Metabolic Engineering for the Biosynthesis of Styrene and its Derivatives

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved March 2014 by the
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May 2014

ABSTRACT

Metabolic engineering is an extremely useful tool enabling the biosynthetic production of commodity chemicals (typically derived from petroleum) from renewable resources. In this work, a pathway for the biosynthesis of styrene (a plastics monomer) has been engineered in *Escherichia coli* from glucose by utilizing the pathway for the naturally occurring amino acid phenylalanine, the precursor to styrene. Styrene production was accomplished using an *E. coli* phenylalanine overproducer, *E. coli* NST74, and over-expression of *PAL2* from *Arabidopsis thaliana* and *FDC1* from *Saccharomyces cerevisiae.* The styrene pathway was then extended by just one enzyme to either (*S*)-styrene oxide (*StyAB* from *Pseudomonas putida* S12) or (*R*)-1,2-phenylethanediol (*NahAaAbAcAd* from *Pseudomonas* sp. NCIB 9816-4) which are both used in pharmaceutical production. Overall, these pathways suffered from limitations due to product toxicity as well as limited precursor availability. In an effort to overcome the toxicity threshold, the styrene pathway was transferred to a yeast host with a higher toxicity limit. First, *Saccharomyces cerevisiae* BY4741 was engineered to overproduce phenylalanine. Next, *PAL2* (the only enzyme needed to complete the styrene pathway) was then expressed in the BY4741 phenylalanine overproducer. Further strain improvements included the deletion of the phenylpyruvate decarboxylase (ARO10) and expression of a feedback-resistant choristmate mutase ($ARO4^{K229L}$). These works have successfully demonstrated the possibility of utilizing microorganisms as cellular factories for the production styrene, (*S*)-styrene oxide, and (*R*)-1,2-phenylethanediol.

ACKNOWLEDGEMENTS

This work would not have been possible without the support of my advisor, committee members, fellow labmates, friends, and collaborators. I would like to thank my advisor Dr. David Nielsen for his support and guidance. I am very thankful to have been given the opportunity to work in his lab. Also, I would like to thank my committee members Dr. Michael Caplan, Dr. Karmella Haynes, Dr. Laura Jarboe, and Dr. Cesar Torres for their assistance and agreeing to be on my committee. I would like to thank Dr. Kristala Prather at MIT and Dr. Bradley Moore from UCSD for their donations of number plasmids and strains that contributed greatly to these works. I would like to thank Tom Colella in the GEL lab for his help with GC-MS. I would like to thank my lab members, both former and current, as well as those from Dr. Sierks' and Dr. Rege's lab for their help and assistance. I would like to thank Shawn Pugh, Warinsinee Phusitkanchana, Luis Moya, Michael Wiehn, Vick Syradi, Ibrahim Halloum, and Thomas Levario for making everyday an enjoyable day to go to lab. I am not sure how I would have made it through this without our occasional racquetball games. I am also grateful for the funding provided by ASU as well as the donors to the Ford, Barrett and Materials, ARCS, and PEO Fellowships.

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CHAPTER 1

INTRODUCTION

Abstract

This chapter describes background information on the field of metabolic engineering and the motivation for my project on the construction of pathways from L-phenylalanine. The metabolic pathway for styrene and its derivatives (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol will be provided. This chapter concludes with the dissertation organization.

1.1 Background and motivation

A demand for commodity chemicals by renewable means rather than fossil fuels has been increasing in recent years. Microorganisms are capable of naturally producing various useful products including pharmaceuticals, fuels, and polymers; however, often at too small of quantities for industrial economic viability. Metabolic engineering enables efficient construction of both high yield pathways that already exist in nature and novel pathways that may be constructed from the enzymatic parts of various microorganisms. Utilizing a diversity of enzymes through the tools of metabolic engineering, researchers have successfully catalyzed a wide array of biochemical reactions producing important biofuels, pharmaceuticals, and other fine chemicals.

The most prominent application of metabolic engineering is currently in the transportation fuel sector with a heavy focus on bio-ethanol and bio-butanol production. Though these biofuels represent the highest volume of biologically produced chemicals, they also have the lowest profit margin due to the discrepancy between the high cost of

processing suitable carbon substrates (sugars such as glucose) and the value of the final product (Keasling 2010). While the biofuels sector has been of primary interest for the last several decades, recent interest in utilizing metabolic engineering for the production of pharmaceuticals and fine chemicals has developed. Not only does a biosynthetic route offer a renewable means of producing commodity chemicals, it also gives us the ability to circumvent the high energetic requirements of chemocatalytic production (as is the case of styrene from ethylbenzene), control the stereospecificity of chiral compounds (a necessity for pharmaceutical precursors), as well as manufacture compounds which can only be produced naturally (such as amino acids, vitamins, flavors, and fragrances). Several examples of fine chemicals synthesized from microbial platforms have recently been reported, most notably monomers of polymers and co-polymers such as 1,5-diaminopentane and 1,4-diaminopentane (Qian, Xia, and Lee 2011) (used to make polyamids like Nylon) as well as isoprene (Lindberg, Park, and Melis 2010) (a monomer for rubber and copolymer synthesis). Since these products are indistinguishable from those of petrochemical origin, no change in industry infrastructure is necessary for their incorporation into existing polymer and co-polymer synthesis methods. Thus, metabolic engineering strategies provide new opportunities to develop products of commercial interest by biosynthetic means thereby reducing our dependence on nonrenewable resources and offering a ‘green chemistry’ approach to producing essential commodity chemicals.

Monoaromatic compounds are an important and diverse class of fine chemicals with applications ranging from use as solvents to monomers for polymer synthesis. Several monoaromatic compounds have been microbially synthesized to date, but the

origin of these compounds have been mainly derived from *p*-coumarate via the L-tyrosine pathway, making them phenolics(Boudet 2007). This approach simply replicates the natural pathways commonly found in plants. In natural systems, phenolics play vital roles in plant physiology and fitness. For example, plants produce phenolic polymers, like lignin and suberin, as structural components of their cellular wall, as well as flavanoids for pigmentation which is used to attract pollinators and as UV filtration(Boudet 2007). With such an apparent necessity for phenolics in natural systems, there exists a plethora of enzymes and enzymatic sources for phenolic based reactions. Examples of phenolics which have been heterologously synthesized include phenol (a monomer for phenolic resins)(Wierckx et al. 2005), *p*-hydroxybenzoate (a precursor to parabens)(Verhoef et al. 2007), caffeate (an antioxidant and antitumor agent)(Zhang and Stephanopoulos 2012), tyrosol (an antioxidant)(Satoh et al. 2012), and *p*-hydroxystyrene (a useful copolymer)(Qi et al. 2007; Verhoef et al. 2009). However, the number of biosynthesized non-phenolic monoaromatics remains limited to-date. This, in part, is due to the fact that non-phenolics are relatively uncommon in natural systems; therefore, pathway design is more difficult and requires novel approaches. In addition, the pool of known enzymes which express activity on non-phenolics is also greatly limited. Enzymes which demonstrate activity on phenolics often utilize the *para*-hydroxyl group of the substrate for stability serving as a hydrogen bond donor or acceptor(Brownlee et al. 2008; Serre et al. 1999; Caruso et al. 2004; Rodríguez et al. 2010). Due to the necessity of the *para*-hydroxyl group, these phenolic enzymes often possess limited, if any, substrate promiscuity thereby limiting their potential use on non-phenolic homologues.

As previously mentioned, the number of non-phenolic monoaromatic compounds engineered from renewable resources remains limited, and thus the focus of this work will be on the biosynthetic production of monoaromatics derived via phenylalanine, namely styrene and its derivatives, (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol. Styrene is a high-value monomer used in the production of polymers and copolymers, most notably polystyrene, acrylonitrile-butadiene-styrene (ABS), styrene-acrylonitrile (SAN), and styrene-butadiene rubber. As one of the most important monomers in the plastics industry, its annual production exceeds 6 million metric tons per year representing a $28 billion market(McKenna and Nielsen 2011). However, while its uses and need by consumers is apparent, production of styrene via the dehydrogenation of petroleum-derived ethylbenzene is also one of the most energy intensive processes requiring 3 metric tons of steam per metric ton of styrene produced(McKenna and Nielsen 2011). Not only is styrene an essential and valuable precursor to plastics, it is also an important precursor to the fine chemicals (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol. These styrene derivatives are chiral building blocks used for the production of pharmaceuticals as well as other compounds of interest. For example, (*S*)-Styrene oxide is used as a precursor to the biocides levamisole and nematocide(Park, So, et al. 2006) as well as the synthesis of cosmetics(Loprieno et al. 1976), surface coatings, and agricultural(Loprieno et al. 1976) and biological(Panke et al. 2000; Han et al. 2006) chemicals. (*R*)-1,2-Phenylethanediol is used in the synthesis of the pharmaceuticals (*R*)-norfluoxetine and (*R*)-fluoxetine, which are used to treat psychiatric and metabolic disorders, as well as β-lactam antibiotics(Cao et al. 2006; Kumar, Upadhyay, and Pandey 2004). Additionally, (*R*)-1,2-phenylethanediol is also used in the production of various

agrochemicals and pheromones(Gamenara and Dominguez de Maria 2009). In the face of rising costs of oil, alternative, renewable production methods for these compounds will secure the future accessibility to meet the needs of consumers.

1.2 Metabolic pathways

Phenylalanine, the necessary endogenous precursor to the production of styrenics, is produced via the shikimic acid pathway in addition to tyrosine and tryptophan as seen in Fig. 1.1. However, in *E. coli*, the aromatic amino acids are used only for the production of proteins and not as precursors for secondary metabolites; therefore, the pathway is tightly feedback-regulated. The shikimic acid pathway begins with the condensation of phosphoenolpyruvate (PEP), a key intermediate in glycolysis, and erythrose-4-phosphate (E-4P), a key intermediate in the pentose phosphate pathway, to yield the first major precursor of the pathway, 3-deoxy-D-arbino-heptulosonate-7-phosphate (DAHP). This reaction is achieved via the DAHP synthase enzymes AroF, AroG, and AroH which are feedback sensitive to tyrosine, phenylalanine, and tryptophan, respectively. The regulation of these enzymes is two-fold, including regulation at the transcriptional level as well as at the protein level. Firstly, to control transcription, the aromatic amino acids form a complex with their DNA-binding transcriptional regulator (TyrR for phenylalanine and tyrosine, TrpR for tryptophan) which then binds to a palindromic target sequence near the transcriptional promoter region of the genes *aroF*, *aroG*, and *aroH*. The presence of the DNA-binding protein complex interferes with the proper binding of RNA polymerase and thereby inhibits transcription from occurring. Secondly, the proteins themselves are regulated via allosteric enzyme inhibition. Upon binding of the aromatic amino acids to their respective feedback-sensitive DAHP synthase, a

conformational change of the enzyme occurs which represses its activity further. In addition to feedback control of DAHP synthase activity, the shikimic acid pathway is also tight regulated at the metabolite chorismate, which is a key branch point for the three aromatic amino acids. To produce phenylalanine, chorismate is converted to prephenate and subsequently to phenylpyruvate via the bifunctional chorismate mutase/prephenate dehydratase enzyme PheA. However, unlike the DAHP synthases, PheA is only regulated via allosteric enzyme inhibition and is not regulated transcriptionally(Keseler et al. 2005). To achieve high flux through the shikimic acid pathway and obtain high titers of phenylalanine, it is necessary to relieve the feedback regulation imposed on the key enzymes in *E. coli*.

A feedback resistant strain of *E. coli*, NST74 (ATCC 31884), has been previously engineered to overproduce phenylalanine(Tribe 1987). This was accomplished via NTG (N-methyl-N'-nitro-N-nitrosoguanidine) mutagenesis and subsequent selection of mutants in minimal media containing the phenylalanine anti-metabolites β-2-thienylalanine or *p*-fluoro-DL-phenylalanine. In the presence of phenylalanine anti-metabolites, feedback regulation of the shikimic acid pathway is activated and natural production of phenylalanine ceases. In order to survive in the presence of the anti-metabolites, the strain must evolve to de-regulate the feedback sensitive enzymes. This approach resulted in the development of the strain *E. coli* NST74 with the relevant genotype *aroH367(fbr), tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr), pheO352, aroG397(fbr)*. As described, *E.coli* NST74 possesses mutations which relieve the allosteric enzyme inhibition of the DAHP synthase enzymes AroF, AroG, and AroH as well as the chorismate mutase/prephenate dehydratase PheA. In addition, the DNA-binding

transcriptional regulator TyrR and the phenylalanine operator pheO has also been mutated to alleviate repression. As a result of these key mutations, phenylalanine titers as high as 1.98 g/L were achieved in 1 L bioreactor experiments(Tribe 1987). *E. coli* NST74's ability to produce copious amounts of phenylalanine makes it an ideal host platform for the biosynthetic production of the non-natural, monoaromatics styrene, (*S*)-styrene oxide, and (*R*)-1,2-phenylethanediol.

The proposed biosynthesis of styrene and its derivatives (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol may be achieved from endogenously produced phenylalanine, as illustrated in Fig. 1.1. Firstly, phenylalanine is deaminated to *trans*-cinnamate via the expression of a suitable phenylalanine ammonia lyase (PAL). Secondly, *trans*-cinnamate is decarboxylated to styrene via the expression of a suitable phenylacrylate decarboxylase (PADC). To date, the only known PADC which demonstrates activity toward styrene is the ferulate decarboxylase (FDC1) of *Saccharomyces cerevisiae*. Styrene may then be oxidized to (*S*)-styrene oxide via the expression of a styrene monooxygenase, namely StyAB from *Pseudomonas putida* S12, or (*R*)-1,2-phenylethanediol via the expression of a naphthalene dioxygenase which demonstrates activity on styrene, namely NahAaAbAcAd from *Pseudomonas* NCIB 9816. In each enzymatic step of the described pathway, various enzymes were tested for their activity and specificity, which will be discussed in further detail in the proceeding chapters.

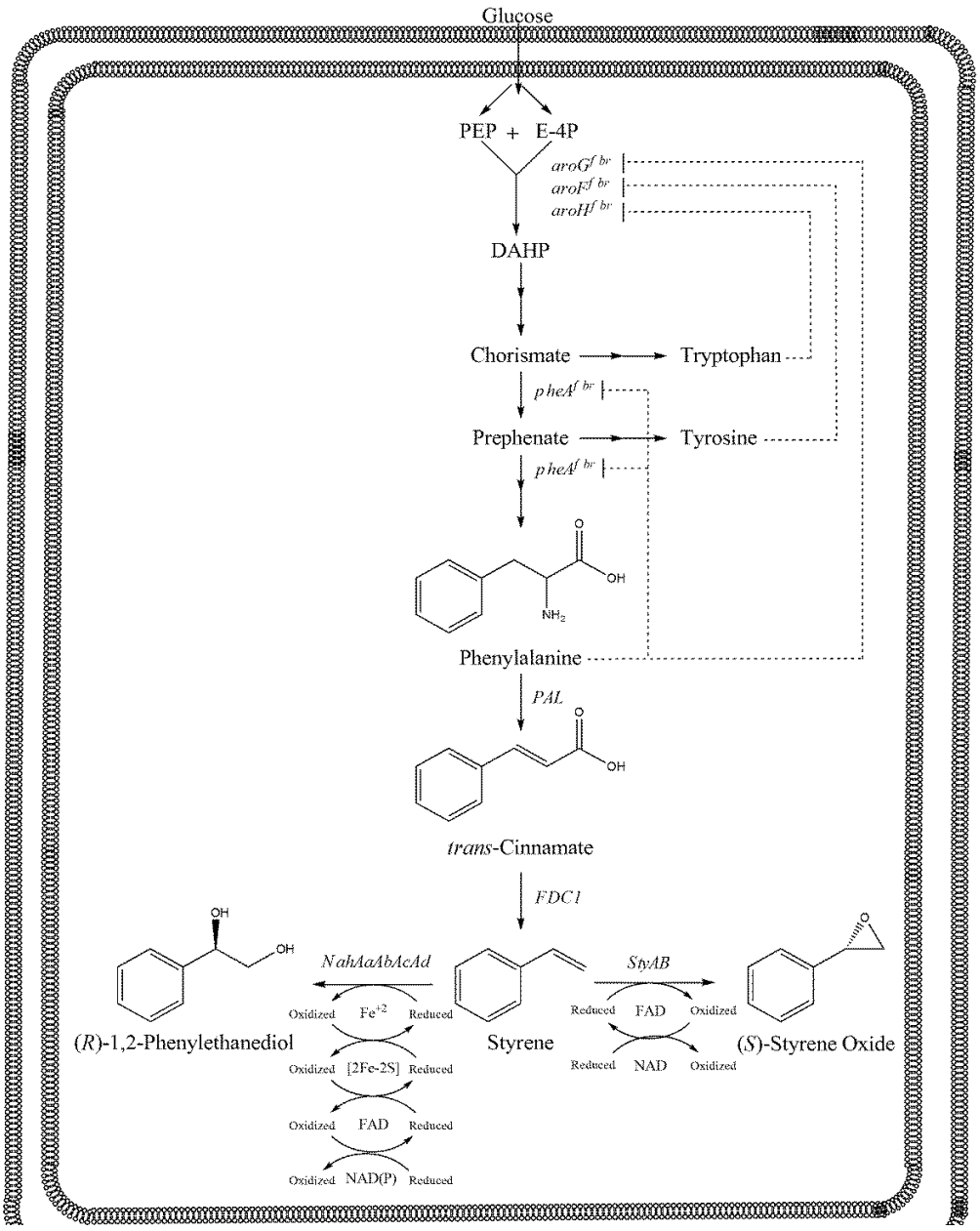

Figure 1.1. Enzymatic pathway to convert glucose to styrene, (S)-styrene oxide and (R)-1,2-phenylethanediol via the shikimic acid pathway through the native metabolite L-phenylalanine. Multiple arrows signify that multiple steps are occurring but are not illustrated. Dotted lines from the aromatic amino acids indicate feedback-repression via transcriptional regulation or allosteric enzyme inhibition, or both. Metabolite and gene abbreviations(Keseler et al. 2005; Krieger et al. 2004): phosphoenolpyruvate (PEP), D-erythrose-4-phosphate (E-4P), 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), 2-dehydro-3-deoxyphosphoheptonate aldolase (*aroF*, *aroG*, *aroH*), chorismate muatse/prephenate dehydratase (*pheA*), phenylalanine ammonia lyase (*PAL*), ferulate decarboxylase (*FDC1*), styrene monooxygenase (*StyAB*), and naphthalene dioxygenase (*NahAaAbAcAd*).

1.3 Dissertation organization

This dissertation is organized into five chapters. Chapter 1 discusses a background into the field of metabolic engineering and the importance of determining methods to create fine chemicals such as styrene, (*S*)-styrene oxide, and (*R*)-1,2-phenylethanediol from renewable resources. This chapter also gives an outline of the metabolic pathways constructed during this project as well as an overview of *E. coli*'s phenylalanine pathway. Chapter 2 introduces us to pathway engineering through the development of a renewable method for the biosynthesis of styrene in *E. coli*. This is the first example of a renewable method for styrene production from renewable resources such as glucose. This chapter will show that the styrene monomer is extremely toxic to the bacteria and severely limits production titers. The styrene pathway is then extended to (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol in Chapter 3. When the endogenously produced styrene is converted to these less toxic compounds, higher productivities are achieved. However, at this point the pathway is limited not only by toxicity but by the ability to make ample amounts of the endogenous precursor phenylalanine. Several methods to improve phenylalanine titers will be discussed. Since the main difficulty in biosynthetically producing monoaromatics in *E. coli* remains the toxicity threshold, Chapter 4, focuses on transferring the styrene pathway to a new yeast host. The work encompasses using *S. cerevisiae* which will be engineered to overproduce phenylalanine. The styrene pathway was incorporated into the *S. cerevisiae* phenylalanine over-producer and tested for styrene production from glucose. Chapter 5 will suggest future directions for the styrene pathway and its derivatives.

CHAPTER 2

BIOSYNTHESIS OF STYRENE BY ENGINEERED *E. COLI*

Abstract

Styrene is a large volume, commodity petrochemical with diverse commercial applications, including as a monomer building-block for the synthesis of many useful polymers. Here we demonstrate how, through the *de novo* design and development of a novel metabolic pathway, styrene can alternatively be synthesized from renewable substrates such as glucose. The conversion of endogenously-synthesized L-phenylalanine to styrene was achieved by the co-expression of phenylalanine ammonia lyase and *trans*-cinnamate decarboxylase. Candidate isoenzymes for each step were screened from bacterial, yeast, and plant genetic sources. Finally, over-expression of *PAL2* from *A. thaliana* and *FDC1* from *S. cerevisiae* (originally classified as ferulate decarboxylase) in an L-phenylalanine over-producing *E. coli* host led to the accumulation of up to 260 mg/L in shake flask cultures. Achievable titers already approach the styrene toxicity threshold (determined as ~300 mg/L). To the best of our knowledge, this is the first report of microbial styrene production from sustainable feedstocks.

This work was published as:

McKenna, R. & Nielsen, D.R. Styrene biosynthesis from glucose by engineered *E. coli*. *Metab Eng* **13**, 544-554 (2011)

2.1 Introduction

Styrene is a versatile, large commodity chemical for which 60% of its global annual consumption supports the production of numerous, industrially-important polymers and co-polymers(SRI 2010). In 2006, over 6 million metric tons of styrene were produced by U.S. manufacturers, a market that was valued at nearly $28 billion and projected to grow by 4.3% per year through 2010(SRI 2010). Today, all commercially-available styrene is derived from the world's dwindling petroleum resources. Conventional styrene synthesis is achieved through the chemocatalytic dehydrogenation of petroleum-derived ethylbenzene(Wu, Koylinski, and Bozik 1981) which requires over 3 metric tons of steam per metric ton of styrene produced. This exorbitant requirement renders styrene production as the most energy-intensive among commodity chemical production routes, consuming nearly 200 trillion BTU of steam for its domestic annual production alone (DoE 2002). With that being said, the goal of this study was to engineer a biocatalyst capable of synthesizing styrene from renewable resources as a more sustainable and greener source of styrene and styrene-derived polymers.

Beyond its petrochemical origins, styrene has been observed as a trace metabolite in foods, in particular cheeses, where it acts as an aroma defect. For instance, the yeast *Penicillium camemberti* has been reported to be capable of synthesizing low levels of styrene from excess L-phenylalanine, but neither a defined pathway nor the requisite genes have thus far been elucidated(Pagot et al. 2007). Styrene is also known to be naturally synthesized by select plant species, including several trees in the *Styracaceae* family (including several *Styrax* sp.). Here, styrene is also synthesized from excess L-phenylalanine where it then subsequently accumulates as a minor constituent (<0.55% of

total dry weight) within benzoin resins (which are predominantly composed of benzoic acid)(Fernandez et al. 2005). Again, however, neither the enzymes nor genes associated with said pathway have been identified to date. Although it is possible to purify styrene from plant resins via distillation or liquid-liquid extraction(Clark 1990), considering the extremely low productivity, poor net yields, and low inherent value of styrene, its potential, large-scale biological production by such a mechanism is rendered as completely uneconomical and unsustainable. A more sustainable and inexpensive approach, however, would involve the engineering of microorganisms that possess the unique ability to synthesize styrene directly from renewable resources.

In recent years, a variety of additional, novel synthetic routes have been proposed and engineered in microorganisms for the production (from renewable substrates such as glucose) of a number of other useful, functionalized monoaromatic compounds with structural similarity to styrene. For example, a biosynthetic pathway for the production of *p*-hydroxystyrene (a monomer used in synthesis of photo-resist polymers) from renewable sugars has been constructed using both *Escherichia coli*(Qi et al. 2007) and *Pseudomonas putida*(Verhoef et al. 2009) as host platforms. Meanwhile, both phenol (a precursor and monomer for phenolic resins)(Wierckx et al. 2005) and *p*-hydroxybenzoate (a precursor to parabens, which are used as preservatives)(Verhoef et al. 2007) have also been synthesized as individual products from glucose by engineered strains of *P. putida*. Interestingly, each of the above non-natural metabolites were derived using L-tyrosine (or its immediate precursor, 4-hydroxyphenylpyruvate) as a pathway precursor, thereby making them each phenolics(Boudet 2007). To date, there remain few examples of engineered biosynthetic pathways for the production of non-phenolic, monoaromatic

compounds using microbial biocatalysts. Moreover, there exist no previous reports regarding the development of a styrene biosynthetic pathway or the engineering of microbes capable of synthesizing styrene from renewable resources. With this in mind, the present study describes the *de novo* design and development of a functional styrene biosynthetic pathway and the engineering of *E. coli* strains capable of styrene biosynthesis from glucose.

The proposed styrene biosynthesis pathway utilizes endogenously synthesized (from glucose) L-phenylalanine as an intermediate precursor which is converted to styrene by a series of two enzymatic steps, as in Fig. 2.1. First, endogenously-occurring L-phenylalanine is converted to *trans*-cinnamic acid (tCA) through its deamination, as catalyzed by phenylalanine ammonia lyase (PAL). Said activity and substrate specificity has been previously reported for a number of PAL isoenzymes that have been identified and characterized in yeast(Vannelli, Xue, et al. 2007; Qi et al. 2007; Gilbert and Tully 1982), plants(Young, Towers, and Neish 1966; Cochrane, Davin, and Lewis 2004), and (although less prevalently) bacteria(Moffitt et al. 2007; Xiang and Moore 2005, 2006; Young, Towers, and Neish 1966). Among previously characterized PAL isoenzymes, considerable variability with respect to both substrate specificity and activity has been reported. The most commonly studied PAL isoenzyme in recombinant systems, including *E. coli*, remains the bifunctional PAL/TAL (TAL: tyrosine ammonia lyase, which also catalyzes the deamination of L-tyrosine to *p*-coumaric acid) of the yeast *Rhodotorula* sp.(Gilbert et al. 1985; Gilbert and Tully 1982; Gilbert, Stephenson, and Tully 1983; Cui, Jia, and Sun 2008; Vannelli, Wei Qi, et al. 2007; Santos, Koffas, and Stephanopoulos 2011). Meanwhile, a number of PAL isoenzymes have been also been studied from plant

sources(Jones 1984) where, for example, *Arabidopsis thaliana* has been characterized as possessing four distinct PALs (encoded by *PAL1*, *PAL2*, *PAL3*, and *PAL4*)(Cochrane, Davin, and Lewis 2004). More recently, a number of prokaryotic PALs have been isolated and characterized, beginning with that which is encoded by *encP* from *Streptomyces maritimus*(Xiang and Moore 2002, 2005). Soon after, two additional PAL isoenzymes were discovered in the cyanobacteria *Nostoc puntiformes* and *Anabaena variabilis*(Moffitt et al. 2007; Xiang and Moore 2005, 2006). Interestingly, each of these prokaryotic PALs was also found to be highly specific for L-phenylalanine, and thus do not also display TAL activity that is so common among yeast PALs.

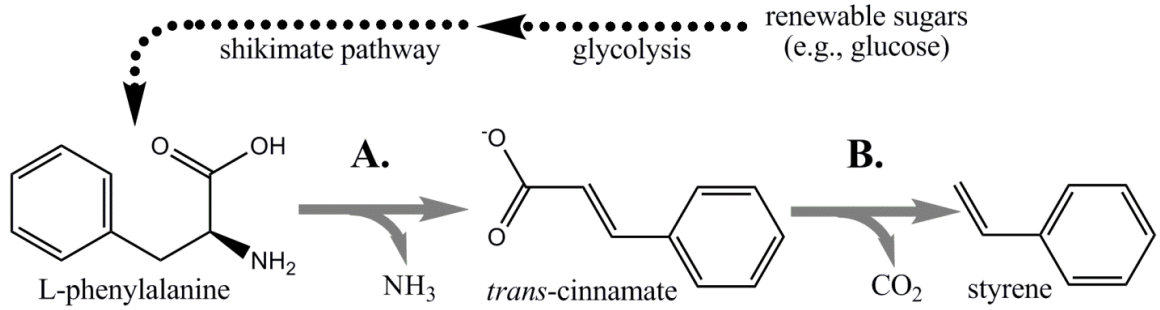

Figure 2.1 Styrene pathway. Enzymatic pathway to convert the precursor L-phenylalanine to the product styrene via the intermediate *trans*-cinnamate. The two-step pathway from L-phenylalanine is achieved by the co-expression of one or more genes which encoded phenylalanine ammonia lyase (PAL) activity (A), and one or more genes which encoded *trans*-cinnamic acid decarboxylase (CADC) activity (B).

The second step in the proposed styrene biosynthesis pathway involves the subsequent decarboxylation of tCA by a phenylacrylate decarboxylase (PADC) displaying *trans*-cinnamate decarboxylase activity to yield styrene as the final product. Several genes encoding PADC activity have been characterized and reported in the literature, including *pdc* from *Lactobacillus plantarum* and *padC* from *Bacillus subtilis*(Tran et al. 2008). It currently remains unknown, however, if their enzyme

products could specifically decarboxylate tCA to produce styrene. Meanwhile, the yeast *Saccharomyces cerevisiae* has demonstrated the ability to synthesize styrene when supplied with exogenous tCA(Clausen et al. 1994); however, the underlying mechanism and genetic basis for said activity presently remains unclear. For instance, previous characterizations of *PAD1* have shown that its over-expression in *S. cerevisiae* results in increased resistance to tCA (as achieved by its conversion to styrene)(Larsson, Nilvebrant, and Jonsson 2001). However, it has also been demonstrated that the expression of *PAD1* alone is insufficient for achieving PADC activity(Clausen et al. 1994), and it was later speculated that the expression of a second enzyme was necessary to convert tCA to styrene(Jiang, Wood, and Morgan 2005). Most recently, it was reported that tCA decarboxylase activity in *S. cerevisiae* maintains an essential dependence on the co-expression of both *PAD1* and *FDC1*, the latter a gene previously characterized as encoding ferulic acid decarboxylase (note that ferulic acid is also a phenylacrylic acid)(Mukai et al. 2010).

The present study describes the *de novo* design of a styrene biosynthetic pathway, as supported through the comprehensive screening of composite pathways enzymes from various genetic sources. The synthesis of styrene from glucose was ultimately achieved through the co-expression of PAL and tCA decarboxylase enzymes in an L-phenylalanine over-producing *E. coli* host platform.

2.2 Materials and Methods

2.2.1 Chemicals.

Chemicals used in this study include L-phenylalanine (98.5%, VWR, Westchester, PA), *trans*-cinnamic acid (99%, MP Biomedicals, Solon, OH), styrene (99%, Alfa Aesar, Ward Hill, MA), methanol (99.8%, VWR, Westchester, PA), and trifluoroacetic acid (99.5%, EMD, Darmstadt, Germany). All other chemicals used in this study are from Sigma-Aldrich (St. Louis, MO).

2.2.2 Strains and Media.

All strains, plasmids, and oligonucleotide primers used in this study are listed in Table 2.1. Custom oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). cDNA of *N. puntiformes* and *A. variabilis* were gifts from Prof. Bradley Moore (UCSD). Strains of *B. subtilis*, *L. plantarum*, and *S. cerevisiae*, as well as the plasmids pSTV28 and pTrc99A were all gifts from Prof. Kristala Prather (MIT). Genomic DNA was prepared from whole cells using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA) according to vendor protocols. Strains were routinely cultured in Luria-Bertani (LB) broth (supplemented with antibiotics as necessary). Cultures were assayed for their ability to synthesize L-phenylalanine, *trans*-cinnamate, and styrene by cultivation in phosphate-limited minimal media (herein referred to as "MM1") with glucose. MM1 was adapted from Qi *et al*. (2007), and is composed of glucose (nominally 15 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ (4.0 g/L), MOPS (24.7 g/L), KH_2PO_4 (0.3 g/L), K_2HPO_4 (0.7 g/L), and 5 mL/L ATCC Trace Mineral Supplement (Catalog No. MD-TMS) (EDTA (0.5 g/L), $MgSO_4 \cdot 7H2O$ (3 g/L), $MnSO_4 \cdot 7H_2O$ (0.5 g/L), NaCl (1 g/L), $FeSO_4 \cdot 7H_2O$ (0.1 g/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.1 g/L), $CaCl_2$ (0.1 g/L), $ZnSO4 \cdot 7H_2O$ (0.1 g/L), $CuSO_4 \cdot 5H_2O$ (0.01 g/L), $AlK(SO_4)_2$ (0.01 g/L),

H_3BO_3 (0.01 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g/L), Na_2SeO_3 (0.001 g/L), $Na_2WO_4 \cdot 2H_2O$ (0.10 g/L), and $NiCl_2 \cdot 6H_2O$ (0.02 g/L)).

Table 2.1. Strains, plasmids, and oligonucleotide primers.

	Description	**Source**
Strains		
E. coli NST74	*aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr),pheO352, aroG397(fbr)*	ATCC 31884
E. coli BL21Star(DE3)	*F- ompT hsdS$_B$ (r_B-m_B-) gal dcm rne131 (DE3)*	Invitrogen
E. coli NEB-10 beta	*araD139 Δ(ara,leu)7697 fhuA lacX74 galK16 galE15 mcrA f80d(lacZΔM15)recA1 relA1 endA1 nupG rpsL rph spoT1Δ(mrr-hsdRMS-mcrBC)*	NEB
S. cerevisiae W303	Source of *pad1* and *fdc1*	MIT
L. plantarum	Source of *pdc*	MIT
B. subtilis 3610	Source of *padC*	MIT
Plasmids		
pTrc99A	P_{trc}, pBR322 ori, *lacI^q*, *Amp^r*	MIT
pSTV28	P_{lac}, pACYC184 ori, *Cm^r*	MIT
pSpal1At	*pal1* of *A. thaliana* inserted into the *EcoRI* and *SphI* sites of pSTV28	This study
pSpal2At	*pal2* of *A. thaliana* inserted into the *EcoRI* and *SphI* sites of pSTV28	This study
pSpalAv	*pal* of *A. variabilis* inserted into the *EcoRI* and *BamHI* sites of pSTV28	This study
pSpalNp	*pal* of *N. punctiforme* inserted into the *EcoRI* and *BamHI* sites of pSTV28	This study
pSencPSm	*encP* of *S. maritimus* inserted into the *EcoRI* and *BamHI* sites of pSTV28	This study
pTpad1Sc	*PAD1* of *S. cerevisiae* inserted into the *NcoI* and *XbaI* sites of pTrc99A	This study
pTpad1Sc-fdc1Sc	*FDC1* of *S. cerevisiae* inserted into pTpad1Sc	This study
pTfdc1Sc	*FDC1* of *S. cerevisiae* inserted into the *SalI* and *HindIII* sites of pTrc99A	This study
pTpadCBs	*padC* of *B. subtilis* inserted into the *BamHI* and *SbfI* sites of pTrc99A	This study
pTpdcLp	*pdc* of *L. plantarum* inserted into the *BamHI* and *SbfI* sites of pTrc99A	This study
pUN15-pal1	Clone U10120 containing AT2G37040 (*pal1*) from *A. thaliana*	ABRC
pUN15-pal2	Clone U12256 containing AT3G53260 (*pal2*) from *A. thaliana*	ABRC
Primers (5' -> 3')		
F_pal_AV_EcoRI	TAAGAATTCAAGGGGATAAATAATGAAGACACTATCTCAAGC	
R_pal_AV_BamHI	ATTGGATCCTTAATGCAAGCAGGGT	
F_pal_NP_EcoRI	TAAGAATTCAAGGGGATAAATAATGAATATAACATCTCTACAAC	
R_pal_NP_BamHI	ATTGGATCCTTACGTTGACTTTAAGCT	
F_pal1_AT_EcoRI	TAAGAATTCAAGGGGATAAATAATGGAGATTAACGGGGCAC	
R_pal1_AT_SphI	ATTGCATGCTTAACATATTGGAATGGGAGCTC	
F_pal2_AT_EcoRI	TAAGAATTCAAGGGGATAAATAATGGATCAAATCGAAGCAATG	
R_pal2_AT_SphI	ATTGCATGCTTAGCAAATCGGAATCGGAG	
F_encP_SM_EcoRI	TAAGAATTCAAGGGGATAAATAATGACCTTCGTCATAGAGCT	
R_encP_SM_BamHI	ATTGGATCCTTAGTGCGCCGCCACG	
F_padC_BS_BamHI	AAAGGATCCCGACTAAGGGAGGATAAGATGGAAAACTTTATCGGAAG	

R_padC_BS_SbfI	ATACCTGCAGGATGTTTATTATAATCTTCCCGCG
F_pdc_LP_BamHI	ATAGGATCCCTCTGGAGGCAGTTCTAATGACAAAAACTTTTAAAACACT
R_pdc_LP_SbfI	ATACCTGCAGGCCAGAATGTTTCACGTGAA
F_pad1_SC_NcoI	TTACCATGGAGGAACCTAGGCACACAATGGTCCTATTTCCAAGAAGAA
R_pad1_SC_XbaI	ATTTCTAGATTACTTGCTTTTTATTCCTTCCC
F_fdc1_SC_SalI	ATAGTCGACAGACATCAAAGGACGGTTCATGAGGAAGCTAAATCCAGCT
R_fdc1_SC_HindIII	ATTAAGCTTTTATTTATATCCGTACCTTTTCCAAT

2.2.3 Toxicity assays.

To determine extent and effects of metabolite toxicity on *E. coli*, the impacts of the exogenous addition of tCA and styrene (at increasing final concentrations) to growing cultures was investigated. Seed cultures of *E. coli* NST74 were prepared in 5 mL of LB broth and grown at 32^{o}C overnight while shaking at 250 rpm. The seed culture (1 ml) was then used to inoculate 50 ml of LB broth in a 250 mL shake flask. Cultures were grown to an optical density (OD_{600}) of either ~0.6 or ~2.0, at which time either tCA or styrene was added to the flasks at an array of final concentrations between 0 to 1 g/L. Two different initial OD_{600} levels were used to investigate the potential effects of product toxicity as a function of cell density and/or growth stage. In all cases, culturing then resumed at 32^{o}C for another 6-8 h while cell growth, as determined by OD_{600} measurements, was periodically monitored using a UV/Vis spectrophotometer (Beckman Coulter DU800, Brea, CA).

2.2.4 Cloning of candidate genes encoding PAL activity from A. variabilis, N. punctiforme, and A. thaliana.

All genes used in this study were PCR amplified using a BioRad iCycler system with Phusion DNA Polymerase (Finnzymes, Espoo, Finland) using custom oligonucleotide primers. PCR cycling and reaction conditions were standardized

according to manufacturer instructions. Candidate PAL encoding genes were amplified from the cDNA of *A. variabilis* and *N. punctiforme*. Candidate PAL encoding genes from *A. thaliana* were derived from cDNA library plasmids containing the specific loci of interest (Table 2.1) obtained from the ABRC (Ohio State University, Columbus, OH). Whereas *A. thaliana* posses four distinct PALs, we focused on those encoded by *PAL1* and *PAL2* as these have displayed the greatest activity when expressed in recombinant *E. coli* (noting also that PAL3 was found to be of 'very low activity')(Cochrane, Davin, and Lewis 2004). Amplified linear DNA fragments were purified using the Zyppy Clean and Concentrator kit (Zymo Research, Orange, CA). Purified fragments were treated by endonuclease digestion using appropriate restriction enzymes (all from New England Biolabs, Ipswich, MA). Amplified fragments containing the *pal* from both *N. punctiforme* and *A. variabilis* were digested with *BamHI* and *EcoRI* whereas fragments containing *PAL1* and *PAL2* from *A. thaliana* were digested with *EcoRI* and *SphI*. All digestions were performed at 37^{o}C according to manufacturer's protocols. The expression vector pSTV28 was similarly digested with either *BamHI* and *EcoRI* or *EcoRI* and *SphI*. Digested fragments were gel purified using the Zyppy Gel DNA recovery kit (Zymo Research, Orange, CA). Linearized fragments were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA) at 4^{o}C overnight. Chemically competent *E. coli* NEB10-Beta (New England Biolabs, Ipswich, MA) cells were used for all transformations. Transformants were selected by plating on LB solid agar containing 34 mg/L chloramphenicol and culturing at 37^{o}C overnight. The transformant pool was screened according to both colony PCR (employing the same primers as used in the initial amplification) and restriction digest mapping of the resultant plasmids to identify those

clones harboring the successful construct. This approach resulted in construction of plasmids pSpalAv, pSpalNp, pSpal1At, and pSpal2At, as listed in Table 2.1.

2.2.5 Cloning of candidate genes encoding PADC activity from L. plantarum, B. subtilis, and S. cerevisiae.

Candidate PADC encoding genes, including *pdc, padC, PAD1*, and *FDC1*, were amplified via PCR using genomic DNA templates derived from *L. plantarum*, *B. subtilis*, and *S. cerevisiae*, respectively. PCR amplified DNA fragments were purified before treatment by endonuclease digestion. Fragments containing *padC* and *pdc* were each digested with *BamHI* and *SbfI.* The *E. coli* expression vector pTrc99A was similarly digested with *BamHI* and *SbfI*. Meanwhile, the amplified fragment containing *PAD1* was digested with *NcoI* and *XbaI* whereas the *FDC1* containing fragment was digested with *SalI* and *HindIII*. The *E. coli* expression vector pTrc99A was similarly digested with either *NcoI* and *XbaI* (for the insertion of *PAD1*) or *SalI* and *HindIII* (for the insertion of *FDC1*). All digested fragments were gel purified then ligated at 4^{o}C overnight before their transformation into chemically competent *E. coli* NEB10-Beta. Selection was then achieved by plating transformed cells on LB solid agar containing 100 mg/L ampicillin and culturing at 37^{o}C overnight. After confirmation of the correct transformant, these works resulted in the generation of plasmids pTpadCBs, pTpdcLp, pTpad1Sc, and pTfdc1Sc, as listed in Table 2.1. The *SalI-HindIII* digested *FDC1* fragment was then also cloned into the same sites in the newly generated plasmid pTpad1Sc by an analogous protocol, resulting in the plasmid pTpad1Sc-fdc1Sc.

2.2.6 Assaying PAL activity in crude lysates of recombinant E. coli.

Each of the newly created PAL harboring plasmids (namely pSpalAv, pSpalNp, pSpal1At, and pSpal2At) were individually transformed into *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA). Seed cultures of each of the resultant strains were prepared (in triplicate) in 5 mL LB broth supplemented with 34 mg/L chloramphenicol and cultured at 32°C while shaking at 250 rpm overnight. 50 µl of each seed was then used to again inoculate 5 ml LB broth. These cultures were grown until reaching an OD_{600} of ~0.6, at which point each was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM. Induced cultures were incubated for an additional 6 h after which an equal number of cells (determined by OD_{600} measurement) were collected by centrifugation at 1400 x *g* for 4 min. The cell pellet was re-suspended in 900 µL distilled water. Cell lysis was achieved using the FastBreak Cell Lysis Reagent kit (Promega, Madison, WI) and the supernatant collected after centrifugation at 11,000 x *g* for 2 min. PAL activity was analyzed at room temperature in pH 7.5 50 mM Tris-HCl buffer containing 250 mM L-phenylalanine. Activity assays were initiated by the addition of 5 µL of crude cell lysate. The production of tCA was followed at 290 nm on a Beckman Coulter UV/Vis Spectrophotometer for a total of 5 min at 20 sec intervals. A molar extinction coefficient of 9,000 M^{-1} cm^{-1} and a 1 cm path length were used to establish enzyme activity in terms of U mg^{-1} protein. The PAL protein content in each crude lysate was determined via first separation by SDS-PAGE using Mini-PROTEAN TGX 4-20% precast gels (Bio-Rad, Hercules, CA) and standard protocols. Concentration was then analyzed using the ImageJ software package (NIH, Bethesda, MD) and calibrated versus Precision Plus Unstained Protein Standards (Bio-Rad, Hercules, CA).

2.2.7 Assaying PAL activity in recombinant E. coli whole cells.

Seed cultures of *E. coli* BL21(DE3) harboring one of pSpalAv, pSpalNp, pSpal1At, or pSpal2At, were prepared in 5 mL LB broth and grown overnight. Shake flasks (250 mL) containing 50 mL of LB were inoculated with 1 mL of each seed culture. Cultures were grown until an OD_{600} of ~0.6 was reached, at which point the cultures were induced by IPTG addition at a final concentration of 0.2 mM. Cultures were then incubated for an additional 6 h (resulting in an OD_{600} of ~2) before an equal number of cells were collected and centrifuged at 1400 x *g* for 5 min. The pellet was washed once with PBS (phosphate buffered saline) at pH 7 before being re-suspended in 12 ml PBS buffer. Finally, the appropriate substrate, L-phenylalanine or L-tyrosine at a final concentration of 1 g/L, was added to the suspension. The suspensions were then shaken at 32°C for a total of three hours. Samples (1 mL) were taken every hour, centrifuged, and 750 µL of supernatant was collected for HPLC analysis to monitor the production of either *tCA* or *p*-coumaric acid.

2.2.8 Assaying PADC activity in recombinant E. coli whole cells.

The plasmids pTpad1Sc-fdc1Sc, pTpad1Sc, pTfdc1Sc, pTpdcLp, and pTpadcBs were each individually transformed into chemically competent *E. coli* BL21(DE3). A seed culture of each strain was then grown in LB broth overnight. Shake flasks (250 mL) containing 50 mL of LB were inoculated with 1 ml of seed culture. Cultures were grown at 32°C until an OD_{600} of ~0.6, at which point they were induced by adding 0.2 mM IPTG and then incubated for an additional 6 h. Cells were then collected and re-suspended in 12 ml PBS buffer (as previously described) and the substrate (*tCA* or *p*-coumaric acid) was added at a final concentration of 1 g/L. Samples (1 mL) were

removed from the culture at both the time of initiation as well as after 12 h of incubation at 32^{o}C and analyzed by HPLC using the methods described herein.

2.2.9 Co-expression of PAL and CADC isoenzymes in E. coli NST74 to convert L-phenylalanine to styrene.

The L-phenylalanine over-producing strain *E. coli* NST74 (Table 2.1) was co-transformed with the plasmids pSpal2At and pTfdc1Sc and selected for on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. The resultant transformant was then grown overnight at 32°C in 5 mL LB broth. Shake flasks (250 mL) containing 50 mL LB were inoculated with 1 mL of seed culture. The culture was then grown at 32°C until an OD_{600} of ~0.6, at which time it was induced with 0.2 mM IPTG and then incubated for an additional 8 h. Cells were then collected and re-suspended in 12 ml PBS buffer (as previously described) and L-phenylalanine added at a final concentration of either 400 or 900 mg/L. Samples were taken periodically and analyzed by HPLC to determine the content of L-phenylalanine, tCA, and styrene, according to the methods presented below.

2.2.10 Co-expression of PAL and CADC isoenzymes in E. coli NST74 to convert glucose to styrene in shake flask cultures.

The L-phenylalanine over-producing strain *E. coli* NST74 was co-transformed with each of the following combinations of plasmids: pSpalAv and pTfdc1Sc, pSpalNp and pTfdc1Sc, pSpal1At and pTfdc1Sc, and pSpal2At and pTfdc1Sc.Single colonies were then selected from the resulting transformants and grown in 5 mL LB broth for 12 h at 32°C while shaking at 250 rpm to prepare a seed culture. Each seed (1 mL) was then used to inoculate 50 mL MM1. These cultures were performed in 100 mL serum bottles

outfitted with septa caps that were tightly sealed upon inoculation. A closed system was used to avoid volatile product (i.e., styrene) losses. A large headspace was used to preclude the depletion of oxygen from the bottle. Cultures were grown for 10 h prior to being induced by the addition of IPTG at a final concentration of 0.2 mM. Culturing continued for 29 to 48 h post induction while 1 ml samples were periodically taken from each culture and analyzed for relevant metabolite contents via HPLC, using the methods described herein.

2.2.11 Metabolite analysis by HPLC.

Samples were prepared by removing 1 mL of culture from a shake flask culture and pelleting the cells at 11,000 x *g* for 2 min. The supernatant (0.75 mL) was then transferred to a glass HPLC vial and sealed with a Teflon-lined cap. HPLC analysis was carried out using a Hewlett Packard 1100 series HPLC system equipped with an auto sampler, diode array (UV/Vis) detector, and reverse-phase Hypersil Gold SBC18 column (4.6mm x 150 mm; Thermo Fisher, USA). Samples (5 µL) were injected for analysis at a total constant flow rate of 1.0 ml/min and constant column temperature of 45^{o}C. The column was eluted with 'solvent A' (consisting of double-distilled water) and 'solvent B' (consisting of methanol (99.8% grade) plus 0.1% trifluoroacetic acid (TFA)). The eluent began as a mixture of 95% solvent A and 5% solvent B before a linear gradient was applied over 8 min to then reach a mixture of 20% solvent A and 80% solvent B. This eluent composition was then held constant for 2 min before a second linear gradient was then applied over the course of 4 min to achieve a final mixture of 95% solvent A and 5% solvent B. The eluent was monitored at each of 215 nm for L-phenylalanine and 258 nm for *t*CA, *p*-coumaric acid, hydroxystyrene and styrene. Under these conditions L-

phenylalanine, *p*-coumaric acid, *tCA*, *p*-hydroxystyrene, and styrene were eluted at 4.5, 6.7, 8.67, 8.78, and 10.4 min, respectively.

2.2.12 Confirmation of styrene biosynthesis by GC-MS.

Culture supernatant (1 ml) was added to hexane (1 ml) and vortexed for 20 min at maximum speed. The biphasic mixture was centrifuged for 1 min at 11,000 x *g* to settle. 750 µl of the hexane layer was removed for analysis by GC-MS. GC-MS analysis was performed on a Hewlett Packard 5890 Series II gas chromatograph with a flame ionizing detector and Supelco MDN-5 S (30 m × 0.25 mm id) fused-silica capillary column using helium as the carrier gas. The injector, column, and detector temperatures were initial set at 280, 240, and 220 °C, respectively. The column temperature was then increased from 40 to 320 °C at 14 °C/min.

2.3 Results and discussion

2.3.1 Assaying styrene toxicity.

Under the studied conditions, maximal titers of L-phenylalanine produced by *E. coli* NST74 after 48 h of culture in MM1 (with 1.5% glucose) reached about 700-1000 mg/L (results not shown). Whereas the theoretical yield of L-phenylalanine on glucose is 0.55 g/g(Baez-Viveros et al. 2004), achievable yields by *E. coli* NST74 under the present conditions in shake flask cultures were only 0.052-0.074 g/g (or about 10% of theoretical). Assuming complete conversion of all endogenously produced L-phenylalanine to styrene were possible (if our engineered pathway could achieve a particularly high flux), we would expect to be able to synthesize up to 440-630 mg/L of styrene under the same culture conditions (corresponding to a glucose yield of 0.051-

0.072 g/g), barring any physiological limitations such as product toxicity. Through a preliminary screening study, however, we have found that the products *trans*-cinnamate ($logK_{ow}$ = 2.13) and styrene ($logK_{ow}$ = 3.05) can each inhibit *E. coli* growth when added to cultures at final concentrations above ~800 mg/L and ~300 mg/L, respectively, as demonstrated in Fig. 2.2.

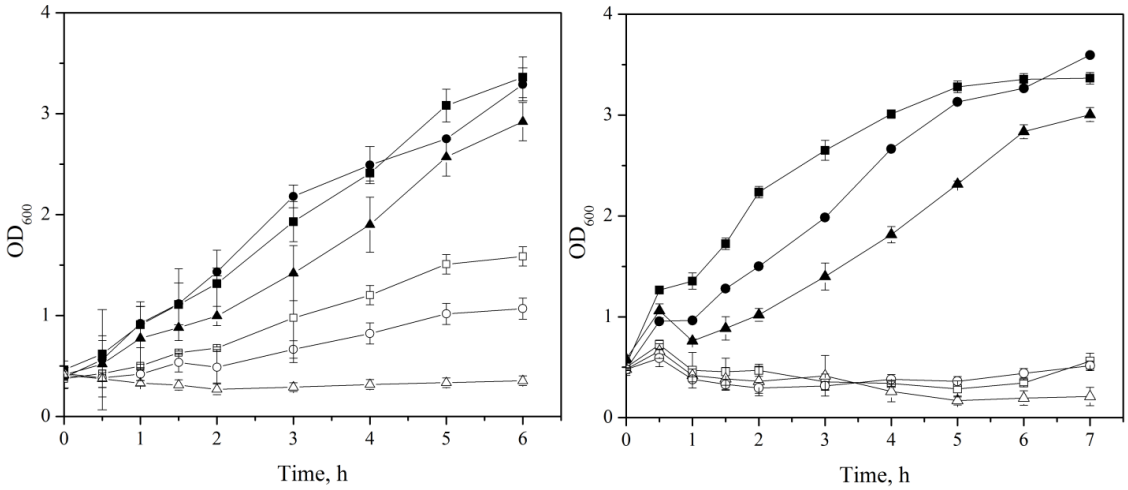

Figure 2.2 Toxicity of styrene and trans-cinnamate. Left: Growth response of *E. coli* NST74 to tCA at concentrations of 0 mg/L (solid square), 200 mg/L (solid circle), 400 mg/L (solid triangle), 600 mg/L (open square), 800 mg/L (open circle), and 1000 mg/L (open triangle). Right: Growth response of *E. coli* NST74 to styrene at concentrations of 0 mg/L (solid square), 100 mg/L (solid circle), 200 mg/L (solid triangle), 300 mg/L (open square), 400 mg/L (open circle), and 500 mg/L (open triangle). Error bars reported at one standard deviation from triplicate experiments.

For reference, *p*-hydroxystyrene ($logK_{ow}$ = 2.28) has previously been shown to inhibit *P. putida* at concentrations of ~540 mg/L(Verhoef et al. 2009). Solvent hydrophobicity is typically a good indicator of its toxicity towards microorganisms and may be quantified by $logK_{ow}$, the log of the octanol-water partition coefficient(Ramos, Duque, Gallegos, Godoy, Ramos-González, et al. 2002). Even at low concentrations, compounds with a $logK_{ow}$ between 1.0 to 5.0 tend to be very toxic(Heipieper, Weber, Sikkema, Keweloh, and de Bont 1994). Accumulation of hydrophobic aromatics within

the cytoplasmic membrane has been shown to disrupt its integrity, resulting in the leakage of ions, metabolites, lipids, and proteins, as well as affecting the cells ability to maintain its internal pH and an appropriate transmembrane proton gradient(Ramos, Duque, Gallegos, Godoy, Ramos-González, et al. 2002; Weber et al. 1993). The measured toxicity thresholds were found to be consistent at both low (OD_{600} 0.6) and high (OD_{600} 2, data not shown) initial cell densities (reflecting early and late exponential growth, respectively), indicating growth inhibition was independent of cell density and growth stage. As a result of the inherent toxicity of styrene we anticipated that its biosynthesis using an NST74 host platform would be limited to below its maximum potential under our culture conditions. While no prior studies have specifically explored either the effects of styrene toxicity on *E. coli* or strategies to improve its tolerance, several *Pseudomonas* sp. have been shown to display enhanced styrene tolerance characteristics(Weber et al. 1993). Whereas the present study is solely focused on the prototyping of a novel pathway for styrene biosynthesis, it is clear from our preliminary assays that styrene toxicity must eventually be overcome or effectively circumvented if renewable styrene production is ever to become viable or sustainable.

2.3.2 Screening candidate PAL isoenzymes for activity in recombinant E. coli.

The activities of recombinant PALs from various genetic sources were analyzed according to both *in vitro* (crude lysate) and *in vivo* (whole, resting cell) assays. All recombinant PALs, expressed from plasmids pSencPSm, pSpalAv, pSpalNp, pSpal1At, and pSpal2At (Table 2.1) in an *E. coli* BL21(DE3) background were recovered as crude lysates. According to our *in vitro* assay results, all of the tested PALs showed comparable levels of activity on L-phenylalanine as substrate (Table 2.2), with the exception of EncP

from *S. maritimus* whose activity was non-measureable. It has previously been shown through kinetic studies on prokaryotic PALs that those derived from *N. puntiformes* and *A. variabilis* possessed 500-1000 times greater activity than EncP from *S. maritimus*; furthermore, the k_{cat}/K_m value of PAL from *A. variabilis* was found to be greater than that of *N. puntiformes* (72.2 and 43.8 $mM^{-1}s^{-1}$, respectively)(Moffitt et al. 2007). Thus, it is possible that EncP was in fact functionally expressed in our study; however, its activity was too low to measure according to the protocols employed. The relative activities of candidate PALs were then further explored through the use of whole-cell assays in recombinant *E. coli*. Resting cells suspended in PBS buffer (pH 7) were supplemented with L-phenylalanine or L-tyrosine and product (tCA or *p*-coumaric acid, respectively) formation was monitored over the course of 3 h. Although the PALs from *A. thaliana* were found to possess the greatest specific activity in crude lysates (Table 2.2), assays of *in vivo* function provided a more stark contrast into the relative activities among all candidate PALs, as shown in Fig. 2.3.

Table 2.2 PAL Specific Activity. Specific activity of PAL isoenzymes from *A. variabilis*, *N. punctiforme*, and *A. thaliana* on L-phenylalanine and L-tyrosine when expressed in recombinant *E. coli* BL21(DE3).

Strain	Substrate	Activity (U mg^{-1} protein)
BL21(DE3)	L-phenylalanine	n.d.
	L-tyrosine	n.d.
pSencPSm	L-phenylalanine	n.d.
	L-tyrosine	n.d.
pSpalAv	L-phenylalanine	2.38 ± 0.64
	L-tyrosine	n.d.
pSpalNp	L-phenylalanine	0.91 ± 0.32
	L-tyrosine	n.d.
pSpal1At	L-phenylalanine	2.42 ± 1.07
	L-tyrosine	n.d.
pSpal2At	L-phenylalanine	4.08 ± 0.11
	L-tyrosine	n.d.

n.d. – not detected

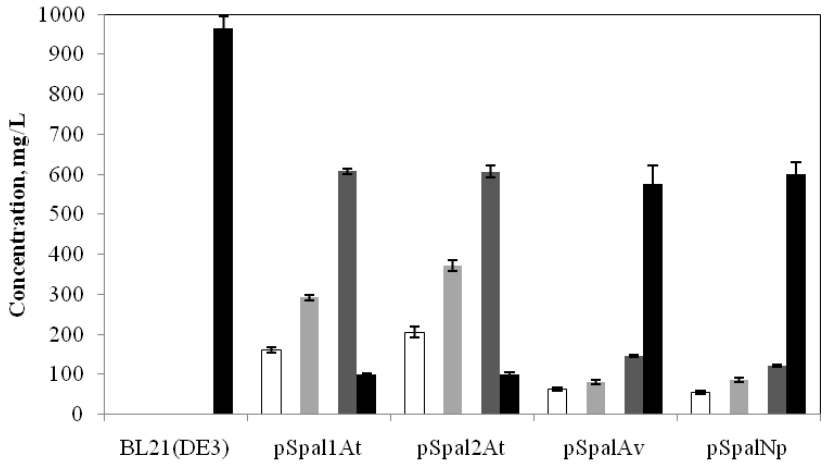

Figure 2.3 PAL Activity. Phenylalanine ammonia lyase activity from candidate genes cloned from *A. thaliana*, *A. variabilis*, and *N. punctiformes* in recombinant *E. coli* BL21(DE3) whole cells. 50 ml cultures were grown for 8 h (induced with 0.2 mM IPTG after 1.5 h), spun down and resuspended in 10 ml PBS buffer. The conversion of 1 g/L L-phenylalanine (black) to *trans*-cinnamic acid after 1 h (no color), 2 h (light gray), and 3h (dark gray). Error bars reported at one standard deviation from triplicate experiments.

Under the studied conditions, the PALs of *A. thaliana* were found to display the greatest activity (being significantly higher that of either prokaryotic PAL) although being comparable for both isoenzymes. This result is more consistent with previous studies wherein k_{cat}/K_m values of PAL1 and PAL2 were estimated as 25,500 and 51,200 $M^{-1}s^{-1}$ (or six orders of magnitude greater than those reported for *N. puntiformes* and *A. variabilis*) when expressed in recombinant *E. coli* (Cochrane, Davin, and Lewis 2004). The elevated relative activity of PAL2 is also consistent with the observation of higher initial rates of tCA accumulation when expressed from BL21(DE3) pSpal2At, as seen in Fig. 2.3. Again, however, none of the tested PAL isoenzymes were found to display any activity on L-tyrosine as the substrate (data not shown), which is consistent with the results of our *in vitro* assays (Table 2.2). Taken together, these analyses demonstrate that all of the studied PALs display rigid substrate specificity for L-phenylalanine. This important result contrasts numerous prior works on aromatic pathway development in

recombinant *E. coli* which have solely relied upon the use of bifunctional yeast PAL/TALs(Gilbert et al. 1985; Gilbert and Tully 1982; Gilbert, Stephenson, and Tully 1983; Cui, Jia, and Sun 2008) and, which was specifically used to synthesize *p*-hydroxystyrene(Qi et al. 2007). As these results illustrate that the first committed step in our pathway is highly specific for the intended substrate (L-phenylalanine) alone, we anticipate that this advantageous outcome will ultimately help to control product purity (specifically styrene over *p*-hydroxystyrene or a mixture of products) while also improving the activity and flux of our desired pathway. Meanwhile, the same cannot be assured had the first committed step of our pathway been catalyzed by PAL/TAL, as was the case for all engineered *p*-hydroxystyrene pathways reported to date(Qi et al. 2007; Verhoef et al. 2009).

2.3.3 Screening candidate PADC isoenzymes for tCA decarboxylase activity in recombinant E. coli.

Candidate PADC isoenzymes from *L. plantarum*, *B. subtilis*, and *S. cerevisiae* were screened for their ability to decarboxylate tCA to produce styrene when expressed in *E. coli*. Plasmids harboring the candidate PADC-encoding genes (Table 2.1) were individually transformed into *E. coli* BL21(DE3), as described above. Whole, resting cells were prepared in PBS buffer supplemented with 1g/L of tCA acid or *p*-coumaric acid. The production of styrene or *p*-hydroxystyrene, respectively, was then followed periodically, and the results after 12 h of culture are compared in Fig. 2.4. With the exception of the strain expressing *PAD1* from *S. cerevisiae* alone, all other strains displayed decarboxylase activity on *p*-coumaric acid, leading to *p*-hydroxystyrene biosynthesis. These results are consistent with previous reports on the functional

expression of *pdc* and *padC* in recombinant *E. coli*(Qi et al. 2007) to support p-hydroxystyrene biosynthesis from glucose. However, our findings further and importantly show that the enzymes from *L. plantarum* and *B. subtilis* cannot catalyze the conversion of tCA to styrene (thus making them more specific for *p*-coumaric acid). In the present study it was instead found that the sole expression of *FDC1* from *S. cerevisiae* (which was previously characterized to encode a ferulic acid decarboxylase) was sufficient for achieving decarboxylase activity on either tCA or *p*-coumaric acid (with perhaps a slight preference towards tCA, based on overall conversion). Though it was previously reported that the co-expression of both *FDC1* and *PAD1* is necessary to achieve tCA decarboxylase activity in the native *S. cerevisiae* (Mukai et al. 2010), we now report that functional tCA decarboxylase activity in *E. coli* depends solely upon *FDC1* over-expression and is not dependent upon the co-expression of *PAD1*. Furthermore, as can be seen in Fig. 2.4, comparable styrene titers were achieved when either *FDC1* was expressed alone or together with *PAD1*, indicating that the co-expression of *PAD1* does not increase (or otherwise alter) tCA decarboxylase activity in *E. coli*, as has previously been suggested in the native *S. cerevisiae* (Larsson et al., 2001). It is, however, plausible that the expression of *ubix* (which has been shown to be 50% similar to *PAD1*(Mukai et al. 2010)) in our *E. coli* background could have served to compensate for the absence of *PAD1* expression, enabling tCA decarboxylase activity to be achieved in *E. coli* expressing *FDC1* alone. Future studies will explore the deletion of *ubix* from the background of *E. coli* to further investigate the recombinant function of *FDC1*. Most importantly, these results have demonstrated that *FDC1* over-expression in *E. coli* uniquely enables the decarboxylation of tCA to styrene.

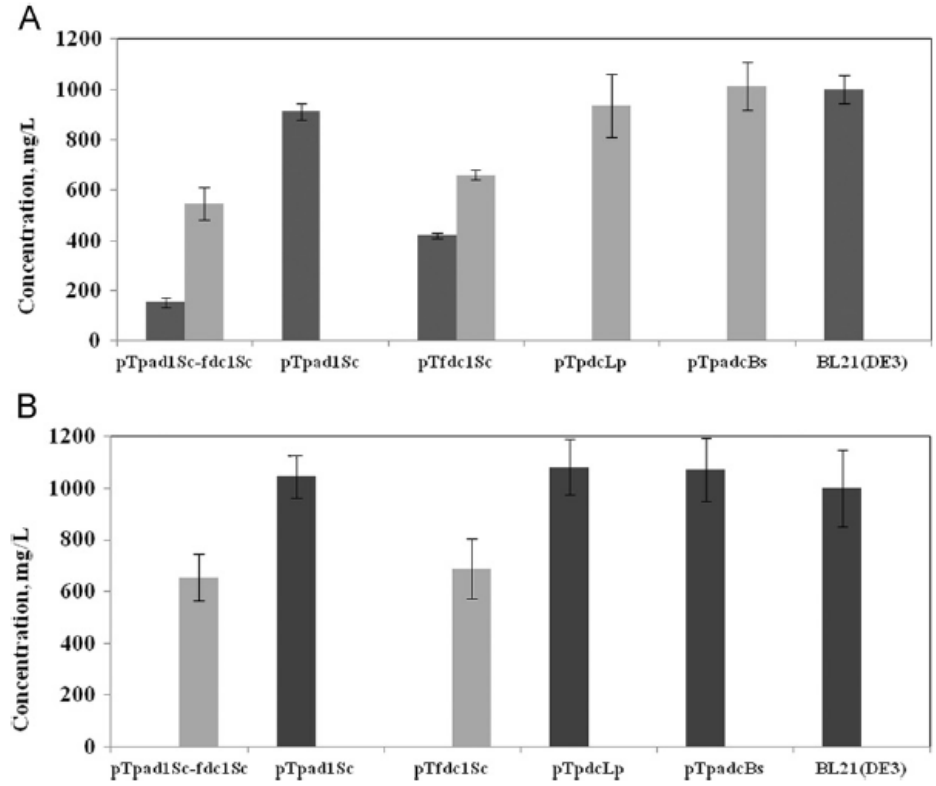

Figure 2.4 PADC Activity. Phenylacrylic acid decarboxylase activity from candidate genes cloned from *S. cerevisiae*, *L. plantarum*, and *B. subtilis* in recombinant *E. coli* BL21(DE3) whole cells. The conversion of A) 1 g/L *p*-coumaric acid (dark gray) to *p*-hydroxystyrene (light gray) and B) 1 g/L *trans*-cinnamic acid (dark gray) to styrene (light gray) after 12 h. Error bars reported at one standard deviation from triplicate experiments.

Although the proposed styrene biosynthesis pathway is somewhat analogous to that which was previously developed for *p*-hydroxystyrene biosynthesis(Qi et al. 2007; Verhoef et al. 2009), it is essential to realize that the specific enzyme 'parts' used in those two prior studies are wholly inadequate for achieving the present objective of styrene biosynthesis from glucose. In other words, said previously-characterized enzymes were not simply transferrable among the two (styrene and *p*-hydroxystyrene) distinct pathways, but rather that styrene biosynthesis could only be supported through the identification and characterization of a specific tCA decarboxylase.

2.3.4 Biosynthesis of tCA and styrene from glucose in recombinant E. coli.

The biosynthesis of tCA from glucose was first investigated using strains of *E. coli* NST74 that individually carried the plasmids pSpalAv, pSpalNp, pSpal1At, and pSpal2At. Cultures were grown in MM1 media with 1.5% glucose. 48 h after induction with 0.2 mM IPTG, the production of tCA was detected in each culture at final titers of 600 mg/L, 473 mg/L, 648 mg/L, and 918 mg/L, respectively. Neither styrene nor *p*-coumaric acid were detected in any samples.

To test for styrene biosynthesis from glucose, *FDC1* from *S. cerevisiae* was then co-expressed with each of the PAL-encoding genes according to the following constructed strains: NST74 pSpalAv pTfdc1Sc, NST74 pSpalNp pTfdc1Sc, NST74 pSpal1At pTfdc1Sc, and NST74 pSpal2At pTfdc1Sc. Conversion of glucose to styrene was followed after IPTG induction and results for the strain NST74 pSpal2At pTfdc1Sc are shown in Table 2.3 (other results not shown as this dataset was representative of the results and trends observed with all other strains). Substantial L-phenylalanine accumulation was not observed until 17 h post induction, after which time styrene titers then also rose considerably, reaching a final titer of 260 mg/L in the culture medium 29 h post-induction. The final styrene titers for the strains NST74 pSpalAv pTfdc1Sc, NST74 pSpalNp pTfdc1Sc, and NST74 pSpal1At pTfdc1Sc were found to be 210, 183, and 188 mg/L, respectively.

Table 2.3 Styrene Fermentation Titers. Biosynthesis of L-phenylalanine, *trans*-cinnamic acid, and styrene by *E. coli* NST74 pSpal2At pTfdc1Sc from glucose in MM1 media. Errors reported at one standard deviation from triplicate experiments.

time (h)	L-phenylalanine (mg/L)	t*rans*-cinnamic acid (mg/L)	styrene (mg/L)
0	13.9 ± 0.03	0	0
13	56.4 ± 0.13	3.7 ± 0.01	7.3 ± 0.12
17	236.5 ± 0.57	6.3 ± 0.01	23 ± 0.38
21	167.4 ± 0.40	9.1 ± 0.02	205 ± 3.40
25	152.2 ± 0.36	13.5 ± 0.02	243 ± 4.03
29	179.5 ± 0.43	14.0 ± 0.03	260 ± 4.31

As styrene-producing strains were cultured, a strong ‘hydrocarbon’ aroma was readily detected upon opening the sealed culture bottles. The high volatility of styrene necessitated the use of sealed jars for culturing. To ensure that sufficient oxygen remained available to the culture, a large headspace volume (200 mL headspace vs. 50 mL media) was used. Thus, cultures were maintained under aerobic conditions throughout the culture (as confirmed by the inclusion of the indicator dye resazurin). However, the use of such a large headspace volume also allows for the significant accumulation of styrene vapor, which should also be accounted for when assessing productivity. Under dilute and near-ambient conditions, the equilibrated headspace vapor composition can be estimated by application of Henry’s Law. Using a dimensionless Henry’s Law constant of 0.113(Yang 1992), it can be predicted that a headspace equilibrated with an aqueous phase containing 260 mg/L styrene will contain an additional 29 mg/L styrene, for a total of 18.9 mg in the 250 mL flask (note this would be equivalent to an aqueous titer of 376 mg/L if no volatilization had occurred). In an analogous manner, the final styrene production by strains NST74 pSpalAv pTfdc1Sc,

NST74 pSpalNp pTfdc1Sc, and NST74 pSpal1At pTfdc1Sc would be estimated as 15.2, 13.4, and 13.6 mg, respectively.

Following these fermentation studies, culture supernatants were extracted using hexane, and the extracts were analyzed by GC-MS to confirm that it was in fact styrene that was being synthesized by our cultures. Comparing the spectra of the dominant metabolite peak which was recovered from the extract to the NIST08 spectral database(Babushok et al. 2007), styrene was found to be the most probable compound. As can be seen from Fig. 2.5, the mass spectra of the extracted sample and the library reference provide an excellent match, providing confirmation that styrene was in fact synthesized by our engineered strains of *E. coli*.

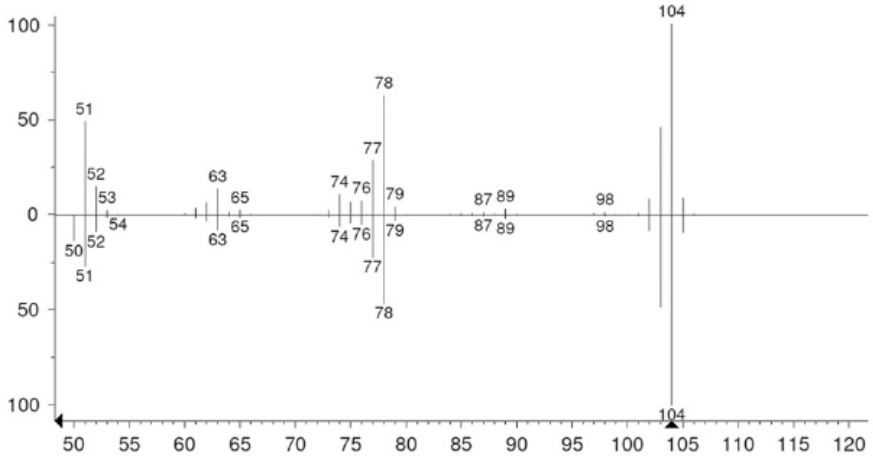

Figure 2.5 Mass spectra of styrene. Head to tail comparison of the standard mass spectra showing the relative abundance of the mass-to-charge ratio of styrene from the NIST08 library (lower) with that of the dominant metabolite peak obtained in hexane extractions of the culture broth (upper).

Throughout the duration of culture, never was all of the L-phenylalanine observed to be fully assimilated into the styrene pathway (as seen in Table 2.3). Furthermore, tCA titers were observed to remain low throughout, indicating that almost all of the

synthesized tCA could be quickly converted to styrene. Taken together, these observations suggest that low PAL activity presently remains as the flux limiting condition in the engineered styrene biosynthesis pathway. As the first committed step in the styrene pathway, high PAL activity is essential and must be improved in subsequent generations of our strains. To start, the expression of PAL can likely be improved through the use of codon optimized variants. This approach has shown to be useful, particularly in cases for which pathways involve the expression of plant-derived genes in *E. coli*. For example, codon optimization was applied to the amorphadiene oxidase from *Artemisia annua* (a plant species) as part as a synthetic pathway to produce artemisinic acid(Keasling 2008). Whereas the native protein originally showed neither *in vivo* nor *in vitro* activity when expressed in *E. coli*, a codon optimized variant resulted in its functional expression in *E. coli*, contributing to successful pathway development.

Whereas improvements in PAL expression can lead to enhancements in the specific metabolite flux, the net flux can also be improved by increasing the pathway 'driving force'. That is, by promoting the increased availability of the pathway's immediate precursor, L-phenylalanine. This notion was tested in resting cells assays wherein *E. coli* NST74 pSpal2At pTfdc1Sc cell suspensions were supplemented with exogenous L-phenylalanine at initial concentrations of either 400 or 950 mg/L. As shown in Fig. 2.6, the conversion of 400 mg/L L-phenylalanine to styrene occurred rapidly and completely (i.e., no residual L-phenylalanine or tCA was detectable), yielding a final styrene titer of 250 mg/L after 30 h. Meanwhile, when 950 mg/L L-phenylalanine was added to the resting cell cultures, nearly 500 mg/L styrene could be produced as all of the L-phenylalanine was consumed. However, in this case, nearly an additional 250 mg/L

tCA also remained in the culture. Noting that such a final styrene titer actually surpasses the toxicity threshold of growing cells, it is not altogether surprising that the reaction was unable to proceed to completion. The final styrene titer was only able to surpass the toxicity threshold of 300 mg/L because the cells grew to stationary phase and have already assembled the necessary enzymes. In this case, 500 mg/L represents an enzymatic inhibition imposed by styrene rather than a cellular growth inhibition as previously described.

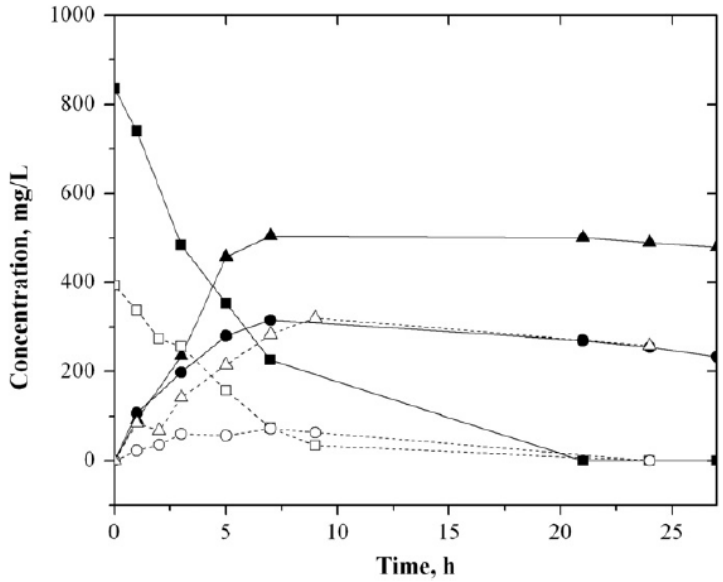

Figure 2.6. Whole cell styrene production. Whole cell production of styrene (triangle) from *trans*-cinnamic acid (circle) after exogenous L-phenylalanine (square) addition by *E. coli* NST74 pSpal2At pTfdc1Sc. Open and filled shapes correspond to the initial addition of 400 and 950 mg/L L-phenylalanine.

These results demonstrate that if the L-phenylalanine precursor pool can be enhanced the net production of styrene can also be increased. Clearly, however, the addition of exogenous L-phenylalanine is not a sustainable approach towards enhancing styrene biosynthesis, but rather endogenous L-phenylalanine production must be enhanced in our host platform. Subsequent studies will explore the incorporation of

additional modifications into the genome of the *E. coli* NST74 host that have previously been shown by other groups to result in L-phenylalanine yield enhancements. For instance, it has been reported that the over-expression of endogenous transketolase I (encoded by *tktA*) supports elevated L-phenylalanine biosynthesis(Gosset, Yong-Xiao, and Berry 1996). TktA over-expression enables the enhanced biosynthesis of erythrose 4-phosphate (E4P) which, when condensed with phosphoenolpyruvate (PEP), yields 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), the first committed intermediate in the aromatic amino acid biosynthesis pathway(Flores et al. 1996; Lütke-Eversloh and Stephanopoulos 2008). Meanwhile, disruption of the global carbon storage regulatory system of *E. coli* through deletion of *csrA*, increases PEP biosynthesis, leading to up to a 2-fold enhancement in L-phenylalanine yield(Tatarko and Romeo 2001).

For microbially-derived, renewable styrene to become an economically-viable and sustainable alternative to petroleum-derived predecessor, titers and productivity must ultimately be improved. Although the styrene titers achieved by our 1st generation strains were modest, in comparison to the toxicity assays performed above it can be seen that they are already approaching the inhibitory threshold. Thus, whereas increasing L-phenylalanine yields could eventually translate into elevated styrene production, product toxicity would soon become the subsequent limiting factor and must be addressed. As complex phenotypes such as solvent tolerance are simply not monogenic in nature(Alper et al. 2006), numerous specific mutations must applied in concert to achieve the specifically desired result. Since little is presently known of styrene toxicity or tolerance, rational approaches to engineering more tolerant strains may be less suitable than higher throughput, combinatorial strategies. Although commonly employed, combinatorial

approaches towards enhancing desired phenotypes often rely upon the aggressive use of chemomutagenesis, such techniques are less desirable as they can also result an unforeseen and difficult to understand negative impacts on host fitness and/or productivity(Bonomo et al. 2006), in addition to requiring laborious screening and selection procedures. A more effective approach for the present application might involve the use of an alternative host platform which inherently boasts greater tolerance(Fischer, Klein-Marcuschamer, and Stephanopoulos 2008), a strategy which has been applied for the production of biofuels, such as n-butanol(Nielsen et al. 2009). The bacterium *P. putida* S12 has been engineered, for example, as a solvent tolerant platform for the biosynthesis of both *p*-hydroxybenzoate and *p*-hydroxystyrene(Verhoef et al. 2007; Verhoef et al. 2009), and might also make excellent starting point for styrene production. Since our pathway is derived from the ubiquitous, proteinogenic amino acid L-phenylalanine, its transference to an alternative host platform remains wholly compatible. Alternatively, improved product tolerance can also be achieved through genome evolution, as accomplished through serial adaptations to increasing styrene concentrations. Such an approach has worked well for achieving tolerance to biofuels like n-butanol and could also be coupled with genomic library screens to provide the first comprehensive view of styrene inhibition and tolerance in *E. coli*(Reyes, Almario, and Kao 2011). In an interesting example, prior works have shown that *Pseudomonas* sp. may be adapted to styrene when also grown in the presence of acetate or similar carboxylic acids as the sole carbon source(Weber et al. 1993). It has been suggested that this evolved phenotype may be associated with genetic changes leading to reductions in membrane fluidity, as well as through the activation of genes believed to be specifically associated

with enhanced tolerance to aromatic compounds. As an alternative strategy, combinatorial procedures such as the global Transcription Machinery Engineering (gTME)(Alper et al. 2006; Nicolaou, Gaida, and Papoutsakis 2010) may be implemented to improve styrene tolerance in *E. coli* or alternative host platforms selected due to their elevated tolerance baseline. The principals of gTME have been successfully employed to enhance ethanol tolerance in both yeast(Alper et al. 2006) and *E. coli*(Alper and Stephanopoulos 2007).

In addition to the development of styrene tolerant phenotypes, product toxicity can also be enhanced through the use of *in situ* product recovery (ISPR). Common ISPR approaches involve solvent extraction(Gyamerah and Glover 1996; Malinowski 2001; Weilnhammer and Blass 1994), adsorption(Nielsen, Amarasiriwardena, and Prather 2010; Nielsen and Prather 2009), gas and vacuum stripping(Loser et al. 2005), and membrane pervaporation(Vane 2005). Each of these approaches has been successfully applied for the continuous recovery of biofuel compounds such as ethanol, for example, and even aromatics like L-phenylacetylcarbinol(Khan and Daugulis 2010) and benzaldehyde(Jain, Khan, and Daugulis 2010). The latter three approaches may be particularly well-suited for styrene recovery given its volatile nature, as previously discussed.

2.4 Conclusion

For the first time, the present study has demonstrated the biosynthesis of styrene from renewable resources using an engineered microbial platform. Whereas low activity of pathway enzymes and product toxicity remain as challenges which limit the

productivity of our styrene-producing strains, continued improvements will lead to the development of robust biocatalysts for the sustainable production of this important, large volume, commodity chemical.

Acknowledgements

We thank Prof. Bradley Moore (UCSD) and Prof. Kristala Prather (MIT) for their kind gifts. We also thank Tom Colella (ASU) for his skillful technical assistance in GC-MS analysis.

CHAPTER 3

BIOSYNTHESIS OF (S)-STYRENE OXIDE AND (R)-1,2-PHENYLETHANEDIOL FROM GLUCOSE

Abstract

(*S*)-Styrene oxide and (*R*)-1,2-phenylethanediol are chiral aromatic molecular building blocks used commonly as precursors to pharmaceuticals and other specialty chemicals. Two pathways have been engineered in *E. coli* for their individual biosynthesis directly from glucose. The novel pathways each constitute extensions of the previously engineered styrene pathway, developed by co-expressing either styrene monooxygenase (SMO) or styrene dioxygenase (SDO) to convert styrene to (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol, respectively. StyAB from *Pseudomonas putida* S12 was determined to be the most effective SMO. SDO activity was achieved using NahAaAbAcAd of *Pseudomonas* sp. NCIB 9816-4, a naphthalene dioxygenase with known broad substrate specificity. Production of phenylalanine was enhanced through a number of mutations, most notably via deletion of *tyrA* and over-expression of *tktA*. As a result, (*R*)-1,2-phenylethanediol reached titers as high as 1.23 g/L, and at 1.32 g/L (*S*)-styrene oxide titers already approach their toxicity limit. This study additionally demonstrates that greater flux through the styrene pathway can be achieved if its toxicity is addressed, as achieved in this case by reacting styrene to less toxic products.

This work was published as: McKenna, R., Pugh, S., Thompson, B. & Nielsen, D.R. Microbial production of the aromatic building-blocks (S)-styrene oxide and (R)-1,2-phenylethanediol from renewable resources. *Biotechnology Journal* **8**, 1465-1475 (2013).

3.1 Introduction

(*S*)-Styrene oxide and (*R*)-1,2-phenylethanediol (also referred to as (*R*)-styrene glycol) are chiral aromatic molecules used in the synthesis of numerous high value pharmaceuticals and specialty chemicals. For example, (*S*)-styrene oxide is used as a precursor to the biocides levamisole and nematocide (Park, So, et al. 2006), as well as in the synthesis of cosmetics (Loprieno et al. 1976), surface coatings, and numerous agricultural and biological chemicals (Panke et al. 2000; Han et al. 2006). Meanwhile, (*R*)-1,2-phenylethanediol is an optically active diol and precursor to the pharmaceuticals (*R*)-norfluoxetine and (*R*)-fluoxetine (used to treat psychiatric and metabolic disorders), as well as *β*-lactam antibiotics (Cao et al. 2006; Kumar, Upadhyay, and Pandey 2004). Additionally, (*R*)-1,2-phenylethanediol is also used in the production of various agrochemicals and pheromones (Gamenara and Dominguez de Maria 2009), as well as chiral catalysts (King et al. 1979). Conventional production of (*S*)-styrene oxide occurs by the partial oxidation of styrene, most often over a heavy metal catalyst. This chemocatalytic process offers little control over product stereochemistry, and yields (*S*)-styrene oxide at only 48 to 57% enantiomeric excess (Groves and Myers 1983; Zhang et al. 1990) resulting in a mixture of isomers that must then be further processed to resolve and isolate the desired (*S*)-enantiomer. As (*R*)-1,2-phenylethanediol is conventionally produced from (*S*)-styrene oxide via a hydrolytic ring opening reaction, its enantiopurity ultimately depends upon that of the substrate.

In contrast to heavy metal and other chemocatalysts, more stereoselective enzyme biocatalysts can often provide greater control over product enantiopurity, while offering additional benefits such as a reduced environmental footprint. For example, the

enzymatic conversion of styrene to (*S*)-styrene oxide via styrene monooxygenase (SMO) has been extensively studied, with high conversion and enantiopurity (>99% e.e.) routinely reported (Panke et al. 1998; Archelas and Furstoss 1997; Panke et al. 2000). Two distinct biosynthetic routes to (*R*)-1,2-phenylethanediol, meanwhile, have been previously described in the literature, each involving a single-step enzyme biotransformation. For example, it has been shown that (*S*)-styrene oxide can be transformed to (*R*)-1,2-phenylethanediol via the expression of an epoxide hydroxylase (EH). Well-characterized EH homologs include those from bacteria such as *Caulobacter crescentus* (Min and Lee 2012) and *Agrobacterium radiobacter* AD1 (Rui et al. 2005), as well as potato (*Solanum tuberosum*) (Monterde et al. 2004). Alternatively, it has also been shown that styrene itself can instead be directly dihydroxylated to (*R*)-1,2-phenylethanediol at high conversion/yields and up to 74% e.e. via the expression of a *Pseudomonas* sp. naphthalene dioxygenase that also displays broad substrate specificity (Lee and Gibson 1996).

However, just as with conventional industrial production of (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol, such single-step enzymatic biotransformations are similarly limited by the need to supply substrates (i.e., styrene or (*S*)-styrene oxide) derived from non-renewable, petroleum feedstocks. Accordingly, previous enzymatic biotransformation studies have yet to address concerns over renewability and sustainability. In view of declining petroleum reserves and their ever-increasing costs, the ability to produce chiral aromatic building blocks such as (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol directly from renewable resources using microbial biocatalysts would represent a more sustainable approach. The engineering of a styrene biosynthesis

pathway in *Escherichia coli* (McKenna and Nielsen 2011) has recently, and for the first time, enabled such a prospect. Building upon the styrene bioproduction platform, the focus of this study was to systematically engineer novel enzyme pathways and microbial biocatalysts to enable both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol to be synthesized as individual products from renewable sugars alone.

As shown in Fig. 3.1, the proposed (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol pathways essentially represent extensions of the previously-engineered styrene pathway, and thus similarly utilize L-phenylalanine as their immediate endogenous precursor (McKenna and Nielsen 2011). As has been shown, phenylalanine can be deaminated to *trans*-cinnamate by a phenylalanine ammonia lyase (PAL); for example, *PAL2* from *Arabidopsis thaliana* was previously found to display both high recombinant activity in *E. coli* and strict substrate specificity for phenylalanine (McKenna and Nielsen 2011). *trans*-Cinnamate can then be decarboxylated to styrene by a phenylacrylate decarboxylase (PADC), a reaction that to date has only been proven to be catalyzed by *FDC1* (originally characterized as a ferulate decarboxylase) from *Saccharomyces cerevisiae* (McKenna and Nielsen 2011). Once synthesized endogenously, styrene could potentially then be oxidized to either (*S*)-styrene oxide or (*R*)-1,2-phenylethanediol via co-expression of a third, appropriate oxidase. (*S*)-Styrene oxide production, for instance, could be achieved by co-expressing a suitable styrene monooxygenase (SMO), whereas (*R*)-1,2-phenylethanediol could be achieved by co-expressing a suitable styrene dioxygenase (SDO).

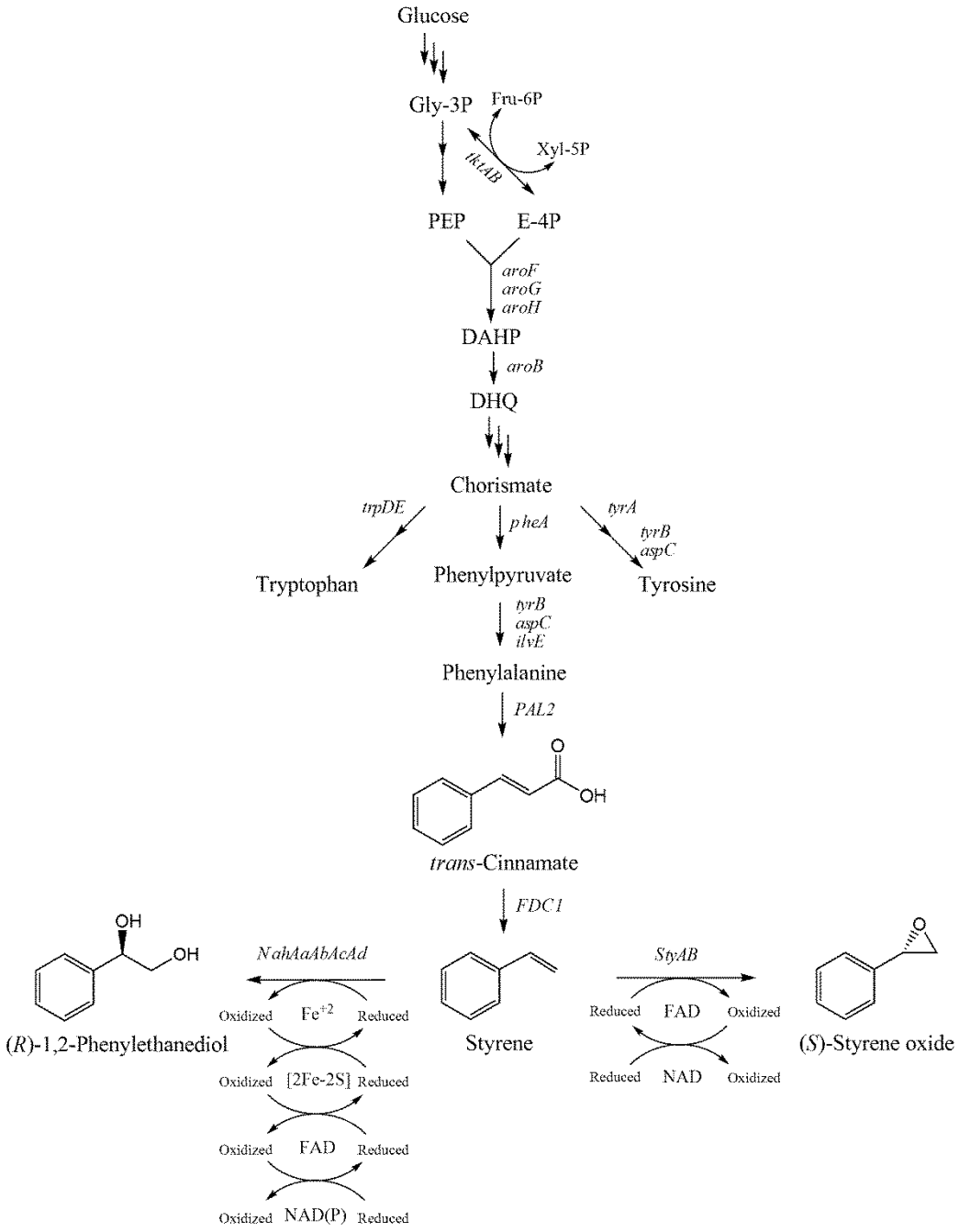

Figure 3.1. (S)-styrene oxide and (R)-1,2-phenylethanediol pathway. The proposed, non-natural pathways for the individual production of (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol from glucose. Pathways are shown in relation to *E. coli*'s native glycolysis and aromatic amino acid biosynthesis pathways. Multiple arrows represent multiple steps not illustrated. (Gly-3P: glycerol-3-phosphate, Fru-6P: fructose-6-phosphate, Xyl-5P: xylose-5-phosphate, PEP: phosphoenolpyruvate, E-4P: erythrose-4-phosphate, DAHP: 3-deoxy-D-arabino-heptulosonate-7-phophate, DHQ: 3-dehydroquinate).

Enzymatic oxidation is typically the first step associated with the aerobic biodegradation of styrene (as well as other aromatics), a process that numerous *Pseudomonas* sp. have been naturally evolved to perform and for which they particularly are well renowned (Smith 1990; Stanier, Palleroni, and Doudoroff 1966). Accordingly, pseudomonads represent a useful genetic repository for SMO and SDO encoding genes. To date, several isoenzymes displaying SMO activity have been identified and characterized in *Pseudomonas* sp., including, for example, StyAB from *P. putida* S12, XylMA (originally characterized as xylene monooxygenase) from *P. putida* mt-2, and CymA1A2 (originally characterized as cymene monooxygenase) from *P. putida* F1 (Nishio et al. 2001; Wubbolts, Reuvekamp, and Witholt 1994; Panke et al. 1998). All three are two-component monooxygenases consisting of an FAD-dependent hydroxylase (StyA, XylM, CymA1, respectively) and an NADH-FAD oxidoreductase (StyB, XylA, CymA2, respectively) (Nishio et al. 2001; Wubbolts et al. 1994; Wubbolts, Reuvekamp, and Witholt 1994; Otto et al. 2004). The epoxidation of styrene to (*S*)-styrene oxide accordingly requires $FADH_2$, which is in turn regenerated through NADH oxidation (Otto et al. 2004). On the other hand, whereas no specific SDOs have been reported in the literature to date, as a result of its very relaxed substrate specificity, naphthalene dioxygenase of *P. putida* NCIB 9816-4 (encoded by the *nahAaAbAcAd* operon) has been shown capable of utilizing styrene as an alternative substrate, oxidizing it directly to (*R*)-1,2-phenylethanediol (Lee and Gibson 1996). NahAaAbAcAd is also a multi-component enzyme, containing an iron-sulfur flavoprotein reductase, an iron-sulfur ferredoxin, and a two-subunit oxygenase. Similar to SMO, oxidized cofactors are ultimately regenerated by the consumption of NAD(P)H.

3.2 Materials and Methods

3.2.1 Strains and media

All strains and plasmids used in this study are listed in Table 1. Custom oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Genomic DNA (gDNA) templates were prepared from whole cells using the Zyppy Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA) according to vendor protocols. Strains were routinely cultured in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, as necessary, at the following final concentrations: 100 mg/L ampicillin, 34 mg/L chloramphenicol, and 50 mg/L kanamycin. As required, auxotrophic strains were additionally supplemented with tyrosine and tryptophan, each initially at 50 mg/L. All cultures were grown aerobically at 32^{o}C with shaking at 250 rpm, unless otherwise noted. Seed cultures were prepared in 5 mL of LB broth with appropriate antibiotics and grown overnight. A 1%v./v. seed was then used to inoculate all flask cultures. To test for production of aromatic species, strains were cultivated in shake flasks (250 mL) containing 50 mL phosphate-limited minimal media (MM1) with 1.5%w./v. glucose, as previously described (McKenna and Nielsen 2011). Cultures were grown until an OD_{600} of ~0.6, at which point they were induced by 0.2 mM IPTG addition and incubated an additional 72 h. Samples were taken at 24 h intervals and analyzed by HPLC to determine concentrations of phenylalanine, *trans*-cinnamate, styrene, (*S*)-styrene oxide, and (*R*)-1,2-phenylethanediol.

Table 3.1. Strains and plasmids used in this study.

	Description	Source
Strains		
E. coli NST74	*aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr),pheO352, aroG397(fbr)*	ATCC 31884
E. coli BL21Star(DE3)	*F- ompT hsdS$_B$ (r$_B$-m$_B$-) gal dcm rne131 (DE3)*	Invitrogen
E. coli NEB-10 beta	*araD139 Δ(ara,leu)7697 fhuA lacX74 galK16 galE15 mcrA f80d(lacZΔM15)recA1 relA1 endA1 nupG rpsL rph spoT1Δ(mrr-hsdRMS-mcrBC)*	NEB
S. cerevisiae W303	Source of *FDC1*	Prather Lab, MIT
P. putida S12	Source of *styAB*	Prather Lab, MIT
P. putida mt-2	Source of *xylMA*	DSMZ 3931
P. putida F1	Source of *cymA1A2*	ATCC 700007
P. putida NCIB 9816-4	Source of *nahAaAbAcAd*	DSMZ 8368
N74SO	*E. coli* NST74 harboring the plasmids pTpal-fdc and pTKstyAB	This study
N74PED	*E. coli* NST74 harboring the plasmids pTpal-fdc and pTKnah	This study
NST74aroB	*E. coli* NST74 harboring the plasmid pSaroB	This study
NST74tktA	*E. coli* NST74 harboring the plasmids pStktA	This study
NST74aroBtktA	*E. coli* NST74 harboring the plasmids pSaroBtktA	This study
N74dA	*E. coli* NST74 *ΔtyrA*	This study
N74dAdE	*E. coli* NST74 *ΔtyrA ΔtrpE*	This study
N74dAtktA	*E. coli* NST74 *ΔtyrA* harboring the plasmid pStktA	This study
N74dAaroB	*E. coli* NST74 *ΔtyrA* harboring the plasmid pSaroB	This study
N74dAaroBtktA	*E. coli* NST74 *ΔtyrA* harboring the plasmid pSaroBtktA	This study
N74dAdEtktA	*E. coli* NST74 *ΔtyrA ΔtrpE* harboring the plasmid pStktA	This study
N74dAdEaroB	*E. coli* NST74 *ΔtyrA ΔtrpE* harboring the plasmid pSaroB	This study
N74dAdEaroBtktA	*E. coli* NST74 *ΔtyrA ΔtrpE* harboring the plasmid pSaroBtktA	This study
N74dASO	*E. coli* NST74 *ΔtyrA* harboring the plasmids pTpal-fdc and pTKstyAB	This study
N74dAPED	*E. coli* NST74 *ΔtyrA* harboring the plasmids pTpal-fdc and pTKnah	This study
Plasmids		
pTrc99A	P_{trc}, pBR322 ori, *lacI^q*, *Amp^r*	Prather Lab, MIT
pSTV28	P_{lac}, p15A ori, *lacI^q*, *Cm^r*	Prather Lab, MIT
pTrcColaK	P_{trc}, ColA ori, *lacI^q*, *Kan^r*	This study
pTpal	*PAL2* of *A. thaliana* inserted into the *NcoI* and *XbaI* sites of pTrc99a	This study
pTpal-fdc	*FDC1* of *S. cerevisiae* inserted into the *SbfI* and *HindIII* sites of pTpal	This study
pTKxylMA	*xylMA* of *P. putida* mt-2 inserted into the *XbaI* and *HindIII* sites of pTrcColaK	This study
pTKcymAB	*cymAB* of *P. putida* F1 inserted into the *XbaI* and *HindIII* sites of pTrcColaK	This study
pTKstyAB	*styAB* of *P. putida* S12 inserted into the *XbaI* and *HindIII* sites of pTrcColaK	This study
pTKnah	*nahAaAbAcAd* of *P. putida* NCIB 9816 inserted into the sites of pTrcColaK	This study
pUN15-pal2	Clone U12256 containing AT3G53260 (*PAL2*) from *A. thaliana*	ABRC
pStktA	*tktA* of *E. coli* inserted in the *BamHI* and *SphI* sites of pSTV28	This study
pSaroB	*aroB* of *E. coli* inserted in the *EcoRI* and *BamHI* sites of pSTV28	This study
pSaroBtktA	*aroB* of *E. coli* inserted in the *EcoRI* and *BamHI* sites of pStktA	This study

3.2.2 Toxicity assays

The toxicity effects of (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol on *E. coli* were determined by monitoring the impacts of the exogenous addition of increasing final concentrations of (*S*)-styrene oxide or (*R*)-1,2-phenylethanediol to growing cultures. Approximately 1 ml of an *E. coli* NST74 seed was used to inoculate 50 ml of LB broth in a 250 mL shake flask. At the time when the cultures reached an optical density (OD_{600}) of ~0.6, (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol were added to the flasks at an array of final concentrations between 0 to 1.8 g/L and 0 to 9 g/L, respectively. Culturing then resumed for another 6 h while cell growth, as determined by OD_{600} measurements, was periodically monitored using a UV/Vis spectrophotometer (Beckman Coulter DU800, Brea, CA).

3.2.3 Cloning of PAL2 from A. thaliana, FDC1 from S. cerevisiae, and tktA and aroB from E. coli

All genes were PCR amplified using a BioRad iCycler system, Phusion DNA Polymerase (New England Biolabs, Ipswich, MA), and custom oligonucleotide primers (see Supplementary Information; note: primer names indicate restriction sites used for cloning). PCR cycling and reaction conditions were standardized according to manufacturer instructions. All PCR amplified DNA fragments were purified using the Zyppy DNA Clean and Concentrator kit (Zymo Research, Irvine, CA). Gene fragments and plasmids were treated by endonuclease digestion according to manufacturer's protocols. All digested fragments were first gel purified using the Zyppy DNA purification kit (Zymo Research, Irvine, CA) and then ligated with T4 DNA Ligase (New England Biolabs, Ipswich, MA) at 4^{o}C overnight before the mixture was then

transformed into chemically competent *E. coli* NEB10-Beta. Transformants were selected on LB solid agar with appropriate antibiotics and cultured at 37^{o}C overnight. Transformant pools were screened using colony PCR with final confirmation by gene sequencing. *PAL2* was amplified from cDNA of clone U12256 from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH) and cloned into pTrc99a, resulting in construction of the plasmid pTpal. *FDC1* was amplified from gDNA of *S. cerevisiae* and cloned into pTpal, resulting in construction of the plasmid pTpal-fdc (Table 3.1). Using methods analogous to those described above, *tktA* and *aroB* were each PCR amplified from gDNA template prepared from *E. coli* NST74 and cloned into pSTV28. These works resulted in the generation of the plasmids pStktA and pSaroB (Table 3.1).

3.2.4 Construction of the plasmid pTrcColaK and the cloning of SMO- and SDO-encoding genes

The plasmid pTrcColaK was constructed by ligating a PCR amplified expression cassette from pTrc99a (containing the *trc* promoter, *laqIq* repressor, and multi-cloning site) together with a PCR amplified fragment from pCOLADuet-1 (EMD Millipore, Billerica, MA) containing the ColA origin of replication and kanamycin resistance marker. Using methods analogous to those described above, *xylAM*, *cymA1A2*, *styAB*, and *nahAaAbAcAd* were PCR amplified from gDNA templates from *P. putida* mt-2, F1, S12, and NCIB 9816-4, respectively, and individually cloned into pTrcColaK. These works resulted in the generation of the plasmids pTKstyAB, pTKcymAB, pTKxylMA, and pTKnah (Table 3.1).

3.2.5 Construction of tyrA and trpE deletion mutants in E. coli NST74

Chromosomal in-frame gene deletion was accomplished using an approach adapted from the methods of Datsenko and Wanner (Datsenko and Wanner 2000). Deletion cassettes harboring the kanamycin resistance gene flanked by the FLP recognition target sites were PCR amplified from the gDNA of the Keio collection mutants *E. coli* JW2581-1 and JW1256-1 (Baba et al. 2006) for the chromosomal integration of *tyrA*::FRT-Kan-FRT and *trpE*::FRT-Kan-FRT, respectively. This resulted in the construction of the phenylalanine over-producing strains N74dA (NST74 *ΔtyrA*) and N74dAdE (NST74 *ΔtyrA ΔtrpE*).

3.2.6 Construction of phenylalanine, (S)-styrene oxide, and (R)-1,2-phenylethanediol producing strains

The previously engineered phenylalanine over-producing strain *E. coli* NST74 was individually transformed with pStktA, pSaroB, and pSaroBtktA to generate strains NST74tktA, NST74aroB, and NST74aroBtktA, respectively. *E. coli* NST74 and N74dA were also co-transformed with the plasmids pTpal-fdc and pTKsty or pTKnah resulting in strains N74SO, N74PED, N74dASO, and N74dAPED, respectively. Analogous methods were used to construct the strains N74dAtktA, N74dAaroB, N74dAaroBtktA, N74dAdEtktA, N74dAdEaroB, and N74dAdEaroBtktA (Table 3.1).

3.2.7 Assaying SMO activity in recombinant E. coli whole resting cells

The three SMO harboring plasmids were individually transformed into *E. coli* BL21(DE3) to generate strains *E. coli* BL21:pTKstyAB, *E. coli* BL21:pTKcymAB, and *E. coli* BL21:pTKxylMA. Shake flasks (250 mL) containing 50 mL of LB with 1%w./v.

glucose were inoculated with 1 mL of overnight seed culture. Cultures were grown until an OD_{600} of ~0.6, induced by addition of 0.2 mM IPTG, and then incubated for 6 h before an equal number of cells (~0.5 g/L dry cell weight) were collected from each by centrifuging at 2000 x *g* for 5 min. The pellet was washed once with pH 7 PBS (phosphate buffered saline) before being re-suspended in 25 ml PBS buffer with 1%w./v. glucose (for cofactor regeneration) in a 250 ml shake flask. Finally, 0.22 g/L styrene was added to the suspension before sealing the flask. Cultures were shaken at 32°C for 3 h and sampled every hour to monitor both styrene depletion and (*S*)-styrene oxide production via HPLC.

3.2.8 HPLC analysis

Samples (1 mL) were centrifuged at 11,000 x *g* for 2 min to pellet cells and supernatants (0.75 mL) were then transferred to glass HPLC vials with Teflon-lined caps. All HPLC analysis was carried out using a Hewlett Packard 1100 series HPLC system. For aromatics analysis, separation was performed on a reverse-phase Hypersil Gold SBC18 column (4.6mm x 150 mm; Thermo Fisher, USA) operated at 45^{o}C. The column was eluted at a total constant flow rate of 1.0 ml/min using 'solvent A' (consisting of double-distilled water) and 'solvent B' (consisting of methanol (99.8% grade) plus 0.1%v./v. trifluoroacetic acid (TFA)). The eluent began as a mixture of 95% solvent A and 5% solvent B before a linear gradient was applied over 8 min to then reach a mixture of 20% solvent A and 80% solvent B. This eluent composition was then held constant for 2 min before a second linear gradient was then applied over the course of 4 min to achieve a final mixture of 95% solvent A and 5% solvent B. The eluent was monitored using a diode array detector set at 215 nm for L-phenylalanine, (*S*)-styrene oxide, and

(*R*)-1,2-phenylethanediol and 258 nm for *trans*-cinnamate and styrene. Under these conditions L-phenylalanine, *trans*-cinnamate, styrene, (*S*)-styrene oxide, and (*R*)-1,2-phenylethanediol were eluted at 4.3, 9.5, 11.1, 8.2 and 6.0 min, respectively. Glucose analysis was performed on the same HPLC system, however now using an RID detector and an anion exchange column (Aminex HPX-87H; BioRAD, Hercules, CA) operated at 35^{o}C. The column was eluted with 0.005 M H_2SO_4 at a constant flow rate of 0.8 ml/min. External calibrations were used to determine concentrations for each analyte.

3.3 Results and Discussion

3.3.1 Assessing and investigating the mechanisms of product toxicity

Prior to investigating their production from glucose, preliminary toxicity screens were first performed for both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol to better anticipate the effects of their accumulation on *E. coli* growth. By screening for changes in *E. coli* growth rate and yield under the stress of the added chemicals, toxicity thresholds of ~1.6 and ~8 g/L were approximated for (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol, respectively, as shown in Fig. 2A and 2B. While it is certainly plausible that exogenous chemical addition does not offer a complete view of cell response to their presence relative to when the same compounds are instead synthesized endogenously within the cell, experience indicates that, at least in the case of aromatic solvents, said approach can provide a reasonable first approximation (McKenna and Nielsen 2011). By this same approach, toxicity limits for the pathway intermediates styrene and *trans*-cinnamate against *E. coli* were previously approximated as 300 and 800 mg/L, respectively (McKenna and Nielsen 2011). For many organic solvents, the octanol-

water partition coefficient (reported as log$K_{O/W}$) has been found to strongly correlate with cytotoxicity, and thus is commonly used as predictor of solvent toxicity towards *E. coli* and other bacteria (Heipieper, Weber, Sikkema, Keweloh, and Debont 1994; Ramos, Duque, Gallegos, Godoy, Ramos-Gonzalez, et al. 2002). Thus, with a log$K_{O/W}$ of 1.61 and 0.92, (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol were anticipated to be less toxic than both styrene and *trans*-cinnamate (whose log$K_{O/W}$ value are 3.05 and 2.13, respectively), as is consistent with the present findings.

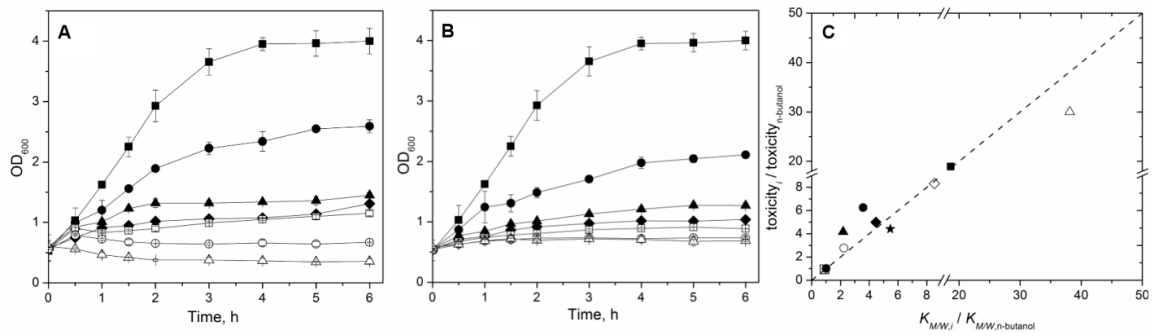

Figure 3.2. (S)-styrene oxide and (R)-1,2-phenylethanediol toxicity. A) The effects of (*S*)-styrene oxide to the growth of *E. coli* cultures as measured by OD_{600}. (*S*)-Styrene oxide was added at final concentrations of 0 (squares), 0.5 (circles), 1 (triangles), 1.2 (diamonds), 1.4 (open squares), 1.6 (open circles), and 1.8 g/L (open triangles). B) The effects of (*R*)-1,2-phenylethanediol on *E. coli* cultures when added at final concentrations of 0 (squares), 4 (circles), 5 (triangles), 6 (diamonds), 7 (open squares), 8 (open circles), and 9 g/L (open triangles). C) A comparison of toxicity versus $K_{M/W}$ (both relative to n-butanol as a reference) for several aromatics of interest, including: styrene (open triangle), *p*-hydroxystyrene (solid square), *trans*-cinnamate (open diamond), (*S*)-styrene oxide (solid star), phenol (solid diamond), 2-phenylacetate (solid circle), 2-phenylethanol (solid triangle), n-pentanol (open circle; included as additional reference), (*R*)-1,2-phenylethanediol (open square), and n-butanol (solid circle). Error bars reported at one standard deviation from triplicate experiments.

It has been hypothesized that the toxicity of aromatics against *E. coli*, like many other solvents (De Carvalho et al. 2004; Knoshaug and Zhang 2009; Nielsen et al. 2009), stems from their proclivity to accumulate in the cytoplasmic membrane, disrupting its integrity and ultimately cell function (McKarns et al. 1997; Sardessai and Bhosle 2002).

Testing this hypothesis, membrane-water partitioning coefficients ($K_{M/W}$) were predicted for (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol, as well as other aromatics that have been examined by our group (unpublished data) or for which prior literature reports were available (Keweloh, Heipieper, and Rehm 1989; Park, Buehler, et al. 2006), according to the model of Sikkema *et al.* (Sikkema, de Bont, and Poolman 1994):

$$logK_{M/W} = 0.97 \cdot logK_{O/W} - 0.64$$

Predictions of $K_{M/W}$ for each species, as well as their toxicity thresholds for *E. coli*, are also compared in Fig. 2C. To provide a frame of reference, however, the results are compared relative to the predicted $K_{M/W}$ and previously report toxicity of n-butanol (note: *E. coli*'s n-butanol toxicity limit is ~1%w./v.) (Nielsen et al. 2009; Winkler, Rehmann, and Kao 2010), as it is generally accepted that *n*-butanol's toxicity arises due to its membrane accumulation (Bowles and Ellefson 1985; Osborne et al. 1990). The observed strong linear correlation existing between the relative toxicity of aromatics versus their predicted relative tendency to accumulate in the membrane provides direct support for the hypothesis that membrane accumulation/disruption is the predominant toxicity mechanism of aromatics against *E. coli*.

3.3.2 Screening and selecting pathway enzymes

In addition to providing preliminary targets for maximal end-product accumulation, these findings further suggest that a high turnover of *trans*-cinnamate, and especially styrene, must be achieved to avoid the inhibitory effects of their intermediate accumulation on (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol producing strains. Furthermore, if this condition can be achieved, it may then also be possible to achieve

higher final titers of (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol than have been previously reported for either styrene or *trans*-cinnamate under otherwise equivalent conditions (McKenna and Nielsen 2011). To achieve this, however, high metabolite flux through the entire pathway must be realized, including at the final pathway steps (i.e., SMO and SDO). Thus, identifying a SMO and SDO with the greatest inherent activity in *E. coli* was an important initial aim. Based on previous reports, SMO candidates encoded by *styAB* from *P. putida* S12, *xylMA* from *P. putida* mt-2, and *cymA1A2* from *P. putida* F1 were selected for screening and analysis in *E. coli*. Recombinant SMO activity was screened via whole resting cell assays for each of *E. coli* BL21(DE3) pTKstyAB, *E. coli* BL21(DE3) pTKxylMA, and *E. coli* BL21(DE3) pTKcymAB. The results are compared in Fig. 3.3, where (*S*)-styrene oxide production via the biotransformation of exogenously supplied styrene was monitored for a period of 3 h. As can be seen, *E. coli* BL21(DE3) pTKstyAB displayed the highest rates of styrene degradation and (*S*)-styrene oxide accumulation, suggesting that StyAB possessed the greatest recombinant activity; followed by XylMA from mt-2 then CymA1A2 from F1. These findings are consistent with previous reports which found the specific activity of StyAB for styrene to be 180 U/g (Panke et al. 1998), whereas XylMA (22 U/g (Wubbolts et al. 1994)) and CymA1A2 (2.30 U/g (Nishio et al. 2001)) were much less active. Furthermore, this result is not altogether surprising as styrene is the natural substrate of StyAB, whereas XylMA and CymA1A2 only incidentally display SMO activity as a result of their relaxed substrate specificities. StyAB was accordingly selected for use in the proposed pathway and in all subsequent studies. Meanwhile, as only a single SDO candidate was identified, the

recombinant activity of NahAaAbAcAd was not analogously screened in isolation, but instead was directly evaluated in styrene-producing cultures, as described below.

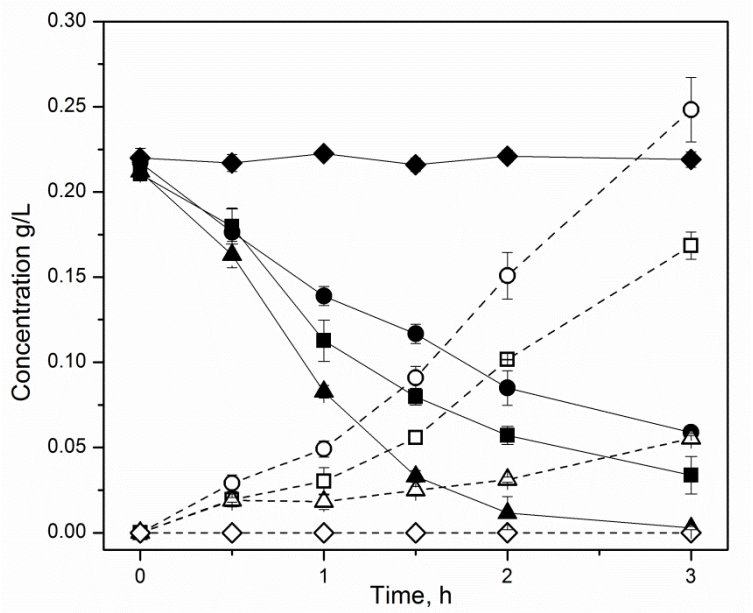

Figure 3.3. SMO activity. Comparing the activity of candidate SMOs in whole resting cell assays of *E. coli* BL21(DE3) (diamonds) individually transformed with the plasmids pTKstyAB (triangles), pTKxylMA (squares), and pTKcymAB (circles). Styrene (solid symbols) was added at an initial concentration of 0.22 g/L and the accumulation of (*S*)-styrene oxide (open symbols) was monitored as a function of time. No styrene monooxygenase activity was observed in the *E. coli* BL21(DE3) background. Error bars reported at one standard deviation from triplicate experiments.

3.3.3 Evaluating pathway function and performance

Having been previously-engineered to overproduce phenylalanine (Tribe 1987) and successfully employed as a platform for styrene production (McKenna and Nielsen 2011), *E. coli* NST74 was selected as the initial production host for both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol. *E. coli* NST74 was co-transformed with pTpal-fdc and pTKstyAB or pTKnah, resulting in strains N74SO and N74PED, respectively. Together with NST74, these first generation strains were analogously evaluated. with

accumulation of the respective end-products being monitored in each case. As seen in Fig. 3.4, after 72 h, up to 0.97±0.03 g/L (8.08±0.25 mM) of (*S*)-styrene oxide was produced by N74SO at a yield of 0.083±0.005 g/g (0.125±0.008 mol/mol). N74PED, meanwhile, produced 0.41±0.02 g/L (2.95±0.14 mM) (*R*)-1,2-phenylethanediol at 0.035±0.002 g/g yield (0.046±0.003 mol/mol). It should be noted that phenylalanine, *trans*-cinnamate, and styrene were undetectable in these strains at all times, indicating rapid turnover of intermediates and efficient flux through the pathway. In comparison, phenylalanine production by NST74 reached up to 1.1±0.03 g/L (6.66±0.18 mM) at a yield of 0.095±0.005 g/g (0.104±0.008 mol/mol), or 25% of theoretical. Relative to phenylalanine, it is hypothesized that higher molar titers and yields of (*S*)-styrene oxide resulted due to the rapid turnover of phenylalanine by StyAB, which in turn increased flux through the amino acid biosynthesis pathway. By this logic, the relatively lower titers and yields of (*R*)-1,2-phenylethanediol by N74PED could perhaps have stemmed from low SDO activity displayed by NahAaAbAcAd. If this is the case, identifying or engineering for more active SDO isoenzymes could result in future improvements. A BlastP search based on NahAaAbAcAd reveals several additional, four-subunit candidate dioxygenases from *Pseudomonas* sp. (including *P. fluorescens*, *P. stutzeri*, *P. chlororaphis*, *P. xanthomarina*, *P. monteilii*, *P. balearica*), as well as one from *Rhanella* sp. LCY15. Whereas all are similarly classified as naphthalene dioxygenases, their future screening could lead to the identification of more robust enzyme 'parts' for the (*R*)-1,2-phenylethanediol pathway.

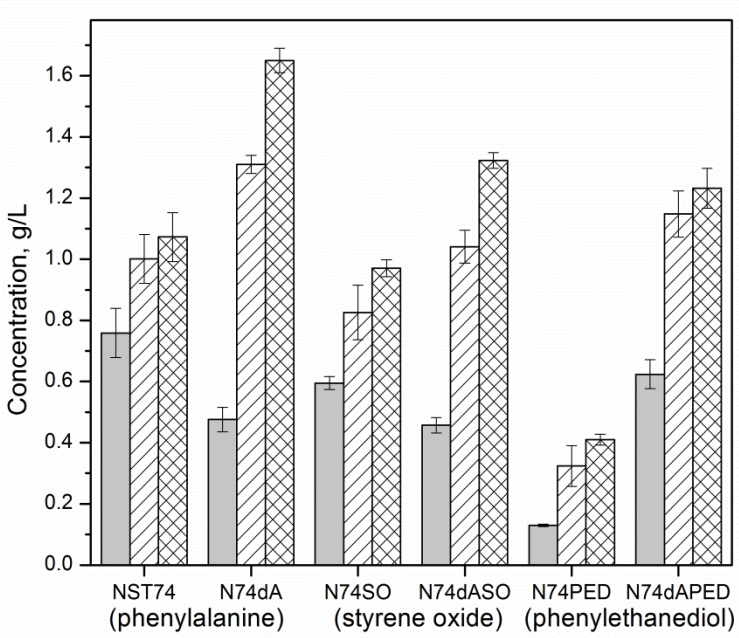

Figure 3.4. Comparing phenylalanine, (S)-styrene oxide, and (R)-1,2-phenylethanediol titers after 24 (gray), 48 (diagonals), and 72 h (hashed) by strains NST74, N74SO, and N74PED and the tyrosine auxotrophs N74dA, N74dASO, and N74dAPED, respectively. Note that additional accumulation of phenylalanine, *trans*-cinnamate, or styrene was undetected at all times in all N74SO, N74PED, N74dASO, and N74dAPED cultures. Error bars reported at one standard deviation from triplicate experiments.

3.3.4 Promoting availability of phenylalanine, the pathway precursor

As maximum titers remained below their anticipated inhibitory thresholds and no excess phenylalanine was detectable in N74SO and N74PED cultures, it was hypothesized that biosynthesis of the phenylalanine precursor was perhaps the predominant limiting factor in our pathways and strains. Thus, multiple strategies were systematically investigated to improve endogenous phenylalanine production by the host strain. As seen in Fig. 3.1, the first step of the shikimic acid pathway consists of the condensation of phosphoenolpyruvate (PEP; derived via glycolysis) and erythrose 4-phosphate (E4P; derived via the pentose phosphate pathway) to yield 3-deoxy-D-arabino-heptulosonate-7-phophate (DAHP). Although DAHP synthase has already been

effectively feedback-deregulated in *E. coli* NST74, it has been shown that this initial condensation step is typically limited by low E4P availability. As such, overexpression of transketolase I (TktA) promotes increased E4P production (Gosset, Yong-Xiao, and Berry 1996; Kambourakis, Draths, and Frost 2000; Draths et al. 1992). Also reported, flux through the shikimic acid pathway is improved by preventing DAHP accumulation, as achieved by overexpressing 3-dehydroquinate synthase (AroB) (Kambourakis, Draths, and Frost 2000; Keseler et al. 2005). Meanwhile, in addition to serving as a precursor to phenylalanine, chorismate is also the key branch point metabolite from which all other aromatic amino acids, namely tryptophan (via anthranilate synthase; TrpED) and tyrosine (following its conversion to prephenate via chorismate dehydratase; TyrA). While *trpE* and *tyrA* deletion will lead to tryptophan and tyrosine auxotrophies (necessitating their supplementation to cultures), said mutations will enable an assessment of maximum achievable production levels by our strains. The individual and combined effects of these strategies on phenylalanine production were next investigated with the goal of developing a more robust host. As seen in Fig. 3.5, relative to NST74, deletion of *tyrA* (N74dA) led to a 1.5-fold increase in phenylalanine production after 72 h at a similar yield, whereas the additional deletion of *trpE* (N74dAdE) actually negated this improvement. Then, by combining *tyrA* deletion with *tktA* overexpression (N74dAtktA), phenylalanine titer and yield were each improved by >50%, reaching 37% of the theoretical yield. However, *tktA* overexpression also imposed a marked negative effect on cell growth, causing an initial lag period that lasted nearly 16 h (data not shown). The impact on phenylalanine production too is seen in Fig. 3.5, where titers at 24 h are lowest for *tktA* overexpressing strains. In contrast, *tyrA* deletion rendered no impact on cell growth (data not shown).

Meanwhile, overexpression of *aroB*, either alone or in combination, provided no significant impact. N74dA and N74dAtktA were accordingly selected for further evaluation as hosts for (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol production upon pathway introduction.

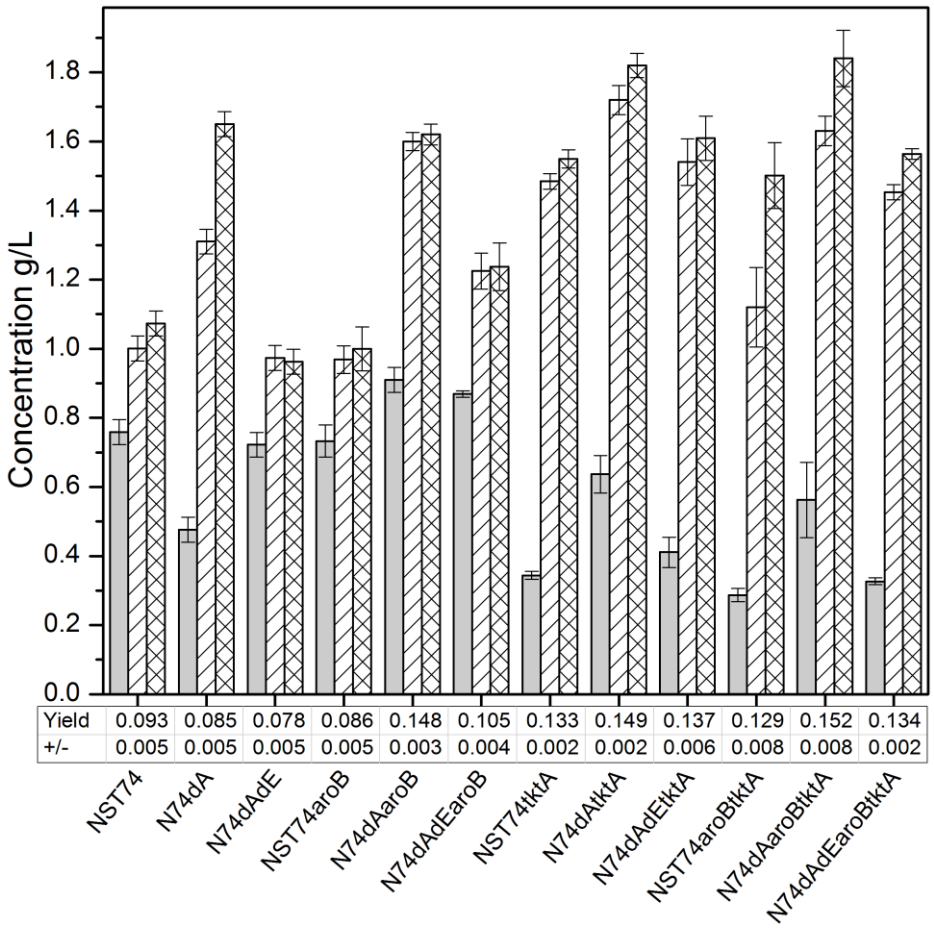

Figure 3.5 Phenylalanine titers. Comparing phenylalanine titers after 24 (gray), 48 (diagonals), and 72 h (hashed). Inset table shows final (72 h) phenylalanine yields on glucose (g/g). The maximum theoretical phenylalanine yield from glucose is 0.55 g/g (Juminaga et al. 2012). Error bars reported at one standard deviation from triplicate experiments.

3.3.5 Improving production using more robust host strains

Despite producing the most phenylalanine, with N74dAtktA as host both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol titers were greatly reduced, reaching only

0.62±0.07 g/L and 0.32±0.02 g/L, respectively. This was likely due to the compromised fitness of this strain, as indicated by its poor growth phenotype (noted above), which may have been further exacerbated by the increased burden associated with introducing the pathway plasmids. In contrast, with N74dA as host (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol production was greatly improved (Fig. 3.4). Relative to N74SO, (*S*)-styrene oxide production by N74dASO was improved by ~30%, to a final titer of 1.32±0.03 g/L (11.0±0.25 mM) at a yield of 0.115±0.005 g/g (0.172±0.007 mol/mol) or 39% of theoretical, and now approaches its estimated toxicity limit (~1.6 g/L). However, with a final titer of 1.23±0.07 g/L (8.9±0.51 mM) and a yield of 0.109±0.002 g/g (0.142±0.003 mol/mol) or 32% of theoretical, a more striking difference, was the ~3-fold increase in (*R*)-1,2-phenylethanediol production by N74dAPED (relative to N74PED).

Another important outcome of this study is the demonstration that enhanced flux through the styrene pathway can be realized if styrene's toxic effects can be addressed or overcome, as was achieved in this case by its *in vivo* transformation to a less toxic product, namely (*S*)-styrene oxide or (*R*)-1,2-phenylethanediol. For example, under analogous conditions, prior works have shown that *E. coli* can produce up to 260±4.31 mg/L (2.5±0.04 mM) styrene before reaching its toxicity limit, representing a yield of 0.07 g/g (0.12 mol/mol) or 27% of its theoretical maximum (McKenna and Nielsen 2011). However, by simply extending the styrene pathway by one step to efficiently convert styrene to (*S*)-styrene oxide, for example, net flux through the pathway was increased by 4.4-fold while the yield was improved by over 40%. Though slightly less dramatic, the same is also true of (*R*)-1,2-phenylethanediol production. Thus, as styrene is the precursor to both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol, these findings also

imply that it is possible for the molar flux to styrene in *E. coli* to reach at least these levels. This realization provides important motivation to the continued development of the styrene pathway, and the development of styrene tolerant microbes as platforms to produce this important monomer at high levels from renewable resources.

3.4 Conclusion

A novel method for the direct production of the chiral aromatic building blocks (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol from renewable glucose has been established. While initial experiments demonstrated that low availability of precursor phenylalanine limited production of both compounds, a systematic approach towards enhancing flux through the phenylalanine biosynthesis pathway ultimately resulted in the improved bioproduction of both (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol. Toxicity of both compounds was strongly correlated with a model of membrane accumulation and disruption, and was likely a limiting factor in the case of (*S*)-styrene oxide. These works demonstrate the versatility of the styrene pathway as a platform for producing other useful and valuable aromatic fine chemicals, and show that greater flux through the styrene pathway is possible if styrene's toxicity can first be effectively addressed.

CHAPTER 4

ENGINEERING THE STYRENE PATHWAY IN YEAST

Abstract

Here we report the first heterologous production of styrene in yeast from glucose. This was achieved from the native aromatic amino acid phenylalanine in a two-step biosynthetic pathway by expressing the phenylalanine ammonia lyase *PAL2* from *Arabidopsis thaliana* and the ferulate decarboxylase *FDC1*, which is native to *Saccharomyces cerevisiae*. A phenylalanine overproducing strain of *S. cerevisiae* was first engineered via EMS mutagenesis and selection on antimetabolites resulting in a strain capable of producing 357±32.5 mg/L phenylalanine. This strain, 22A74D, was further engineered to knockout the Ehrlich pathway (Δ*ARO10*) and to incorporate the feedback-resistant DAHP synthase $ARO4^{K229L}$. After expressing *PAL2* and relying on native expression of *FDC1*, styrene titers reached 28.8±2.1 mg/L.

4.1 Introduction

Like most monomers used in conventional plastics production, at present, all commercially-available styrene is solely derived from non-renewable petroleum feedstocks. More specifically, the predominant means by which styrene is synthesized is the chemocatalytic dehydrogenation of petroleum-derived ethylbenzene (Wu, Koylinski, and Bozik 1981; Mimura and Saito 2000). With the global annual demand of styrene to surpass 41 million tons by 2020 (James and Castor 2011) (a >$28 billion U.S. market(SRI 2010)), the net energy requirements for just this single transformation step amount to over 200 trillion BTU of steam each year (DoE 2002). However, driven by concerns over depleting feedstock availability and deleterious environmental impacts,

there is growing interest in the development of 'green' processes for the production of biorenewable replacements to conventional petroleum products, including numerous monomers and plastics.

Advances in metabolic and pathway engineering have been paramount to the continuously expanding range of conventional monomer compounds that can now be synthesized from renewable biomass feedstocks (Adkins et al. 2012; Curran and Alper 2012; Erickson, Nelson, and Winters 2012; Lee et al. 2011). Along these lines, in prior works a novel and non-natural pathway for styrene biosynthesis from biomass-derived glucose was recently engineered in the bacterium *Escherichia coli* (McKenna and Nielsen 2011). The previously-engineered pathway, depicted in Fig. 4.1, utilizes phenylalanine, a naturally occurring proteinogenic amino acid, as its precursor (McKenna and Nielsen 2011). Phenylalanine is first deaminated to *trans*-cinnamate by phenylalanine ammonia lyase (PAL). It was found that *PAL2* from *Arabidopsis thaliana* displays both the greatest activity and highest substrate specificity (i.e., for phenylalanine) when recombinantly expressed in *E. coli* (McKenna and Nielsen 2011). Next, *trans*-cinnamate is decarboxylated to styrene via the expression of the phenylacrylate decarboxylase *FDC1* (originally characterized as a ferulate decarboxylase) from *Saccharomyces cerevisiae* (McKenna and Nielsen 2011; Mukai et al. 2010). When PAL2 and FDC1 were subsequently co-expressed in a previously-engineered L-phenylalanine over-producing *E. coli* background grown in glucose minimal media, the resultant styrene titers reached ~260 mg/L in 29 h at a yield of about 0.030 g/g (McKenna and Nielsen 2011). As *E. coli* was found to possess a low toxicity threshold for styrene (between only 200 to 300 mg/L), this suggested that end-product toxicity (likely caused by membrane

accumulation/disruption (McKenna et al. 2013)) was the principal limiting factor (McKenna and Nielsen 2011).

As sensitivity to solvent-like products – including, for example, n-butanol(Atsumi, Hanai, and Liao 2008) and (*S*)-styrene oxide(McKenna et al. 2013), and 2-pentanone(Lan et al. 2013),– commonly arises as a productivity-limiting factor in *E. coli*, engineering more robust hosts for renewable chemical production is an important aim in industrial biotechnology. Relative to *E. coli*, the yeast biosynthetic platforms can offer several inherent advantages of importance to robust and large–scale renewable chemicals production. The most significant of these attributes often includes: faster growth rates, elevated solvent tolerance, and the ability to withstand low temperatures and pH (Demain and Vaishnav 2009; Sudbery 1996; Curran et al. 2013; Nevoigt 2008; Ostergaard, Olsson, and Nielsen 2000). Among yeast, *S. cerevisiae* is particularly attractive for metabolic engineering studies due to its well characterized genetics, physiology, and metabolism, as well as the plethora of diverse genetic toolkits for the stable expression and introduction of heterologous enzymes and pathways (Alberti, Gitler, and Lindquist 2007).

With an eye towards evaluating and ultimately improving the prospects of industrial scale renewable styrene production, the objective of the present study was to engineer the yeast *S. cerevisiae* to synthesize styrene from glucose. In addition to the promising phenotypic traits previously mentioned, several additional and complementary factors were further responsible for motivating this specific aim. First, past studies have shown that *S. cerevisiae* is a suitable host for the engineering of other, non-native aromatic biosynthesis pathways, including for the production of protocatechuate,

catechol, vanillin, naringenin, and 2-phenylethanol, among others (Curran et al. 2013; Hansen et al. 2009; Jiang, Wood, and Morgan 2005; Stark et al. 2002). Second, in addition to displaying general solvent tolerance (Matsui et al. 2008; Kawamoto, Kanda, and Tanaka 2001), *S. cerevisiae* has specifically been shown to display elevated tolerance to aromatics such as 2-phenylethanol (Etschmann et al. 2002). Third, it was hypothesized that improved function of styrene pathway enzymes might be achieved in *S. cerevisiae* since: *i) PAL2* is of eukaryotic origin, and *ii) FDC1* is native to *S. cerevisiae.* Contingent upon the native regulation of *FDC1* (to be addressed later in this study) this suggests that a functional pathway could be in fact constructed via the expression of a single heterologous enzyme, thus minimizing the prospects of metabolic burden. Lastly, *S. cerevisiae* does not naturally possess the transporter required for phenylalanine efflux. For example, in previous works it has been shown that although intracellular concentrations of phenylalanine in *S. cerevisiae* exceeded 60 mM, its content in the associated supernatant was undetectable (Luttik et al. 2008). Retaining phenylalanine intracellularly may enhance its availability to the styrene pathway.

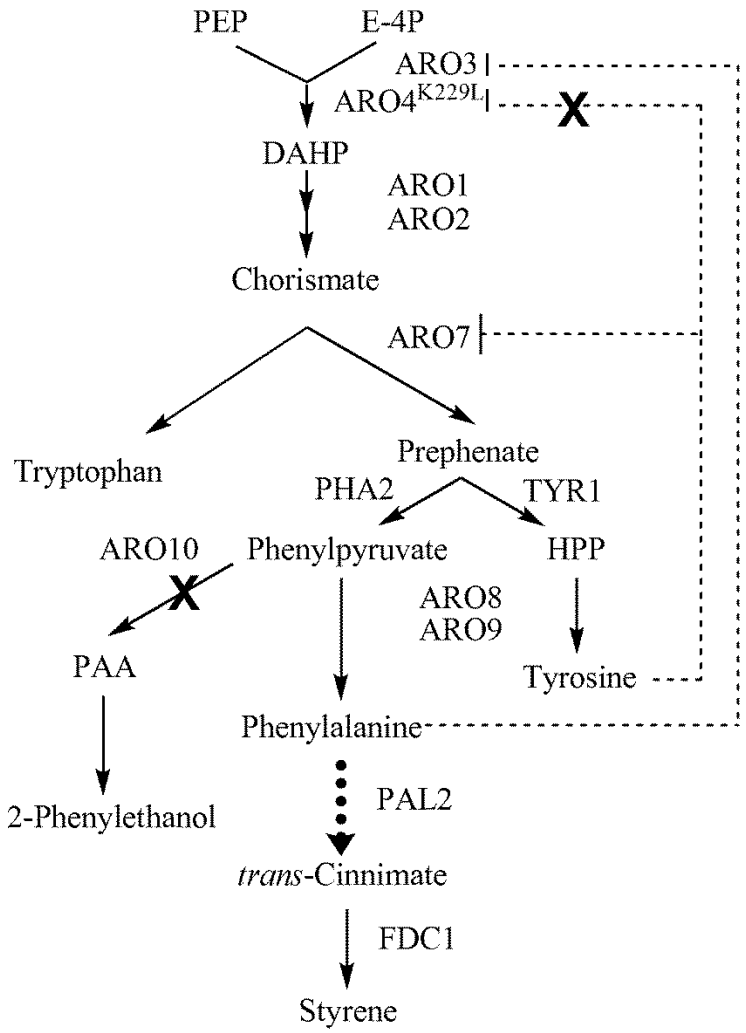

Figure 4.1 Styrene biosynthesis by *S. cerevisiae*. Multiple arrows signify that multiple steps are occurring but are not illustrated. The dotted arrow represents heterologous gene expression of PAL2. Dotted lines from the aromatic amino acids indicate feedback-repression via transcriptional regulation or allosteric enzyme inhibition, or both. Metabolite abbreviations: phosphoenolpyruvate (PEP), erythrose-4-phosphate (E-4P), 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), hydroxyphenylpyruvate (HPP), phenylacetaldehyde (PAA).

4.2 Materials and Methods

4.2.1 Strains and media.

All strains and plasmids used in this study are listed in Table 4.1. Custom oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) and presented in the supplementary material. All *S. cerevisiae* strains were purchased from Thermo Scientific (Waltham, MA). Yeast plasmids used were derived from the Gateway vector collection and purchased from AddGene (Cambridge, MA). Genomic DNA was prepared from whole cells using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA) according to vendor protocols. *E. coli* strain NEB10β (New England Biolabs, Ipswich, MA) was used for all cloning and plasmid propagation, except for pDONR221 which was propagated in One Shot *ccdB* Survival 2 T1 *E. coli* (Life Technologies, Grand Island, NY). *E. coli* strains were routinely cultured at 37ºC in Luria-Bertani (LB) broth (supplemented with appropriate antibiotics, as necessary). Yeast strains were routinely cultured at 32ºC in Yeast Extract Peptone Dextrose (YPD) medium, yeast synthetic dextrose (SD) medium, or yeast synthetic minimal (SD-Leu) medium. YPD medium was composed of of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. SD medium was composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose, and 20 mg/L of each Uracil, Histidine, Leucine, and Methionine. SD-Leu medium was composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose, 20 mg/L each Uracil, Histidine, and Methionine.

Table 4.1. List of strains and plasmids.

Strain	Genotype	Source
E. coli NEB-10 beta	*araD139 Δ(ara,leu)7697 fhuA lacX74 galK16 galE15 mcrA f80d(lacZΔM15)recA1 relA1 endA1 nupG rpsL rph spoT1Δ(mrr-hsdRMS-mcrBC)*	New England Biolabs
One Shot *ccdB* Survival 2 T1 *E. coli*	F¯*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697*gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::*IS2*	Life Technologies
BY4741	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Thermo Scientific
BY4741ΔFDC1 (YDR539W)	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 fdc1Δ*	Thermo Scientific
BY4741-PAL	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*-425GPDPAL	This Study
22A75D	BY4741 phenylalanine overproducer	This Study
22A75D-PAL	22A75D-425GPDPAL	This Study
22A75D10-PAL	22A75D *aro10Δ*-425GPDPAL	This Study
22A75D104-PAL	22A75D *aro10Δ::aro4*^{K229L}-425GPDPAL	This Study
Plasmids		
pTrc99A	P_{trc}, pBR322 ori, *lacI*^{q}, *Amp*^{R}	Prather Lab, MIT
pFA6-KanMX	KanR2, pBR322 ori, *Amp*^{R}	AddGene
pDONR221	*att*P1-*ccdB*-*Cm*^{R}-*att*P2 cassette, pUC ori, *Kan*^{R}	Life Technologies
pDONR-PAL	*PAL2* from *A. thaliana* inserted into pDONR221	This study
425GPD	P_{GPD}, *att*R1-*ccdB*-*Cm*^{R}-*att*R2 cassette, pBR322 ori, *LEU2*, *Amp*^{R}	AddGene
425GPDPAL	*PAL2* from *A. thaliana* inserted into 425GPD	This study
pACYCDuet-1	P_{T7}, p15A ori, *lacI*^{q}, *Cm*^{R}	Novagen
pACYC- ARO4^{K229L}-KanMX	pACYC with the integration cassette *aro4*^{K229L}-KanMX	This Study
pUN15-PAL2	Clone U12256 containing AT3G53260 (*PAL2*) from *A. thaliana*	ABRC

4.2.2 Toxicity assays.

To determine impact of styrene on *S. cerevisiae* growth, the effect of its exogenous addition to growing cultures at increasing final concentrations was investigated. Seed cultures of *S. cerevisiae* BY4741 were prepared in 5 mL of YPD broth and grown at 32^{o}C overnight while shaking at 250 rpm. The seed culture (1 ml) was then used to inoculate 50 ml of YPD broth in a 250 mL shake flask. Cultures were grown to an

optical density at 600 nm (OD_{600}) of ~0.6, at which time styrene was added to the flasks at an array of final concentrations ranging between 0 to 1 g/L. Culturing resumed at 32^{o}C for an additional 6-8 h while cell growth, as determined by OD_{600} measurements, was periodically monitored using a UV/Vis spectrophotometer (DU800, Beckman Coulter, Brea, CA).

4.2.3 Evolution of phenylalanine overproducing strains.

Evolution of a phenylalanine over-producing phenotype in *S. cerevisiae* was achieved through random mutagenesis and high-throughput selection using the phenylalanine anti-metabolite *m*-fluoro-DL-phenylalanine to provide selective pressure. *S. cerevisiae* BY4741 was first treated with ethylmethanesulphonate (EMS) according to standard protocols(Winston 2001) before then being plated on minimal media supplemented with *m*-fluoro-DL-phenylalanine. In the first round of mutagenesis, selection occurred on SD media plates supplemented with either 18 or 22 mg/L *m*-fluoro-DL-phenylalanine. Note that the BY4741's minimum inhibitory concentration of *m*-fluoro-DL-phenylalanine was previously found to be approximately 15 mg/L (data not shown). Two mutants (designated 18A and 22A) were subsequently isolated from the first round of mutagenesis before then being cultured in SD media for 48 h at 32^{o}C. Supernatants were analyzed by high performance liquid chromatography (HPLC; as described below) to test for their comparative ability to produce 2-phenylethanol. Note that 2-phenylethanol, which is endogenously produced from phenylpyruvate via ARO10, was used as a surrogate to indicate phenylalanine over-production because phenylalanine is not exported to the extracellular media(Luttik et al. 2008). Strains 18A and 22A were then subjected to a second round of mutagenesis as preformed above, however, in this

case the resultant mutants were selected for on SD media plates supplemented with 25, 50, or 75 mg/L *m*-fluoro-DL-phenylalanine. From this, 18 mutants were then isolated before then being similarly cultured and characterized with respect to their 2-phenylethanol production abilities. The key regulatory genes (as well as 500 bp upstream of the start codon) of the phenylalanine pathway *ARO3*, *ARO4*, *ARO7*, *ARO8*, *GCN4*, and *PHA2* were sequenced in the strains BY4741, 22A, and 22A75D.

4.2.4 Transcriptional analysis of phenylalanine over-producing mutants.

Relative transcription levels of each of *ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO7*, *ARO8*, *ARO9*, and *PHA2* were quantified at mid-log phase in the strains BY4741, 22A, and 22A75D. Approximately 1.5×10^8 cells of each strain were collected by centrifugation at 17,000 x *g* for 1 min. The supernatant was discarded and RNA was extracted from the cell pellet using the YeaStar RNA Extraction Kit (Zymo Research, Irvine, CA). cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) and RT-qPCR was performed using SYBR Green (Life Technologies) based quantitative PCR according to manufacturer's protocols. Custom oligonucleotide primers for RT-qPCR experiments, including those for the reference housekeeping gene 26S (Martorell, Querol, and Fernández-Espinar 2005), were designed and synthesized, the sequences of which are provided in Table S1 (see Supplementary Information). RT-qPCR was performed on an Applied Biosystems StepOne Real-Time PCR (Applied Biosystems) using a 60ºC annealing temperature. Data analysis was performed using StepOne software and relative transcriptional levels were calculated using the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen 2001).

4.2.5 Investigating native expression and activity of FDC1 in S. cerevisiae.

S. cerevisiae BY4741 seed cultures were prepared (in triplicate) in 5 mL YPD broth and cultured at 32°C while shaking at 250 rpm overnight. 1 ml of each seed was then used to again inoculate 50 ml SD media. These cultures were grown until reaching an OD_{600} of ~0.6, at which point each was induced by either *trans*-cinnamate, ferulic acid, *p*-coumaric acid, or phenylalanine at a final concentration of 0.2 mM. Induced cultures were incubated for an additional 12 h after which an equal number of cells (OD_{600} = 4) were collected by centrifugation at 2800 x *g* for 4 min. Cells were lysed using Zymolyase (Zymo Research) and the supernatant collected after centrifugation at 11,000 x *g* for 2 min. FDC1 activity was assayed at room temperature in pH 7.5 50 mM Tris-HCl buffer containing 250 mM *trans*-cinnamate, and initiated by the addition of 5 µL of crude cell lysate. The accumulation of styrene was then followed at 247 nm on a UV/Vis Spectrophotometer for a total of 5 min at 20 sec intervals. A molar extinction coefficient of 10,000 M^{-1} cm^{-1} and a 1 cm path length were used to establish enzyme activity in terms of U mg^{-1} total protein. The total protein content in each lysate sample was determined by Bradford Assay using bovine serum albumin (BSA) as an external standard.

4.2.6 Cloning of PAL2 from A. thaliana.

The *PAL2* encoding gene from *A. thaliana* was derived from cDNA library plasmids containing the specific loci of interest (Table 4.1) obtained from the Arabidopsis Biological Research Center (ABRC; Ohio State University, Columbus, OH). *PAL2* was PCR amplified using Phusion DNA Polymerase (Finnzymes, Espoo, Finland) using custom oligonucleotide primers (supplementary material). Using Gateway Cloning Technology (Alberti, Gitler, and Lindquist 2007), amplified linear DNA fragments

flanked with *att*B sequences were purified using the Zyppy Clean and Concentrator kit (Zymo Research). The BP reaction between the DNA fragment and pDONR221 (Life Technologies) was created using Gateway BP Clonase II Enzyme Mix (Life Technologies) following manufacturer's protocols. Transformants were selected by plating on LB solid agar containing kanamycin and culturing at 37^{o}C overnight. The resultant donor plasmid, pDONR-PAL, was mixed with the desired destination plasmid, 425-GPD, using the Gateway LR Clonase II Enzyme Mix (Life Technologies). Transformants were selected by plating on LB solid agar containing ampicillin and confirmed using colony PCR. This approach resulted in construction of the constitutively induced, high copy number (2μ) plasmid 425GPDPAL, as listed in Table 4.1.

4.2.7 Assaying the extracellular transport of trans-cinnamate.

S. cerevisiae BY4741 and the *FDC1* knockout mutant BY4741*ΔFDC1* (YDR539W, clone 5834 (Thermo Scientific, Waltham, MA)) were each transformed with plasmid 425GPDPAL. Cultures grown in SD-Leu media supplemented with 200 mg/L phenylalanine while the extracellular accumulation of *trans*-cinnamate, styrene, and 2-phenylethanol were periodically monitored via HPLC over the course of 24 h.

4.2.8 Chromosomal disruption of ARO10 and integration of ARO4^{K229L}.

Targeted chromosomal disruption of *ARO10* in strain 22A75D was performed via homologous recombination. Gene disruption cassettes were generated via PCR to contain 40 base pairs of homology on both sides of the targeted integration site (i.e., *ARO10*) in addition to the KanMX selectable marker (as obtained from pFA6-KanMX4). Following transformation, colonies were selected on YPD solid agar plates containing 200 mg/L G418. Clones carrying the successful *ARO10* disruption cassette were further confirmed

by PCR. This resulted in the strain 22A75D10. In addition, a copy of the feedback resistant mutant *ARO4*^{K229L}, whose expression was driven by the native *ARO4* promoter, was likewise integrated into 22A75D at the *ARO10* locus, thereby also and simultaneously resulting in its chromosomal disruption. In this case, however, the gene disruption cassette was first constructed in the *E. coli* expression vector pACYCDuet-1. The ARO4 point mutation (K229L) was generated (and confirmed by sequencing) via overlap extension using the primers listed in Table S1 and inserted into the *BamHI* and *EcoRI* sites of pACYCDuet-1. Subsequently, the KanMX selectable marker together with its promoter was inserted downstream of *ARO4*^{K229L} to generate the plasmid pACYC-ARO4^{K229L}-KanMX. The entire cassette was then PCR amplified using primers whose overhangs each contained 40 base pairs of homology to *ARO10*. The resultant fragment was then transformed into 22A74D as described above, resulting in the strain 22A75D104.

4.2.9 Styrene production from glucose in S. cerevisiae shake flask cultures.

Each of the *S. cerevisiae* strains BY4741, 22A75D, 22A75D10, and 22A75D104 were individually transformed with the plasmid 425GPDPAL. The transformants were each grown in 5 mL SD-Leu broth for 12 h at 32°C while shaking at 250 rpm to prepare seed cultures. Each seed (1 mL) was then used to inoculate 50 mL SD-Leu media in 250 ml shake flask fitted with a glass cap. A closed system with large headspace was used to avoid volatile product (i.e., styrene) losses while also precluding the exhaustion of oxygen. Culturing continued for 48 h while 1 ml samples were periodically taken for analysis of cell growth and metabolite production.

4.2.10 Metabolite analysis.

Samples were prepared for metabolite analysis via HPLC by first removing 1 mL of culture from a shake flask culture and pelleting the cells at 11,000 x *g* for 2 min. The supernatant (0.75 mL) was then transferred to a glass HPLC vial and sealed with a Teflon-lined cap. Analysis was carried out using a Hewlett Packard 1100 series HPLC system (Palo Alto, CA) equipped with an auto sampler, diode array (UV/Vis) detector, and reverse-phase Hypersil Gold SBC18 column (4.6mm x 150 mm; Thermo Fisher, USA). Samples (5 µL) were injected for analysis according to the methods of McKenna and Nielsen(McKenna and Nielsen 2011). The eluent was monitored at 215 nm for L-phenylalanine and 2-phenylethanol and 258 nm for *trans*-cinnamate and styrene. Under these conditions L-phenylalanine, 2-phenylethanol, *trans*-cinnamate, and styrene were eluted at 4.5, 7.1, 8.67, and 10.4 min, respectively.

4.3 Results

4.3.1 Assaying styrene toxicity

To assess if *S. cerevisiae* BY4741 would indeed be a suitable host for styrene production, a cursory evaluation of its ability to tolerate exogenous styrene at increasing concentrations was first performed. Due to its hydrophobic nature, styrene readily accumulates within the hydrophobic core of the membrane lipid-bilayer(Juan L. Ramos 2002; Heipieper, Weber, Sikkema, Keweloh, and de Bont 1994; Isken and de Bont 1998; McKenna et al. 2013).

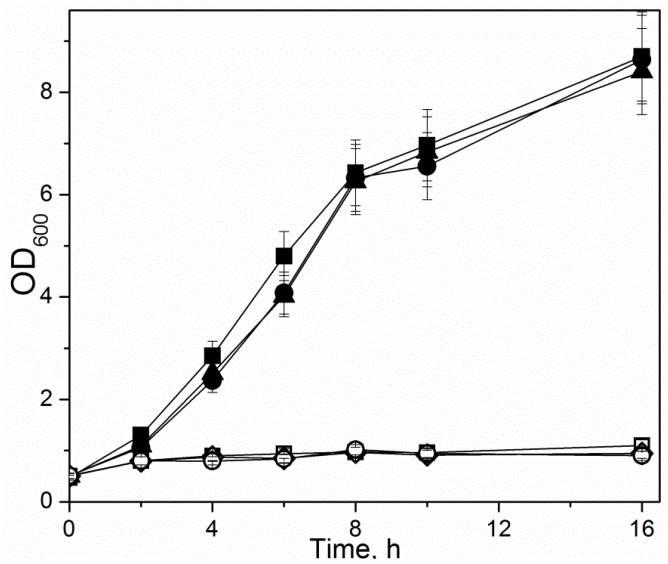

Figure 4.2. Toxicity of exogenous styrene against *S. cerevisiae* BY4741 growing cells. Growth response of *S. cerevisiae* BY4741 to styrene added at final concentrations of 0 mg/L (solid square), 200 mg/L (solid circle), 400 mg/L (solid triangle), 600 mg/L (open square), 800 mg/L (open circle), and 1000 mg/L (open triangle). Error bars reported at one standard deviation from triplicate experiments.

As illustrated in Fig. 4.2, although growth rates and biomass yields were reduced as a function of added styrene concentration, growth of BY4741 was still possible up to 400 mg/L. At 600 mg/L and above, however, no growth was observed following styrene addition, suggesting that the toxicity limit exists between 400-600 mg/L. Although still relatively low, this represents a ~2-fold improvement over that of *E. coli* (McKenna and Nielsen 2011). Most importantly, for the purposes of this initial study, these results suggest that styrene accumulation to appreciable levels is at least possible in *S. cerevisiae*. For longer-term applications, however, it is expected that the development or discovery of an industrial yeast strain with an improved solvent tolerance phenotype over BY4741 (a laboratory strain) will be required for robust styrene bioproduction.

4.3.2 Evolving and engineering phenylalanine over-production in S. cerevisiae

As phenylalanine serves as the immediate endogenous pathway precursor, development of a phenylalanine over-production phenotype in *S. cerevisiae* is an essential pre-requisite to styrene biosynthesis. The native aromatic amino acid biosynthesis pathways of *S. cerevisiae* are shown in Fig. 4.1, wherein it can be seen that two principal control points must be deregulated to promote increased metabolite flux toward phenylalanine. The first, DAHP synthase (ARO3 and ARO4), is allosterically feedback inhibited by phenylalanine and tyrosine, respectively (Caspi et al. 2006; Fukuda et al. 1991; Teshiba et al. 1986). The second, meanwhile, occurs at the chorismate branch point where chorismate mutase (ARO7) converts chorismate to prephenate (the intermediate precursor to phenylalanine and tyrosine), but ARO7 is inhibited by as little as 0.5 mM tyrosine (Schmidheini et al. 1989; Caspi et al. 2006; Kradolfer et al. 1977).

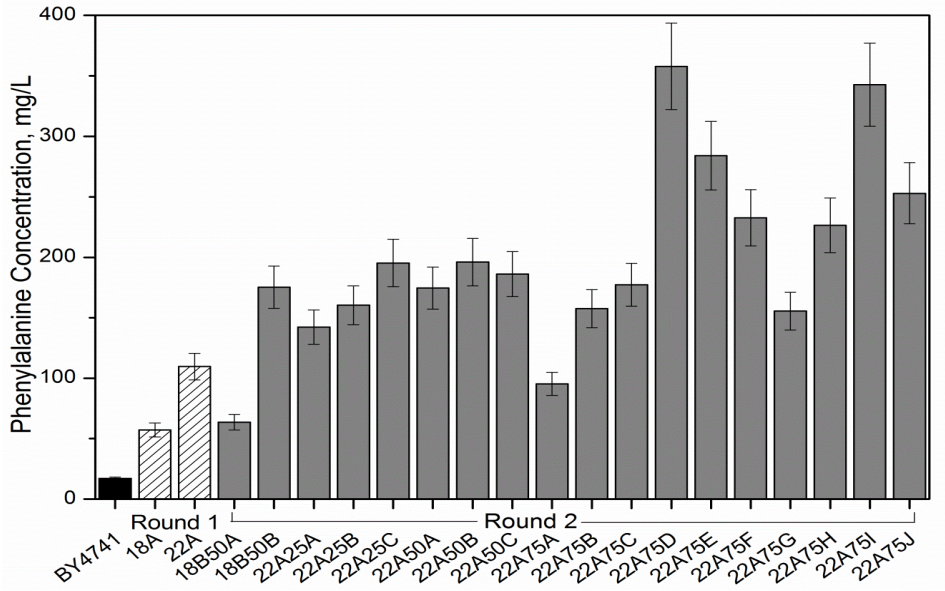

Figure 4.3. Evolution of phenylalanine overproducing mutants of *S. cerevisiae*. Mutants were evolved through the use of EMS mutagenesis and high-throughput selection on anti-metabolite plates. Mutants were grown in SD media and phenylalanine production was analyzed after 48 h by measuring the concentration of 2-phenylethanol in the supernatant. Error bars reported at one standard deviation from triplicate experiments.

To overcome feedback resistance in the phenylalanine biosynthesis pathways, the use of antimetabolites, in this case *m*-fluoro-DL-phenylalanine, in parallel with EMS mutagenesis provided the needed selection pressure for evolving phenylalanine over-producing mutants(Drake 1976). Two mutants were isolated from the first round of selection on SD plates containing 18 mg/L (strain 18A) and 22 mg/L (strain 22A) *m*-fluoro-DL-phenylalanine. Both mutants were subsequently tested for their respective phenylalanine production capacities in shake flask cultures (Fig.4.3). As phenylalanine is not exported from *S. cerevisiae*, the extracellular accumulation of 2-phenylethanol, which is naturally and readily produced as a degradation product of phenylpyruvate (the precursor to phenylalanine; Fig. 4.1), was used as a measure of increased flux through the phenylalanine biosynthesis pathway. 18A and 22A showed 3.3- and 6.4-fold improvements in phenylalanine production potential (57.24±5.20 and 109.56±9.24 mg/L), respectively, relative to the parent control (BY4741).

A second round of mutagenesis and selection was performed with the objective of further deregulating phenylalanine biosynthesis. In this case, the isolated mutants 18A and 22A were themselves subjected to further mutagenesis where the selective pressure was elevated by application of *m*-fluoro-DL-phenylalanine at increased concentrations (namely 25 mg/L, 50 mg/L, or 75 mg/L). In contrast to the first round, this second round of mutagenesis netted numerous colonies (over 50) at all three selection pressures. Thus, to screen for the best performers, only the fastest growing mutants were selected (in this case, a total of 18) and subsequently tested for their ability to overproduce phenylalanine. Amongst the pools, the top performing mutant, named 22A75D, was able to produce an estimated 357±32.5 mg/L of phenylalanine in 48 h, representing a ~21-fold improvement

over BY4741. The performance of mutant 22A75D was followed closely by 22A75I, which was able to produce up to 342±28.9 mg/L phenylalanine.

In an effort to better understand the genetic bases responsible for the evolved phenylalanine over-production phenotype in the most productive mutant (22A75D), key genes (as well as 500 bp upstream of the start codon) involved in the phenylalanine biosynthesis pathway were sequenced and compared with those of its parent (22A) and the control (BY4741). The subset of genes of interest included those associated with the known bottleneck enzymes DAHP synthase (*ARO3* and *ARO4*) and chorismate mutase (*ARO7*), as well as phenylalanine prephenate dehydratase (*PHA2*), aromatic aminotransferase (*ARO8*), and the activator protein *GCN4*, a transcriptional activator involved in the expression of all steps in the phenylalanine biosynthesis pathway (Caspi et al. 2006). Interestingly, however, no mutations were observed in any of the genes investigated, or in their associated upstream sequences. It is possible, perhaps, that a global transcription factor was the cause of the increase rather than single point mutations in the individual phenylalanine pathways genes themselves. As no changes in any open reading frame tested were detected, this would imply that the evolved phenotype was not accrued as a result of relieving allosteric inhibition.

Accordingly, attention was next directed towards understanding if it were instead transcriptional changes that were responsible for the observed phenotypic changes. Changes in the transcription level of each of the genes of interest were measured with the use of RT-qPCR, and quantified relative to that of the wild-type control (BY4741). As illustrated in Fig. 4.4, in 22A75D up-regulation of *ARO8* was found to be most significant (a 9.3-fold increase), followed by *ARO1* (6.8-fold), *ARO2* (5.8-fold), and

ARO3 (4.5-fold). It is possible that up-regulation of ARO8 was necessary to compete with the ARO10 (converts phenylpyruvate to phenylacetaldehyde) activity of the Ehrlich pathway to drive the flux towards phenylalanine rather than phenylacetaldehyde. A similar trend was also observed in the parent strain 22A, though the level of up-regulation was much less in all cases. In both mutant strains, meanwhile, almost no changes in transcription levels were observed for each of *ARO4*, *ARO7*, *ARO9*, and *PHA2*.

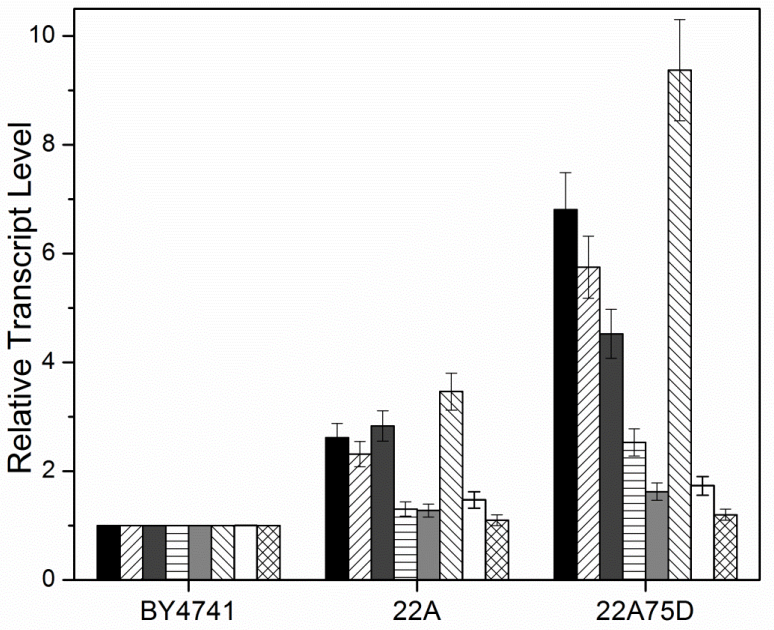

Figure 4.4. Transcriptional analysis of top phenylalanine overproducing *S. cerevisiae* mutants. Relative transcript levels of the first round yeast mutant 22A and the second round yeast mutant 22A75D, normalized to their parental strain BY4741, for the genes ARO1 (black), ARO2 (right diagonal), ARO3 (dark gray), ARO4 (horizontal), ARO7 (light gray), ARO8 (left diagonal), ARO9 (no fill), and PHA2 (hashed). Error bars reported at one standard deviation from triplicate experiments.

The four up-regulated genes (ARO1, ARO2, ARO3, and ARO8) were further analyzed for their similarities in promoter sequences and transcriptional regulators. First, a common promoter sequence was investigated by aligning the sequences of 1000 bp prior to the start codon of the four genes. A possible consensus sequence was found to be

AACATC (located at ARO1,-292; ARO2,-307; ARO3, -289; ARO8,-290) though this sequence does not match any known transcription binding sequences. Next, common regulators of the four genes were determined from known regulators listed in the Yeast Genome Database and listed in Table 4.2. Eleven transcriptional regulators were found to be in common of these four up-regulated genes, but sequencing will be necessary to determine if any of these regulators are responsible for the increase in transcription level.

Table 4.2. Common regulators of ARO1, ARO2, ARO3, and ARO8

Regulator	Association Type	Description	Reference
ACE2	Negative	Transcription factor required for septum destruction after cytokinesis; phosphorylation by Cbk1p blocks nuclear exit during M/G1 transition, causing localization to daughter cell nuclei, and also increases Ace2p activity; phosphorylation by Cdc28p and Pho85p prevents nuclear import during cell cycle phases other than cytokinesis; part of RAM network that regulates cellular polarity and morphogenesis; ACE2 has a paralog, SWI5, that arose from the whole genome duplication	(Di Talia et al. 2009)
BAS1		Myb-related transcription factor; involved in regulating basal and induced expression of genes of the purine and histidine biosynthesis pathways; also involved in regulation of meiotic recombination at specific genes	(Venters et al. 2011)
GCN4	Positive	bZIP transcriptional activator of amino acid biosynthetic genes; activator responds to amino acid starvation; expression is tightly regulated at both the transcriptional and translational levels	(Moxley et al. 2009) (Natarajan et al. 2001) (Uluisik et al. 2011)
LEU3	Negative	Zinc-knuckle transcription factor, repressor and activator; regulates genes involved in branched chain amino acid biosynthesis and ammonia assimilation; acts as a repressor in leucine-replete conditions and as an activator in the presence of alpha-isopropylmalate, an intermediate in leucine biosynthesis that accumulates during leucine starvation	(Boer et al. 2005) (Venters et al. 2011)

Regulator	Assoc. Type	Description	Reference
RAD3		5' to 3' DNA helicase; involved in nucleotide excision repair and transcription; subunit of RNA polII initiation factor TFIIH and of Nucleotide Excision Repair Factor 3 (NEF3); homolog of human XPD protein; mutant has aneuploidy tolerance; protein abundance increases in response to DNA replication stress	(Venters et al. 2011)
SOK2	Positive	Nuclear protein that negatively regulates pseudohyphal differentiation; plays a regulatory role in the cyclic AMP (cAMP)-dependent protein kinase (PKA) signal transduction pathway; relocalizes to the cytosol in response to hypoxia; SOK2 has a paralog, PHD1, that arose from the whole genome duplication	(Rossouw, Jacobson, and Bauer 2012) (Vachova et al. 2004)
SSL1		Subunit of the core form of RNA polymerase transcription factor TFIIH; has both protein kinase and DNA-dependent ATPase/helicase activities; essential for transcription and nucleotide excision repair; interacts with Tfb4p	(Venters et al. 2011)
SWI3		Subunit of the SWI/SNF chromatin remodeling complex; SWI/SNF regulates transcription by remodeling chromosomes; contains SANT domain that is required for SWI/SNF assembly; is essential for displacement of histone H2A-H2B dimers during ATP-dependent remodeling; required for transcription of many genes, including ADH1, ADH2, GAL1, HO, INO1 and SUC2; relocates to the cytosol under hypoxic conditions	(Venters et al. 2011)
TAF1		TFIID subunit, involved in RNA pol II transcription initiation; possesses in vitro histone acetyltransferase activity but its role in vivo appears to be minor; involved in promoter binding and G1/S progression; relocalizes to the cytosol in response to hypoxia	(Venters et al. 2011)
VPS72		Htz1p-binding component of the SWR1 complex; exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A; may function as a lock that prevents removal of H2AZ from nucleosomes; required for vacuolar protein sorting	(Venters et al. 2011)
YRM1		Zinc finger transcription factor involved in multidrug resistance; Zn(2)-Cys(6) zinc finger transcription factor; activates genes involved in multidrug resistance; paralog of Yrr1p, acting on an overlapping set of target genes	(Lucau-Danila et al. 2003)

While this level of analysis offers some clues as to changes associated with the evolved trait, elucidating the entire picture will likely only be possible through whole-genome sequencing. However, given that the achievable phenylalanine titers are still quite modest (only up to ~350 mg/L), such an undertaking does not seem warranted at this time. For now, however, and for the purpose of this study, a phenylalanine over-producing host has been developed that can now serve as a test platform for engineering styrene biosynthesis.

4.3.3 Investigating native FDC1 activity and factors influencing its expression

The fact that FDC1 – the second enzyme in the established styrene pathway (Fig. 4.1) – is native to *S. cerevisiae* was an important factor motivating its selection as a potential host platform for styrene biosynthesis. However, as factors controlling its native regulation have not yet been fully resolved, it was initially unclear if: *i)* background expression of FDC1 would occur in the specific strain and under the culture conditions of interest, or *ii)* expression levels would be sufficient for supporting ample flux through the last step of the pathway. With regards to the first point, it has previously been shown that PAD1 expression was induced in the presence of ferulic acid, coumaric acid, and *trans*-cinnamate, when said species were added to growing cultures(Larsson, Nilvebrant, and Jonsson 2001). The potential role of *trans*-cinnamate (a styrene pathway intermediate and known substrate of FDC1) as an inducer of FDC1 expression, however, was not tested and has not before been reported. Thus, to better understand native expression of FDC1, particularly within the context of the styrene pathway, an investigation of factors influencing its induction was performed.

Potential inducers of interest included both phenylalanine and *trans*-cinnamate, as well as coumaric acid and ferulic acid as both structural homologs to *trans*-cinnamate. BY4741 was cultured in minimal media supplemented with 200 mg/L of each species for 12 h before collecting the cells by pelleting and preparing crude cell lysate. Cellular extracts were then each tested for *in vitro* decarboxylase activity on *trans*-cinnamate, as measured spectrophotometrically. As seen in Table 4.3, a positive result was observed for cells cultured in the presence of each of ferulic acid, *trans*-cinnamate, or coumaric acid, indicating that all three are suitable inducers of FDC1 expression. Interestingly, lysates prepared from cells grown in the presence of *trans*-cinnamate displayed the greatest specific *trans*-cinnamate decarboxylase activity. In contrast, phenylalanine was not an inducer of FDC1, nor was FDC1 activity detected in the control. This implies that FDC1 expression is not constitutive. Furthermore, its expression in the final strain will be contingent upon PAL2 expression, which could be of benefit with respect to minimizing metabolic burden. Further experiments, however, are certainly necessary before the mechanism of transcription initiation can be fully understood.

Table 4.3. Assaying the in vitro decarboxylase activity of FDC1 against a pool of structurally-related, phenylacrylic acid substrates.

Compound	Induced activity	mU/mg total protein
trans-cinnamic acid	Positive	0.46±0.02
p-coumaric acid	Positive	0.39±0.02
ferulic acid	Positive	0.21±0.03
phenylalanine	Negative	Not Detected
control	Negative	Not Detected

4.3.4 Probing the styrene pathway via the exogenous addition of phenylalanine

Preliminary studies were next performed to begin probing the functionality of the styrene pathway, with the particular objectives of determining: *i)* if styrene can be

produced from exogenously supplied phenylalanine, and *ii)* if native expression levels of FDC1 are sufficient for supporting flux through the pathway. To test this, *S. cerevisiae* BY4741 was first transformed with 425GPDPAL (a high copy (2μ) number, constitutive plasmid) to enable the heterologous expression of PAL2 from *A. thaliana*. Cultures grown in SD-Leu minimal media were then supplied phenylalanine (200 mg/L) while the extracellular accumulation of key pathway metabolites *trans*-cinnamate and styrene, as well as the natural degradation product 2-phenylethanol, were monitored via HPLC for a period of 24 h. In BY4741, although only less than half of the phenylalanine was consumed, as shown in Fig. 4.5, styrene and 2-phenylethanol constituted the major accumulated end-products (20.4±0.8 and 43±0.45 mg/L, respectively). Importantly, no *trans*-cinnamate was detected in the culture medium at any time. This finding suggested that sufficient FDC1 activity was present so as to swiftly convert all *trans*-cinnamate to styrene as it was produced (i.e., implying that native levels of FDC1 expression are sufficient for circumventing the creation of a flux bottleneck).

However, the question still remained as to whether the reason for not detecting *trans*-cinnamate in the culture medium was alternatively due to its potential inability to be exported by the cells. Whereas it has previously been shown that *trans*-cinnamate can be imported by *S. cerevisiae* (Mukai et al. 2010), its natural ability to be exported has never been explicitly reported. To test this, the control strain BY4741*ΔFDC1* was similarly transformed with 425GPDPAL and analogously assayed. In this case, as seen in Fig. 4.5, 2-phenylethanol was the major product (98±2.98 mg/L) and no styrene production was observed. More importantly, however, in this case extracellular accumulation of *trans*-cinnamate (26.2±1.6 mg/L) was in fact observed, implying that it

is in fact naturally exported by *S. cerevisiae*. Thus, taken together, this study suggests that native FDC1 expression should in fact be sufficient for supporting flux through the styrene pathway, at the very least under the specific PAL2 expression levels considered.

Furthermore, in the absence of *FDC1* to drive flux from phenylalanine through *trans*-cinnamate to styrene, a higher accumulation of phenylethanol was observed. This demonstrates that even though PAL2 is being constitutively expressed on a high copy number plasmid, it is insufficient for competing with the reverse reaction of ARO8 which degrades excess phenylalanine to phenylpyruvate and subsequently phenylethanol via the Ehrlich pathway. These results demonstrate the necessity for deletion of the Ehrlich pathway in order to drive flux through the styrene pathway.

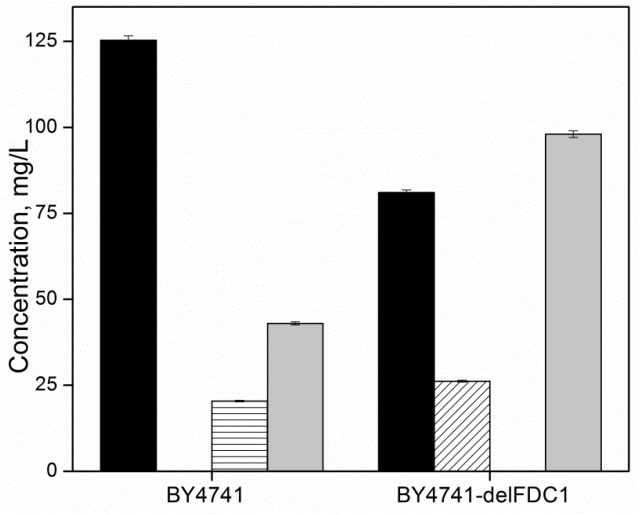

Figure 4.5. Assessing the trans-membrane export of trans-cinnamate. Phenylalanine (200 mg/L) fed to cultures of wild type *S. cerevisiae* BY4741 and BY4741 ΔFDC1. Results are shown at 48h for phenylalanine (black), *trans*-cinnamate (diagonal), styrene (horizontal), and 2-phenylethanol (gray). Error bars reported at one standard deviation from triplicate experiments.

4.3.5 Styrene production from glucose

Having demonstrated both the development of phenylalanine over-producing *S. cerevisiae* as well as the functionality of the styrene pathway enzymes in said host, the

final objective of this study was to demonstrate that styrene could by produced directly from glucose by *S. cerevisiae*. When the plasmid 425GPDPAL was transformed into BY4741 (strain BY4741-PAL) and cultured for 48 h in SD-Leu media for production of styrene, minimal amounts (less than 5 mg/L, Fig. 4.6) of styrene were detected. This was not unexpected since the concentration of phenylalanine was minimal in BY4741. Once the plasmid 425GPDPAL was transformed into the phenylalanine overproducer 22A75D (to generate strain 22A75D-PAL) and cultured for styrene production, styrene biosynthesis levels increased to 18.2±1.5 mg/L with 54.4±4.6 mg/L co-production of phenylethanol. The co-production of phenylethanol demonstrates that even though PAL is being over-expressed, it cannot compete with native metabolism and the natural regulation of phenylalanine production. In *S. cerevisiae*, the production of phenylalanine is not only regulated via feedback-inhibition but also via the Ehrlich pathway which converts the phenylalanine to phenylpyruvate in order to utilize the nitrogen from the amino acid (Hazelwood et al. 2008; Dickinson, Salgado, and Hewlins 2003). At this point, further strain improvements were necessary to increase styrene titers.

Due to the endogenous Ehrlich pathway present in yeast, phenylpyruvate is subsequently converted to phenylacetaldehyde and phenylethanol rather than styrene by means of *ARO10*(Caspi et al. 2006; Hazelwood et al. 2008). To prevent degradation of phenylpyruvate, *ARO10* was subsequently deleted from strain 22A75D to create strain 22A75D10. The *ARO10* knockout strain hosting the 425GPDPAL plasmid, strain 22A75D10-PAL, had a modest improvement in styrene synthesis achieving a final titer of 22.7±1.9 mg/L. Since *trans*-cinnamate was not observed in the culture media, it was

believed that the styrene titers suffered from a lack of precursor availability, and to improve titers, an increase in phenylalanine was needed.

To enhance flux through the phenylalanine pathway, it has been previously reported that the tyrosine sensitive DAHP synthase mutant ARO4^{K229L} alleviates feedback inhibition from tyrosine and can increase flux through the shikimic acid pathway by as much as 4.5 fold(Curran et al. 2013; Luttik et al. 2008). In an attempt to relieve the feed-back regulation, we incorporated the tyrosine-sensitive feedback resistant DAHP synthase mutant *ARO4*^{K229L} onto the genome while simultaneously deleting *ARO10*. The strain 22A75D104 was then transformed with the plasmid 425GPDPAL to achieve the strain 22A75D104-PAL (expressing both ARO4^{K229L} and *PAL2*). 22A75D104-PAL achieved a 25% increase (Fig. 4.6) in styrene production to 28.8±2.1 mg/L with a glucose yield of 0.00144±0.00011 g/g. It is important to note, however, that ARO4 is feed-back sensitive to tyrosine (not phenylalanine) (Caspi et al. 2006); therefore, it is not surprising that it did not have a more impactful result. To date, a feedback-resistant mutant of the phenylalanine sensitive DAHP synthase homolog ARO3 has not been discovered. While this point-mutation demonstrates a modest increase in flux through the shikimic acid pathway, achievable phenylalanine yields are still quite low in yeast suggesting that the pathway is tightly regulated beyond the DAHP synthase. In order for higher titers to be realized, further de-regulation of the phenylalanine biosynthesis pathway in *S. cerevisiae* must be achieved.

With only a modest improvement from its predecessor, we believe that the bottleneck is a result of poor PAL activity. Although flux through the phenylalanine pathway greatly improved in mutant 22A75D, we believe that the activity of PAL is not

sufficient enough to draw flux through the styrene pathway. The poor activity of PAL is further supported by the fact that during the fermentation, no *trans*-cinnamate is detected in the supernatant. As a result of the low PAL activity, we believe that phenylalanine accumulates within the cell (recall phenylalanine cannot be exported) and subsequently imposes further feedback regulation upon the pathway. Furthermore, since PAL activity is low, it most likely cannot compete with the reverse reaction of ARO8 and ARO9 for phenylalanine, and thus phenylalanine undergoes the transamination reaction freeing the nitrogen for use elsewhere within the cell.

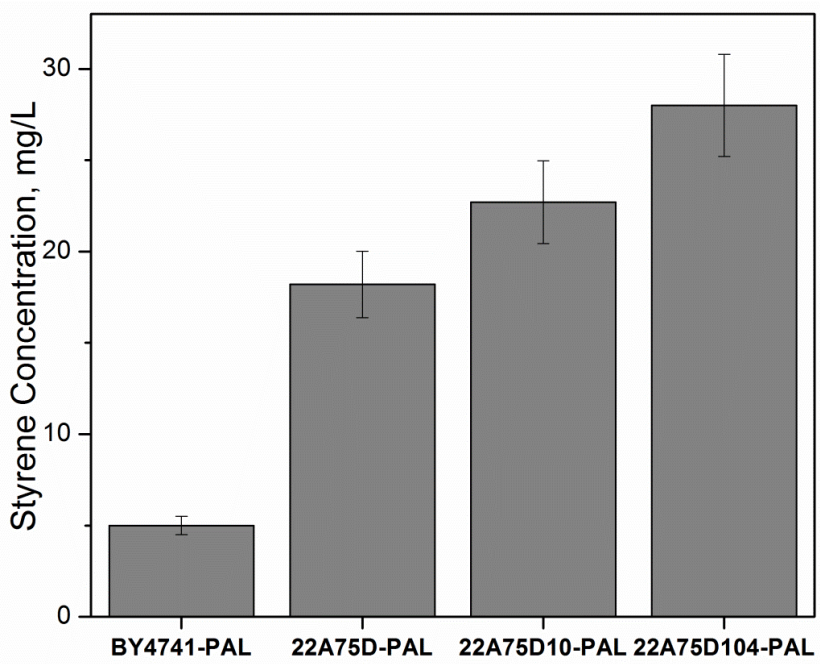

Figure 4.6. Styrene biosynthesis from glucose by engineered *S. cerevisiae*. Styrene concentration for the strains BY4741-PAL, 22A75D-PAL, 22A75D10-PAL, and 22A75D104-PAL after 48 h in shake flasks. Error bars reported at one standard deviation from triplicate experiments.

4.4 Conclusion

Unlike *E. coli*, for which a phenylalanine overproducer capable of producing phenylalanine in excess of 1 g/L has already been engineered(McKenna and Nielsen 2011; Tribe 1987), little work has been done previously to generate a robust

phenylalanine overproducer in *S. cerevisiae*. Through systematic strain and pathway engineering, the biosynthetic production of styrene from glucose by *S. cerevisiae* has been demonstrated for the first time. Although limited PAL activity as well as precursor availability of the pathway precursor phenylalanine limits the overall productivity and remains a challenge, continued improvements on engineering a more robust phenylalanine overproducer as well as enhancing PAL activity will lead to greater yields and a more sustainable approach for styrene biosynthesis.

CHAPTER 5

DISCUSSION AND FUTURE WORK

Abstract

The styrene pathway has successfully been engineered in both *E. coli* and *S. cerevisiae.* Furthermore, the styrene pathway has been extended for production of the styrene derivatives (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol in engineered *E. coli*. Currently, however, product toxicity, poor precursor availability, and poor PAL activity remain a challenge. This chapter discusses those weaknesses of the current processes and suggests potential future directions.

5.1 Introduction

These works have demonstrated the possibility of using microorganisms for the production of styrene, a plastics monomer commercially produced from petroleum derivatives. This renewable approach has created a 'green' process for not only styrene but has also opened the door for the biosynthetic production of the styrene derivatives (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol by extension of the styrene pathway. In our endeavors to maximize both titers and yields of styrene and its derivatives, we have pushed the boundaries of our *E. coli* host and are limited by product toxicity and/or precursor availability. Though we have demonstrated that the yeast *S. cerevisiae* may be a more robust, solvent tolerant host, precursor availability remains a limiting factor. Thus, in order to further improve upon the achievable titers and yields as well as establish a robust, economical process for styrene production we must continue to explore routes for

process improvements, including *i)* relieving or circumventing product toxicity, *ii)* enhancing precursor availability, and *iii)* improving recombinant PAL activity.

5.2 *In situ* product removal as a method to overcome toxicity

Due to the hydrophobic nature of styrene and its derivatives, it is believed that product accumulation occurs within the lipid bi-layer of the cell membrane resulting in reduced membrane fluidity and integrity. According to the model of *Sikkema et al.*, there exists a linear relationship between a compounds hydrophobicity, as measured by its octanol-water partition coefficient (*log* K_{OW}), and its propensity to accumulate in the membrane lipid bi-layer of an organism, as measured by the membrane-water partition coefficient (*log* K_{MW})(Sikkema, de Bont, and Poolman 1994). In order to understand the toxic effects of a particular solvent, we have previously demonstrated that the toxicity limit may be estimated by adding the compound directly to the culture medium and monitoring the growth response. When the concentration of a product reaches a threshold at which the cells can no longer proliferate, we designate this threshold as the toxicity limit. When we compared the toxicity limit of styrene and its derivatives with their predicted membrane-water partition coefficient ($K_{M/W}$), we found that there was a strong linear correlation (Fig 3.2) providing further evidence to support the hypothesis that the intra-membrane accumulation of hydrophobic entities is the cause of reduced host fitness.

Table 5.1: Toxicity Limit Analysis of Styrene and its Derivatives

Product (*Log* K_{OW})	**Host**	**Highest Titer**	**Maximum Toxicity Threshold**	**% of Toxicity Limit Achieved**
Styrene (3.05)	*E. coli*	260 ± 4.3mg/L	300 mg/L	87%
	S. cerevisiae	28.8 ± 2.1 mg/L	600 mg/L	5%
(*S*)-Styrene Oxide (1.61)	*E. coli*	1.32 ± 0.03 g/L	1.6 g/L	83%
(*R*)-1,2-Phenylethanediol (0.92)	*E. coli*	1.23 ± 0.07 g/L	8 g/L	15%

Product toxicity remains the greatest hindrance in achieving a high titer biosynthetic process for styrene production. In *E. coli*, styrene titers are currently limited to 260±4.3 mg/L, approximately 87% of the estimated toxicity limit of ~300 mg/L. It is important to note however, that it is unlikely that a product could ever truly reach the toxicity limit, rather in actuality, there must exist a balance between host fitness and the metabolic burden associated with expressing a heterologous pathway. Similar to styrene, the (*S*)-styrene oxide titers also approach the toxicity limit achieving 1.32 ± 0.03 g/L, approximately 83% of the estimated toxicity limit of 1.6 g/L. In the case of (*S*)-styrene oxide however, precursor availability of phenylalanine was also a limiting factor. In an attempt to overcome our toxicity obstacle, a new host, *S. cerevisiae*, was evaluated for its potential to produce styrene. Though the toxicity limit of *S. cerevisiae* was twice higher (~600 mg/L), other factors including poor precursor availability and low PAL activity limited the final titer to 28.8 mg/L, 5% of the toxicity limit. While finding a more solvent-tolerant host and improving its ability to produce styrene remains a goal, a more attractive option to addressing product toxicity may be *in situ* product removal (ISPR).

Not only would ISPR circumvent the toxicity issue, it would also, by nature, recover the compound of interest. Some examples of ISPR approaches include liquid-liquid extraction (or solvent extraction)(Gyamerah and Glover 1996; Malinowski 2001; Weilnhammer and Blass 1994), adsorption(Nielsen, Amarasiriwardena, and Prather 2010; Nielsen and Prather 2009), gas and vacuum stripping(Loser et al. 2005), and membrane pervaporation(Vane 2005). These approaches have been successfully applied for the continuous recovery of many chemical compounds including ethanol as well as the aromatics L-phenylacetylcarbinol(Khan and Daugulis 2010) and benzaldehyde(Jain,

Khan, and Daugulis 2010). Although the hydrophobic and volatile nature of styrene proved to negatively limit titers due to the associated toxicity, these qualities conversely make styrene amenable to ISPR.

Taking advantage of styrene's volatility, we first attempted to utilize gas stripping coupled to a cold trap for retention of styrene in a bioreactor model. The outlet gas of the bioreactor was monitored for the concentration of stripped styrene by taking 100 µL gas samples from the effluent using a Hamilton gas tight syringe (Hamilton, Reno NV) for subsequent analysis by gas chromatography (GC). GC analysis of styrene gas was performed on a Hewlett Packard 5890 Series II gas chromatograph with a flame ionizing detector and Agilent DB-5 (30 m × 0.25 mm id) fused-silica capillary column using helium as the carrier gas. The injector, column, and detector temperatures were set at 250 °C.

The accumulated concentration of styrene that was produced by the culture after 42 h was determined from the gas samples to be ~464 mg/L, refer to Fig. 5.1 for time course production of styrene. While gas stripping proved effective for removing styrene from the culture medium and increasing the yield by almost two-fold, our lab-scale cold-trap was ill-equipped for retaining stripped styrene. Due to the quick rate of loss of styrene during gas stripping, other approaches such as solvent extraction (via overlays) or pervaporation are most likely the best option for styrene removal and recovery.

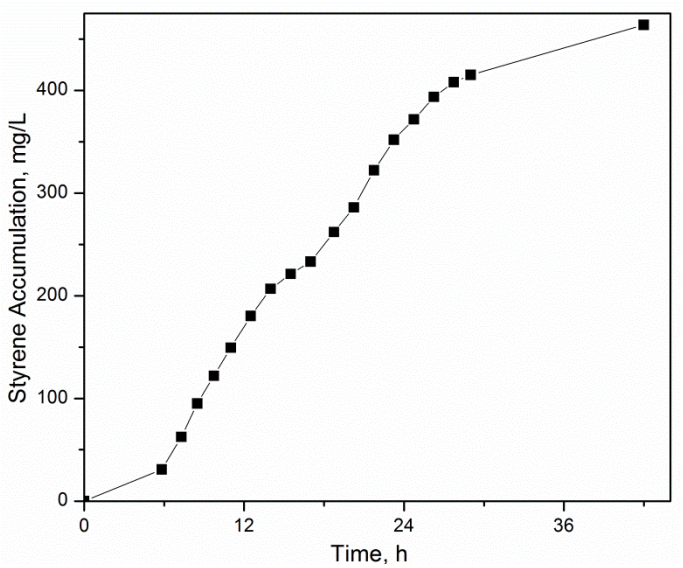

Figure 5.1. Styrene accumulation in bioreactor. The styrene effluent gas of the bioreactor was analyzed by GC for 42 h to determine the amount of styrene produced.

Solvent extraction has been used successfully for the extraction of many microbial-derived products, most notably the terpene amorpha-4,11-diene, a precursor to the anti-malarial drug artemisinin. Similar to styrene, amorpha-4,11-diene is both hydrophobic and volatile. In experiments by *Newman et al.*, it was found that amorpha-4,11-diene evaporated at a half life of 50 min from the culture, but using an organic dodecane solvent overlay, researchers were able to improve retention of the product achieving titers of over 0.5 g/L cultures(Newman et al. 2006). Although dodecane is hydrophobic, it tends to not interact with the hydrophilic surface of the membrane lipid bi-layer making its contribution to toxicity minimal. Furthermore, its extremely low water solubility makes it useful for subsequent separation from the aqueous solution. To test the feasibility of solvent overlays for the ISPR of styrene, we evaluated the organic solvents dodecane, hexadecane, and bis(2-ethylhexyl) phthalate (BEHP). In our experiment, we used 10 mL of solvent per 50 mL of culture of our styrene producing

strain, *E. coli* NST74 harboring pSpal1At and pTfdc1Sc. As illustrated in Fig. 5.2, in the presence of hexadecane, styrene titers reached 436 mg styrene/L culture (2.18 g styrene/L hexadecane), a modest 1.7-fold improvement over the 260 mg/L produced with no solvent overlay. Cultures with dodecane overlays performed slightly better achieving 544 mg styrene/L culture (2.72 g styrene/L dodecane), a 2-fold improvement. The highest titers however were achieved by utilizing BEHP as the overlay solvent, for which an impressive 836 mg styrene/L culture (4.18 g styrene/L BEHP) was produced. This represents a 3.2-fold improvement in styrene production and is the highest titer of styrene that has ever been achieved in *E. coli*. In order to reach higher titers, further strain improvements, in particular increased phenylalanine production, must be realized.

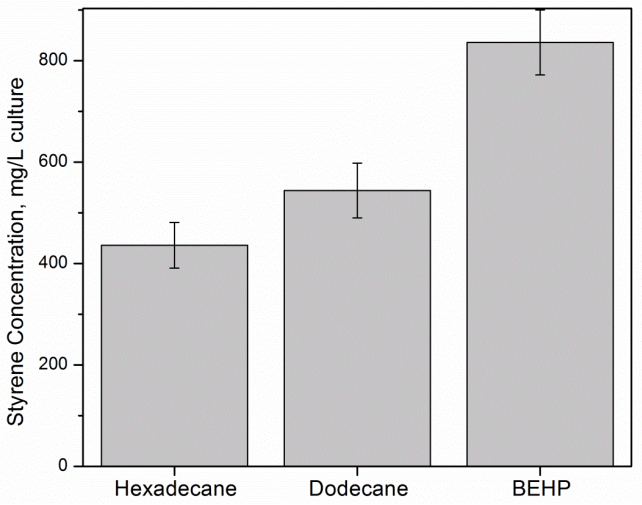

Figure 5.2. *In situ* product removal of styrene via solvent extraction *E.coli* NST74 harboring the styrene pathway plasmids pSpal1At and pTfdc1Sc was cultured in 50 mL MM1 media with 10 mL of solvent: hexadecane, dodecane, or (BEHP).

5.3 Improving Precursor Availability

Although it was demonstrated that the toxicity of styrene may be circumvented via ISPR, the availability of the precursor phenylalanine is also a limiting factor and needs to be further enhanced for titers to approach the theoretical yield. Several different

theoretical yields have been previously calculated from known stoichiometry of phenylalanine biosynthesis from glucose based on different metabolic scenarios(Baez-Viveros et al. 2004; Takagi et al. 1996; Patnaik and Liao 1994; Förberg, Eliaeson, and Häggström 1988). In wild type *E. coli*, the phosphotransferase system (PTS) imports glucose and converts one mole of phosphoenolpyruvate (PEP) to one mole of pyruvate (PYR). If the competing aromatic amino acid pathways for tyrosine and tryptophan are knocked out and there is no feed-back inhibition imposed upon the phenylalanine pathway, the maximum theoretical yield of phenylalanine is 0.275 g/g (assuming carbon dioxide is produced at a rate of 3.33 mol/mol glucose)(Förberg, Eliaeson, and Häggström 1988). If, however, *E. coli* is engineered to recycle PYR to PEP, resulting in less carbon being lost as carbon dioxide (2.62 mol/mol glucose), the yield is increased to 0.34 g/g (Förberg, Eliaeson, and Häggström 1988). Recycling PYR requires the expression of the PEP synthetase (PpsA) which is not typically induced by growth on glucose. The maximum PYR able to be recycled was determined by the break point when the TCA (citric acid) cycle balanced NADPH. The maximum theoretical yield possible would be if all PYR is recycled to PEP or the PTS was inactivated resulting in a yield of 0.55 g/g(Förberg, Eliaeson, and Häggström 1988). This theoretical yield is highly improbable to ever experimentally reach though since it assumes no carbon enters the TCA cycle which would severely hinder cell viability. Current experimental yields from this project have yet to reach the most probable theoretical yield of 0.275 g/g, though some strain improvements have proven effective in working toward that goal.

Table 5.2. Summary of titers and yields for styrene and its derivatives

Product	Host	Highest Titer	Max Yield (styrene/glucose)	% of Theoretical Yield[1]
Styrene	*E. coli* NST74	260 ± 4.3mg/L, 25.0 ± 0.04 mM	0.03 ± 0.005 g/g, 0.05 ± 0.009 mol/mol	17%
	S. cerevisiae 22A75D	28.8 ± 2.1 mg/L, 0.28 ± 0.02 mM	0.0014 ± .0001 g/g 0.0024 ± .0001 mol/mol	-
(*S*)-Styrene Oxide	*E. coli* NST74ΔtyrA	1.32 ± 0.03 g/L, 11.0 ± 0.25 mM	0.12 ± 0.005 g/g, 0.17 ± 0.01 mol/mol	60%
(*R*)-1,2-Phenylethanediol	*E. coli* NST74ΔtyrA	1.23 ± 0.07 g/L, 8.9 ± 0.51 mM	0.11 ± 0.002 g/g, 0.14 ± 0.01 mol/mol	47%

[1] Assumes a maximum yield of 0.275 g-phenylalanine/g-glucose

Through strain development, we have been able to achieve as high as 60% of the theoretical yield. In Chapter 3, it was shown that (*S*)-styrene oxide titers were raised in *E. coli* NST74 from 1.1 g/L to 1.32 g/L (resulting in a yield of 0.12 g/g, Table 5.2) by deleting the competing tyrosine pathway. While effective, the drawback of this approach is that tyrosine must now be supplemented to the culture medium. In addition to deleting the competing tyrosine pathway, it was also shown that enhancing the production of the phenylalanine precursor erythrose-4-phosphate, via overexpression of the transketolase *tktA*, had a significant impact on improving phenylalanine titers achieving ~1.55 g/L, a 40% increase. However, when *tktA* was overexpressed in combination with the heterologous pathway, an improvement in product titers was not observed, presumable due to increased metabolic burden. Single gene mutations, deletions, or overexpression of pathway genes, in efforts to drive more flux through the pathway, are not only time consuming but often result in minimal yield improvements.

In order to have a more global impact on the phenylalanine pathway, methods beyond single gene alterations needs to be considered. For example, other approaches may include media optimization or modifying the sugar transport system. For example,

lycopene titers were increased 7-fold in *E. coli* by *i)* removing the PTS to increase PEP availability, *ii)* feeding glycerol, a non-PTS sugar, and *iii)* optimizing media components via statistical design of experiments(Zhang et al. 2013). While modifying the PTS has shown to be effective in *E.coli*, yeast rely on facilitated diffusion for glucose import so conservation of PEP is not a concern(Lagunas 1993).

As an alternative approach, procedures such as global Transcription Machinery Engineering (gTME)(Alper et al. 2006; Nicolaou, Gaida, and Papoutsakis 2010) may be implemented to increase phenylalanine titers as well as perhaps improve styrene tolerance in our hosts *E. coli* and *S. cerevisiae*. The principals of gTME have been successfully employed to enhance ethanol tolerance in both yeast(Alper et al. 2006) and *E. coli* (Alper and Stephanopoulos 2007). Furthermore, gTME was also applied in *S. cerevisiae* to increase ethanol tolerance through mutagenesis of the Spt15p transcription factor encoding the TATA-binding protein resulting in a 15% increase in ethanol yield(Alper et al. 2006). Furthermore, in *E. coli* gTME was used to mutate RpoD, encoding sigma factor σ^{70}, to increase ethanol tolerance as well as increase lycopene production in parallel(Alper and Stephanopoulos 2007). In theory, gTME could be applied to RpoD as well as the sigma factor responsible stationary phase activity, σ^{38} encoded by RpoS, in hope that phenylalanine titers would increase during both the exponential and stationary phases (when synthesis is typically stagnant) given a high-throughput selection method was used.

Investigating strategies for yeast specifically, there are several strategies that can be made to the aromatic amino acid pathway in *S. cerevisiae* to further increase phenylalanine titers. First, the transketolase *TKL1* may be overexpressed to increase

production of E-4P. This may be especially advantageous since *TKL1* is believed to be feedback regulated by hydroxyphenylpyruvate, the precursor to tyrosine. Second, the glucose-6-phosphate dehydrogenase, *ZWF1*, may be knocked out to force entry into the pentose phosphate pathway via E-4P. When these two modifications were combined for muconic acid production, their synergistic effect resulted in a two-fold increase in titers (Curran et al. 2013). In addition to increasing E-4P precursor availability, further deregulation of the phenylalanine pathway would likely increase flux. For example, the tyrosine sensitive chorismate mutase ARO7 may be de-regulated via single point mutation $ARO7^{G141S}$(Curran et al. 2013); however, this is likely to increase titers of both phenylalanine as well as tyrosine.

It is possible based on the results of increasing phenylalanine in *E. coli* presented in Chapter 3 (Fig. 3.5) that deletion of TYR1 to create a tyrosine auxotroph may direct flux to phenylalanine. This approach has not yet been investigated in *S. cerevisiae* to enhance phenylalanine concentrations, but the TYR1 knockout is available from Thermo Scientific (Strain 852464). However, since this strategy would require supplementation of exogenous tyrosine to the culture media at relatively high concentrations, it is likely that tyrosine would impose feedback regulation upstream in the pathway. Alternatively, it may be more optimal to use a gene knock-down approach which has been previously shown to be possible in yeast with as little as 21 bp hairpin structured and silence genes through translational repression which has been shown to be capable of reducing activity up to 60%(Elbashir, Lendeckel, and Tuschl 2001; McManus et al. 2002).

5.4 Improving PAL Activity

PAL activity was shown to be the rate limiting step for the styrene pathway in both hosts, *E. coli* and *S. cerevisiae*. Strategies to improve enzyme expression include tuning the ribosomal binding site (RBS) and translation initiation region, using a stronger promoter (such as *trc* or T7), increasing the copy number of the protein expression system, changing the transcriptional promoter (constitutive versus inducible), or changing the concentration of the inducer. Previous attempts at improving PAL activity in *E. coli* involved using a stronger promoter (*trc* versus *lac*) and a plasmid with a higher copy number (20 verses 10 copies/cell); although, this resulted in minimal increase of activity (data not shown). With the exception of tuning the RBS, the aforementioned strategies specifically address transcriptional improvements, as opposed to translational improvements. One of the key problems associated with expressing heterologous enzymes in engineered hosts is that the codon usage is foreign and generally not optimal for high level expression. Not only could improper codon usage create a potential bottleneck, it may also unnecessarily increase the metabolic burden on the host. As a result of the redundancy of the genetic code, each organism demonstrates preferences for certain codon usage which is dependent on the relative abundance and diversity of tRNA within the host(Jana and Deb 2005). Codon optimization offers the chance to alter the codon usage of a protein without altering the protein's sequence. Not only can codon optimization tune the tRNA usage and improve protein translation, it can also be used to optimize the GC content as well as remove unwanted RNA secondary structures(Gustafsson, Govindarajan, and Minshull 2004; Wu et al. 2004). Codon optimization has proven useful for increasing product titers, for example mevalonate in *E.*

coli(Anthony et al. 2009) and muconic acid in *S. cerevisiae(Curran et al. 2013)*. While PAL activity remains an obstacle, particularly in *S. cerevisiae*, codon optimization, as well as other previously mentioned techniques, may prove effective in improving titers.

While the previously suggested enzyme activity improvement techniques rely on improving PAL2 from *A. thaliana*, it is possible that this specific isoenzyme may not have the highest activity in all hosts. While it was previously demonstrated that PAL2 had the highest recombinant activity in *E. coli*, the pool of candidate enzymes was small consisting of only four isozymes (Chapter 2). In future works, it is suggested that alternative PAL isoenzymes be tested for their possible greater PAL activity. Examples of other PAL plant sources include *Nicotiana tabacum*(O'Neal and Keller 1970; Fukasawa-Akada, Kung, and Watson 1996), *Bambusa oldhamii*(Hsieh et al. 2011; Hsieh et al. 2010), *Lycoris radiata*(Jiang et al. 2011), *Salvia Miltiorrhiza*(HU et al. 2009), *Cynara cardunculus*(De Paolis et al. 2008; Pandino et al. 2011), and *Daucus carota*(De Lorenzo et al. 1987), to name a few.

5.5 Conclusion

These works constitute a 'proof of concept' that it is possible to engineer microorganisms with the pathways necessary to replace chemical products of petrochemical origin with those biosynthesized from renewable resources. Though these processes are not currently at an economical scale, there is immense opportunity for future improvement. Through *in situ* product removal, enhanced precursor availability, and increased enzyme activity there is potential for increased titers that may one day be able to compete with the petroleum industry.

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