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Styrene biosynthesis from glucose by engineered *E. coli*

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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 *Arabidopsis thaliana* and *FDC1* from *Saccharomyces cerevisiae* (originally classified as ferulate decarboxylase) in an L-phenylalanine over-producing *Escherichia 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.

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 et al., 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

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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. 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 (Gilbert and Tully, 1982; Qi et al., 2007; Vannelli et al., 2007b), plants (Cochrane et al., 2004; Young et al., 1966), and (although less prevalently) bacteria (Moffitt et al., 2007a; Xiang and Moore, 2005; Xiang and Moore, 2006; Young et al., 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. (Cui et al., 2008; Gilbert et al., 1985, 1983; Gilbert and Tully, 1982; Santos et al., 2011; Vannelli et al., 2007a). 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 et al., 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., 2007a; 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.

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 et al., 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 et al., 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. Materials and methods

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. Strains and media

All strains, plasmids, and oligonucleotide primers used in this study are listed in Table 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

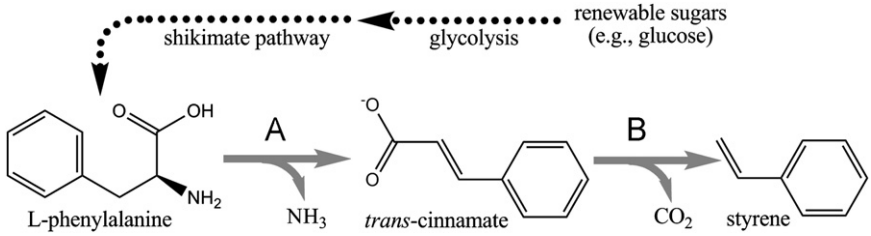

Fig. 1. Enzymatic pathway to convert the precursor L-phenylalanine to the product styrene via the intermediate *trans*-cinnamate. The two-step pathway from L-phenylalanine is achieved by the co-expression of one or more genes which encoded phenylalanine ammonia lyase (PAL) activity (A), and one or more genes which encoded *trans*-cinnamic acid decarboxylase (CADC) activity (B).

Table 1
Strains, plasmids, and oligonucleotide primers used in the present 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 spoT1*Δ(*mrr-hsdRMS-mcrBC*)	New England Biolabs
S. cerevisiae W303	Source of *pad1* and *fdc1*	Prather Lab, MIT
L. plantarum	Source of *pdc*	Prather Lab, MIT
B. subtilis 3610	Source of *padC*	Prather Lab, MIT
Plasmids		
pTrc99A	P_{trc}, pBR322 ori, *lacI^q*, *Amp^r*	Prather Lab, MIT
pSTV28	P_{lac}, pACYC184 ori, *Cm^r*	Prather Lab, 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	

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), $MgSO_4 \cdot 7H_2O$ (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), $Na_2WO_4 \cdot 2\,H_2O$ (0.10 g/L), and $NiCl_2 \cdot 6H_2O$ (0.02 g/L)).

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 °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 °C for another 6–8 h while cell growth, as determined by OD_{600} measurements, was periodically monitored using a UV/vis spectrophotometer (Beckman Colter DU800, Brea, CA).

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 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 et al., 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 °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 °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 °C overnight. The transformant pool was subsequently 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 1.

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 °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 °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 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.

2.6. 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 $1400 \times g$ 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 $11{,}000 \times g$ 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 Colter UV/vis spectrophotometer for a total of 5 min at 20 s intervals. A molar extinction coefficient of 9000 $M^{-1}\,cm^{-1}$ 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 vs. Precision Plus Unstained Protein Standards (Bio-Rad, Hercules, CA).

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 $1400 \times g$ 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 3 h. 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.

2.8. 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 °C and analyzed by HPLC using the methods described herein.

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 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.

2.10. 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 29–48 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.

2.11. 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 × *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 (4.6 mm × 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 °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 *tCA*, *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.

2.12. 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 × *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-5S (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.

3. Results and discussion

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 (for example, 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 ($\log K_{ow}$=2.13) and styrene ($\log K_{ow}$=3.05) can each inhibit *E. coli* growth when added to cultures at final concentrations above ~800 and ~300 mg/L, respectively, as demonstrated in Fig. 2. For reference, *p*-hydroxystyrene ($\log K_{ow}$=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

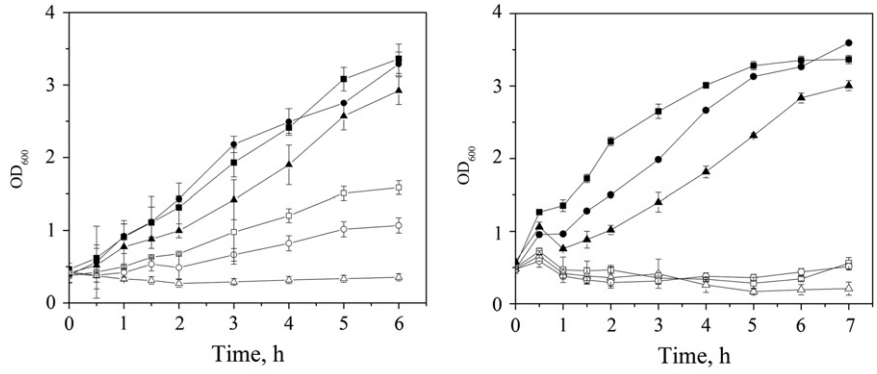

Fig. 2. Left: growth response of *E. coli* NST74 to tCA at concentrations of 0 mg/L (solid square), 200 mg/L (solid circle), 400 mg/L (solid triangle), 600 mg/L (open square), 800 mg/L (open circle), and 1000 mg/L (open triangle). Right: growth response of *E. coli* NST74 to styrene at concentrations 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.

indicator of its toxicity towards microorganisms and may be quantified by log K_{ow}, the log of the octanol–water partition coefficient (Ramos et al., 2002). Even at low concentrations, compounds with a log K_{ow} between 1.0 to 5.0 tend to be very toxic (Heipieper et al., 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 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.

Table 2
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). Errors reported at one standard deviation from triplicate experiments.

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.

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 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), 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., 2007b). 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), assays of *in vivo* function provided a more stark contrast into the relative activities among all candidate PALs, as shown in Fig. 3. 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 et al., 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. 3.

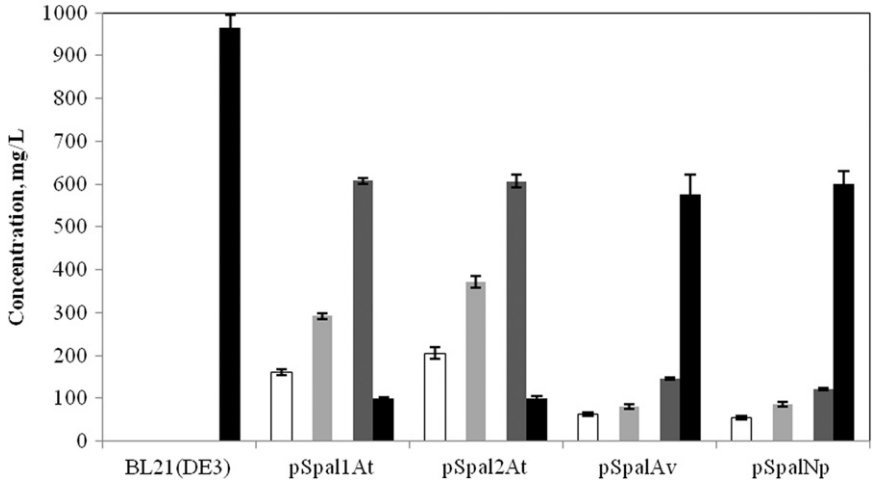

Fig. 3. Phenylalanine ammonia lyase activity from candidate genes cloned from *A. thaliana*, *A. variabilis*, and *N. punctiformes* in recombinant *E. coli* BL21(DE3) whole cells. 50 ml cultures were grown for 8 h (induced with 0.2 mM IPTG after 1.5 h), spun down and re-suspended in 10 ml PBS buffer.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

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). 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 (Cui et al., 2008; Gilbert et al., 1985; Gilbert et al., 1983; Gilbert and Tully, 1982) 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).

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 1) were individually transformed into *E. coli* BL21(DE3), as described above. Whole, resting cells were prepared in PBS buffer supplemented with 1 g/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. 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. 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.

Although the proposed styrene biosynthesis pathway is somewhat analogous to that which was previously developed for

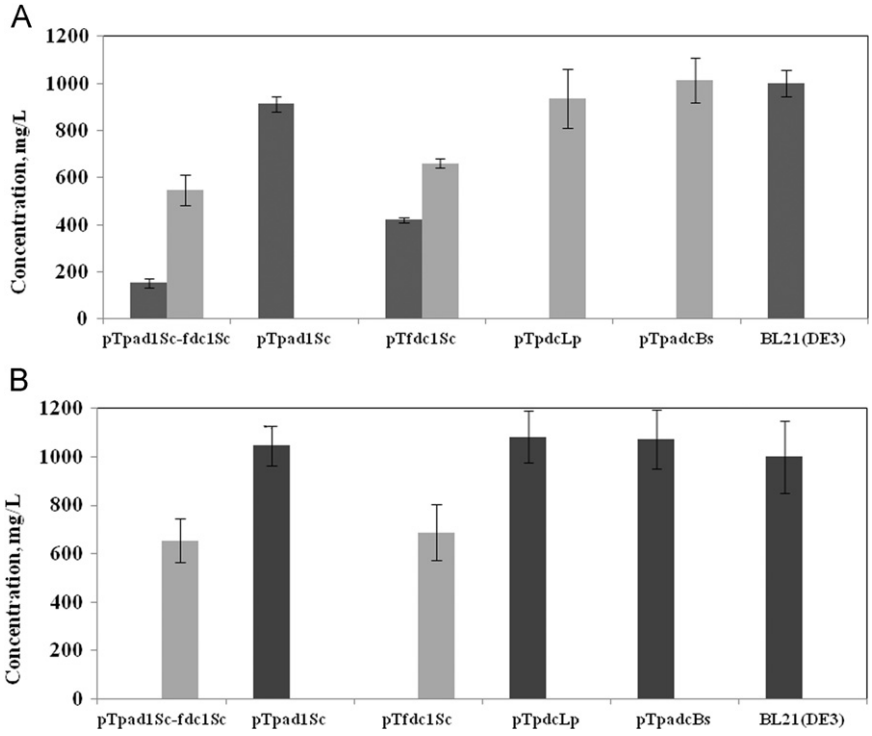

Fig. 4. Phenylacrylic acid decarboxylase activity from candidate genes cloned from *S. cerevisiae*, *L. plantarum*, and *B. subtilis* in recombinant *E. coli* BL21(DE3) whole cells. The conversion of (A) 1 g/L *p*-coumaric acid (dark gray) to *p*-hydroxystyrene (light gray) and (B) 1 g/L *trans*-cinnamic acid (dark gray) to styrene (light gray) after 12 h. Error bars reported at one standard deviation from triplicate experiments.

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.

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, 473, 648, 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 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. 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. 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*.

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 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

Table 3
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)	*trans*-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

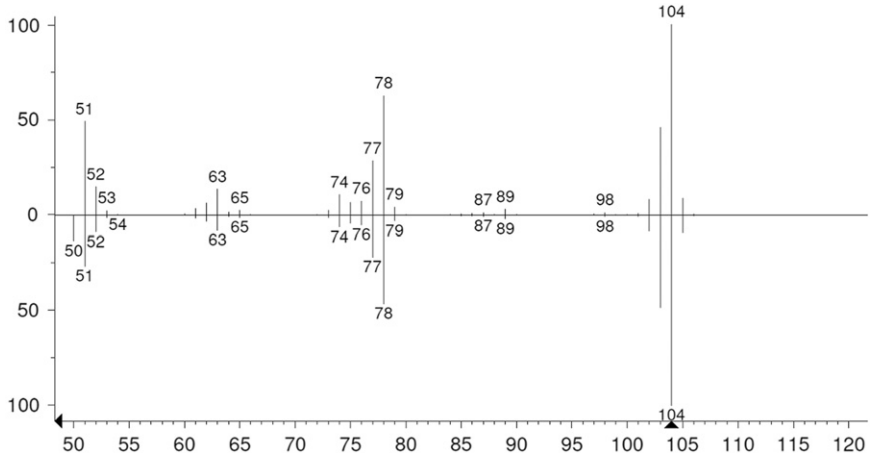

Fig. 5. Head to tail comparison of the standard mass spectra showing the relative abundance of the mass-to-charge ratio of styrene from the NIST08 library (lower) with that of the dominant metabolite peak obtained in hexane extractions of the culture broth (upper).

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. 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. It is speculated that the final styrene titer was only capable of surpassing the previously-determined toxicity threshold in resting cell studies because the biotransformation in this case was no longer growth dependent. That is to say that the stationary phase resting cells had previously assembled the necessary pathway enzymes and were not reliant upon endogenously synthesized L-phenylalanine as a precursor. Thus, the impediment of conversion observed at further elevated styrene titers most likely represents an inhibition imposed at the level of the pathway enzymes rather than an impairment of cell growth, as was observed in our previous characterization experiments. Further experiments will be required to characterize and understand this potential inhibitory mechanism. 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 et al., 1996). TktA overexpression 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).

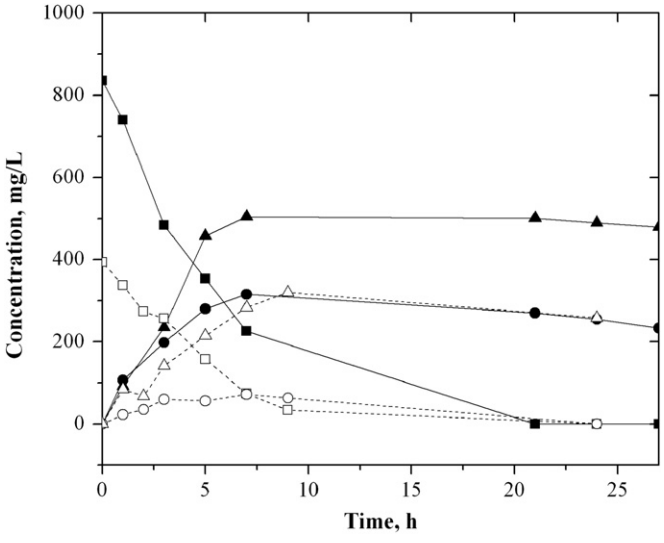

Fig. 6. Whole cell production of styrene (triangle) from *trans*-cinnamic acid (circle) after exogenous L-phenylalanine (square) addition by *E. coli* NST74 pSpal2At pTfdc1Sc. Open and filled shapes correspond to the initial addition of 400 and 950 mg/L L-phenylalanine.

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 et al., 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, 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 et al., 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 et al., 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 et al., 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 et al., 2010). The latter three approaches may be particularly well-suited for styrene recovery given its volatile nature, as previously discussed.

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.

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References

Alper, H., Moxley, J., Nevoigt, E., Fink, G.R., Stephanopoulos, G., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314, 1565–1568.

Alper, H., Stephanopoulos, G., 2007. Global transcription machinery engineering: a new approach for improving cellular phenotype. Metabolic Engineering 9, 258–267.

Babushok, V.I., Linstrom, P.J., Reed, J.J., Zenkevich, I.G., Brown, R.L., Mallard, W.G., Stein, S.E., 2007. Development of a database of gas chromatographic retention properties of organic compounds. Journal of Chromatography A. 1157, 414–421.

Baez-Viveros, J.L., Osuna, J., Hernandez-Chavez, G., Soberon, X., Bolivar, F., Gosset, G., 2004. Metabolic engineering and protein directed evolution increase the yield of L-phenylalanine synthesized from glucose in *Escherichia coli*. Biotechnology and Bioengineering 87, 516–524.

Bonomo, J., Warnecke, T., Hume, P., Marizcurrena, A., Gill, R.T., 2006. A comparative study of metabolic engineering anti-metabolite tolerance in *Escherichia coli*. Metabolic Engineering. 8, 227–239.

Boudet, A.M., 2007. Evolution and current status of research in phenolic compounds. Phytochemistry 68, 2722–2735.

Clark, G., 1990. Phenethyl alcohol. Perfumer Flavor 15, 37–44.

Clausen, M., Lamb, C.J., Megnet, R., Doerner, P.W., 1994. PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. Gene 142, 107–112.

Cochrane, F.C., Davin, L.B., Lewis, N.G., 2004. The Arabidopsis phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. Phytochemistry 65, 1557–1564.

Cui, J.D., Jia, S.R., Sun, A.Y., 2008. Influence of amino acids, organic solvents and surfactants for phenylalanine ammonia lyase activity in recombinant *Escherichia coli*. Letters in Applied Microbiology 46, 631–635.

DoE, Steam system opportunity assessment for the pulp and paper, chemical manufacturing, and petroleum refining industries. In: Energy, O. o. E. E. a. R. E. U. S. D. o. (Ed.), 2002.

Fernandez, X., Lizzani-Cuvelier, L., Loiseau, A.M., Perichet, C., Delbecque, C., Arnaudo, J.F., 2005. Chemical composition of the essential oils from Turkish and Honduras Styrax. Flavour and Fragrance Journal 20, 70–73.

Fischer, C.R., Klein-Marcuschamer, D., Stephanopoulos, G., 2008. Selection and optimization of microbial hosts for biofuels production. Metabolic Engineering 10, 295–304.

Flores, N., Xiao, J., Berry, A., Bolivar, F., Valle, F., 1996. Pathway engineering for the production of aromatic compounds in *Escherichia coli*. Nature Biotechnology 14, 620–623.

Gilbert, H.J., Clarke, I.N., Gibson, R.K., Stephenson, J.R., Tully, M., 1985. Molecular-cloning of the phenylalanine ammonia-lyase gene from rhodosporidium-toruloides in *Escherichia-coli* K-12. Journal of Bacteriology 161, 314–320.

Gilbert, H.J., Stephenson, J.R., Tully, M., 1983. Control of synthesis of functional messenger-rna coding for phenylalanine ammonia-lyase from rhodosporidium-toruloides. Journal of Bacteriology 153, 1147–1154.

Gilbert, H.J., Tully, M., 1982. Synthesis and degradation of phenylalanine ammonia-lyase of rhodosporidium-toruloides. Journal of Bacteriology 150, 498–505.

Gosset, G., Yong-Xiao, J., Berry, A., 1996. A direct comparison of approaches for increasing carbon flow to aromatic biosynthesis in *Escherichia coli*. Journal of Industrial Microbiology 17, 47–52.

Gyamerah, M., Glover, J., 1996. Production of ethanol by continuous fermentation and liquid-liquid extraction. Journal of Chemical Technology and Biotechnology 66, 145–152.

Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H., de Bont, J.A.M., 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends in Biotechnology 12, 409–415.

Jain, A.N., Khan, T.R., Daugulis, A.J., 2010. Bioproduction of benzaldehyde in a solid–liquid two-phase partitioning bioreactor using *Pichia pastoris*. Biotechnology Letters 32, 1649–1654.

Jiang, H., Wood, K.V., Morgan, J.A., 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 71, 2962–2969.

Jones, D.H., 1984. Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. Phytochemistry 23, 1349–1359.

Keasling, J.D., 2008. Synthetic biology for synthetic chemistry. ACS Chemical Biology 3, 64–76.

Khan, T.R., Daugulis, A.J., 2010. Application of solid–liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. Biotechnology and Bioengineering 107, 633–641.

Larsson, S., Nilvebrant, N.O., Jonsson, L.J., 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Applied Microbiology and Biotechnology 57, 167–174.

Loser, C., Schroder, A., Deponte, S., Bley, T., 2005. Balancing the ethanol formation in continuous bioreactors with ethanol stripping. Engineering in Life Sciences 5, 325–332.

Lütke-Eversloh, T., Stephanopoulos, G., 2008. Combinatorial pathway analysis for improved L-tyrosine production in *Escherichia coli*: identification of enzymatic bottlenecks by systematic gene overexpression. Metabolic Engineering 10, 69–77.

Malinowski, J.J., 2001. Two-phase partitioning bioreactors in fermentation technology. Biotechnology Advances 19, 525–538.

Moffitt, M.C., Louie, G.V., Bowman, M.E., Pence, J., Noel, J.P., Moore, B.S., 2007a. Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry 46, 1004–1012.

Moffitt, M.C., Louie, G.V., Bowman, M.E., Pence, J., Noel, J.P., Moore, B.S., 2007b. Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry 46, 1004–1012.

Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., Iefuji, H., 2010. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. Journal of Bioscience and Bioengineering 109, 564–569.

Nicolaou, S.A., Gaida, S.M., Papoutsakis, E.T., 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metabolic Engineering 12, 307–331.

Nielsen, D.R., Amarasiriwardena, G.S., Prather, K.L.J., 2010. Predicting the adsorption of second generation biofuels by polymeric resins with applications for in situ product recovery (ISPR). Bioresource Technology 101, 2762–2769.

Nielsen, D.R., Leonard, E., Yoon, S.H., Tseng, H.C., Yuan, C., Prather, K.L., 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metabolic Engineering.

Nielsen, D.R., Prather, K.J., 2009. In situ product recovery of n-butanol using polymeric resins. Biotechnology and Bioengineering 102, 811–821.

Pagot, Y., Belin, J.M., Husson, F., Spinnler, H.E., 2007. Metabolism of phenylalanine and biosynthesis of styrene in *Penicillium camemberti*. Journal of Dairy Research 74, 180–185.

Qi, W.W., Vannelli, T., Breinig, S., Ben-Bassat, A., Gatenby, A.A., Haynie, S.L., Sariaslani, F.S., 2007. Functional expression of prokaryotic and eukaryotic genes in *Escherichia coli* for conversion of glucose to p-hydroxystyrene. Metabolic Engineering 9, 268–276.

Ramos, J.L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-González, M.I., Rojas, A., Terán, W., Segura, A., 2002. Mechanisms of solvent tolerance in gram-negative bacteria. Annual Review of Microbiology 56, 743–768.

Reyes, L.H., Almario, M.P., Kao, K.C., 2011. Genomic library screens for genes involved in n-butanol tolerance in *Escherichia coli*. PLoS ONE 6, e17678.

Santos, C.N.S., Koffas, M., Stephanopoulos, G., 2011. Optimization of a heterologous pathway for the production of flavonoids from glucose. Metabolic Engineering 13, 392–400.

SRI, 2010. Styrene. Access Intelligence LLC Inc.

Tatarko, M., Romeo, T., 2001. Disruption of a global regulatory gene to enhance central carbon flux into phenylalanine biosynthesis in *Escherichia coli*. Current Microbiology 43, 26–32.

Tran, N.P., Gury, J., Dartois, V., Nguyen, T.K.C., Seraut, H., Barthelmebs, L., Gervais, P., Cavin, J.-F., 2008. Phenolic acid-mediated regulation of the padC gene, encoding the phenolic acid decarboxylase of *Bacillus subtilis*. Journal of Bacteriology 190, 3213–3224.

Vane, L.M., 2005. A review of pervaporation for product recovery from biomass fermentation processes. Journal of Chemical Technology and Biotechnology 80, 603–629.

Vannelli, T., Wei, Qi, W., Sweigard, J., Gatenby, A.A., Sariaslani, F.S., 2007a. Production of p-hydroxycinnamic acid from glucose in *Saccharomyces cerevisiae* and *Escherichia coli* by expression of heterologous genes from plants and fungi. Metabolic Engineering 9, 142–151.

Vannelli, T., Xue, Z.X., Breinig, S., Qi, W.W., Sariaslani, F.S., 2007b. Functional expression in *Escherichia coli* of the tyrosine-inducible tyrosine ammonia-lyase enzyme from yeast Trichosporon cutaneum for production of p-hydroxycinnamic acid. Enzyme and Microbial Technology 41, 413–422.

Verhoef, S., Ruijssenaars, H.J., de Bont, J.A., Wery, J., 2007. Bioproduction of p-hydroxybenzoate from renewable feedstock by solvent-tolerant *Pseudomonas putida* S12. Journal of Biotechnology 132, 49–56.

Verhoef, S., Wierckx, N., Westerhof, R.G., de Winde, J.H., Ruijssenaars, H.J., 2009. Bioproduction of p-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. Applied and Environmental Microbiology 75, 931–936.

Weber, F.J., Ooijkaas, L.P., Schemen, R.M., Hartmans, S., de Bont, J.A., 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Applied and Environmental Microbiology 59, 3502–3504.

Weilnhammer, C., Blass, E., 1994. Continuous fermentation with product recovery by in-situ extraction. Chemical Engineering & Technology 17, 365–373.

Wierckx, N.J., Ballerstedt, H., de Bont, J.A., Wery, J., 2005. Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. Applied and Environmental Microbiology 71, 8221–8227.

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

Xiang, L.K., Moore, B.S., 2002. Inactivation, complementation, and heterologous expression of encP, a novel bacterial phenylalanine ammonia-lyase gene. Journal of Biological Chemistry 277, 32505–32509.

Xiang, L.K., Moore, B.S., 2005. Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. Journal of Bacteriology 187, 4286–4289.

Xiang, L.K., Moore, B.S., 2006. Biochemical characterization of a prokaryotic phenylalanine ammonia lyase (187, pg4286, 2005). Journal of Bacteriology 188, 5331.

Yang, C.L.Y. a.H.C., 1992. Henry's Law Constant for Compound in Water. Gulf Publishing Company, Houston.

Young, M.R., Towers, G.H.N., Neish, A.C., 1966. Taxonomic distribution of ammonia-lyases for L-phenylalanine and L-tyrosine in relation to lignification. Canadian Journal of Botany 44, 341.