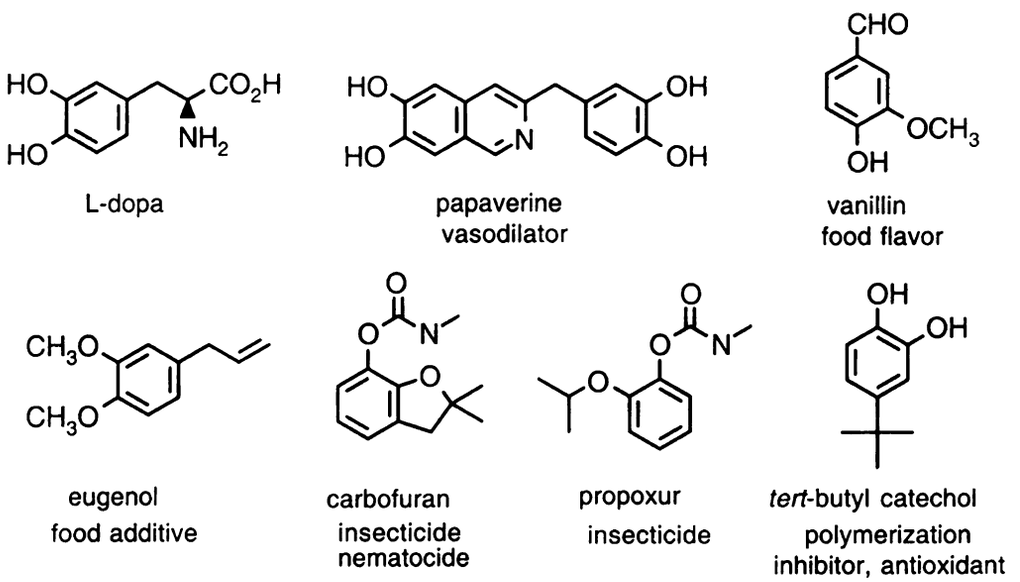


Figure 101. Derivatives of catechol.

Catechol is another important industrial intermediate. Chemical products derived from catechol (Figure 101) include pharmaceuticals (L-Dopa, adrenaline, papaverine),

flavors (vanillin, eugenol, isoeugenol), agrochemicals with insecticide activities (carbofuran, propoxur), and polymerization inhibitors and antioxidants (4-*tert*-butylcatechol, veratrol).[15] In addition, catechol is an important intermediate in the biocatalytic synthesis of adipic acid (Figure 9 Chapter 2).[20] Most of the catechol production begins with Friedel-Crafts alkylation of benzene to afford cumene (Figure 102). 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 (Figure 102). Catechol and hydroquinone are separated by distillation.[15]

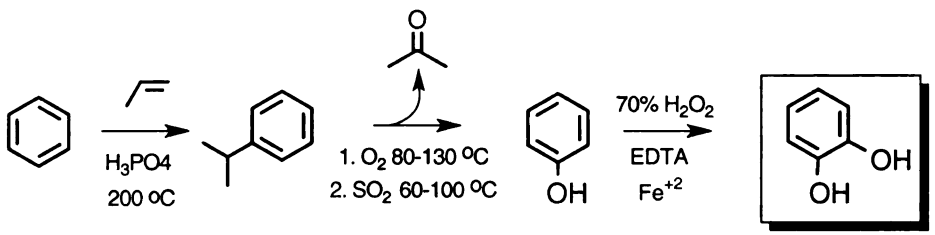


Figure 102. Chemical synthesis of catechol

While in aerobic environments the microbial degradation of aromatic compounds is initiated by oxygenases that hydroxylate and cleave the aromatic nucleus through addition of molecular oxygen,[16] the degradation of aromatic compounds in the absence of molecular oxygen has also been documented.[17] The first step in the anaerobic degradation of gallic acid by various organisms involves pyrogallol formation with the action of GA decarboxylase, followed by formation of phloroglucinol, reduction with NADPH dependent enzymes, and ring cleavage after H_2O addition.[17] This PGL decarboxylase activity has been identified in various organisms including *Pantoea agglomerans*,[18] *Eubacterium oxidoreducens*,[17a,b] *Pelobacter acidigallici*[17c] and *Citrobacter sp.*[19] Although some enzymes can only accept gallic acid as the substrate,[18] other bacteria posses

decarboxylases with broader substrate specificity including protocatechuic acid.[19] Protocatechuic acid is the primary substrate for a decarboxylase that exist in *Klebsiella sp.*[20] The *aroY*-encoded PCA decarboxylase enzyme has been isolated from a *K. pneumonae* genomic library, and has been successfully expressed in *E. coli* bacteria.[20] Although AroY's primary substrate was protocatechuic acid, other aromatic acids including gallic acid could also be decarboxylated.[22]

In this Chapter, an efficient biocatalytic synthesis of pyrogallol and catechol is described. The decarboxylation reaction is based on the PCA decarboxylase enzyme(s) that exist in *Klebsiella sp.* Recombinant *E. coli* expressing *aroY*-encoded[20] PCA decarboxylase isolated from *K. pneumonae,* or intact *Klebsiella oxytoca M5a1* are used for the decarboxylation of gallic acid and protocatechuic acid. In both cases, cells were grown under fed-batch fermentor conditions prior to addition of gallic acid and protocatechuic acid. Straightforward purification gives pyrogallol and catechol in high yields and purities. Biocatalytic synthesis of the gallic acid and protocatechuic acid precursor from glucose using *E. coli* bacteria has already been discussed in Chapters 5 and 2. Therefore, an overall synthesis of pyrogallol and catechol from glucose is being achieved.

II. DECARBOXYLATION OF GALLIC ACID AND PROTOCATECHUIC ACID

A. Biocatalyst and Plasmid Construction

Recombinant *Escherichia coli* or intact *Klebsiella oxytoca M5a1* were utilized as the host strains for the decarboxylation of both gallic acid and protocatechuic acid. The key enzyme in both cases was PCA decarboxylase, an enzyme that exists in *Klebsiella sp.*, and has been cloned as *aroZ* from a *K. pneumonae* genomic library.[20] Expressing of plasmid-localized *aroZ* in *E. coli* led to similar results for the decarboxylation of protocatechuic acid as well as gallic acid. Decarboxylation and formation of catechol and pyrogallol were also observed when *K. oxytoca M5a1* was cultured in the presence of gallic acid or protocatechuic acid.

The host strain employed for heterologous expression of *aroY* in *E. coli* was RB791*serA::aroB,* which contained an extra copy of the *aroB* gene inserted in the *serA* locus of the genome.[22] As already discussed in Chapter 5, disruption of the *serA* locus of the host strain was designed for plasmid maintenance. Bacterial growth can only be achieved when the *serA* gene, which is essential for biosynthesis of L-serine (Figure 64, Chapter 5), is plasmid-localized. Plasmid pSK6.234 was therefore constructed carrying both *serA* and *aroY*. Digestion of pKD9.069A[20] with *Hin*dIII gave a 2.3 kb DNA containing the *aroY* gene with its native promoter. Ligation of the latter to the *Hin*dIII site of pSU18 created pSK6.228 (Figure 103). Ligation of the 1.9 kb DNA containing the *serA* gene in to the *Sma*I site of pSK6.228 gave pSK6.234 (Figure 104). The *serA* containing DNA was isolated from pD2625 after a double *Dra*I/*Eco*RV digestion.

The previous biocatalysts, *K. oxytoca M5a1* and RB791*serA::aroB*/pSK6.234, are both designed to produce pyrogallol and catechol after external addition of gallic acid and protocatechuic acid. Catalysts that could potentially synthesize these molecules from glucose in one step were also constructed. For the catechol-producing biocatalyst,

aroF^{FBR} was plasmid-localized along with *aroY* and *serA*. The host strain, KL7, as already discussed in Chapters 2 and 5, was engineered to accumulate protocatechuic acid when *aroF*^{FBR} was expressed from a plasmid. Plasmid pSK7.126 was constructed after ligating the *serA* (*Dra*I/*Eco*RV fragment from pD2625) gene into the *Sma*I/CIAP treated site of pWN1.079A (Figure 105).

Plasmid pSK6.232 that contained *aroY* in addition to *aroF*^{FBR}, and *pobA** was constructed to produce pyrogallol from glucose when transformed in KL7. Plasmid pSK4.232B was digested with *Hin*dIII, treated with CIAP and ligated with the *Hin*dIII digested 2.3 kb *aroY*-containing DNA fragment, giving pSK6.224 (Figure 106). Subsequent ligation of the *serA* (*Dra*I/*Eco*RV fragment from pD2625) into the *Sma*I/CIAP site of pSK6.224, gave plasmid pSK6.232 (Figure 107)

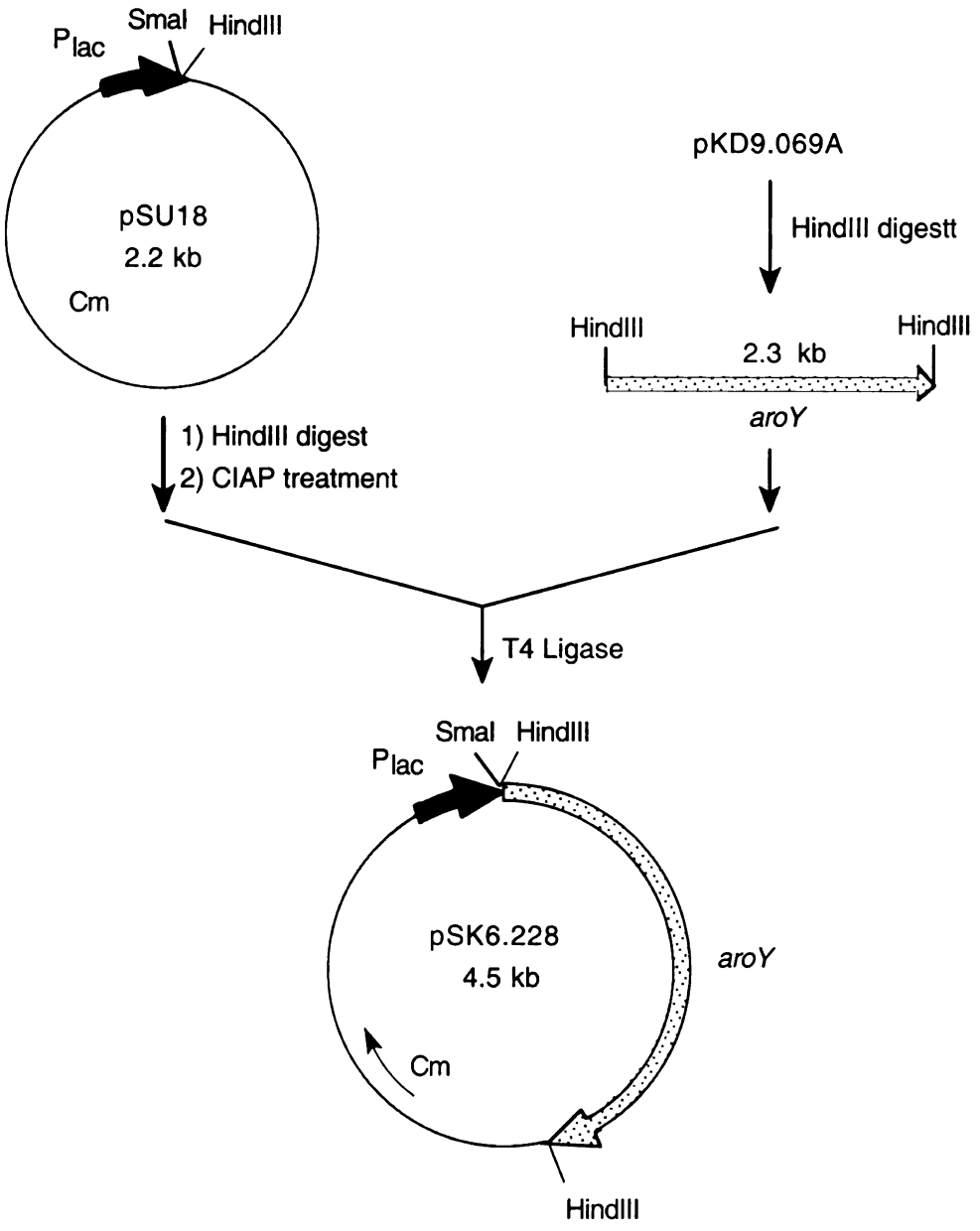


Figure 103. Construction of plasmid pSK6.228

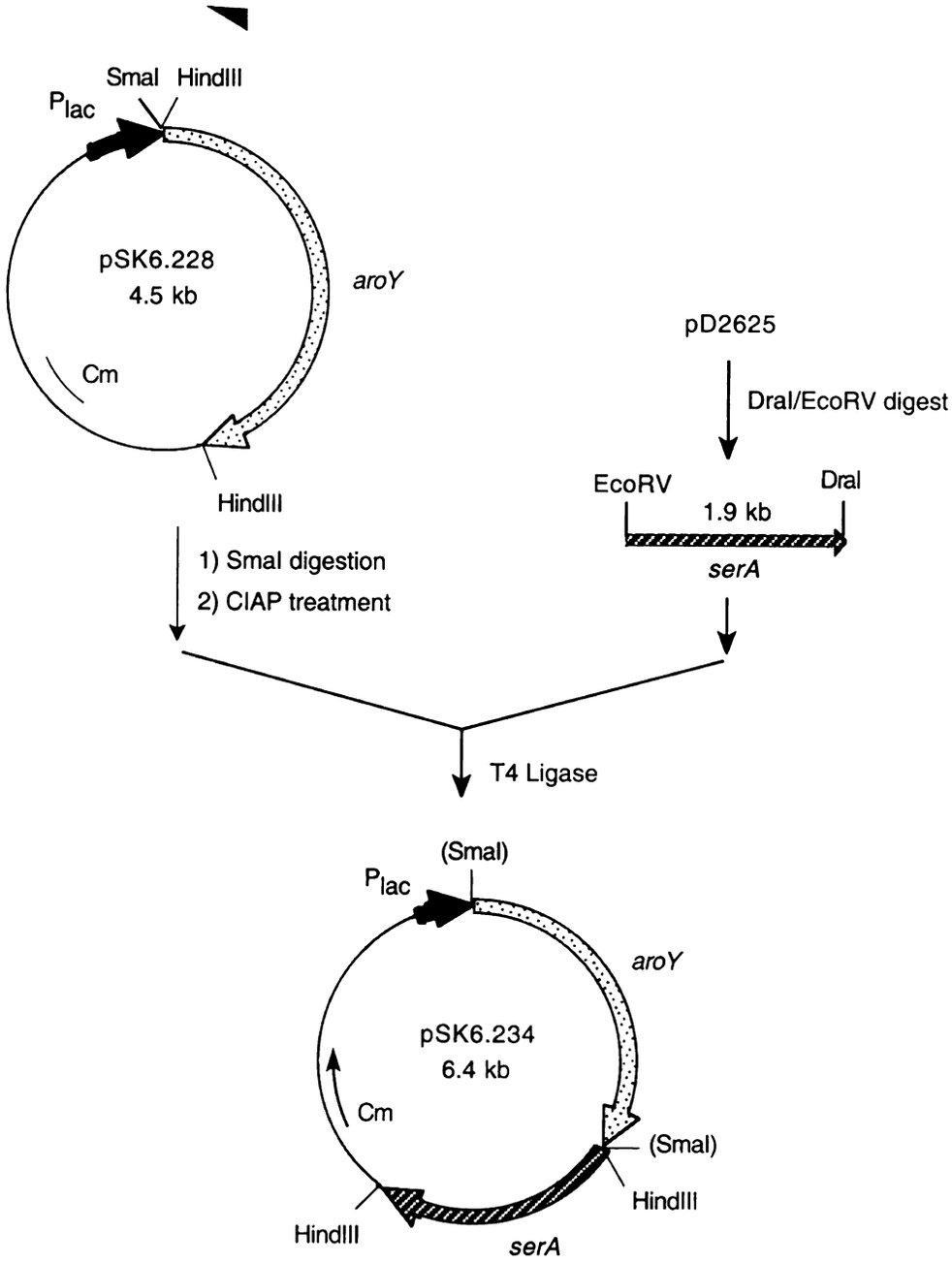


Figure 104. Construction of plasmid pSK6.234

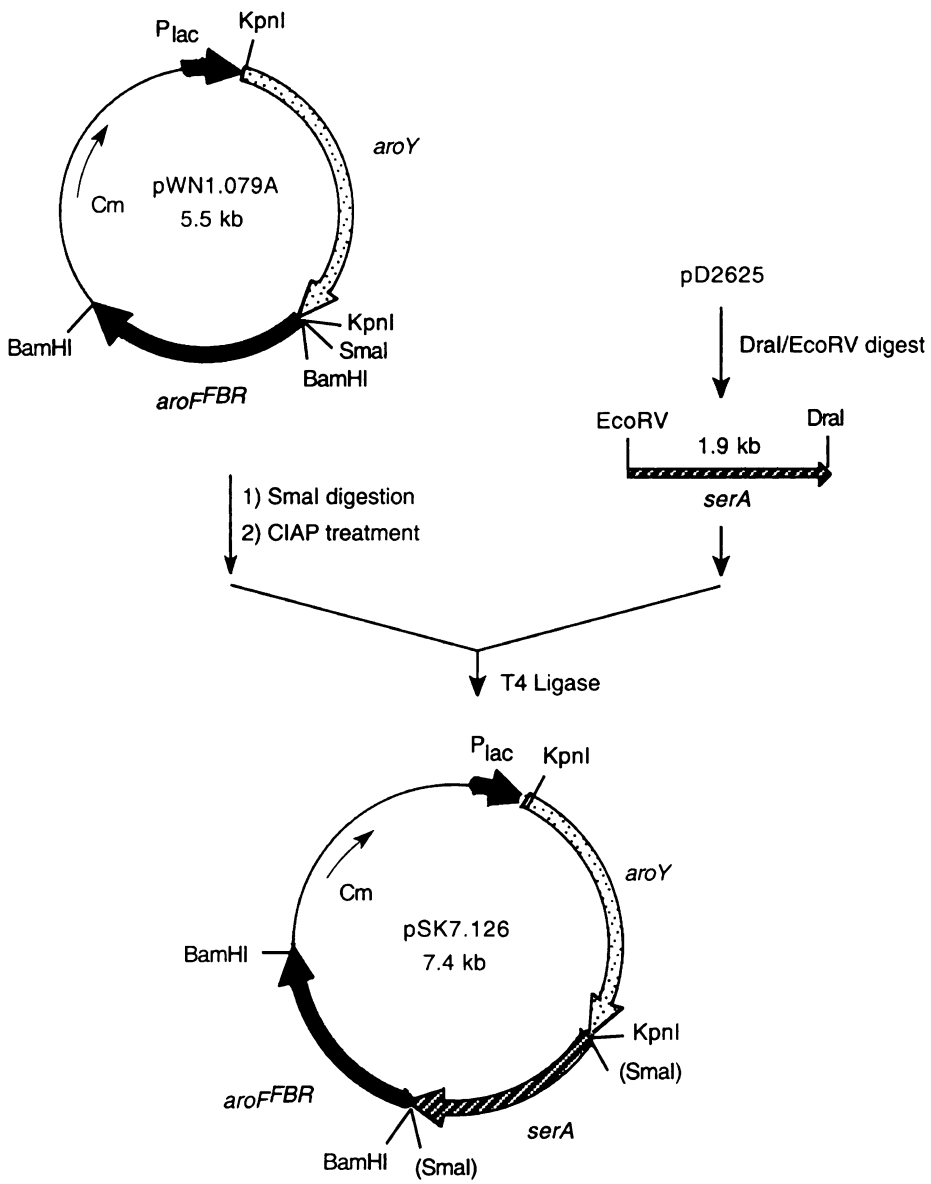


Figure 105. Construction of plasmid pSK7.126

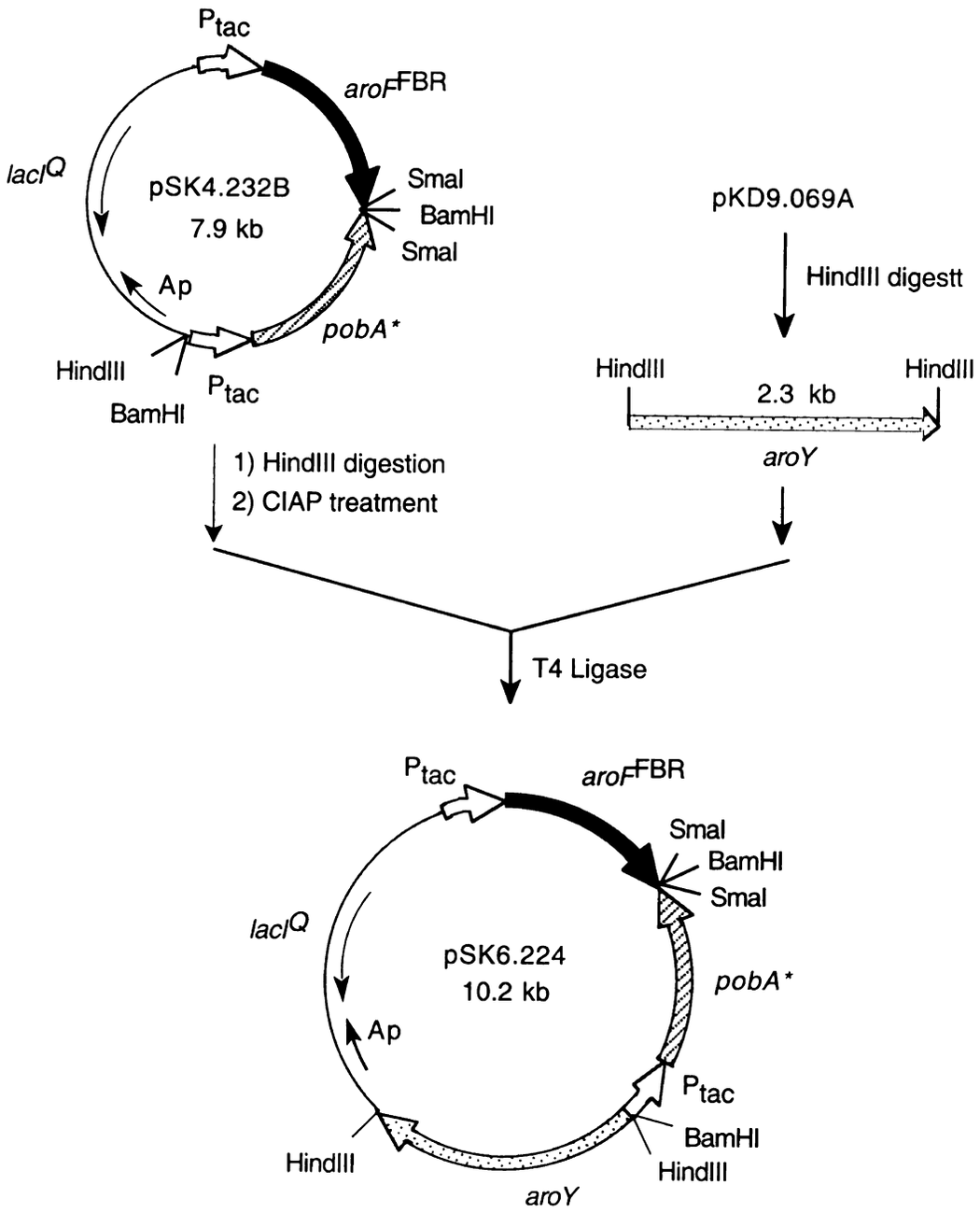


Figure 106. Construction of plasmid pSK6.224

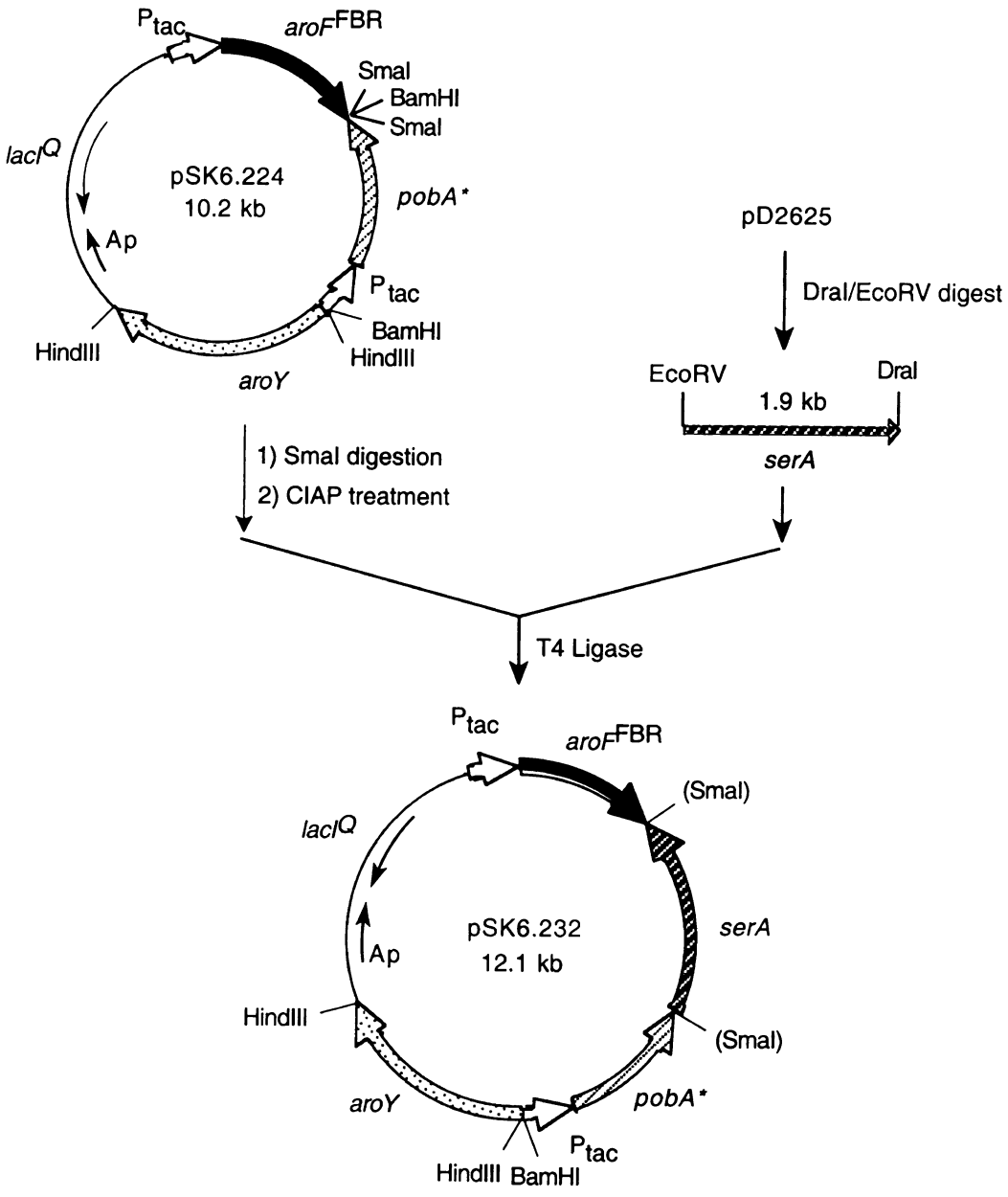


Figure 107. Construction of plasmid pSK6.232

B. PGL and catechol formation using *E. coli* bacteria

One-step synthesis of catechol and pyrogallol from glucose required the expression of PCA decarboxylase in the PCA-synthesizing (Chapter 2) and GA-synthesizing (Chapter 5) biocatalysts. Pyrogallol formation was the intended result in cells carrying $aroF^{FBR}$, *pobA** and *aroY* genes in the same plasmid (Figure 107). KL7/pSK6.232 cultured under fed-batch fermentor conditions reached the third phase of D.O. control at 11 h when the first IPTG (30 mg) was added. Expression of *pobA** and $aroF^{FBR}$ was induced at this point since both genes were under the control of *tac* promoters. Having both genes repressed during the first 12 h avoided any toxicity problems associated with pyrogallol or catechol formation during this early phase of growth. ^{1}H NMR analysis of the cell supernatant after 12 h showed formation of only catechol and 3-dehydroshikimic acid, without any protocatechuic acid or gallic acid. The concentration of catechol was 31 mM at 24 h and 34 mM at 30 h, whereas cell growth significantly declined after the 18 h time point (Table 24). This growth inhibition was accompanied by a loss of the D.O. control after 24 h. D.O. levels reached 75% of air saturation at 36 h. Protocatechuic acid is the precursor metabolite in the synthesis of catechol using *aroY* and in the synthesis of gallic acid using *pobA** (Figure 113). The exclusive formation of catechol without any gallic acid or pyrogallol indicates that protocatechuic acid reacts faster with AroY to form catechol compared to reaction with PobA* to afford gallic acid.

Table 24. KL7/pSK6.232 and KL7/pSK7.126. Product distribution and cell mass.

	KL7/pSK6.232		KL7/pSK7.126	
time (h)	catechol (mM)	cell mass (g/L)	catechol (mM)	cell mass (g/L)
12	2.3	10	–	15
18	18	17	13	26
24	31	18	28	29
30	34	20	34	29

The same loss of D.O. control and inhibition of cell growth was observed when a construct, KL7/pSK7.126, which is capable of synthesizing catechol from glucose was cultured under fermentor conditions. Vigorous growth until 18 h was observed with KL7/pSK7.126 reaching the third phase of D.O. control at 11 h. However, when catechol concentration approached 30 mM (24 h) a growth inhibition and a loss in the ability to maintain D.O. levels at 10% air saturation was observed. Apparently, catechol is toxic to *E. coli* cultured under fermentor conditions at concentrations of approximately 30 mM (3.4 g/L). During the attempted one-step synthesis of pyrogallol from glucose, protocatechuic acid was undergoing PCA decarboxylase-catalyzed conversion to catechol before *pobA** had a chance to oxidize it into gallic acid (Figure 113).

An alternative method for pyrogallol formation utilized addition of gallic acid to the supernatant of *E. coli* expressing plasmid-localized *aroY*. Cells first grew to early stationary phase under shake-flask conditions in a rich medium before they were resuspended in a minimal salt medium containing glucose and gallic acid. Pyrogallol formation showed an increase during the first 6 h, but the rate of pyrogallol formation decreased substantially during the next 12 h with the formation of a black solution (Figure 108). The host strain, *E. coli* BL21(DE3)[24] carried a copy of the T7 RNA polymerase gene inserted on the genome, whereas pT7-6(AroY)[24] is a plasmid that carries the *aroY* expressed from a T7 promoter. Transforming a host strain that carry a genomic insertion of the T7 RNA polymerase with a plasmid that contains a T7 promoter is usually used for achieving high expression levels of proteins cloned after the T7 promoter. The decarboxylation rates of gallic acid utilizing BL21(DE3)/pT7-6(aroY) or RB791*serA::aroB*/pSK6.234 were very similar.

Rapid decomposition of pyrogallol upon reaction with oxygen in phosphate-buffered solutions of pH range 6.9-10.5 has been well documented.[23] The observed decrease of pyrogallol concentration in bacterial shake-flask cultures was likely the result of this oxidation. Initial accumulation of the pyrogallol was possible during the first 6 h

probably because the high rate of bacterial respiration was keeping the D.O. levels low thereby protecting pyrogallol from decomposition.

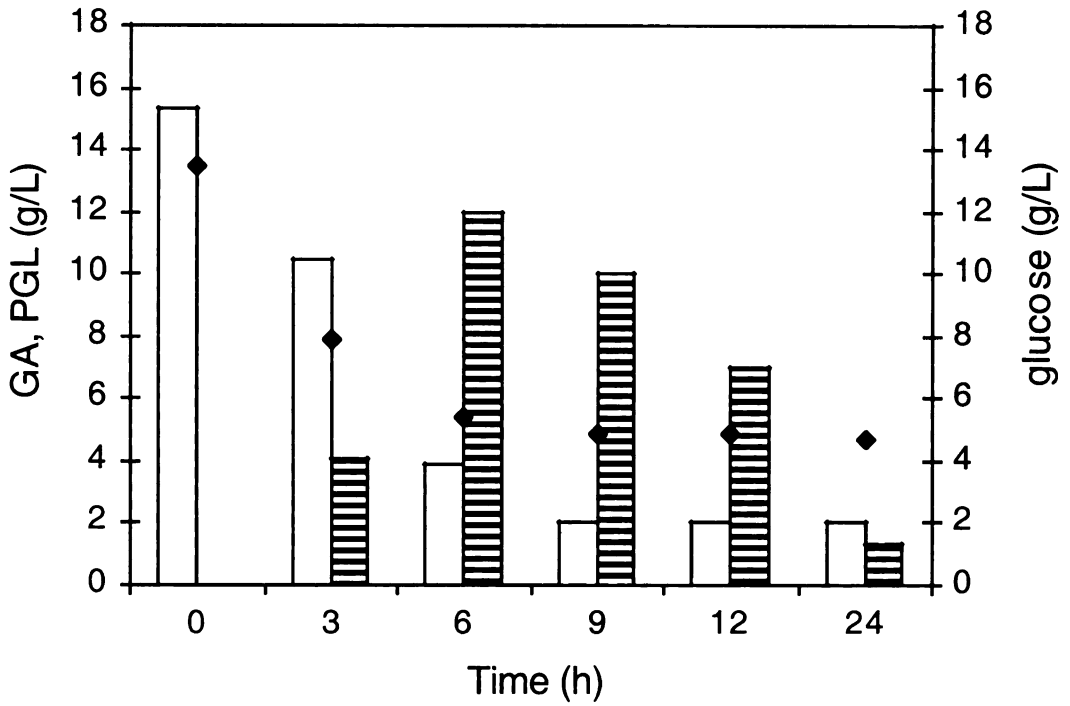


Figure 108. Shake flask production of pyrogallol using BL21(DE3)/pT7-6(*aroY*). Gallic acid (▭), pyrogallol (▤), and glucose (◆) concentrations.

Since both oxygenation levels and pH were difficult to control in shake flasks, culturing strains expressing PCA decarboxylase under fermentor conditions was examined. Under these conditions, controlled addition of glucose or replacement of O_2 with N_2 could be employed to minimize D.O. levels. D.O. levels were monitored via an oxygen probe. Another advantage for using microbes to decarboxylate gallic acid and protocatechuic acid, was the high cell densities that were produced under fermentor conditions. Cell densities for microbes cultured under fermentor conditions were in excess of 15-fold higher relative to cell densities for the same microbes cultured under shake flask conditions.

RB791*serA::aroB*/pSK6.234 was allowed to reach early stationary phase at 24 h prior to addition in one portion of 28 g of GA·H_2O and 1.5 g PCA dissolved in 150 mL of

water under pH~7 adjusted with NH_4OH addition. The usual conditions of 20% D.O., 1L/L/min airflow and a stirring rate of 940 rpm were utilized for cell growth as described in detail in Chapter 5. Once either gallic acid or protocatechuic acid was added, aeration was terminated and the fermentor was sparged with N_2 at 0.5 L/L/min. Glucose addition was also terminated at this point. Quantitative transformation of gallic acid (115 mM, 19.6 g/L) and protocatechuic acid (4.5 mM, 0.7 g/L) into pyrogallol (112 mM, 14.1 g/L) and catechol (4 mM, 0.44 g/L), respectively, was observed within 12-14 h (Figure 109A). The total amount of glucose that was consumed for cell growth was around 72 g. The same decarboxylation rate and reaction yield were observed if instead of a GA/PCA mixture prepared from pure gallic acid and protocatechuic acid, 1 L of fermentation supernatant containing gallic acid (20 g/L), protocatechuic acid (0.7 g/L), 3-dehydroshikimic acid (4.1 g/L), 3-deoxy-D-*arabino*-heptulosonic acid (6.6 g/L) and glutamate (15 g/L) was added when RB791*serA::aroB*/pSK6.234 reached stationary phase. The 2.3 L fermentation supernatant that was produced contained 51 mM (8.7 g/L) gallic acid, 2 mM (0.3 g/L) protocatechuic acid, 14 mM (2.9 g/L) 3-deoxy-D-*arabino*-heptulosonic acid and 44 mM (6.5 g/L) glutamate. DHS, DAH, and glutamate remained unchanged when cultured under fed-batch fermentor conditions and did not affect the decarboxylation reaction, whereas 47 mM (5.2 g/L) pyrogallol, and trace amounts of catechol were formed by the end of the fermentation run (Figure 109B).

Purification of pyrogallol was straightforward, high-yielding, and independent of the source of gallic acid added to the culture medium. Pyrogallol was extracted at neutral pH with EtOAc followed by back extraction with brine to give a yellow pyrogallol-containing solution. After stirring the solution with activated charcoal and filtering through Celite, the EtOAc solution was evaporated to dryness to give an off-white powder. The total recovery of purified pyrogallol from the fermentation broth was 92-95%.

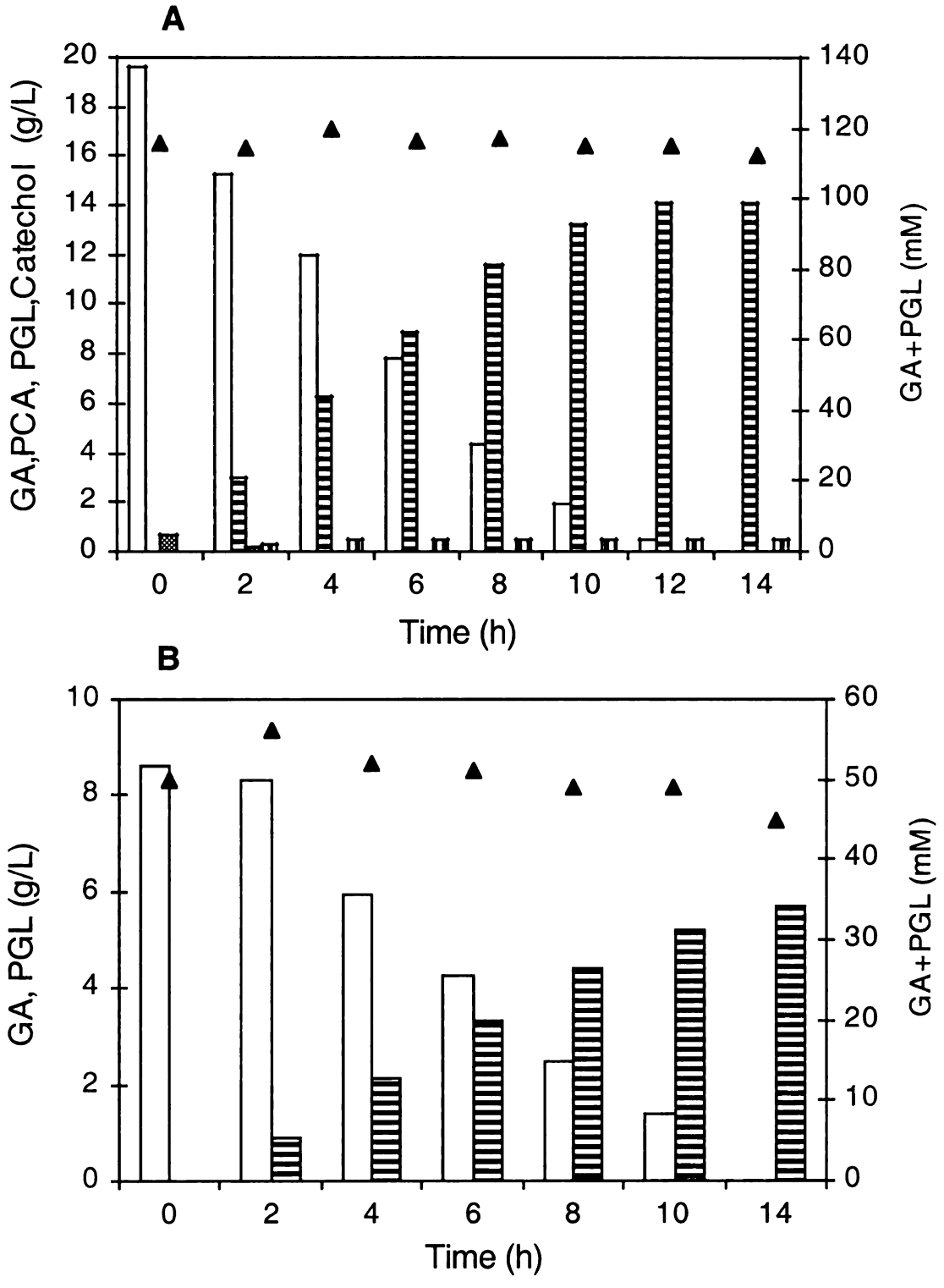


Figure 109. RB791*serA::aroB*/pSK6.234. PGL formation using cells cultured under fed-batch conditions; A. Supplementation of pure GA; B. Supplementation of GA-containing fermentation supernatant. Time in the graphs is measured after the addition of the GA/PCA mixture. Gallic acid (▭), pyrogallol (▬), protocatechuic acid (▩) catechol (▥) and total GA and PGL concentration (▲).

All the GA-containing fermentation supernatants that were described in Chapter 5, contained small impurities of the gallic acid biosynthetic precursor protocatechuic acid. Decarboxylation of this molecule along with gallic acid, resulted in catechol contamination of pyrogallol. Catechol could be sublimed away from pyrogallol using a Kugelrohr apparatus at 62 °C under high vacuum, to afford pure pyrogallol (Yield 90%).

In the case of one-step synthesis of catechol from glucose loss of D.O. control and cessation of cell growth was observed after 18-24 h at catechol concentrations of 30-35 mM. No growth of *E. coli* was also observed when plated on petri dishes containing 2.5 mM of catechol.[20] These results suggest that catechol may be more toxic towards growing bacteria as opposed to bacteria that have reached the stationary phase of their growth. Indeed, addition of protocatechuic acid to bacteria cultured under fermentor conditions to stationary phase significantly increased catechol formation. After growing RB791*serA::aroB*/pSK6.234 to stationary phase at 24 h in a fermentor, 80 mL of an autoclaved solution containing 22 g of protocatechuic acid (pH adjusted to 6.5 with NH_4OH addition) was added all at once giving a concentration of 110 mM (17 g/L) in the fermentation broth. At the same time, aeration was terminated and the fermentor sparged with N_2 (0.5 L/L/min). Glucose addition was also terminated. Protocatechuic acid was decarboxylated with the formation of 89 mM (9.8 g/L, 82% mol/mol) catechol (Figure 110A). This concentration of catechol is almost three times higher than the levels of catechol established to be toxic towards growing *E. coli*.

Attempts were made to identify the upper limit of catechol concentrations tolerated by *E. coli*. While adding 35 g/L of protocatechuic acid in one portion yielded no catechol, addition of 17.5 g/L of protocatechuic acid at 24 h followed by addition of another 17.5 g/L protocatechuic acid at 28 h, resulted in 120 mM (13.2 g/L) catechol and 72 mM unreacted protocatechuic acid (Figure 110B). The yield for catechol formation was 53%, whereas 32% of the added protocatechuic acid was still present at the end of the run. Therefore, approximately 85% of the protocatechuic acid, which had been added, could be accounted

for as product catechol and unreacted protocatechuic acid. Purification of catechol followed the same procedure as that used for purification of pyrogallol. Extraction with EtOAc, decolorization with activated charcoal, and solvent evaporation gave an off-white powder. The total recovery of catechol from the fermentation broth was 92%.

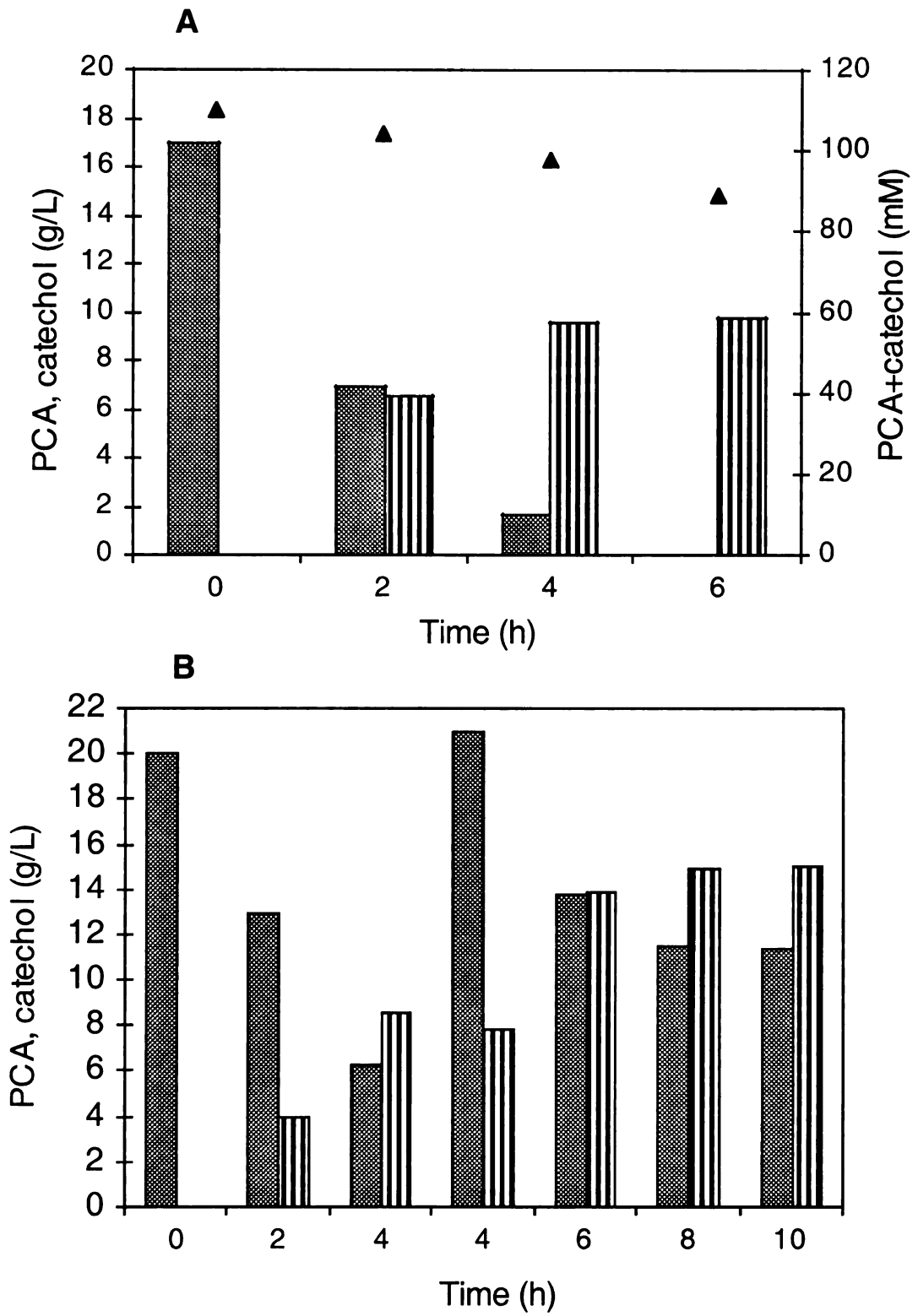


Figure 110. RB791*serA::aroB*/pSK6.234. Catechol formation using cells cultured on a fermentor; A. Addition of PCA in one portion; **B**. Addition of PCA in two portions. Time in the graphs is measured after the first PCA addition. In graph B the second 4 h point represents the concentration of substrates after the second PCA addition. Protocatechuic acid (▒▒▒), catechol (▥▥▥).

C. PGL and catechol formation using *Klebsiella oxytoca M5a1* bacteria

In the gallic acid decarboxylation reactions, cleaner pyrogallol product may be expected if the contaminating protocatechuic acid was metabolized by *K. oxytoca M5a1* as a result of this organism's ability to use protocatechuic acid as a sole source of carbon for growth. As discussed in Chapter 5, protocatechuic acid was always present in the gallic acid fermentation solutions and resulted in catechol contamination of product pyrogallol.

Initial protocatechuic acid decarboxylations using *K. oxytoca M5a1* utilized the standard conditions that were previously employed for *E. coli* biocatalysts. *K. oxytoca M5a1* was grown to stationary phase in a fermentor, before addition of a mixture (150 mL) of GA•H_2O (28 g) and PCA (1.5 g). No pyrogallol or catechol was observed after 6 h of reaction and the concentration of gallic acid and protocatechuic acid remained unchanged. Interestingly, protocatechuic acid was not metabolized, even though the glucose feed was terminated prior to addition of the GA/PCA mixture. Clearly, the fermentation conditions employed for *E. coli*-catalyzed decarboxylations were not appropriate for *K. oxytoca M5a1*.

K. oxytoca M5a1 cells cultured under fermentor conditions until maximum airflow was reached at 6 h, and then grown for 2 more hours to mid-log phase. The GA/PCA mixture was then added. Glucose addition was continued after GA/PCA addition to maintain an excess of glucose and the airflow was decreased to 0.5 L/L/min. Approximately 2-4 h later, D.O. levels begun to increase and could not be controlled by glucose addition. At this point, airflow was terminated and N_2 sparging initiated and maintained at 0.25 L/L/min until the end of the run. In a typical experiment gallic acid (114 mM, 19.4 g/L) and protocatechuic acid (12 mM, 1.8 g/L), were decarboxylated in 12 h to pyrogallol (105 mM, 13.2 g/L) and catechol (10 mM, 1.5 g/L) in 95% (mol/mol) yield (Figure 111). Purification of pyrogallol from the *K. oxytoca M5a1* solutions was identical to that as previously described for *E. coli*. Contrary to expectations, catechol accumulation

was not avoided, probably because of catabolic repression of protocatechuate metabolism by the excess of glucose (30-50 mM) always present.

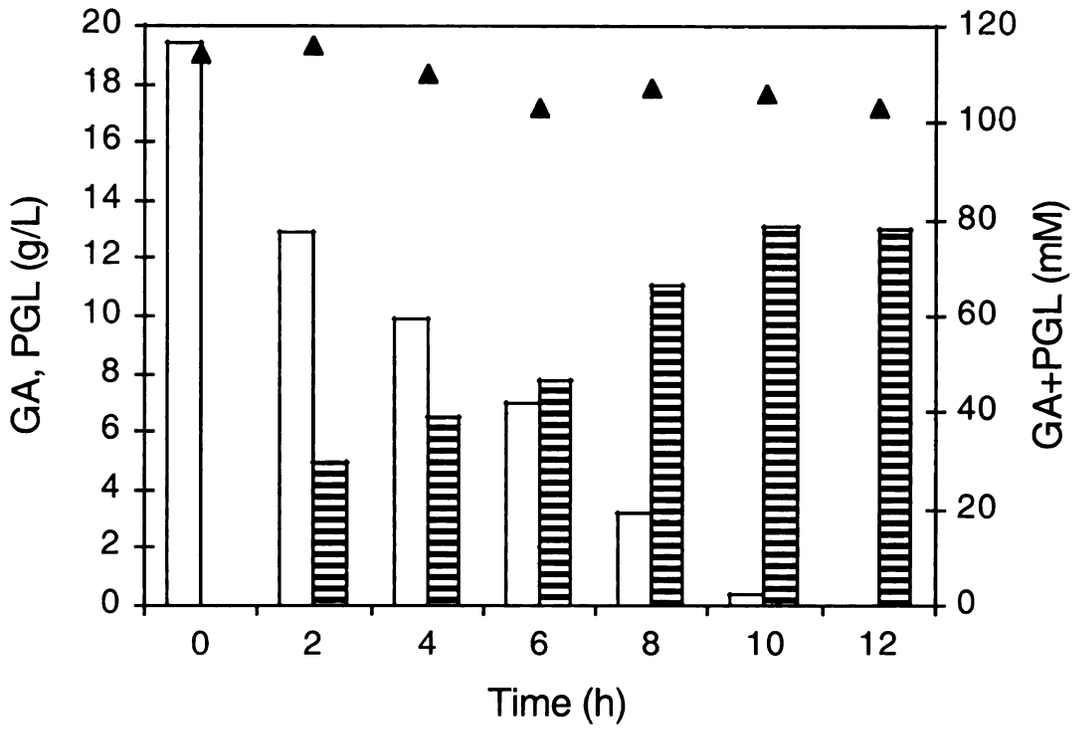
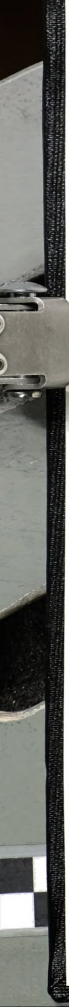


Figure 111. *Klebsiella oxytoca M5a1*. Decarboxylation of GA to PGL using bacteria cultured under fed-batch fermentor conditions. Time is measured from the addition of gallic acid. Gallic acid (▭), pyrogallol (▬) and total GA and PGL concentration (▲).

Under the fermentation conditions employed for gallic acid decarboxylation, an excess of glucose was always present. However, its rate of utilization by the microbe declined as the pyrogallol concentration increased. Growth inhibition was usually observed after 3-4 h after addition of the GA/PCA mixture. In experiments where the glucose feed was terminated before decarboxylation was finished (80 mM PGL, 25 mM GA, 160 mM glucose) at 7 h, complete decarboxylation by 14 h was observed without any further glucose consumption. In similar experiments where glucose was consumed by 3 h (50 mM PGL, 62 mM GA) as a result of one time addition, incomplete reaction was observed even after 19 h of reaction time (78 mM PGL, 34 mM GA) when no additional glucose was added.



Protocatechuic acid (12 mM) was not metabolized even under these glucose-limited conditions. It appears, that although glucose is not necessarily consumed in large amounts during decarboxylation, its presence is important for completing the reaction. In a typical run, around 70 g of glucose were consumed, a number almost identical to the 72 g that were consumed by *E. coli* for the same reaction.

Decarboxylation of protocatechuic acid by *K. oxytoca* opened the way for utilizing this microbe for protocatechuic acid decarboxylation. Using the same fermentation conditions employed for gallic acid decarboxylation, protocatechuic acid (124 mM, 19 g/L) was completely consumed to catechol (102 mM, 11.2 g/L) 8 h after its addition in a total yield of 90% (mol/mol) (Figure 112A). Identifying the maximum catechol tolerance by this organism was done after addition of one portion of protocatechuic acid at 8 h (80 mL, 22 g,) and a second portion at 12 h (80 mL, 19 g). Under these conditions, 15 g/L (136 mM) of catechol were formed after 8 h, in 61% (mol/mol) yield while 33.5 % of the added protocatechuic acid (74 mM, 11.4 g/L) remained unreacted (Figure 112B).

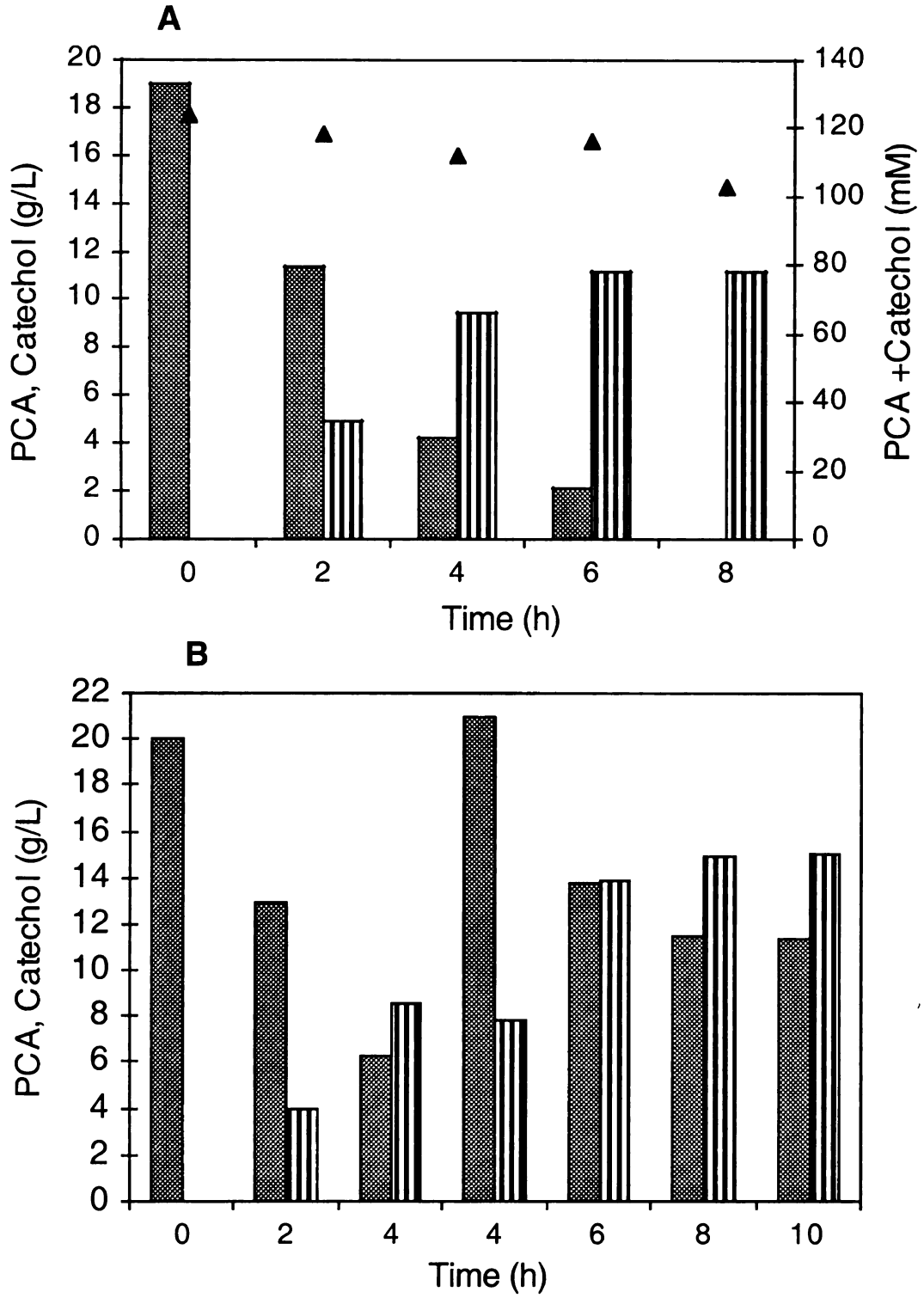


Figure 112. ***K. oxytoca M5a1*****. Catechol formation using intact cells cultured in a fermentor; A** Addition of PCA in one portion; **B**. Addition of PCA in two portions. The time in both graphs is measured from the first PCA addition. In graph B the second 4 h point represents the concentration of susbstrates after the second PCA addition. Protocatechuic acid (▒▒▒), catechol (▮▮▮▮).

III. DISCUSSION

In this Chapter, decarboxylation of protocatechuic acid and gallic acid into catechol and pyrogallol, respectively, using intact cells of either recombinant *E. coli* or wild-type *K. oxytoca M5a1* was discussed. The decarboxylation reaction was due to an *aroY*-encoded PCA decarboxylase gene that is native to *Klebsiella*. The *aroY* gene, which encodes PCA decarboxylase, was originally isolated from a genomic library of *K. pneumonae*.[20] Plasmid-localized *aroY* expressed in *E. coli* RB791*serA::aroB* successfully catalyzed the decarboxylation of both gallic acid and protocatechuic acid. Although this enzyme's primary substrate was protocatechuic acid, complete decarboxylation of gallic acid by recombinant *E. coli* expressing *aroY* from a plasmid was achieved. Using partially purified enzyme, the activity of AroY towards the decarboxylation of both gallic acid and protocatechuic acid has been previously established.[22] This substrate specificity was also illustrated from the difference on the reaction times of gallic acid versus protocatechuic acid using either biocatalyst (Table 25).

Table 25. Comparison of PGL and catechol production between recombinant *E. coli* bacteria and intact *k. oxytoca M5a1*.

Catalyst	Substrate	Product	Reaction Yield %	Reaction Time (h)	Titer (g/L)
E. coli	GA	PGL	95	14	14
	PCA	catechol	85	6	10 (13)[a]
K. oxytoca M5a1	GA	PGL	95	12	13
	PCA	catechol	90	8	11 (15)[a]

a. The number in parenthesis represents the maximum titer acheived as a result of PCA addition in two portions.

While catechol is industrially produced completely from petrochemicals (phenol, *o*-chlorophenol), pyrogallol's major production involves thermal decarboxylation of gallic

acid. The latter is produced from acid-catalyzed hydrolysis of Chinese gallnuts. An industrial synthesis of pyrogallol from cyclohexanone has also been utilized. The environmental factors associated with the chemical synthesis, as well as the utilization of non-renewable fossil fuel feedstocks to obtain the starting materials are important considerations in the chemical synthesis of both molecules. Even when the starting material is plant-derived gallic acid, such as in the major synthesis of pyrogallol, weather conditions and the amount of land that can be utilized for plant growth may cause availability limitations and price instability.

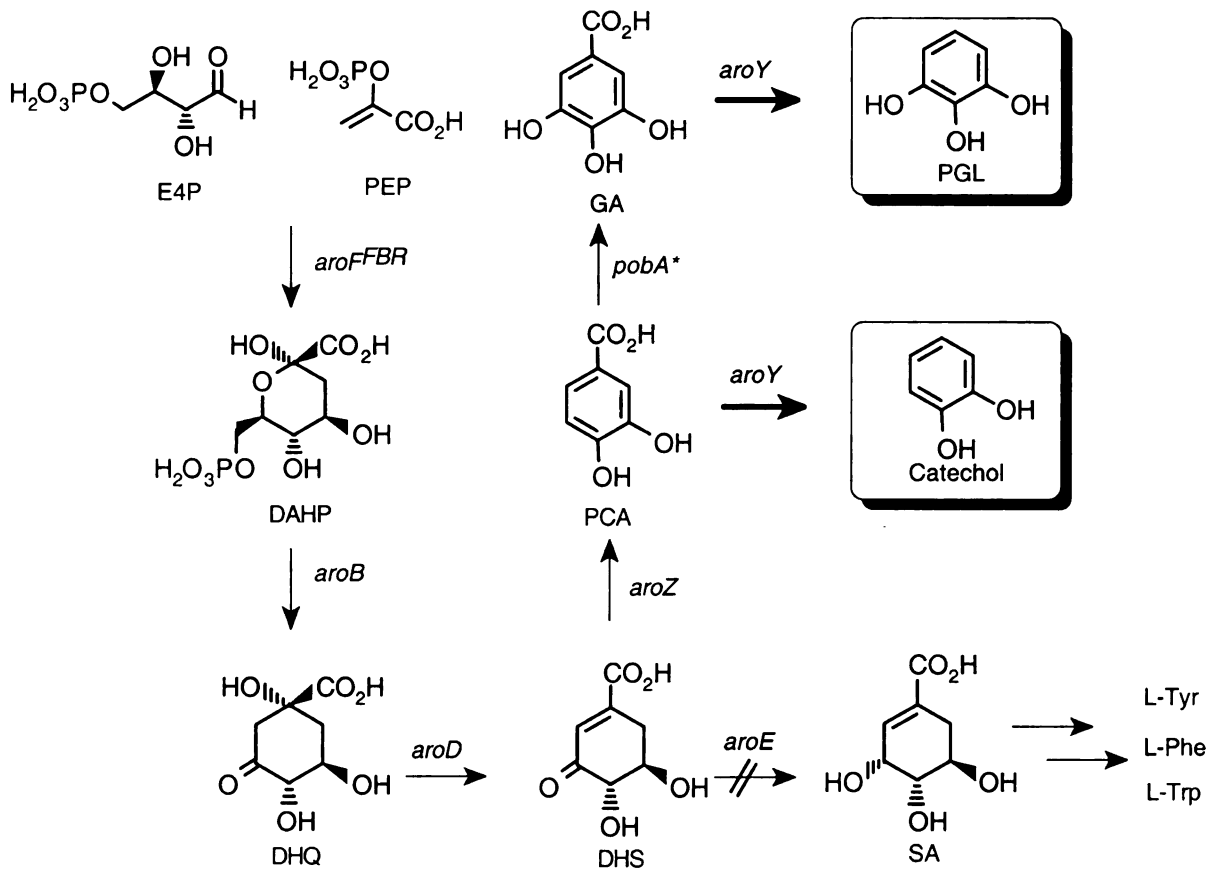


Figure 113. Biocatalytic synthesis of PGL and catechol from glucose

In this thesis, the construction of biocatalysts that produce both gallic acid (Chapters 3, 4, 5) and protocatechuic acid (Chapter 2) from glucose was discussed. Microbial-catalyzed decarboxylation of both these products, therefore provides a route for the

synthesis of pyrogallol and catechol with glucose being the ultimate starting material (Figure 113). The high-yielding, short reaction times, and mild reaction conditions required for decarboxylation are advantageous. Coupled with the high recoveries of products during purification, microbe-catalyzed synthesis is superior compared to the abiotic thermal decarboxylation of gallic acid.

Attempts to synthesize either pyrogallol or catechol in one step from glucose were unsuccessful for various reasons. A biocatalyst that could potentially synthesize pyrogallol was constructed after cloning the *aroY* gene in a construct capable of gallic acid biosynthesis. Accumulation of catechol instead of pyrogallol was observed (Figure 113), causing loss of the D.O. levels and inhibition of cell growth at a concentration of 31 mM of recombinant *E. coli* cultured under fermentor conditions. Catechol formation was the result of the initial accumulation of protocatechuic acid that is always observed in the GA-synthesizing biocatalysts (Chapter 5). The same loss of D.O. control and cessation of cell growth was observed at the same concentrations of catechol when a catechol-synthesizing biocatalyst was cultured under fed-batch fermentor conditions.

Catechol is toxic to growing *E. coli* at relatively low concentrations (30 mM) when it is synthesized in one step from glucose. On the other hand, concentrations of catechol as high as 120 mM accumulated in the supernatants of recombinant *E. coli* cultured under fed-batch fermentor conditions as a result of protocatechuic acid addition in bacteria that have already reached stationary phase. The difference in the intracellular concentration of catechol between these two different conditions might be one of the reasons for the observed different tolerance of *E. coli* for catechol. If *aroY* is a cytosolic protein, the rate of catechol formation inside the cell will be controlled by the rate of protocatechuic acid uptake, which as discussed in Chapter 4 is likely slow and proceeds via diffusion rather than by active transport. When catechol is synthesized in one step from glucose, its intracellular concentration is controlled by the rate of the enzymatic formation of protocatechuic acid. The rate of protocatechuic acid synthesis is controlled by the rate of carbon that is

channeled into the common pathway and in the formation of 3-dehydroshikimic acid. If *aroY* is a membrane protein, catechol will be forming extracellularly or during its export from the cell. Whatever the precise location of protocatechuic acid decarboxylase, synthesis of catechol in two steps gave product concentrations that were three to four times higher than the concentrations of catechol obtained when it was synthesized in one step from glucose.

In Chapter 4, recombinant *E. coli* was utilized to convert 3-dehydroshikimic acid and protocatechuic acid into gallic acid. In contrast to the decarboxylation reactions that were discussed in this Chapter, lower yields accompanied by unreacted starting material rendered these microbe-catalyzed conversions of little value. A chemical reaction that was developed in Chapter 3 proved to be a better method to synthesize gallic acid from 3-dehydroshikimic acid. In this Chapter, utilization of microbes to catalyze a reaction gave product in high yield and purity. These two different results clearly demonstrate the utility and limitations of microbes when employed as catalysts for chemical synthesis.

Figure 114. ^{1}H NMR of standard catechol. δ 6.85 (m, 2H), 9.95 (m, 2H) in D_2O.

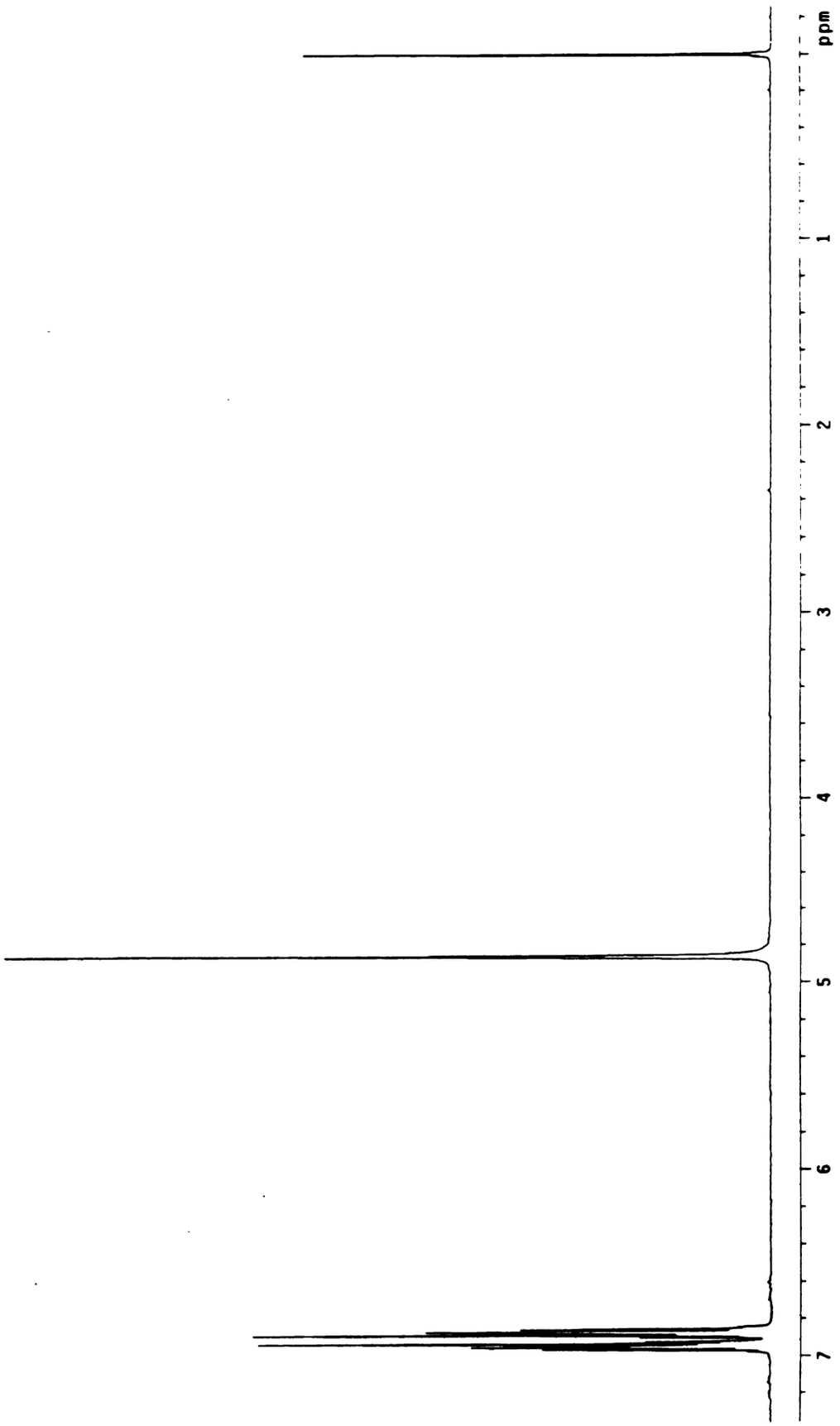

ppm
1
2
3
4
5
6
7

Figure 115. ^{1}H NMR of the fermentation supernatant of RB791*serA::aroB*/ pSK6.234. Pyrogallol formation after supplementation of the cells with pure gallic acid.

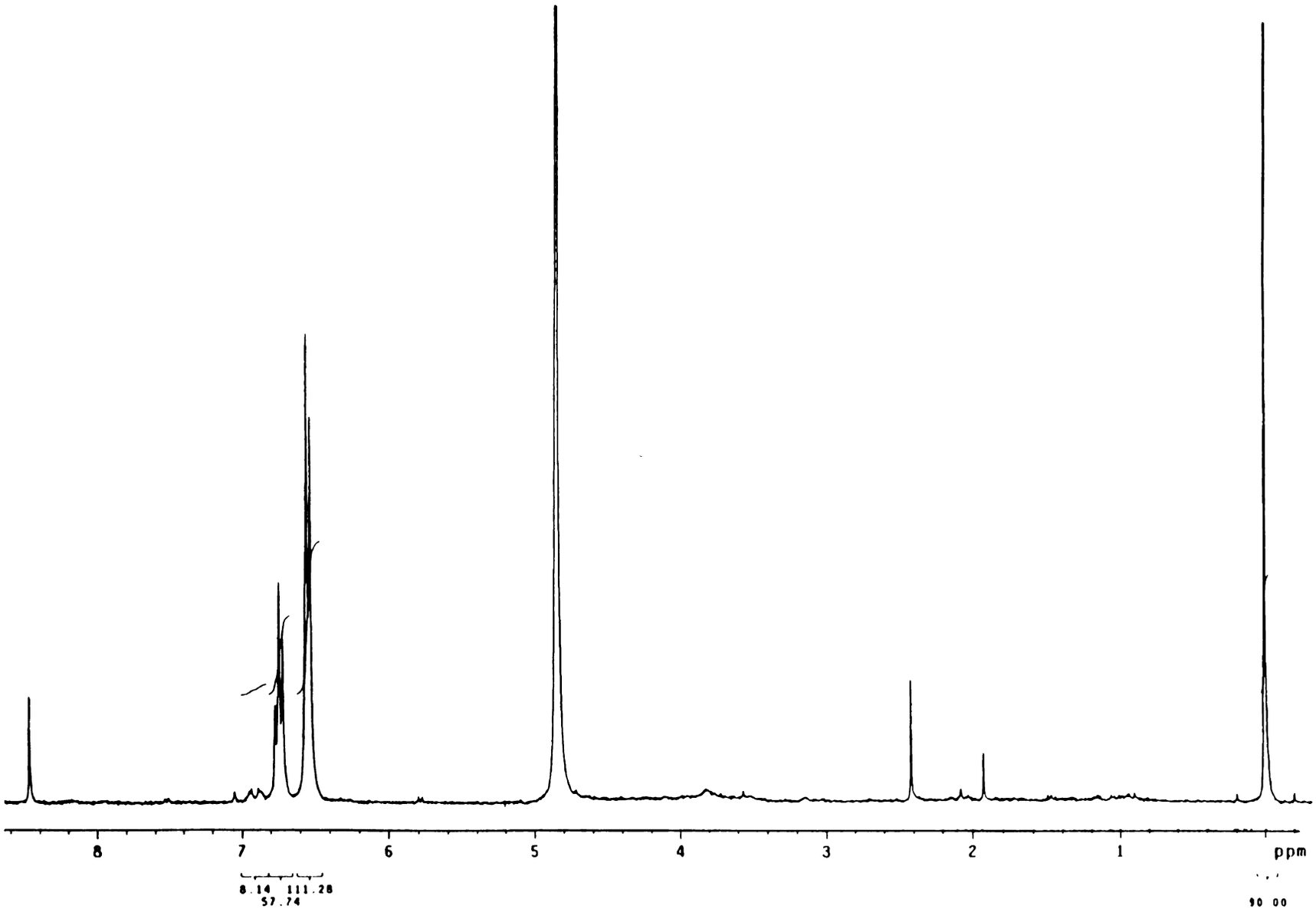

8
7
6
5
4
3
2
1
ppm
8.14
111.28
57.74
90 00

Figure 116. ^{1}H NMR of the fermentation supernatant of RB791*serA::aroB*/ pSK6.234. Catechol formation after supplementation of the cells with pure protocatechuic acid.

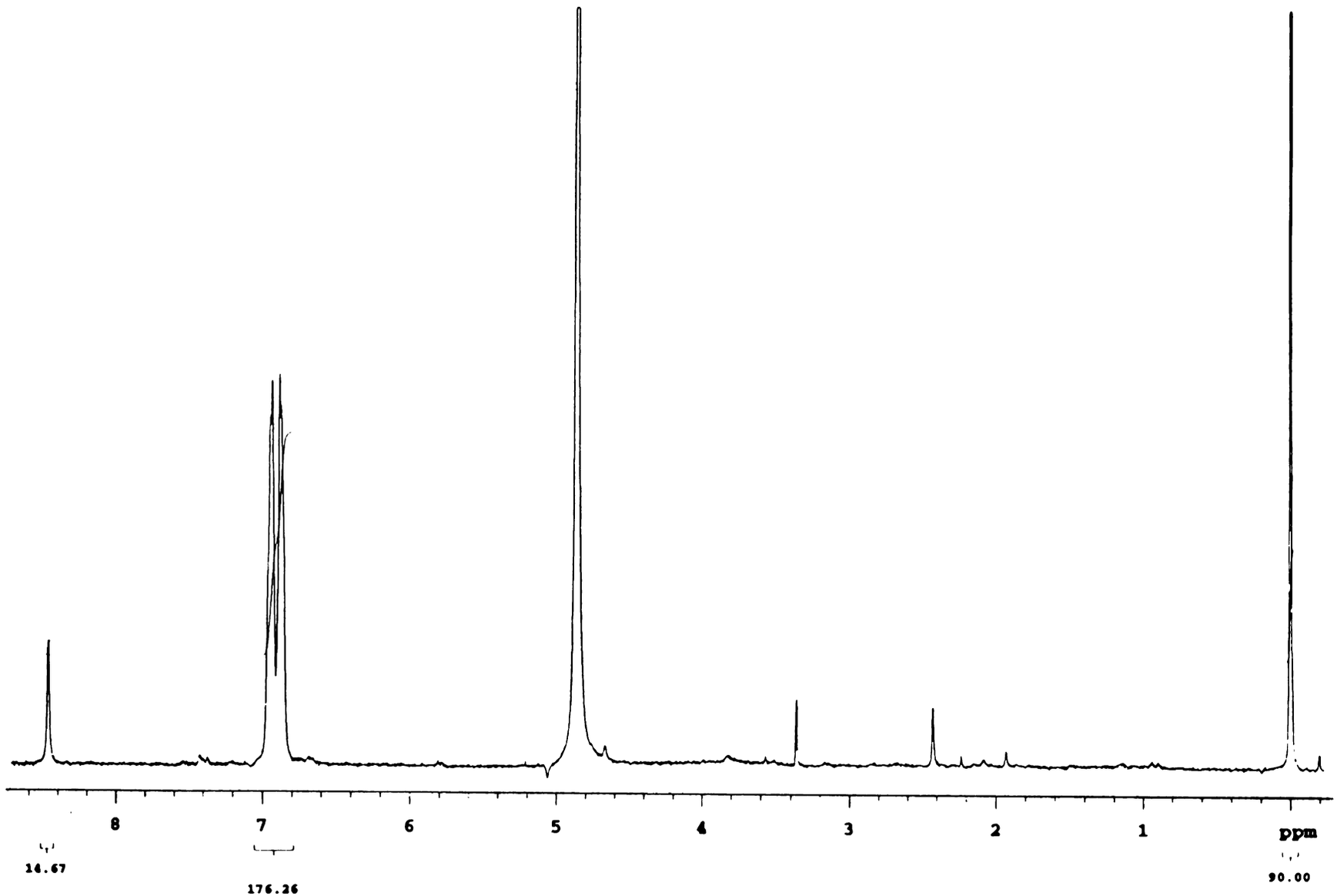

8
7
6
5
4
3
2
1
ppm
16.67
176.26
90.00

Figure 117. ^{1}**H NMR of the fermentation supernatant of** ***Klebsiella oxytoca M5a1*****. Pyrogallol formation after supplementation of the cells with pure gallic acid**

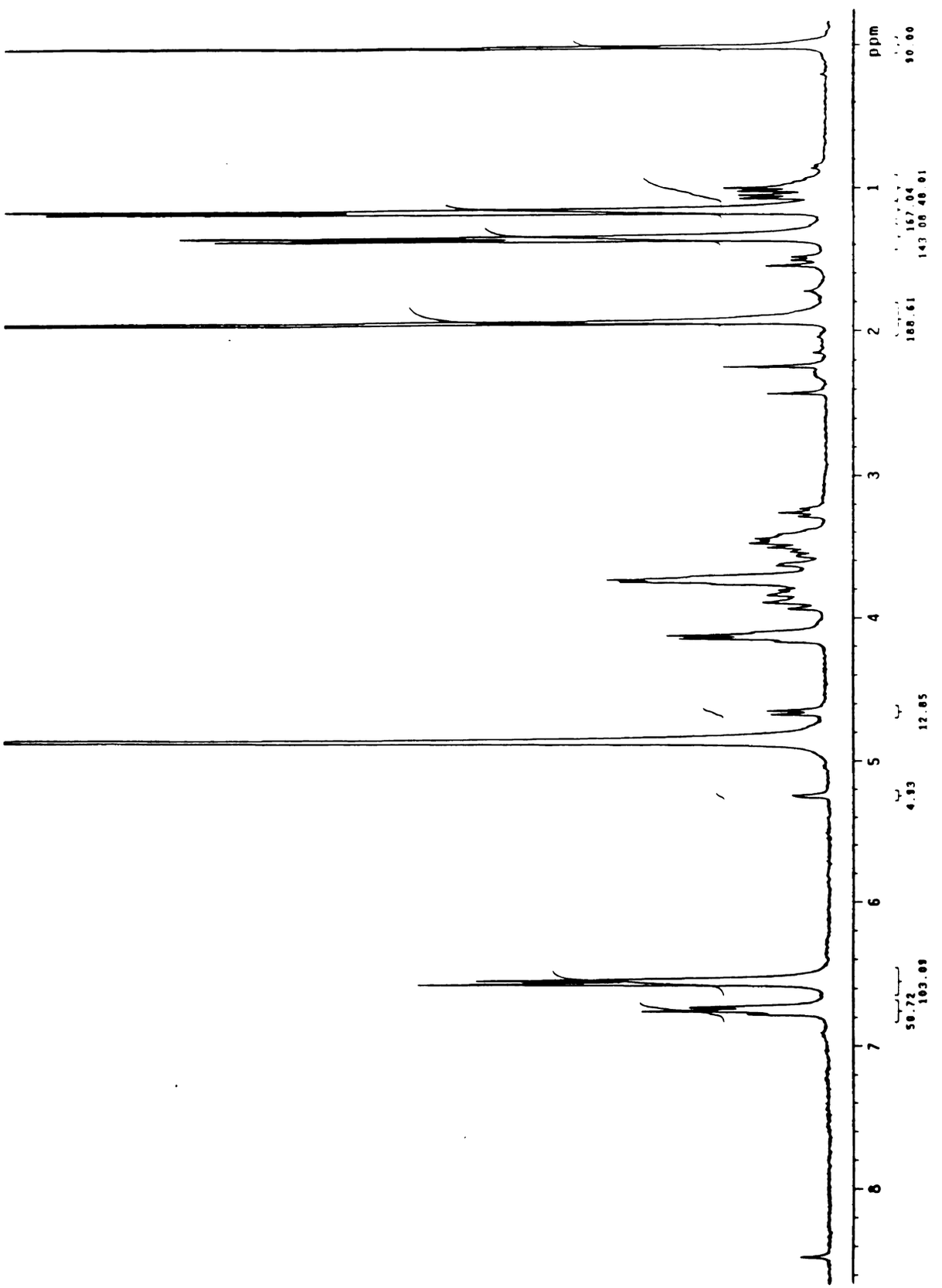

ppm
8
7
6
5
4
3
2
1
0
50.72
103.00
4.33
12.85
188.61
167.04
143.00
40.01
90.00

Figure 118. ^{1}H NMR of the fermentation supernatant of *Klebsiella oxytoca M5a1*. Catechol formation after supplementation of the cells with pure protocatechuic acid

ppm
8
7
6
5
4
3
2
1
0
1.00
204.33
51.50
94.46
127.68
79.32
26.58 25.93
90.00

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CHAPTER 7

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 μm, Rainin Microsrobe-MV™, 4.6 x 250 mm) was used for all HPLC separations. Anion and cation exchange resins AG1x8 and Dowex-50H were purchased from Bio-Rad.

Spectroscopic measurements

^{1}H 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 D_2O as solvent. TSP was purchased from Lancaster. UV and visible measurements were recorded on a Perkin-Elmer Lambda 3b UV-vis spectrophotometer connected to a R100A chart recorder or on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with HP 89532A UV-Visible Operating Software. IR spectra were obtained in a IR/42 Nicolet instrument.

Bacterial strains and plasmids

E. coli DH5α [*F' endA1 hsdR17($r^-K m^+K$) supE44 thi-1 recA1 gyrA relA1 φ80lacZΔM15 Δ(lacZYA-argF)U169*] and RB791 (W3110 *lacL8I^q*) were obtained previously by this laboratory. AB2834 [*tsx-352 supE42 λ$^-$ aroE353 malA352 (λ$^-$)*] and AB2848 [*aroD*352] were obtained from the *E. coli* Genetic Stock Center at Yale University. Plasmid pNE130 that carried the *Pseudomonas aeroginosa p*-hydroxybenzoate hydroxylase (*pobA*) wild type enzyme under the control of a *lac* promoter, was a pUC19 derivative which also posseses Ampicillin antiobiotc resistance. Plasmid pIE130 was also constructed in a pUC19 plasmid vector and carried the mutant (Tyr385→Phe) PHB hysroxylase (*pobA**) under the control of a *lac* promoter. Both pNE130 and pIE130 were obtained from Prof. David P. Ballou from the University of Michigan, Ann Arbor. Plasmid pHJD110 contained the open reading frame of *p*-hydroxybenzoate transporter protein (*pcaK*) from *Pseudomonas putida*, cloned after the *tac* promoter of a pKK223-3 plasmid. Plasmid pHJD110 was obtained from Prof. Caroline S. Harwood from University of Iowa, Iowa city.

Storage of bacterial strains and plasmids

All bacterial strains were stored at -78 °C in glycerol. Plasmids were transformed into DH5α 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 medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), and NaCl (0.5 g). M9 medium contained D-glucose (10 g), $MgSO_4$ (0.12 g), and thiamine (0.001 g) in 1 L of M9 salts. Solutions of inorganic salts, magnesium salts, and carbon sources were autoclaved separately and then mixed. Fermentation medium (1 L) contained K_2HPO_4 (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 H_2SO_4 (1.2 mL). The culture medium was adjusted to pH 7.0 by addition of concentrated NH_4OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: glucose (18 g, 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 $(NH_4)_6(Mo_7O_{24})\cdot 4H_2O$ (0.0037 g), $ZnSO_4\cdot 7H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4\cdot 5H_2O$ (0.0025 g), and $MnCl_2\cdot 4H_2O$ (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 μg/mL; ampicillin, 50 μg/mL; kanamycin, 50 μg/mL; tetracycline, 12.5 μg/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, L-tryptophan were added to M9 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 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 x *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.

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 30 °C as specified. pH was maintained at 7.0 by addition of concentrated NH_4OH or 2 N H_2SO_4. Dissolved oxygen

was measured using a Braun polarographic probe and was set at 20% or 10 % air saturation. Antifoam (Sigma 204) was pumped in manually as needed.

The fermentation supernatant was always inoculated with bacteria that were grown in minimal (M9) solution. Inoculants started by introduction of a single colony into 5 mL of either LB or M9 medium. When LB was utilized, 0.02 g glucose (100 μL of sterile 20 % w/v glucose solution) in addition to the appropriate antibiotic was included. When M9 was utilized, the appropriate antibiotic was also added to double guarantee plasmid maintenance. After growth for 12 h to 14 h for LB and 16-24 h for M9, 100 mL of M9 also containing the appropriate antibiotic was inoculated with 3 to 5 mL of the previous culture, and grew at 37 °C with agitation at 250 rpm for another 10-12 h before an adequate cells mass (OD_{600}=2-2.5) was reached. When LB was the starting growth medium, cells were resuspended to an equal volume of M9 medium prior to their addition into the 100 mL of M9 solution. For the data reported in Chapter 2, 4 and 5, inoculants were supplemented with L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid. When *Klebsiella oxytoca M5a1* was utilized as discussed in Chapter 6, cells grew at 30 °C without any aromatic amino acid or vitamin supplementation. Addition of amino acids and vitamins was also unnecessary in the inoculants of RB791*serA::aroB*/pSK6.234. However, a temperature of 36 °C was utilized. At this point cells were ready for transfer to the fermentor.

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% or 10% air saturation. In the fermentations discussed in Chapters 2, 4, and 6 cells grew under 20% dissolved oxygen set point. In Chapter 5, it was mainly 10% unless otherwise

stated. 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% (or 10%) 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 of 1 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% (or 10%) saturation by oxygen sensor-controlled substrate feeding. At the beginning of this stage, dissolved oxygen levels fell below the preset D.O. set point 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.

A different method to control the dissolved oxygen was utilized under fed-batch fermentor conditions. Increasing the impeller rate to 750 rpm and the airflow from 0.06 to 1 L/L/min was utilized to control the D.O. under the first two phases of growth in this case too. Throughout the whole time at phase three however, a continuous glucose feed was manualy maintained to ensure that 15 to 25 g/L of glucose excess are always present in the fermentation supernatant. During this time the 10% D.O. set point was maintained by varying the impeller rate. Since in the beginning of the phase three the impeller might not solely be capable to keep the D.O. in the 10% set point, the airflow was also

increased to 1.65 L/L/ min to keep the impeller rates lower than 1300 to1400 rpm. Baffles were also utilized under these conditions for more efficient oxygen transfer.

^{1}H NMR analysis of culture supernatants and reaction mixtures

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 (10 mM) of the sodium salt of 3-(trimethylsilyl)propionic-*2,2,3,3*-d4 acid (TSP). Concentrations of metabolites in the supernatant were determined by comparison of integrals corresponding to each metabolite with the integral corresponding to TSP (δ = 0.00 ppm) in the ^{1}H NMR. For the oxidation reactions using metals, an aliquote from the reaction mixture (0.5-1 mL) was filtered through a small pasteur pipette that contained ~0.2 mL of Dowex 50 H^+ resin to remove the dissolved metals. After solvent exchange with D_2O, product quantitication was done using ^{1}H NMR analysis using a known concentration of TSP (10 mM) as internal standard.

Genetic manipulations

General

Recombinant DNA manipulations generally followed methods described in Sambrook *et al.* 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 Na_2EDTA (pH 8.0). Endostop solution (10X concentration) contained 50% glycerol (v/v), 0.1 M Na_2EDTA, 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. Each reaction (0.1 mL) contained 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 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.* 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 Na_2EDTA (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 H_2O) 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 oC). 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 °C). The DNA was rinsed with 70% ethanol, dried, and then redissolved in 200-500 μL of TE.

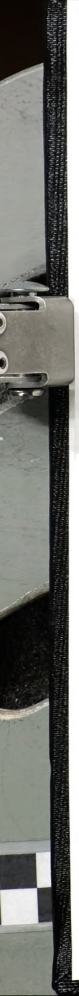
The concentration of DNA in the sample was determined as follows. An aliquot (10 μL) of the DNA was diluted to 1 mL in TE and the absorbance at 260 nm was measured relative to the absorbance of TE. The DNA concentration was calculated based on the fact that the absorbance at 260 nm of a 50 μg mL^{-1} 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 °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 μL 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 μg of DNA in 8 μL TE, 2 μL of restriction enzyme buffer (10X concentration), 1 μL of restriction enzyme, and TE to a final volume of 20 μL. Reactions were incubated at 37 °C for 1 h.



Digests were terminated by addition of 2.2 μL 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 μL of 0.5 M Na_2EDTA (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 μL), 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 $\mu g\ mL^{-1}$) 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 *Hin*dIII (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 *Eco*RI and *Hin*dIII (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

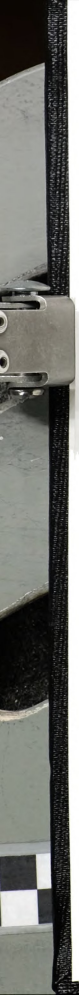
The band of agarose containing DNA of interest was excised from the gel and chopped thoroughly with a razor in a plastic weighing tray. The agarose was then transferred to a spin column consisting of a 500 μL microfuge tube packed tightly with glass wool and with an 18 gauge hole in its bottom. The spin 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 μL). To this sample was added 10 μL of dephosphorylation buffer (10X concentration) and 2 μL 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 μL of 0.5 M Na_2EDTA (pH 8.0) followed by heat treatment (65 °C, 20 min). The sample was extracted with phenol and SEVAG (100 μL 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



μg) restriction digestion was completed in a 20 μL reaction, a solution (1 μL) 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.* 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 $CaCl_2$. 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 °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 °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 °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 was washed once with the same minimal medium. After resuspension in fresh minimal medium (0.1 mL), the cells was 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. 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 °C, 250 rpm) for 12 h. Centrifugation (4000 x *g*, 5 min, 4 °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 °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.

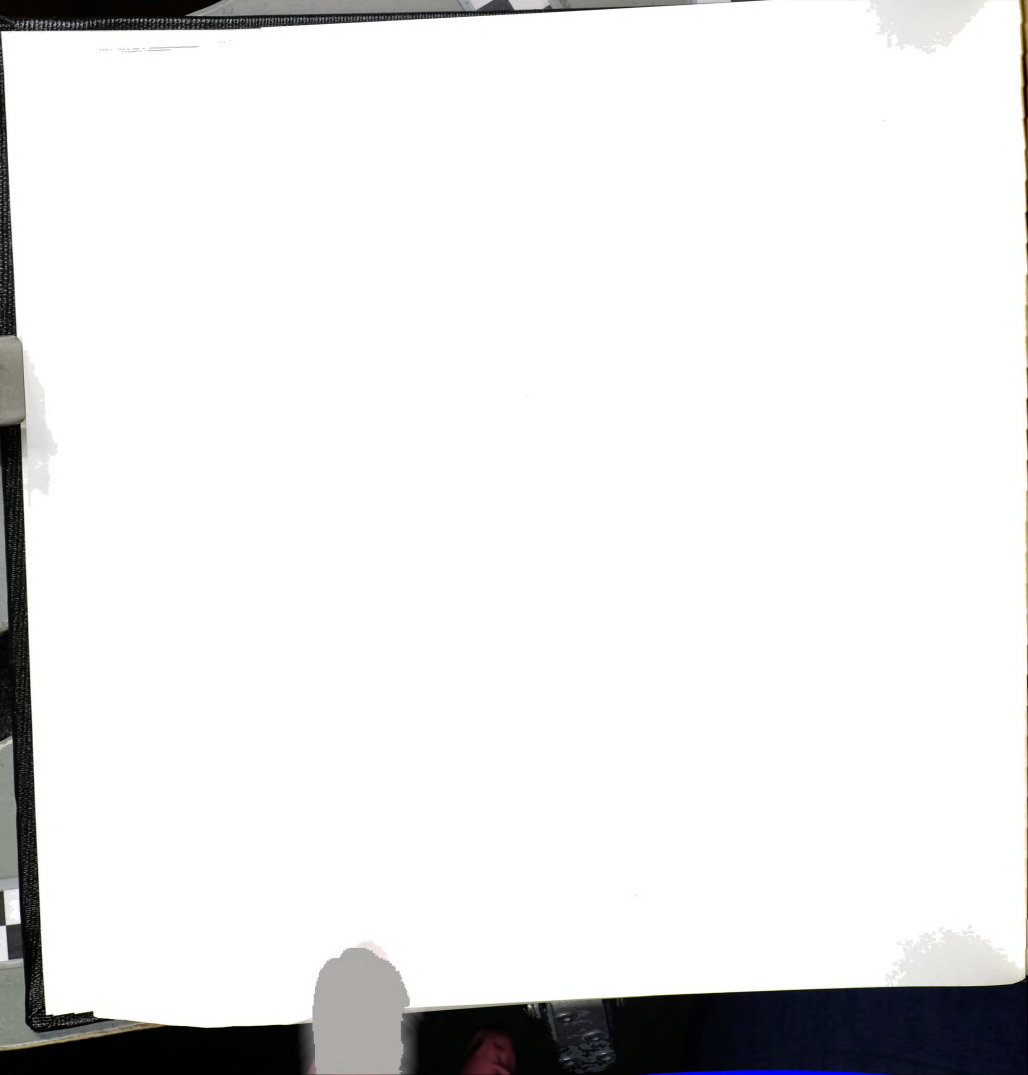
Genomic DNA was precipitated by addition of 0.1 volume of 3 M NaOAc (pH 5.2), gentle mixing, and addition of 2 volumes of 95% ethanol. Threads of DNA were spooled onto a sealed Pasteur pipette and transferred to a Corex tube that contained 5 mL of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), and 1 mg 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). 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. 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 assay 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 (ε = 68000 L mol^{-1} cm^{-1}). One unit of DAHP synthase activity was defined as the formation of 1 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 temperature. The molar extinction coefficient for protocatechuic acid at 290 nm is 3890 L mol^{-1} cm^{-1}.

PHB hydroxylase

PHB hydroxylase activity was assayed by measuring the protocatechuate-dependent when the mutant was assayed, or the *p*-hydroxybenzoate-dependent when the wild type enzyme was assayed, oxidation of NADPH at 340 nm in air saturated buffer at 25 °C. Harvested cells were resuspended in 100 mM Tris-Base (pH 8.0), EDTA (0.8 mM) solution. Cell disruption and protein concentrations were done as described above. Enzyme activity was calculated using a continuous assay of NADPH loss by measuring the absorbance at 340 nm. The assay was performed in 1 mL solutions by mixing 0.4 mL of the resuspension buffer (Tris-Base 100 mM, EDTA 0.8 mM, pH 8.0), 0.4 mL H_2O, 0.1 mL diluted enzyme, 0.05 mL protocatechuic or *p*-hydroxybenzoic acid (30 mM, pH 8), 0.025 mL NADPH (10 mM), and 0.025 mL FAD (0.5 mM). The enzyme was diluted in the same buffer used for resuspension, and the usual dilution required to obtain

detectable activity ranged from 1:5 to 1:10. One unit of enzyme was the amount that oxidized 1μmol of NADPH per minute under the assay conditions

Hydroperoxidase I

Since hydroperoxidase I is both a catalase and a peroxidase enzyme two different assay methods were possible. One was based in direct measurement of loss at 240 nm as a result of H_2O_2 decomposition, whereas the other relied in the oxidation of *o*-dianisidine in the presence of H_2O_2 and enzyme. Oxidized dianisidine absorbs at 460 nm (ε_{460}=11300 L M^{-1} cm^{-1}). The latter peroxidase activity was utilized for activity measurements in our case. Harvested cells were resuspened in a 100 mM $NaHPO_4$ buffer pH, 6.0. The assay was performed in 1 mL solution which was prepared by mixing 0.34 mL H_2O, 0.5 mL buffer (100 mM, NaOAc, pH, 5.5), 0.01 mL H_2O_2 (1M), 0.05 mL *o*-dianisidine (10 mM) and 0.1 mL enzyme lysate. One unit of enzyme was the amount that oxidized 1μmol of *o*-dianisidine or 2 μmol of H_2O_2 per minute under the assay conditions.

Biocatalytic synthesis of protocatechuic acid and 3-dehydroquinic acid

Fermentation process was performed as described previously for the glucose –starving conditions. The initial glucose concentration in the fermentation medium was 22 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 280 mL of water. The dissolved oxygen set point was 20 % and it was controlled by the glucose feeding. No baffles were used.

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, 3-dehydroshikimic acid or 3-dehydroquinic acid components in the supernatant were quantitated by ^{1}H NMR using an internal standart of sodium 3-(trimethylsilyl) propionate (10 mM). 0.5 or 1 mL of fermentation supernatant was used for solvent exchange and ^{1}H NMR analysis. 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.

Purification of protocatechuic acid

Fermentation broth that containing PCA (19.4 g/L) was acidified to pH, 2.5 - 2.8 with addition of conc. H_2SO_4. Some protein precipitated in this pH and was removed by centrifuging the medium for 10 min at 13000 x g. Extraction of 500 mL of this fermentation supernatant (126 mM, 9.7 g) with EtOAc (4x, 200 mL each time) gave a

yellow solution containing only PCA. After concentration of the organic solvent to 100 mL (8.5 g PCA), addition of 200 mL pertoleum ether and chilling at 4 °C for 10 min, precipitated an off white powder consisting only of PCA (7.7 g) that was isolated after filtration (Yield 79 %). This purification applies in both the one and two step PCA synthesis. A charcoal step can be introduced after the extraction to decolorize the solution especially in PCA that is produced using the dehydration reactions. In those experiments the PCA-containing EtOAc solution prior to its concentration was stirred with 3-5 g of activated charcoal (NoritA G-60, 100 mesh) for 5 min and was filtered through a celite containing filter. PCA is not adsorbed in the charcoal under these conditions. ^{1}H NMR (D_2O) for PCA was: δ, 6.9 (d, 1H), 7.4 (m, 2H).

Purification of 3-dehydroquinic acid from the fermentation broth

Purification of 750 mL of fermentation containing 3-dehydroquinic acid (260 mM, 37 g) begun with acidification to pH, 3 with H_2SO_4 and extraction with EtOAc (x2 200 mL) to remove the aromatic acids (GA, PCA). After addition of glacial AcOH (250 mL) in this solution, some residual protein was precipitated and removed by centrifugation (13000 x g, 10 min). This mixture was then filtered through 400 mL of strong anion exchange AG1x8 resin acetate form, and the column washed with another 400 mL of a H_2O/AcOH (v/v, 3/1) mixture. This step removed the inorganic phosphate which was adsorbed by the column, while DHQ was eluted (1.6 L, 110 mM DHQ, 33.2 g). All the metals and positively charged species were removed after filtration through 400 mL of strong cation exchange resin Dowex-50 (H^+). At the end a light yellow solution is obtained containing only DHQ (100 mM, 1.7 L, 32.2 g). Evaporation of the

solvent mixture gave a viscous DHQ containing oil. ^{1}H NMR (D_2O) for 3-dehydroquinic acid : 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).

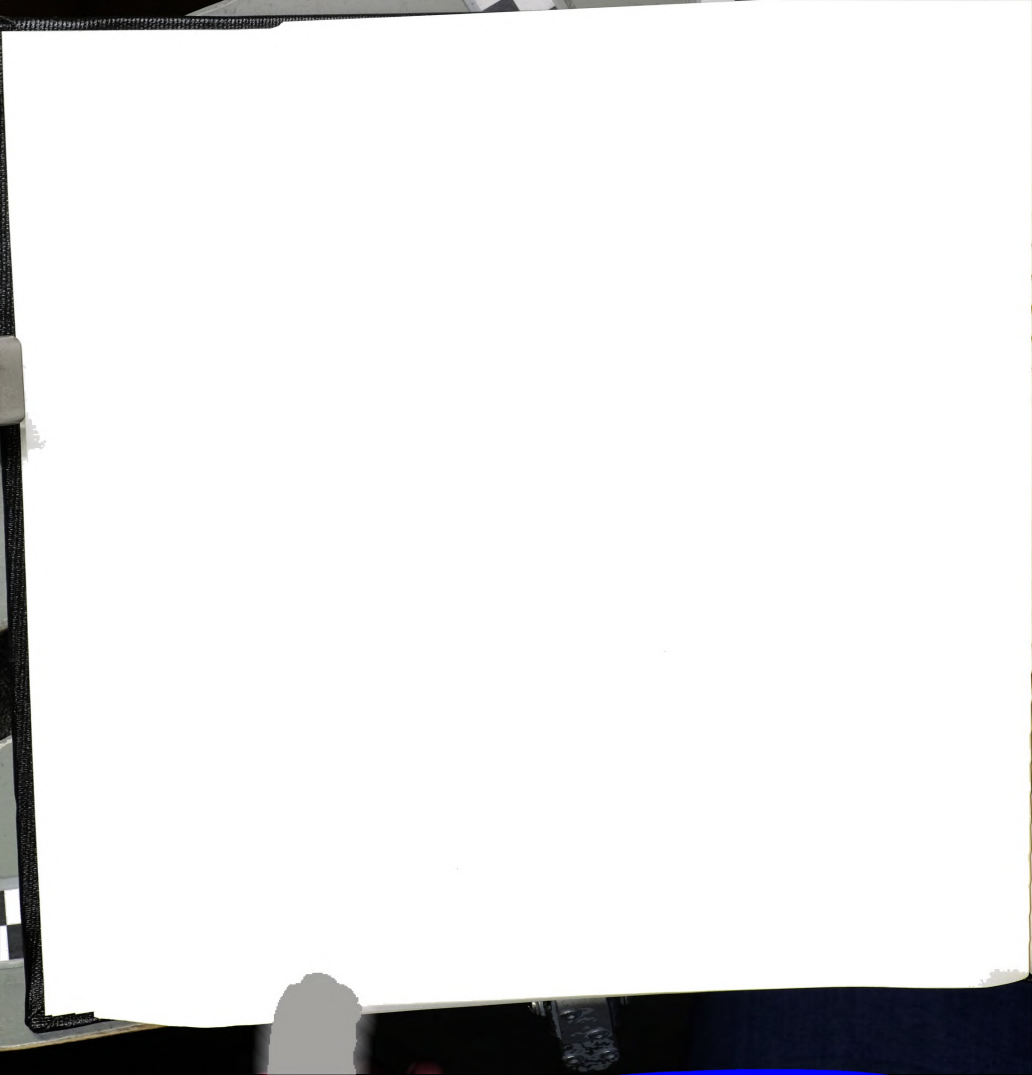
Thermal dehydration of 3-dehydroshikimic acid and 3-dehydroquinic acid

Fermentation supernatants (150-500 mL) containing either 3-dehydroshikimic acid or 3-dehydroquinic acid were filtered through an Amicon membrane to remove the soluble protein, and then refluxed under air or N_2 for 2 – 22 h depending on the reaction conditions. This filtration does not appear to affect the reaction outcome since untreated solutions gave very similar PCA product titers. In the reactions under N_2 atmosphere, the fermentation solution was bubbled with N_2 while stirring for 10 min, and continued for the whole reaction. The reaction progress was monitored by either removing 0.5 – 1 mL of reaction broth and analyzing it with ^{1}H NMR spectroscopy after solvent exchange with D_2O, or by TLC chromatography (EtOAc/Hex, v/v, 2/1)

Plasmid construction

Plasmid pSK7.54

Plasmid pD2625 was digested with *Eco*RV and *Dra*I, and the 1.9 kb *serA*-containing fragment was digested into a *Sma*I/CIAP treated site of pSK4.122 thus forming plasmid pSK7.54 (7.4 kb).



Plasmid pSK7.105

Plasmid pKL4.66B was digested with *Hin*dIII, treated with Klenow fragment to create blund ends and ligated with the 1.9 kb *serA*-containing fragment to create pSK7.105 (8.7 kb). The *serA* insert was isolated from pD2625 after double digestion with *Dra*I/*Eco*RV.

Liquid-Liguid extraction of DHS from culture supernatant

KL3/pKL4.79B was cultured under fed-batch fermentor conditions as described in this thesis (D.O. 20%, glucose limited conditions). Fermentation broth was centrifuged (3000 x g for 10 min) to remove cells and the resulting culture supernatant (500 mL) containing DHS (15.5 g, 0.18 M) acidified to pH 2 with addition of concentrated HCl. Precipitated protein was removed by centrifugation (13000 x g for 10 min). The resulting black-colored solution was then stirred in a continuous liquid-liquid extraction apparatus at a rate to create a translucent colloidal suspension of EtOAc in the aqueous culture supernatant while allowing the colloidal suspension to separate into a clear organic phase at the top of the extraction cylinder. DHS-containing EtOAc was replaced with fresh EtOAc (400 mL) at 2, 5, 8 and 12 h. After the 2 L of DHS-containing EtOAc was filterd through 10-15 g of Darco G-60 (100 mesh) activated charcoal and dried over $MgSO_4$, the yellow-colored solution was concentrated to a volume of 50-60 mL. Chilling this concentrated solution in a ice bath led to the precipitation of DHS as an off-white powder (10.5 g, 68%).

Resin-Based isolation of DHS from culture supernatant.

KL3/pKL4.66A was cultured under fed-batch fermentor conditions as described in this thesis (D.O. 20%, glucose-limited conditions). Fermentation broth was centrifuged (3000 x g for 10 min) to remove cells and the resulting culture supernatant (900 mL) containing DHS (17 g, 0.11 M) acidified to pH 2 with addition of concentrated HCl.

Precipitated protein was removed by centrifugation (13000 x g for 10 min). The resulting black-colored solution was adjusted to pH 5 with the addition of NaOH and then applied to a column containing 400 mL of AG-1 X8 (AcO^-). After washing the column with H_2O (600 mL) and MeOH (600 mL), decolorized DHS (14.5 g, 85%) was selectively eluted with glacial AcOH (500 mL). The acetate form of the resin was regenerated by eluting with NaOH (0.1 N) followed by elution with $HOAc/H_2O$ (v/v, 1/1).

Reaction of DHS with O_2 in phosphate-buffered H_2O

DHS (1 g, 5.81 mmol) was dissolved in 30 mL of 1.0 M $Na_{1.5}H_{1.5}PO_4$ and the solution adjusted to pH 6.7. The reaction was then stirred for 50 h exposed to the atmosphere at 40 °C. At the end of the reaction, 10% of the DHS remained unreacted. Products consisted of gallic acid (14%), protocatechuic acid (12%), tricarballylic acid (14%), and pyrogallol (3%). Extraction (2x, 15 mL each) with EtOAc to remove pyrogallol was followed by acidification of the solution to pH 3.6 and extraction (3x 20 mL each) with EtOAc. Drying of the organic layer and concentration gave a solid (0.26 g) containing of GA and PCA. Chromatography using octadecyl-functionalized silica gel (86 mL) eluted with CH_3OH/H_2O (1/9, v/v, adjusted to pH 2.7) afforded pure gallic acid (0.13 g, 13%).

Reaction of DHS with H_2O_2 in phosphate-buffered H_2O

DHS (1g, 5.81 mmol) and H_2O_2 (2 wt%, 10 mL) were dissolved in 20 mL of 1.0 M $Na_{1.5}H_{1.5}PO_4$ and the solution was adjusted to to pH 6.7. After 36 h reaction at 40 °C, 5% of the DHS starting material remained unreacted. The crude reaction product

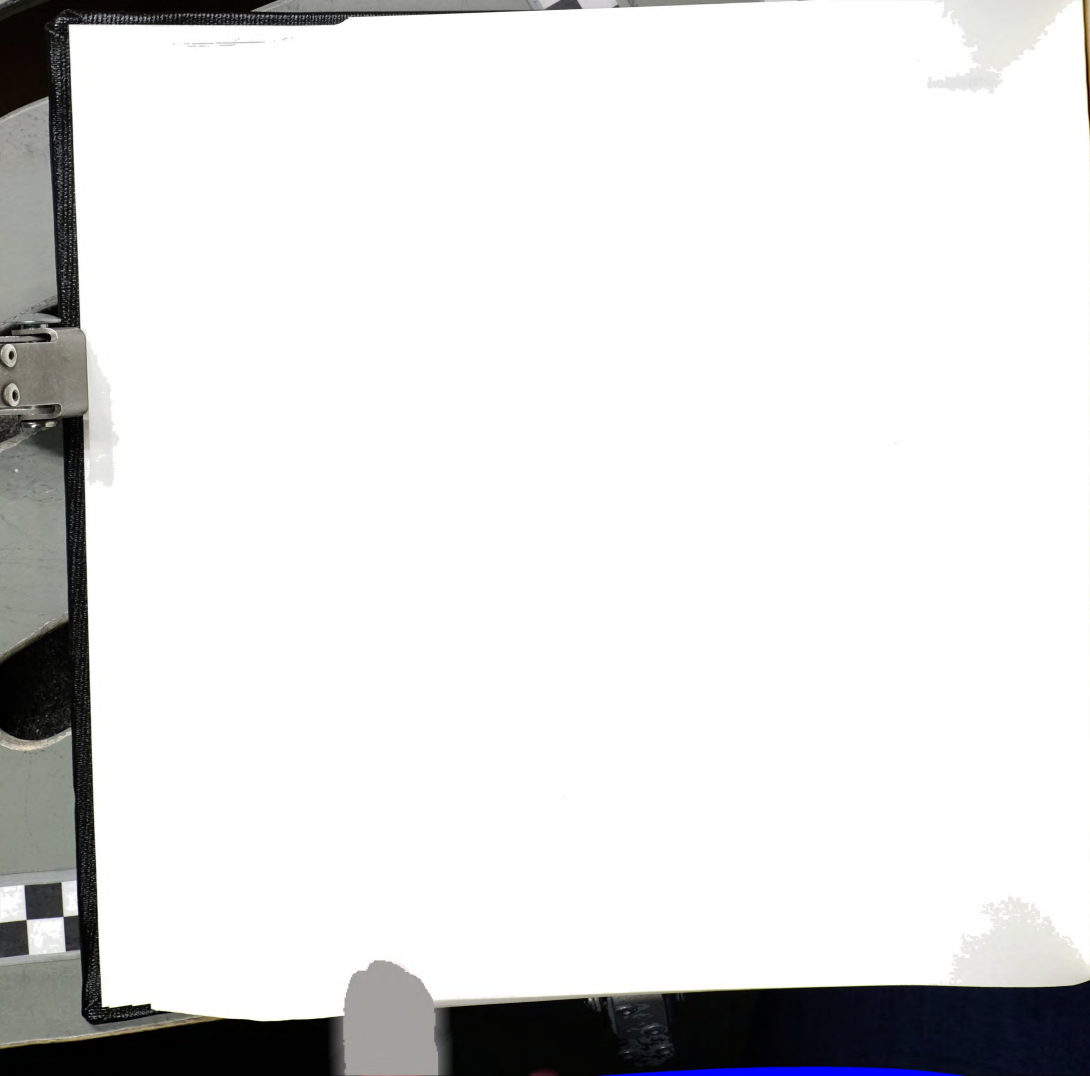
consisted of gallic acid (22%), protocatechuic acid (10%) and tricarballylic acid (2%). After acidification to pH 3, the reaction was extracted (3x, 20 mL each) with EtOAc. Drying of the organic layer and concentration gave a solid (0.33 g) consisting of GA and PCA. Chromatography using octadecyl-functionalized silica gel (86 mL) eluted with CH_3OH/H_2O (3/7, v/v, adjusted to pH 2.8) afforded pure gallic acid (0.18 g, 18%).

$CuSO_4$-Catalyzed oxidation of DHS by H_2O_2 in phosphate-buffered H_2O

DHS (1 g, 5.88 mmol), $CuSO_4 \cdot H_2O$ (0.0147 g, 0.0588 mmol), $NaH_2PO_4 \cdot H_2O$ (4.14 g, 30 mmol) and Na_4HPO_4 (4.26 g, 30 mmol) were dissolved in 49 mL of H2O. After adjusting the solution to pH 6.6, 11 mL of a 2% solution of H_2O_2 (6.40 mmol) was added and the reaction stirred at 40 °C for 35 h. The reaction solution was then adjusted to pH 2.5 by addition of H_2SO_4, extracted with EtOAc (4x 50 mL) and concentrated to a solid consisting of gallic acid (36%) and protocatechuic acid (3%). Chromatography on octadecyl-functionalized silica gel (H_2O/MeOH, v/v, 9/1 adjusted to pH 2.5 with AcOH) provided gallic acid (0.31 g, 31%), which was free of protocatechuic acid contamination.

$FeSO_4$-Catalyzed oxidation of DHS by H_2O_2 in phosphate-buffered H_2O

DHS (1 g, 5.88 mmol), $FeSO_4 \cdot 7H_2O$ (0.016 g, 0.0588 mmol), $NaH_2PO_4 \cdot H_2O$ (4.14 g, 30 mmol) and Na_4HPO_4 (4.26 g, 30 mmol) were dissolved in 49 mL of H_2O. After adjusting the solution to pH 6.6, 11 mL of a 2% solution of H_2O_2 (6.40 mmol) was added and the reaction stirred at 40 °C for 39 h. The reaction solution was then adjusted to pH 2.5 by addition of H_2SO_4, extracted with EtOAc (4x 50 mL) and concentrated to a solid consisting of gallic acid (36%) and protocatechuic acid (9%). Chromatography on



octadecyl-functionalized silica gel (H_2O/MeOH, v/v, 9/1 adjusted to pH 2.5 with AcOH) provided gallic acid (0.18 g,, 31%), which was free of protocatechuic acid contamination

Oxidation of DHS by $CuCO_3 \cdot Cu(OH)_2$ in phosphate-buffered H_2O

E. coli AB2834/pMF63A was cultured in shake flasks as described in the general methods section of this thesis and cells removed from the culture broth by centrifugation (3000 x g for 10 min) to provide a culture supernatant (1 L) containing DHS (7/9 g, 46 mmol). $KH_2PO_4 \cdot H_2O$ (68 g, 500 mmol) and K_2HPO_4 (87 g, 500 mmol) were then added and the culture supernatant adjusted to pH 6.5. Addition of $CuCO_3 \cdot Cu(OH)_2$ (20.3 g, 92 mmol) resulted in a heterogeneous solution, which was vigorously stirred at 50 °C for 5 h. After filtration to remove insoluble coper salts, the solution was acidified to pH 2.5 with addition of concentrated H_2SO_4 and extracted with EtOAc. Concentration of the organic layer gave a red solid, which was dissolved in 150 mL of H_2O/MeOH (9/1, v/v) and filtered through octadecyl-functionalized silica gel. Octadecyl-functionalized solica gel was purchased from Aldrich and activated prior to use by elution with MeOH. AG-1 X8 (AcO^-) anion exchange resin and Dowex 50 (H^+) cation exchange resin were purchased from Bio-Rad. Concentration of the filtrate to 30 mL resulted in formation of a precipitate. Filtration and drying afforded 4.1 g of an off-white solid consisting of gallic acid (51%) and protocatechuic acid (2%).

Oxidation of DHS by $Cu_X(H_{3-X}PO_4)_2$ in phosphate-buffered H_2O.

E. coli AB2834/pMF63A was cultured in shake flasks as described in the general methods of this thesis and cells removed from the culture broth by centrifugation.

KH_2PO_4 (13.5 g) was added to a portion (100 mL) of this culture supernatant containing DHS (0.79 g, 4.6 mmol) and the solution adjusted to pH 6.6 by addition of NaOH. The $Cu_X(H_{3-X}PO_4)_2$ oxidant was prepared by dissolving $CuSO_4$ (1.6 g, 10 mmol) in 50 mL of H_2O followed by adition of$NaHPO_4$ (2.2 g, 16 mmol). The blue-colored precipitate that formed was recovered by filtration and then added to the reaction solution. This heterogeneous solution was stirred under N_2 at 50 °C for 12 h. After filtration to remove the insoluble copper salts, the reaction solution was acidified to pH 2.2 with addition of H_2SO_4 and extracted with EtOAc (3x 60 mL). Concentration of the organic layer aforded 0.35 g of a lightly yellow-colored solid consisting of galic acid (43%) ad protocatechuic acid (2%).

Oxidation of DHS by $Cu(OAc)_2$ in AcOH

A solution of DHS (5.4 g, 31.4 mmol) and $Cu(OAc)_2$ (13.5 g, 67.5 mmol) in 400 mL AcOH/H_2O (3/1 v/v) was stirred at 40 °C under N_2 for 36 h. After filtration to remove the insoluble copper salts, the reaction solution was concentrated to almost gryness and then dissolvde in H_2O (300 mL). This aqueous solution was extracted with EtOAc (4x 100 mL), and the organic layer concentrated. The resulting brown solid consisting of gallic acid (74%) and protocatechuic acid (0.7%) was dissolved in H_2O/MeOH (9/1, v/v) and decolorized by filtration through octadecyl-functionalized silica gel. During concentration of the filtrate, gallic acid precipitated and was recovered as an off-white solid (3.3 g, 62%). Recovery and recycling of $Cu(OAc)_2$ began with combining the filtered copper salts with the aqueous layer after removal of gallic acid by EtOAc extraction. This mixture was reacted overnight with 10 mL of 30% H_2O_2 at rt.

Addition of solid NaOH resulted in precipitation of $Cu(OH)_2$, which was recovered by filtration. Stirring the $Cu(OH)_2$ in AcOH for 2 h led to a homogeneous solution that after concentration and drying gave 12.5 g of $Cu(OAc)_2$ (93%) indistinquishable by IR from the $Cu(OAc)_2$ initially used to oxidize DHS in the culture supernatant.

Catalytic reaction of DHS with $Cu(OAc)_2/NH_3NO_4$.

DHS (1 g, 5.88 mmol), $Cu(OAc)_2$ (0.588 g, 2.94 mmol) and NH_3NO_4 (1.4 g, 1.3 mmol) were dissolved in a mixture of $AcOH/H_2O$ (40 mL, v:v, 4:1). After stirring at 40 ^oC for 22 h, the solution was concentrated to 10 mL. Addition of H_2O (40 mL) followed by extraction with EtOAc (x4, 50 mL each) gave after solvent evaporation a brown powder consisting of GA and PCA (0.44 g, GA/PCA: >48/1). Pure GA (0.38g) as a yellow powder is obtained after recrystalization of that powder from water.

Catalytic reaction of DHS (1 M) with Cu^{+2} and Zn^{+2} (5% and 25%, mol/mol relative to DHS) in $AcOH/H_2O$.

Pure DHS (10.5 g, 61.04 mmol) that was extracted from a fermentation broth using the continuous extraction method described earlier, was dissolved in 61 mL of $AcOH/H_2O$ (v/v, 6/1) giving a concentrated (1 M) solution of DHS. After addition of $Cu(OAc)_2$ (0.61 g, 3.05 mmol) and ZnO (0.995 g, 12.2 mmol), the homogenuous mixture was stirred at 50 ^oC for 4 h under bubbling oxygen giving GA (67%) and PCA (3%) as the only products. The solution was concentrated to 30 mL, chilled in ice bath, and a black solid was collected after filtration consisting of GA, PCA and probably some metal salts. The black solid was redisolved in H_2O (250 mL, pH 2.8-3) and extracted with

EtOAc (4x, 150 mL each). Concentration of the organic layer containing GA to 200 mL addition of 200 mL of petroleum ether (bp=35-60 ^{o}C) gave and chilling in ice bath gave pure GA (5.5 g, 53% yield) as an off white to yellow powder after and filtration. Further decolorization of the product was achieved by dissolving it in H_2O/MeOH (300 mL, v:v, 9:1, pH 2.5) and filtering through octadecyl functionalized silica gel (70 mL). Concentration of the GA solution to 20-30 mL and chilling in ice bath gave pure GA (5.1 g) as an off-white powder (93% recovery after the column).

Catalytic reaction of DHS (1M) with Cu^{+2} and Zn^{+2} (10% and 50% mol/mol relative to DHS) in AcOH/H_2O.

Fermentation broth (KL3/pKL4.66A) containing DHS (0.11 M) was utilized for the isolation of DHS using an AG1 X8 anion exchange resin according to the method described earlier. An AcOH (500 mL) solution containing DHS (14.5 g, 84.3 mmol) was eluted from the column. After addition of 150 mL of AcOH and 150 mL H_2O (AcOH/H_2O, v/v 8/2) to create a DHS concentration of 1 M, $Cu(OAc)_2$ (1.63 g, 8.13 mmol) and ZnO (3.31 g, 40.6 mmol) were dissolved and the mixture was heated at 50 ^{o}C for 15 h under bubbling air. At the end of the reaction, the solution was concentrated under reduced pressure to a final volume of ~30 mL, water was added (300 mL, pH 3), and the homogeneous mixture was extracted with EtOAc (4x, 250 mL each). Evaporation of the organic solvent gave a yellow-red powder consisting only of GA (73%) and PCA (4%).

Catalytic oxidation of DHS with Cu^{+2} and Zn^{+2} (5% and 50% mol/mol relative to DHS) in straight acetic acid.

Fermentation broth (KL3/pKL4.66A) containing DHS (0.11 M) was utilized for the isolation of DHS using an AG1 X8 anion exchange resin according to the method described earlier. An AcOH solution (1.3 L) containing DHS (11.4 g, 66.3 mmol) was eluted from the column. After addition of $Cu(OAc)_2$ (0.66 g, 3.32 mmol) and ZnO (2.7 g, 33.1 mmol) the homogeneous mixture was stirred at 50 °C for 11 h under bubbling air. The reaction mixture was then concentrated to 50 mL and 350 mL of H2O were added to the mixture. Extraction with EtOAc (4x, 200 mL each) and concentration of the organic layer to dryness gave a red solid (9.1 g) consisting only from GA (77%) and PCA (3%). The solid was then dissolved in 350 mL of H_2O/MeOH (v/v, 9/1) and filtered through octadecyl-functionalized silica gel (40 mL). This step removed all the color and an off-white powder (7.7 g) consisting only of GA (67%) was filtered after the solution was concentrated to 60 mL, acidified with H_2SO_4 addition, and chilled in ice-bath.

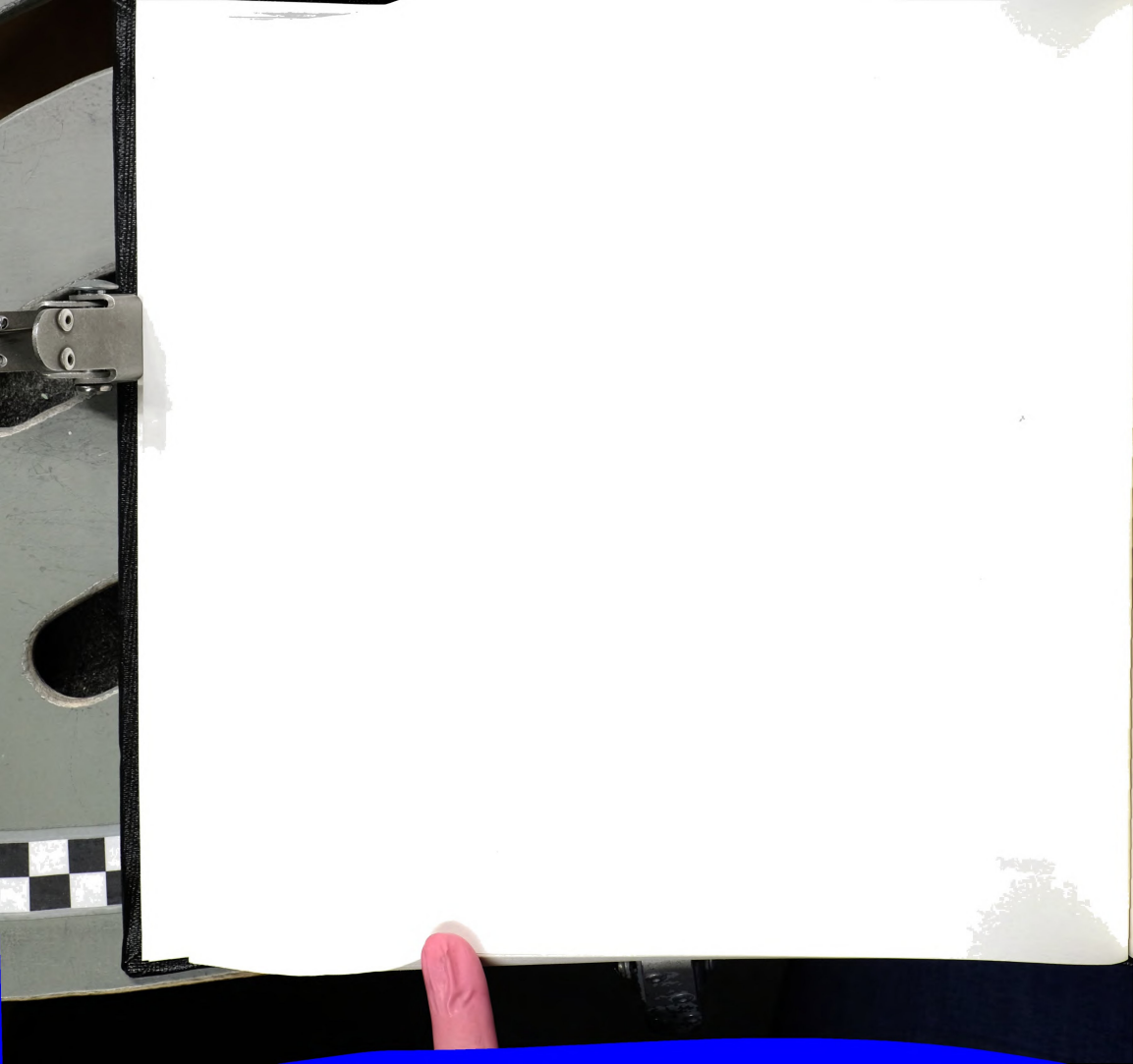
Zn^{+2}, Mg^{+2}, Mn^{+2} acceleration of Cu^{+2}-catalyzed DHS oxidations.

DHS (0.1 g, 0.58 mmol) and $Cu(OAc)_2$ (0.012 g, 0.058 mol) were dissolved in 24 mL of AcOH/H_2O (85/15, v/v) containing ZnO (0.23 g, 0.29 mmol), MgO (0.012 g, 0.29 mmol), or MnO (0.29 g, 0.29 mmol). Rection progress at 50 °C under either air or O2 was determined by withdrawing 0.1 mL aliquots from the reaction solution at timed intervals, diluting with AcOH, and measuring the absorbance at 298 nm for each time interval. To verufy that the absorbance at 298 nm corresponded to the concentration of gallic acid and protocatechuic acids, 1 mL aliquots of the reaction solution at various time intervals were filtered through a pipet containing 0.3 mL of Dowex 50 (H+) to remove

metals. These solutions were concentrated, dissolved in D_2O, a known amount of TSP added, and the concentration of gallic acid and protocatechuic acid determined by ^1H NMR.

Reactions of 3-Dehydroquinic Acid, 3-Dehydroshikimic acid And Glucose Under Catalytic Cu^{+2}/Zn^{+2} Conditions

In 30 mL of $AcOH/H_2O$ mixture (v/v, 4.2/1) 3-dehydroquinic acid (0.260 g, 1.48 mmol) along with $Cu(OAc)_2$ (0.029 g, 0.148 mmol) and ZnO (0.06 g, 0.74 mmol) were dissolved, and the reaction was stirred under bubbling of oxygen at 50 °C for 12 h. At the end of the reaction the mixture consisted of DHQ (14%), gallic acid (26%) and protocatechuic acid (2.9%) as calculated by ^1H NMR analysis of the reaction mixture. When 3-dehydroshikimic acid or glucose reacted under identical conditions, all the starting material was recovered even after 24 h of reaction time.



Fed-batch fermentation conditions

The fermentation process was performed as described previously. The initial glucose concentration was 23 g/L in all cases. The glucose-starving conditions were utilized, where the dissolved oxygen (20% set point) was controlled by the rate of the added glucose. However two different glucose concentrations were utilized depending on the supplementation method of PCA or DHS. When these supplements were added at one portion at the early log phase (10-12 h), the previously described 60% (w/v) glucose solution was prepared. Under these conditions, DHS (10.3 g, 60 mmol) was dissolved in 50 mL H_2O, the pH was adjusted to 6.9 with KOH (5 M) and filter-sterilized prior to its addition to the fermentation supernatant. When PCA was supplemented, 9 g (59 mmol) were suspended in 50 mL H_2O, autoclaved for 8 min (all PCA is now dissolved) and while the solution was still warm (~50-60 °C) the pH was adjusted to 6.8 with NH_4OH, and immediately added in the growing bacteria.

However, when DHS or PCA were supplemented continuously with the glucose feed, the feeding solution was prepared as follows: Glucose (130 g) was added in 110 mL of water to produce a 200 mL mixture which was then autoclaved. Just before the controlled glucose supplementation at the beginning of phase three, 16 g of PCA (0.10 mol) or 18 g of DHS (0.1 mol) were dissolved in water and the pH was adjusted to 6.8 with NH_4OH addition (final volume 100 mL). The DHS solution was filter-sterilized and added to the 200 mL glucose feed, whereas the PCA solution was autoclaved for 7 min

prior to its addition to the 200 mL of glucose. As needed, IPTG was added every 6 h, starting from the time of DHS or PCA supplementation, and continued every 6 h.

Samples (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 (0.5-2 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.

Plasmid construction

Plasmid pSK4.176

The open reading frame (ORF) of the mutant *p*-hydroxybenzoate hydroxylase was cloned in the same orientation after the tac promoter in the *Eco*RI site of plasmid pKK223-3 creating plasmid pSK4.176 (5.8 kb). The ORF of *pobA** (1.0 kb) was isolated by PCR amplification using plasmid pIE130 as the template. The primers were designed to contain terminal *Eco*RI sequences as follows: 5-GGAATTCCATGAAGACTCAA GTCGCC and 5-GGAATTCCGCCGTCTTTCGTCGATAT. This plasmid also contains a copy of the $lacI^Q$ repressor gene and also carries ampicilin resistance.

Plasmid pSK4.232A,B

Digestion of pSK4.176 with *Bam*HI generated a 1.5 kb P_{tac}*pobA** containing DNA fragment which was then ligated into the *Bam*HI/CIAP treated site of pKL4.71 creating pSK4.232A and pSK232B (7.9 kb). Plasmids pSK4.232A and B differ in the orientation of the cloned *pobA** gene relative to $aroF^{FBR}$. In addition while pSK4.232B contains a unique *Sma*I site that can be used for additional blunt-end cloning, pSK4.232A regenerates the 1.5 kb P_{tac}*pobA** fragment after *Sma*I digestion.

Plasmid pSK4.99

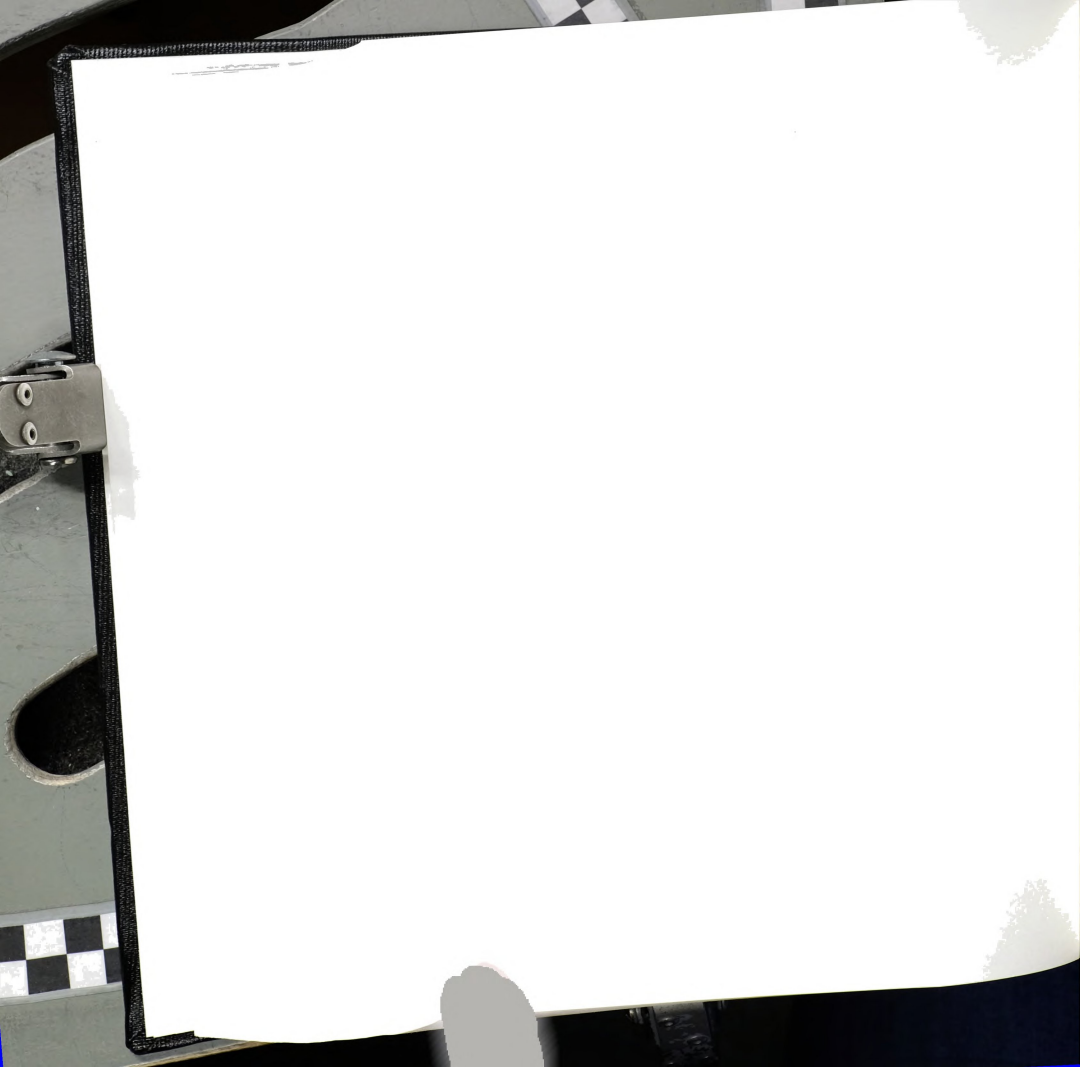
Ligation of the *aroZ* gene into the *Bam*HI site of pSU18, generated plasmid pSK4.99 (4.4 kb). The 2.2 kb *aroZ* fragment containing its native promoter, was isolated by PCR amplification using plasmid pSU1-28 as the template. Primers were designed to contain terminal *Bam*HI sequences as follows: 5-CGGGATCCGCGCATACACATGC and 5-CGGGATCCGGG TACAGAGGGTGTTGT.

Plasmid pSK6.70

Digestion of pSK4.232A with *Sma*I generated a 1.5 kb P_{tac}*pobA** containing fragment that after ligation to pSK4.99, created pSK6.70 (5.9 kb). The vector pSK4.99 was prepared for ligation after *Sma*I digestion and dephosphorelation with CIAP.

Plasmid pSK6.76

Plasmid pSK6.76 (7.8 kb) was prepared after ligation of the 1.9 kb *serA* containing DNA, into pSK6.70. The vector was prepared for cloning after *Hin*dIII



digestion and treatment with the Klenow fragment followed by treatment with CIAP. The *serA* fragment was isolated from pD2625 after *Dra*I/*Eco*RV digestion.

Plasmid pSK4.122

Plasmid pSK4.122 (5.5 kb) was created by ligating the $aroF^{FBR}$ gene into an *Xba*I site of pSK4.99. The 1.1 kb fragment of $aroF^{FBR}$ was isolated from pKL4.66A after digestion with *Xba*I.

Plasmid pSK4.141

Insertion of the $lacI^{Q}$ fragment in the *Eco*RI/CIAP treated site of pSK4.122 created plasmid pSK4.141 (7.5 kb). The $lacI^{Q}$ 2.0 kb insert was isolated by PCR amplification using pKL4.71 as the template. Primers were designed to contain *Eco*RI terminal sequences as follows: 5-CGGAATTCATTTACGTTGACACCATCG 5-CGGAATTC TTAATTGCGTTGCGCTCAC.

Plasmid pSK4.259

Inserting $P_{tac}pobA$* into a *Sma*I/CIAP treated site of pSK4.141 created pSK4.259 (9.0 kb). The 1.5 kb fragment of $P_{tac}pobA$* was isolated from plasmid pSK4.232A after digesting with *Sma*I.

Plasimd pSK6.68

Double digestion of pSK4.259 with *Bam*HI and *Hin*dIII, generated a large 5.7 kb DNA fragment that contained $P_{tac}pobA$* and $lacI^{Q}$, along with the 2.2 kb *aroZ* and the 1.4

kb *aroF*^{FBR} genes. Gel purification of the large fragment and treatment with CIAP, gave after ligation with the 1.8 kb P_{tac}*pcaK*-containing DNA fragment, plasmid pSK6.68 (10.8 kb). The P_{tac}*pcaK* fragment was isolated from pHJD110 after a *Bam*HI/*Hin*dIII double digestion.

Plasmid pSK6.77

Ligation of the 1.9 kb *serA*-containing DNA fragment into the SmaI/CIAP treated site of plasmid pSK6.68 created plasmid pSK6.77 (12.7 kb). The *serA* insert was isolated from pD2625 after *Eco*RV/*Dra*I double digestion.

Plasmid pSK6.118

Digesting pSK6.68 with *Bam*HI gave a 4.8 kb vector fragment containing P_{tac}*pobA** and *lacI*^{Q} along with the 1.8 kb P_{tac}*pcaK* gene. Gel purification of the large fragment, treatment with the Klenow fragment, and ligation with *serA* (*Eco*RV/*Dra*I fragment) afforded plasmid pSK6.118 (7.6 kb).

Fed-batch fermentation conditions

All fermentations were performed as previously described. With the exception of KL7/pSK6.161, all other biocatalysts were grown under the standard glucose-starving conditions where under constant impeller speed (940 rpm) and airflow (1 L/L/min), the dissolved oxygen was maintained at 10% air saturation, by oxygen sensor-controlled glucose feed (60 % w/v, glucose). The impact of the fermentation process on the product titers and yields was demonstrated after taking the best GA producer, KL7/pSK6.161, and growing it under three different sets of fermentation conditions. Air saturation of 20% as well as 10% was utilized in the glucose-starving conditions, while the 10% D.O. set point was used when the organism grew under the glucose rich conditions. In the latter case, under constant airflow and feeding rate, the D.O. was maintained at 10% air saturation by varying the impeller rate. For more efficient oxygen transfer to the liquid medium, baffles were included in the fermentor. The general procedure of those fermentations has been previously described. Glucose assays were performed every 2 to 6 h to ensure that a glucose excess of 15-25 g/L was always present in the fermentation supernatant. The glucose assays were performed using the glucose (HK) 20 kit obtained from Sigma.

Samples (5 mL) of fermentation supernatant were taken at timed intervals and analyzed for product formation using ^{1}H NMR spectroscopy, while cell density was measured by the absorbance of 100-times diluted cells at 600 nm (OD_{600}). Enzyme activities were assayed as previously described. At certain time points 15 mL of

fermentation broth (per assay) was harvested and the cells were resuspended in the appropriate buffer. In the case of DAHP synthase, the resuspended whole cell mixture were stored at –80 °C before they were assyed all together no latter than 2 days after their collection from the fermentation. In all other assays resuspended cells were stored not more than 2 days at –20 °C prior to their assay.

Plasmid construction

Plassmid pSK4.282

Plasmid pSK4.232A was digested with *Sma*I and the 1.5 kb P_{tac}*pobA**-containing fragment that was obtained was ligated to pKD11.291A to create pSK4.282 (7.1 kb). The vector was prepared for cloning after *Hin*dIII digestion, treatment with the Klenow fragment, and dephosphorylation with CIAP.

Plasmid pSK4.272

Insertion of the 1.9 kb *serA*-containing fragment into a *Sma*I/CIAP treated site of pSK4.232B generated plasmid pSK4.272 (9.8 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion.

Plasmid pSK6.225

Plasmid pSK6.225 (9.4 kb) was created after the ligation of the P_{tac}*pobA**-containing fragment into a *Sma*I/CIAP treated site of pSK4.232B. The 1.5 kb DNA insert was isolated from pSK4.232A after a *Sma*I digestion.

Plasmid pSK6.233

Insertion of the 1.9 kb *serA*-containing fragment into pSK6.225 generated plasmid pSK6.233 (11.3 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion, and the vector was prepared after *Hin*dIII digestion of pSK6.225 followed by klenow and CIAP treatment.

Plasmid pSK5.119

Insertion of the 2.4 kb *katG* fragment into the *Xba*I/CIAP treated site of pSU18, created plasmid pSK5.119 (4.6 kb). The *katG* insert was PCR amplified containing its native promoter sequence using RB791 DNA as the templeta. PCR primers were designed to contain *Xba*I digestion sequences as follows: 5-GCTCTAGAT CTCAACTATCGCATCCGTG and 5-GCTCTAGACTTGCTTCATGAAATCCAGC.

Plasmid pSK6.261

Insertion of the 1.9 kb *serA*-containing fragment into pSK5.119 generated plasmid pSK6.261 (6.5 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion, and the vector was prepared after *Sal*I digestion of pSK5.119 followed by klenow and CIAP treatment.

Plasmid pSK6.270

Double digestion of pSK6.261 with *Sma*I and *Hin*dIII released a 4.3 kb fragment that contained the *katG-serA* cassette and the 2.2 kb pSU18 vector. Gel purification of the large fragment, treatment with the Klenow fragment and ligation to pSK6.255 gave

plasmid pSK6.270 (13.7 kb). The latter vector was prepared after *Hin*dIII digestion, followed by treatment with the Klenow fragment and CIAP.

Plasmid pSK6.161

Digestion of pSK4.259 with BamHI released the 2.2 kb *aroZ* fragment and a large 5.9 kb piece of DNA that contained $lacI^{Q}$, $aroF^{FBR}$ and $P_{tac}pobA$*. Ligation of the large fragment with *serA* gave pSK6.161 (7.8 kb). The 1.9 kb *serA* insert was isolated from pD2625 after *Eco*RV/*Dra*I double digestion.

Plasmid pSK7.51

Insertion of the 1.9 kb *serA*-containing fragment into pSK4.259 generated plasmid pSK7.51 (10.9 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion, and the vector was prepared after *Hin*dIII digestion of pSK4.259 followed by Klenow fragment and CIAP treatment.

Plasmid pSK7.52

Plasmid pSK7.52 (9.1 kb) was constructed after ligation of *aroB* into pSK4.141. The 1.6 kb insert was isolated from plasmid pKD14.136 after *Hin*dIII/*Sal*I double digestion. The vector was prepared after digesting with *Hin*dIII/*Sal*I and treating with CIAP.

Plasmid pSK7.61

Plasmid pSK4.232A was digested with SmaI and the 1.5 kb P_{tac}*pobA** fragment that was obtained insetred into a *Sma*I/CIAP treated site of pSK7.52 therefore generating plasmid pSK7.61 (10.6 kb).

Plasmid pSK7.80

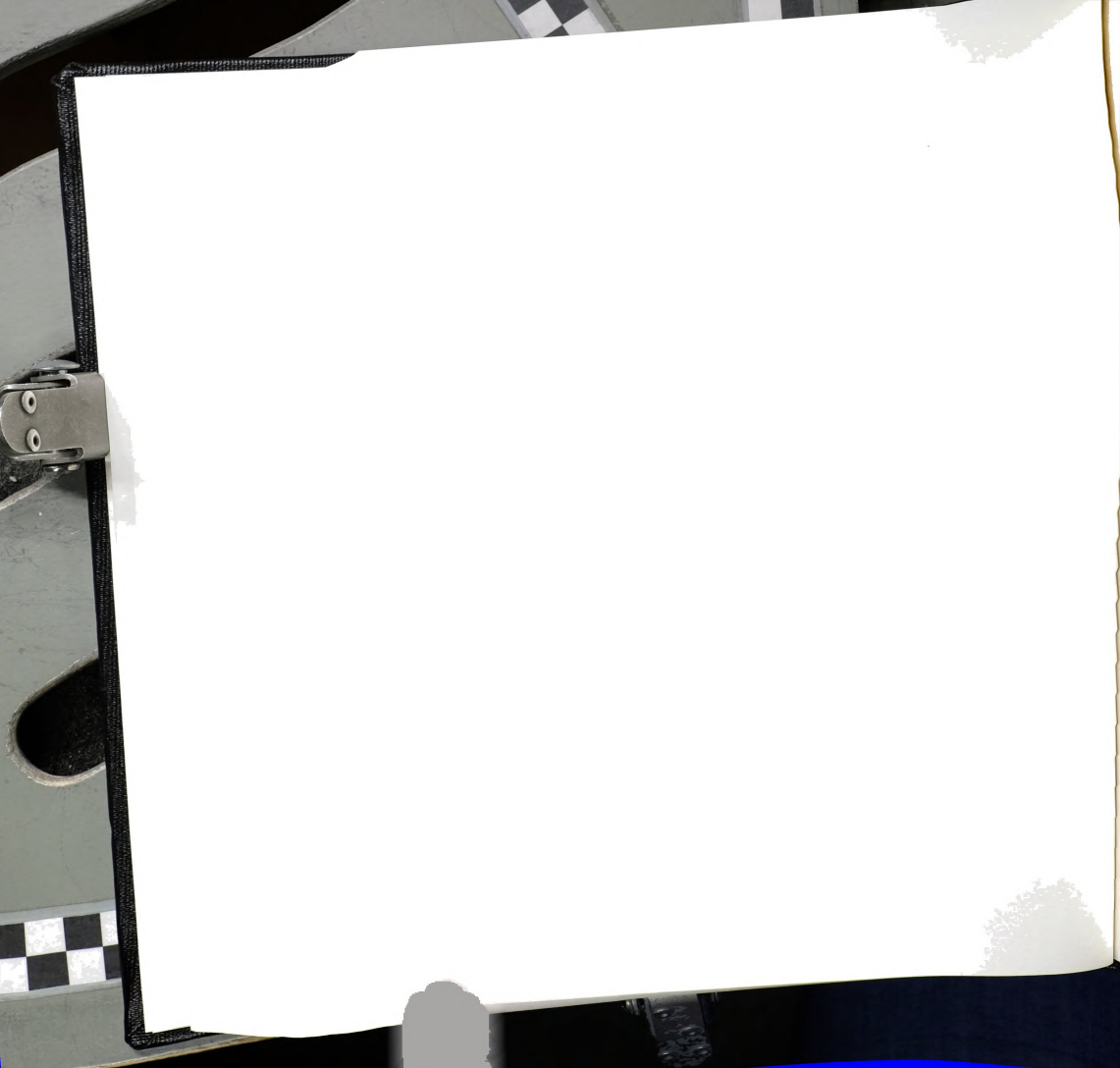
Insertion of the 1.9 kb *serA*-containing fragment into pSK7.61 generated plasmid pSK7.80 (12.8 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion, and the vector was prepared after *Hin*dIII digestion of pSK7.61 followed by Klenow fragment and CIAP treatment.

Plasmid pSK7.104

Plasmid pSK6.161 was digested with SmaI, treated with CIAP and ligated with the *pck* gene generating plasmid pSK7.104 (10.1 kb). The 2.3 kb DNA insert was isolated from pKL2.222 after a triple *Eco*RI/*Bam*HI/*Not*I digestion. While the *Eco*RI/*Bam*HI digestion released the 2.3 kb *pck* gene, the extra *Not*I was added to cut the pSU18 remaining vector into two smaller pieces and simplify the gel purification of the desired DNA piece. After purification the insert was blunt-ended by treatment with the Klenow fragment.

Purification of Gallic Acid From The Fermentation Broth

Fermentation supernatant (450 mL) containing 8 g GA (105 mM), 0.35 g PCA (5 mM), 1.1 g DHS (15 mM), 2.2 g DAH (24 mM) and 6.3 g (95 mM) glutamate acidified,



to pH 2.3 with H_2SO_4 addition, and after centrifugation (13000 x g, 10 min) to remove the precipitated protein, extracted with EtOAc (3x, 350 mL each time). The dark-red organic layer (1 L, 6 g GA, 0.2 g PCA) was filtered through 30 g of Darco G-60 (100 mesh) activated charcoal in a buchner funnel under reduced pressure. White powder consisting only from GA (5.8 g) precipitated after the organic solvent was concentrated to 80 mL, mixed with 100 mL petroleum ether, and chilled in ice bath. The total purification yield was calculated to be 72.5 %. ^{1}H NMR (D_2O): δ 7.1 (s, 2H).

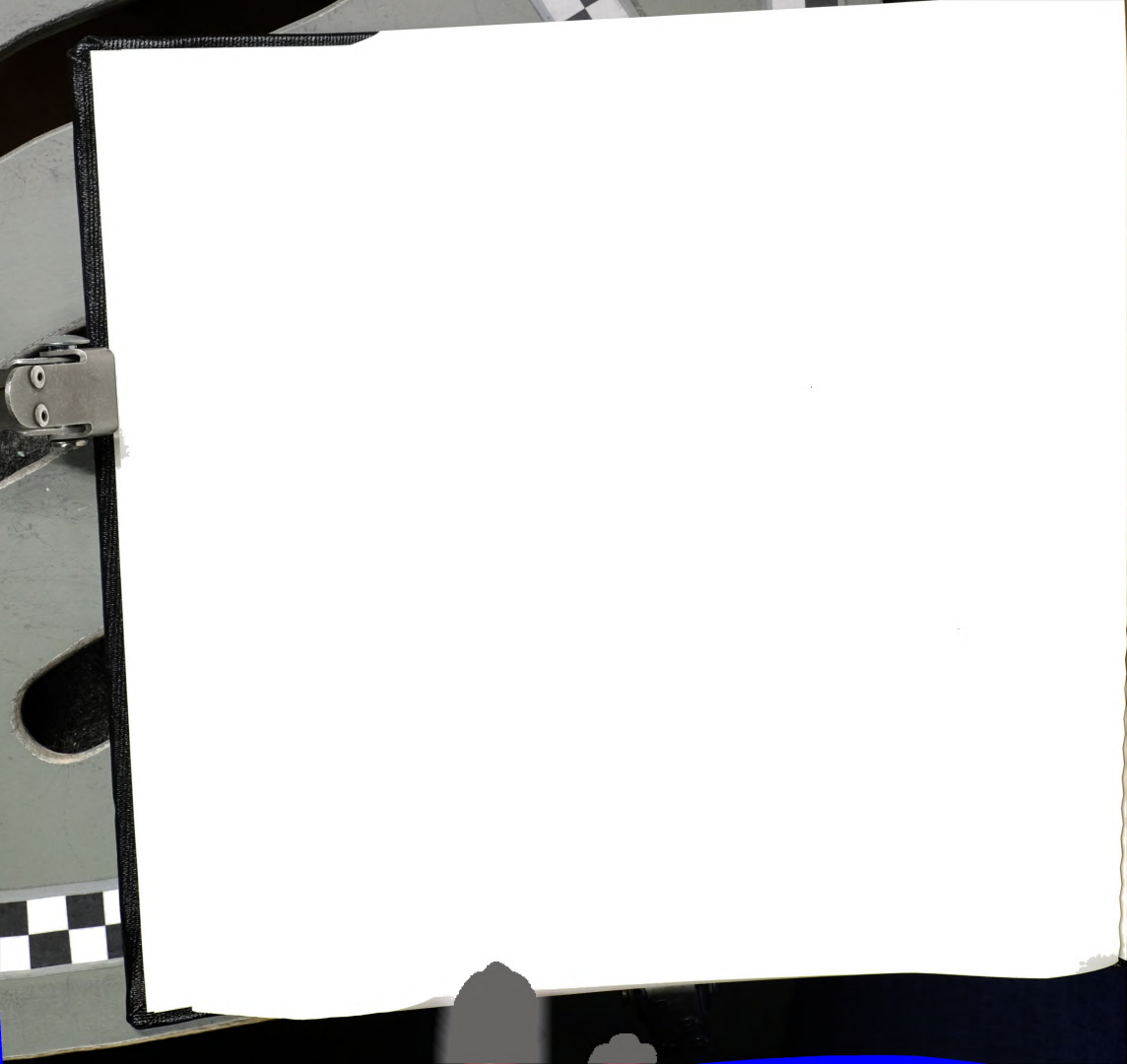
Purification of glutamate from the fermentation broth

Fermentation supernatant containing L-glutamate was refluxed for 2 h to transform all the residual DHS and DAH into PCA. Acidification to pH 2.5 followed by extraction with EtOAc (3x) removed all the aromatic acids (GA, PCA) leaving all glutamate in solution. After filtrating of the remaining water solution through charcoal (Norit A 100 mesh) the pH was adjusted back to 7.0 using KOH (5M) and was filtered through a strong anion exchange AG1x8 (OH form) resin. The adsorbed glutamate was eluted after washing with a H_2O/AcOH (v/v, 85/15) mixture. The latter solution proved to contain only L-glutamate after ^{1}H NMR and ^{13}C NMR analysis and comparison with the spectroscopic data of authentic material. ^{1}H NMR (D_2O): δ 2.1 (m, 2H), 2.5 (t, 2H), 3.8 (t, 1H). ^{13}C NMR (D_2O): δ 20.0, 24.5, 48.3, 168.4, 171.6.

Fermentation conditions

Fermentation conditions were the same as described previously. Both *E. coli* and *K. oxytoca M5a1* grew under the standard glucose-starving conditions where under constant impeller speed (940 rpm) and airflow (1 L/L/min), the dissolved oxygen was maintained at 20% air saturation, by oxygen sensor-controlled glucose feed. When recombinant *E. coli* cells were utilized for decarboxylation, cells were allowed to reach early stationary phase at 24 h, before substrate was added all at one portion. The substrate was prepared as follows: $GA{\cdot}H_2O$ (28 g) or PCA (22 g) were suspended in 60-80 mL of water, and did not all dissolve even after adjusting the pH to 6.5 with NH_4OH addition. A clear brown homogeneous solution was obtained after autoclaving the previous mixture for 7 min, which while still warm (~50-60 °C) was added in the fermenting cells. At the same time the airflow was switched to N_2 (0.5 L/L/min) and the glucose feed was interrupted.

When *K. oxytoca* was utilized for decarboxylation, supplement addition was performed at early log phase (8 h), two hours after the cells had moved into phase three of growth. The substrate was prepared as previously described. After the addition, the airflow was decreased to 0.5 L/L/min and a constant glucose feed was adopted to ensure that an excess of 5 to 10 g was always present in the medium. Although under these conditions the D.O. was less than 2%, two to four hours later, it started to increase. At this point the airflow was switched into N_2 (0.25 L/L/min) and the glucose feed was interrupted. The excess glucose (~10 g) that was usually present at this point did not significantly change by the end of the decarboxylation experiment. The cell mass at the



addition was usually around 17 g/L. A small increase was observed during the first 2 h (~21 g/L) which remained unchanged until the end of the fermentation run.

Plasmid Construction

Plasmid pSK6.228

Plasmid pKD9.069A was digested with *Hin*dIII and the 2.3 kb *aroY* fragment was gel purified. Subsequent ligation into the *Hin*dIII/CIAP treated site of pSU18 gave plasmid pSK6.228 (4.5 kb).

Plasmid pSK6.234

Insertion of the 1.9 kb *serA*-containing fragment into pSK6.228 generated plasmid pSK6.234 (6.4 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I digestion, and the vector was prepared after *Sma*I digestion followed by CIAP treatment.

Plasmid pSK6.224

Plasmid pSK6.224 (10.2 kb) was obtained after ligation of the *aroY* gene into a *Hin*dIII/CIAP treated site of pSK4.232B. The 2.3 kb *aroY* insert was isolated from pKD9.069A after *Hin*dIII digestion.

Plasmid pSK6.232

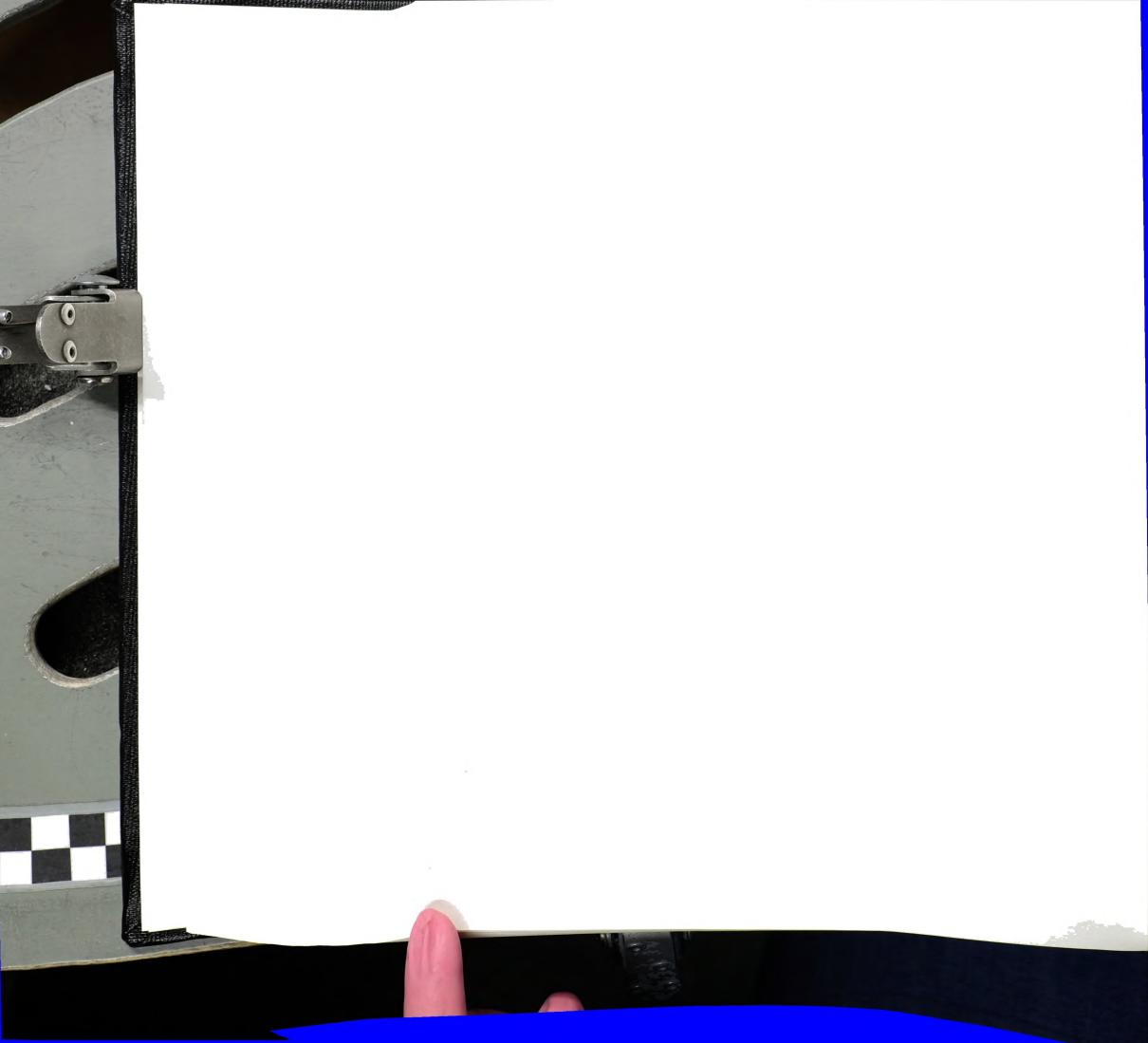
Insertion of the 1.9 kb *serA*-containing fragment into pSK6.224 generated plasmid pSK6.232 (12.1 kb). The insert was isolated from pD2625 after a double *Eco*RV/*Dra*I

digestion, and the vector was prepared after *Sma*I digestion of pSK6.224 followed by CIAP treatment.

Purification of catechol and pyrogallol

Fermentation supernatant (1 L) containing 49 mM PGL (6.2 g), 3 mM catechol (0.33 g), 12 mM DHS (2.0 g), 16 mM DAH (3.3 g), and 51 mM L-glutamate (7.5 g) was used to purify PGL. The DHS, DAH, glutamate and catechol byproducts were present because a fermentation broth of a GA-synthesizing biocatalyst was the original supernatant that was used for decarboxylation by *E. coli* RB791*serA::aroB*/pSK6.234. Extraction of the aforementioned solution at neutral pH with EtOAc (3x, 600 mL each time) followed by one back extraction with brine (150 mL) gave a yellow solution (1.8 L) consisting only from PGL (5.9 g) and catechol (0.24 g). The dark-red organic layer was then stirred with 4 g of Darco G-60 (100 mesh) activated charcoal for 5 min and filtered through celite. Evaporation to dryness of the light-yellow solution that was obtained after filtration, gave an off-white powder (5.9 g) of a PGL/catechol mixture (mol/mol, 33/1) in a total purification yield of 95%. Pure PGL (5.4 g yield 92%) was obtained after selective sublimation of catechol at 61 °C for 1 h under high vacuum using a Kugerlolr apparatus. The purified product and isolated yields were the same irrespective from the original source of the fermentation supernatant (ie *K. oxytoca M5a1* or *E. coli*).

Fermentation supernatant that was coming from the decarboxylation of PCA using *E. coli* RB791*serA::aroB*/pSK6.234, was utilized for the purification of catechol. Extracting 500 mL of catechol-containing (118 mM, 6.5 g) cell broth under neutral pH



with EtOAc (3x, 300 mL each time), and back-extracting with brine, gave a red solution only that only contained catechol. Stirring that organic layer with 4 g of activated charcoal (Darco G-60, 100 mesh) and filtering it through celite gave a light yellow solution which after evaporation to dryness, gave an off-white powder of catechol (6.0 g), in a total purification yield of 92%. As in the case of PGL, the purification yield and product purity was the same when fermentation broth coming from *K. oxytoca*.