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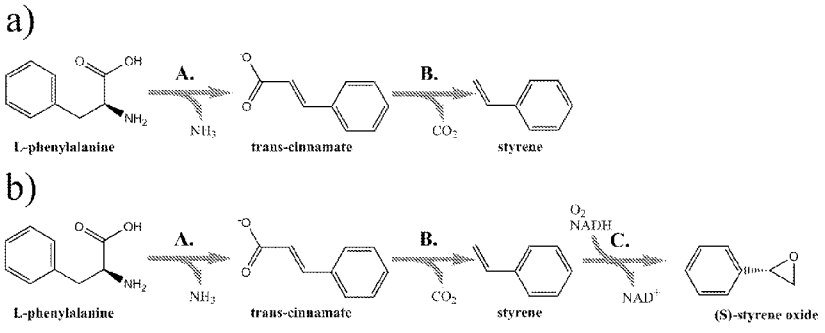
(54) **Title**: MICROBIAL CONVERSION OF GLUCOSE TO STYRENE AND ITS DERIVATIVES

FIGURE 1



(57) **Abstract**: A method for the in vivo production of styrene from renewable substrates using a recombinant microorganism is disclosed. Additionally, a method for the in vivo production of styrene oxide from renewable substrates using a recombinant microorganism is also disclosed. In both cases, the host cell expresses at least one gene encoding a polypeptide that possesses phenylalanine ammonia lyase activity in addition to at least one gene encoding a polypeptide that possesses trans-cinnamic acid decarboxylase activity. In the case of styrene oxide, the host cell must additionally express at least one gene encoding a polypeptide that possesses styrene monooxygenase activity.

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MICROBIAL CONVERSION OF GLUCOSE TO STYRENE AND ITS DERIVATIVES

RELATED APPLICATIONS

[0001] The present application claims the benefit of priority of U.S. Provisional Application No. 61/450,200, which was filed on March 8, 2011. The entire text of the aforementioned application is incorporated herein by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] [Not Applicable]

FIELD OF THE INVENTION

[0003] This invention relates to the fields of molecular biology, microbiology, and biotechnology. More specifically, the present invention relates to a method of producing styrene and styrene oxide from simple renewable substrates such as glucose.

BACKGROUND OF THE INVENTION

Styrene is a useful and versatile monomer for the production of numerous polymers and co-polymers, which accounts for 60% of its total global use[1]. Styrene is most commonly yielded by the chemocatalytic dehydrogenation of petroleum-derived ethylbenzene (U.S. Patent 4,255,599), a process requiring 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[2]. In 2006, over 6 million metric tons of styrene were produced by U.S. manufacturers alone, representing a market that is currently valued at nearly $28 billion and projected to grow by 4.3% per year through at least 2010[1]. A more sustainable and inexpensive approach would involve the engineering of microorganisms that possess the unique ability to synthesize styrene at high levels directly from renewable resources. Presently, however, an inexpensive and sustainable source of styrene has not yet been developed.

[0004] A variety of additional novel synthetic routes have recently been engineered in microorganisms for the production, from substrates such as glucose, of a number of other useful monoaromatic compounds with structural similarity to styrene. For example, a biosynthetic pathway for the production of p-hydroxystyrene (pHS; a monomer used in polymer synthesis) from renewable sugars has been reported using *E. coli*[4] or *P. putida*[5] as the engineered host platform. Meanwhile, both phenol (a precursor and monomer for phenolic resins)[6] and p-hydroxybenzoate (a precursor to parabens, which are used as preservatives)[7] have also been synthesized as individual products from glucose by engineered strains of *P. putida*. These studies further illustrate how, through metabolic engineering strategies, microbial biocatalysts can be developed for the sustainable biosynthesis of a variety of important commodity chemicals of monoaromatic nature from renewable resources. Each of the above non-natural metabolites were derived using L-tyrosine (or its immediate precursor, 4-hydroxyphenylpyruvate) as a precursor (thereby making them all phenolics). There are, however, no previously reported studies on the production of styrene from renewable resources (for example, carbohydrates such as glucose) by either naturally-occurring or recombinant microorganisms.

[0005] L-Phenylalanine is a naturally-occurring, proteinogenic amino acid that is ubiquitous among most all living organisms. Although its natural biosynthesis is often tightly regulated, its overproduction on fermentable sugars has been engineered in several microorganisms, and most notably in *Escherichia coli* (U.S. Patent 4,681,852) and *Corynebacterium glutamicum* (U.S. Patent 3,660,235).

[0006] Phenylalanine ammonia lyase (PAL) activity has been reported in a number of marine bacteria, including *Anabaena variabilis*, *Nostoc punctiforme*, and *Streptomyces maritimus*, and the genes have been identified [8-10]. In addition, the yeast *Rhodotoruloides glutinis* has been well-studied with regards to its phenylalanine ammonia lyase (PAL) activity, however, the identified and characterized gene product is less specific in that it also functions as a tyrosine ammonia lyase (TAL)[4, 11]. It is further known that the yeast *Saccharomyces cerevisiae* is capable of synthesizing styrene when supplied with exogenous trans-cinnamic acid (CA)[12]. That is to say, the yeast *Saccharomyces cerevisiae* is known to naturally display trans-cinnamic acid

decarboxylase (CADC) activity. It has been further demonstrated that this native enzymatic ability has an essential dependence on the combined expression of the enzymes encoded by the genes *PAD1* and *FDC1* [13].

[0007] In light of the foregoing, it would be an advancement in the current state of the art to provide a method by which styrene could be produced from inexpensive and sustainable resources such as carbohydrates or sugars. It would be particularly advantageous if the method produced a high level of styrene at high substrate yields and with a limited diversity and quantity of by-products. The development of such a method will require the ability to manipulate and assemble the appropriate genetic machinery responsible for the conversion of carbohydrates such as glucose to CA, and CA to styrene. It would be exceptionally advantageous if these conversions could all be achieved within a single host cell.

[0008] The above mentioned biological and chemical systems provide both examples of a number of potentially useful genetic elements, as well as a number of pathways that may be useful in the biological production of styrene, however the efficient biological production of styrene has not been achieved. Therefore, the problem to be overcome is to design and develop a method for the efficient production of styrene by a biological source using inexpensive substrates as the carbon source. The applicants have solved the stated problem by engineering a microbial host to produce styrene by expression of foreign genes which encode phenylalanine ammonia lyase (PAL) and trans-cinnamic acid decarboxylase (CADC).

[0009] Furthermore, (S)-styrene oxide may be produced by the enzymatic oxidation of styrene by the additional co-expression of a gene encoding a polypeptide with styrene monooxygenase (SMO) activity. Epoxides are desirable compounds due to their versatile nature as chemical building blocks. More specifically, (S)-styrene oxide is a functional building block that is used as a precursor to a variety of pharmaceutical compounds including levamisole and some analgesics[14]. Enzymatic reactions, as opposed to chemical processes, have the unique ability to yield enantiomerically pure products. The styrene oxygenase activity of several *Pseudomonas sp.* has been identified as the two-component styrene monooxygenase encoded by *styAB*[14]. Activity

has also been reported for the two-component flavoprotein monooxygenase encoded by *styA2B* present in *Rhodococcus opacus* 1CP[15].

BRIEF SUMMARY OF THE INVENTION

[0010] The present invention comprises an *in vivo* method for the production of styrene via a recombinant host cell expressing at least one gene encoding a polypeptide having phenylalanine ammonia lyase (PAL) activity to convert endogenously-synthesized L-phenylalanine to *trans*-cinnamic acid in combination with at least one gene encoding a polypeptide having trans-cinnamate decarboxylase (CADC) activity to then subsequently convert *trans*-cinnamic acid to styrene. This reaction scheme is illustrated in Figure 1A. The present invention also comprises an *in vivo* method for the production of styrene oxide by further engineering said recombinant host cell to additionally co-express at least one gene encoding a polypeptide that displays styrene monooxygenase (SMO) activity. This reaction scheme is illustrated in Figure 1B. This invention provides an inexpensive and sustainable biological route for the conversion of renewable substrates to styrene. Styrene is useful, for example, for the synthesis of numerous polymers and co-polymers. This invention additionally provides an inexpensive and sustainable biological route for the conversion of renewable substrates to (S)-styrene oxide. (S)-Styrene oxide is a molecular building block used in the production of a variety of pharmaceuticals.

[0011] Accordingly, the present invention provides a method for the production of styrene comprising:

[0012] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0013] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0014] b) at least one gene encoding a polypeptide having *trans*-cinnamate decarboxylase activity

[0015] ii) growing said recombinant cell for a time sufficient to produce styrene; and

[0016] iii) optionally recovering said styrene.

[0017] Alternatively, the invention provides a method for the production of styrene comprising:

[0018] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0019] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0020] b) at least one gene encoding a polypeptide having phenylacrylic acid decarboxylase activity

[0021] ii) growing said recombinant cell for a time sufficient to produce styrene; and

[0022] iii) optionally recovering said styrene.

[0023] Alternatively, the invention provides a method for the production of styrene comprising:

[0024] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0025] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0026] b) at least one gene encoding a polypeptide having ferulic acid decarboxylase activity

[0027] ii) growing said recombinant cell for a time sufficient to produce styrene; and

[0028] iii) optionally recovering said styrene.

[0029] Additionally, the present invention also provides a method for the production of styrene oxide comprising:

[0030] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0031] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0032] b) at least one gene encoding a polypeptide having *trans*-cinnamate decarboxylase activity

[0033] c) at least one gene encoding a polypeptide having styrene monooxygenase activity

[0034] ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and

[0035] iii) optionally recovering said styrene oxide.

[0036] Alternatively, the present invention also provides a method for the production of styrene oxide comprising:

[0037] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0038] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0039] b) at least one gene encoding a polypeptide having phenylacrylic acid decarboxylase activity

[0040] c) at least one gene encoding a polypeptide having styrene monooxygenase activity

[0041] ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and

[0042] iii) optionally recovering said styrene oxide.

[0043] Alternatively, the present invention also provides a method for the production of styrene oxide comprising:

[0044] i) contacting a recombinant host cell with a fermentable carbon source, said recombinant host comprising:

[0045] a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0046] b) at least one gene encoding a polypeptide having ferulic acid decarboxylase activity

[0047] c) at least one gene encoding a polypeptide having styrene monooxygenase activity

[0048] ii) growing said recombinant cell for a time sufficient to produce styrene oxide; and

[0049] iii) optionally recovering said styrene oxide.

[0050] Additionally, the invention provides a recombinant host cell comprising:

[0051] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0052] b) a gene encoding a polypeptide having *trans*-cinnamate decarboxylase activity

[0053] Additionally, the invention provides a recombinant host cell comprising:

[0054] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0055] b) a gene encoding a polypeptide having phenylacrylic acid decarboxylase activity

[0056] Additionally, the invention provides a recombinant host cell comprising:

[0057] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0058] b) a gene encoding a polypeptide having ferulic acid decarboxylase activity

[0059] Additionally, the invention provides a recombinant host cell comprising:

[0060] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0061] b) a gene encoding a polypeptide having trans-cinnamate decarboxylase activity

[0062] c) a gene encoding a polypeptide having styrene monooxygenase activity

[0063] Additionally, the invention provides a recombinant host cell comprising:

[0064] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0065] b) a gene encoding a polypeptide having phenylacrylic acid decarboxylase activity

[0066] c) a gene encoding a polypeptide having styrene monooxygenase activity

[0067] Additionally, the invention provides a recombinant host cell comprising:

[0068] a) a gene encoding a polypeptide having phenylalanine ammonia lyase activity; and

[0069] b) a gene encoding a polypeptide having ferulic acid decarboxylase activity

[0070] c) a gene encoding a polypeptide having styrene monooxygenase activity.

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

[0071] **Figure 1. A)** 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). **B)** Enzymatic pathway to convert the precursor L-phenylalanine to the product (S)-styrene oxide via the intermediates *trans*-cinnamate and styrene. The three-step pathway from L-phenylalanine is achieved by the co-expression of one or more genes which encode phenylalanine ammonia lyase (PAL) activity (A), one or more genes which encode

trans-cinnamic acid decarboxylase (CADC) activity (B), and one or more genes which encode styrene monooxygenase (SMO) activity (B).

[0072] **Figure 2.** Phenylalanine ammonia lyase activity from candidate genes cloned from *A. thaliana, A. variabilis,* and *N. punctiforme* 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), centrifuged to obtain a pellet and resuspended in 10 ml pH7 PBS buffer. The conversion of 1 g/L L-phenylalanine (black) to *trans*-cinnamic acid after 1 h (white), 2 h (light gray), and 3h (dark gray). Note that no activity was observed in the control (*E. coli* BL21(DE3)).

[0073] **Figure 3.** *trans*-Cinnamic acid decarboxylase activity from candidate genes cloned from *S. cerevisiae*, *L. plantarum*, and *B. subtilis* 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), centrifuged to obtain a pellet and resuspended in 10 ml pH7 PBS buffer. 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. Not that no activity is observed in the control (*E. coli* BL21(DE3)).

[0074] **Figure 4.** Whole cell production of styrene (triangle) from *trans*-cinnamic acid (circle) after the addition of L-phenylalanine (square) by *E. coli* NST74 pSpal2At pTfdc1Sc.

[0075] **Figure 5.** Concentrations of aromatic metabolites in the aqueous media produced by the strain *E. coli* NST74 pSpal2At pTfdc1Sc when cultivated in a continuously-aerated 1L bioreactor for 33 hours.

[0076] **Figure 6.** Net equivalent styrene titer produced by the strain *E. coli* NST74 pSpal2At pTfdc1Sc when cultivated in a continuously-aerated 1L bioreactor for 33 hours.

[0077] **Figure 7.** Styrene oxide production levels after 24 (dark grey) and 48 (light grey) hours after induction of the strain *E. coli* NST74 pSTV28-pal2At pTrc99a-fdc1Sc-styABPp.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

[0078] SEQ ID NO:1 is the nucleotide sequence of a gene from *A. variabilis* encoding a phenylalanine ammonia lyase.

[0079] SEQ ID NO:2 is the nucleotide sequence of a gene from *N. punctiforme* encoding a phenylalanine ammonia lyase.

[0080] SEQ ID NO:3 is the nucleotide sequence of a gene from *S. maritimus* encoding a phenylalanine ammonia lyase.

[0081] SEQ ID NO:4 is the nucleotide sequence of a gene from *A.thaliana* encoding a phenylalanine ammonia lyase.

[0082] SEQ ID NO:5 is the nucleotide sequence of a gene from *A. thaliana* encoding a phenylalanine ammonia lyase.

[0083] SEQ ID NO:6 is the nucleotide sequence of a gene from *P. putida* encoding a styrene monooxygenase.

[0084] SEQ ID NO:7 is the nucleotide sequence of a gene from *R. opacus* encoding a styrene monooxygenase.

[0085] SEQ ID NO:8 is the nucleotide sequence of a gene from *L. plantarum* encoding a phenylacrylic acid decarboxylase.

[0086] SEQ ID NO:9 is the nucleotide sequence of a gene from *B. subtilis* encoding a phenylacrylic acid decarboxylase.

[0087] SEQ ID NO:10 is the nucleotide sequence of a gene from *S. cerevisiae* encoding a phenylacrylic acid decarboxylase.

[0088] SEQ ID NO:11 is the nucleotide sequence of a gene from *S. cerevisiae* encoding a ferulic acid decarboxylase.

[0089] SEQ ID NO:12 is a primer used to amplify *pal* from *A. variabilis*.

[0090] SEQ ID NO:13 is a primer used to amplify *pal* from *A. variabilis*.

[0091] SEQ ID NO:14 is a primer used to amplify *pal* from *N. punctiforme*.

[0092] SEQ ID NO:15 is a primer used to amplify *pal* from *N. punctiforme*.

[0093] SEQ ID NO:16 is a primer used to amplify *encP* from *S. maritimus*.

[0094] SEQ ID NO:17 is a primer used to amplify *encP* from *S. maritimus*.

[0095] SEQ ID NO:18 is a primer used to amplify *pdc* from *L. plantarum*.

[0096] SEQ ID NO:19 is a primer used to amplify *pdc* from *L. plantarum*.

[0097] SEQ ID NO:20 is a primer used to amplify *padC* from *B. subtilis*.

[0098] SEQ ID NO:21 is a primer used to amplify *padC* from *B. subtilis*.

[0099] SEQ ID NO:22 is a primer used to amplify *PAD1* from *S. cerevisiae*.

[00100] SEQ ID NO:23 is a primer used to amplify *PAD1*from *S. cerevisiae*.

[00101] SEQ ID NO:24 is a primer used to amplify *FDC1* from *S. cerevisiae*.

[00102] SEQ ID NO:25 is a primer used to amplify *FDC1* from *S. cerevisiae*.

[00103] SEQ ID NO:26 is a primer used to amplify *PAL1* from *A. thaliana*.

[00104] SEQ ID NO:27 is a primer used to amplify *PAL1* from *A. thaliana*.

[00105] SEQ ID NO:28 is a primer used to amplify *PAL2* from *A. thaliana*.

[00106] SEQ ID NO:29 is a primer used to amplify *PAL2* from *A. thaliana*.

[00107] SEQ ID NO:30 is a primer used to amplify *styAB* from *P.putida*.

[00108] SEQ ID NO:31 is a primer used to amplify *styAB* from *P.putida*.

[00109] SEQ ID NO:32 is a primer used to amplify *styA2B* from *R. opacus*.

[00110] SEQ ID NO:33 is a primer used to amplify *styA2B* from *R. opacus*.

DETAILED DESCRIPTION OF THE INVENTION

[00111] The following abbreviations and definitions will be used for the interpretation and specification of the claims.

[00112] "Phenylalanine ammonia lyase" is abbreviated PAL.

[00113] "Tyrosine ammonia lyase" is abbreviated TAL.

[00114] "Phenylacrylic acid decarboxylase" is abbreviated PADC.

[00115] "trans-Cinnamic acid decarboxylase" is abbreviated CADC.

[00116] "Ferulic acid decarboxylase" is abbreviated FADC.

[00117] "Styrene monooxygenase" is abbreviated SMO.

[00118] As used herein, the terms "L-phenylalanine", and "phenylalanine" are used interchangeably.

[00119] As used herein, the terms "trans-cinnamic acid", "cinnamic acid", trans-cinnamate", and "cinnamate" are used interchangeably and are abbreviated CA.

[00120] As used herein, the terms "ferulic acid" and "ferulate" are used interchangeably.

[00121] As used herein, the terms "(S)-styrene monooxygenase" and "styrene monooxygenase" are used interchangeably.

[00122] The term "PAL activity" refers to the ability of a protein to catalyze the direct conversion of phenylalanine to CA.

[00123] The term "CADC activity" refers to the ability of a protein to catalyze the direct conversion of CA to styrene.

[00124] The term "SMO activity" refers to the ability of a protein to catalyze the direct conversion of styrene to (S)-styrene monooxygenase.

[00125] The term "phenylalanine over-producing strain" refers to a microbial strain that produces endogenous levels of phenylalanine that are significantly higher than those demonstrated by the wild-type of that strain. Specific examples of an *E. coli* phenylalanine over-producing strains are NST74 and NST37 (US Patent 4,681,852). Meanwhile, still others may include specific strains of *Corynebacterium glutamicum*[16].

[00126] The term "fermentable carbon substrate" refers to a carbon source capable of being metabolized by the host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, organic acids, glycerol, and one-carbon substrates or mixtures thereof.

[00127] The term "host" refers to a suitable cell line such as a strain of bacteria, for example, into which genes can be transferred to impart desired genetic attributes and functions.

[00128] The term "OD_{600}" refers to the measurement of optical density at 600 nm, a standard metric of cell growth used by those familiar in the art.

[00129] The term "gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) and the coding sequence. "Native gene" or "wild type gene" refers to a gene as found in nature with its own regulatory sequences. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. "Foreign gene" refers to a gene not normally found in the host organism but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes.

[00130] The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment used in this invention. Expression may also refer to the translation of the mRNA into a polypeptide. "Overexpession" refers to the production of a gene product in a transgenic organism that exceeds levels of production in the wild-type host or native organisms.

[00131] "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of gene or other a DNA sequence. "Messenger RNA (mRNA)" refers to the RNA that is without introns and can be translated into a protein by the cell. "cDNA" refers to double-stranded DNA that is complimentary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell.

[00132] "Transformation" refers to the transfer of a nucleic acid fragment into the genome of the host organism, resulting in genetically-stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[00133] The terms "plasmid" and "vector" refer to an extra chromosomal genetic element often carrying genes which are not part of host native genome nor the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

[00134] **Genes**

[00135] The key enzymatic activities used in the present invention are encoded by a number of genes known in the art. The principal enzyme activities include phenylalanine ammonia lyase (PAL) and *trans*-cinnamic acid decarboxylase (CADC). These activities may also be displayed by enzymes whose principal natural substrates are not phenylalanine or *trans*-cinnamic acid, respectively, but also those which have the natural capacity to utilize these substrates or which can be engineered to display these activities.

[00136] Phenylalanine ammonia lyase (PAL) and trans-cinnamic acid decarboxylase (CADC) activities

[00137] Genes encoding PAL activity are known in the art and several have been sequenced from both microbial and plant origin (see, for example, EP 321488 [*R. toruoides*]; WO 9811205 [*Eucalyptis grandis* and *Pinus radiata*]; WO 9732023 [Petunia]; JP 05153978 [*Pisum sativum*]; WO 9307270 [potato, rice]; NM_129260.2 GI:30687012 and NM_115186.3 GI:42565889 [*Arabdiposis thaliana*]). The sequence of PAL encoding genes are available (for example, see GenBank AJ010143 and X75967). Where expression of a wild type PAL in a recombinant host is desired, the wild type gene may be obtained from any source including, but not limited to, yeasts such as *Rhodotorula* sp., *Rhodosporidium* sp., and *Sporobolomyces* sp.; bacteria such as *Streptomyces* sp., *Anabaena* sp., and *Nostoc* sp.; and plants such as pea, potato, rice, eucalyptus, pine, corn, petunia, arabidopsis, tobacco, and parsley. It is preferred, but

not necessary, that enzymes should strictly display PAL activity and not TAL activity as well.

[00138] Genes which purportedly encode *trans*-cinnamic acid decarboxylase (CADC) activity have been identified in the literature. In addition, enzymes which have been classified as phenylacrylic acid decarboxylase (PADC) or ferulic acid decarboxylase (FADC) may also display the necessary CADC activity. Genes encoding PADC activity, for example, have been isolated from the bacteria *Lactobacillus plantarum* (AAC45282.1 GI: 1762616), *Lactococcus lactis* (NP_268087.1 GI:15673912), and *Bacillus subtilis* (AF017117.1 GI:2394281). The PADC encoding genes from *Lactobacillus plantarum* and *Bacillus subtilis* are listed herein as SEQ ID NO:8 and SEQ ID NO:9, respectively. Furthermore, CADC activity has been reported in the yeast *Saccharomyces cerevisiae* and it was shown that the display of this native activity required that the genes *PAD1* (L09263.1 GI:393284) and *FDC1* (NP_010828.1 GI:6320748) both be present and undisturbed in the genome[13]. Genomic disruption of either *PAD1* or *FDC1*, whose sequences are provided as SEQ ID NO:10 and SEQ ID NO:11, respectively, resulted in the loss of CADC activity upon exogenously supplied *trans*-cinnamic acid[13]. However, considering the structural similarity between ferulic acid and *trans*-cinnamic acid, we anticipated that enzymes which are known to display ferulic acid decarboxylase (FADC) activity, such as the polypeptide encoded by *FDC1* of *S. cerevisiae*, may also display *trans*-cinnamic acid decarboxylase (CADC) activity as well.

[00139] It will be appreciated that the present invention is not limited to the genes encoding polypeptides having the specific activities mentioned above, but will encompass any suitable homologs of such genes that may be obtained by standard methods. Methods of obtaining homologs to these genes using sequence-dependent protocols are well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction (PCR)).

[00140] For example, genes encoding homologs of the polypeptides that alone or in combination have the above mentioned activities could be isolated directly by using all or a portion of the known sequences as DNA hybridization probes to screen libraries from any desired plant, fungi, yeast, or bacteria using methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the literature nucleic acid sequences can be designed and synthesized by methods known in the art. Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to those skilled in the art, such as random primers DNA labeling, nick translation, or end-labeling techniques or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of or full length of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full-length cDNA or genomic fragments under conditions of appropriate stringency.

[00141] **Microbial Production Hosts**

[00142] The production organisms of the present invention will include any organism capable of expressing the genes required for styrene production. Typically, the production organism will be restricted to microorganisms or plants. Microorganisms useful in the present invention include, but are not limited to enteric bacteria (*Escherichia* and *Salmonella*, for example) as well as *Bacillus, Acinetobacter, Actinomycetes* such as *Streptomyces, Corynebacterium, Methanotrophs* such as *Methylosinus, Methylomonas, Rhodococcus* and *Pseudomonas*; *Cyanobacteria*, such as *Rhodobacter* and *Synechocystis*; yeasts, such as *Saccharomyces, Zygosaccharomyces, Kluyveromyces, Candida, Hansenula, Debaryomyces, Mucor, Pichia,* and *Torulopsis*; and filamentous fungi such as *Aspergillus* and *Arthrobotrys*, and algae, for example. The genes encoding polypeptides with the PAL and CADC activities used in the present invention may be produced or over-expressed in these and other microbial hosts to prepare large quantities of styrene.

[00143] Although any of the above mentioned microorganisms would be useful for the production of styrene, preferred strains would be those that either natively or have

been engineered to over-produce phenylalanine. Phenylalanine over-producing strains are known and include, but are not limited to, *Escherichia* sp., *Corynebacterium* sp., *Microbacterium* sp., *Arthrobacter* sp., *Pseudomonas* sp., and *Brevibacteria* sp.. Particularly useful phenylalanine over-producing strains include, but are not limited to, *Microbacterium ammoniaphilum* ATCC 10155, *Corynebacterium lillium* NRRL-B-2243, *Corynebacterium glutamicum* ATCC 21674, *E. coli* NST74, *E. coli* NST37, and *Arthrobacter citreus* ATCC 11624. A recombinant host may be constructed from a suitable phenylalanine over-producing strain such that it expresses at least one gene encoding a polypeptide having PAL and at least one gene encoding a polypeptide having CADC activity.

[00144] Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins and over-expression of native proteins are well known to those skilled in the art. Any of these could be used to construct chimeric genes for the production of styrene. These chimeric genes could then be introduced into appropriate microorganisms via transformation to allow for expression of high levels of the enzymes.

[00145] Description of the preferred embodiments

[00146] The method of production defined in this invention involves the incorporation of genes encoding polypeptides displaying PAL and CADC activities into a single host organism and the use of those organisms to convert renewable resources, including fermentable carbons sources such as glucose, for example, to styrene. This invention relies upon the identification of genes encoding PAL and CADC activities and, preferably, those genes which when expressed in a recombinant host organism can display such activities. Candidate genes encoding PAL homologs were selected from the open literature and included *pal* from *Anabaena variabilis*, *pal* from *Nostoc punctiforme*, *encP* from *Streptomyces maritimus,* and *PAL1* and *PAL2* from *Arabidopsis thaliana*. Each gene was amplified from genomic DNA samples via PCR, cloned individually into the expression vector pSTV28. This resulted in the generation of plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At, respectively. Each plasmid was then individually transformed into *E. coli*.

[00147] Candidate genes encoding CADC homologs were selected from the open literature and included *pdc* from *Lactobacillus plantarum*, *padC* from *Bacillus subtilis*, *PAD1* from *Saccharomyces cerevisiae*, and *FDC1* from *Saccharomyces cerevisiae*. Each gene was amplified from genomic DNA samples via PCR, cloned individually into the expression vector pTrc99a. This resulted in the generation of plasmids pTpdcLp, pTpadcBs, pTpad1Sc, and pTfdc1Sc, respectively. In addition, *FDC1* from *Saccharomyces cerevisiae* was also cloned into pTrc99a together with *PAD1* as part of a synthetically-assembled, polycistronic operon. This resulted in the generation of plasmids pTpad1Sc-fdc1Sc. Each plasmid was then individually transformed into *E. coli*.

[00148] Screening assays were performed on both whole cells and cell extracts. PAL activity was investigated via the conversion of exogenous phenylalanine to CA, whereas CADC activity was investigated via the conversion of exogenous CA to styrene. As seen in Figure 2 and Table 1, PAL activity was confirmed in recombinant *E. coli* according to both whole cell and cell extract assays in strains expressing *pal* from *Nostoc punctiforme, pal* from *Anabaena variabilis*, *PAL1* from *Arabidopsis thaliana*, or preferably *PAL2* from *Arabidopsis thaliana*. As seen in Figure 3, CADC activity was confirmed in recombinant *E. coli* according to whole cell assays in strains expressing *FDC1* from *Saccharomyces cerevisiae*.

[00149] **Examples**

[00150] The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these following Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

[00151] Procedures required for PCR amplification, DNA modifications by endo- and exonucleases for generating desired ends for cloning of DNA, ligation, and bacterial transformation are well known in the art. The standard molecular biology techniques

used herein are well-known in the art and described by Sambook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual,* 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

[00152] Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Methods and techniques suitable for use in the following set of Examples may be found for example, as described in *Manual of Methods for General Bacteriology;* Gerhardt, P., Murray, R.G.F., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R., and Phillips, G.B., Eds., American Society for Microbiology: Washington, DC, 1994. All reagents used in the Examples were purchased from Sigma Aldrich (St. Louis, MO). Restriction enzymes, polymerases, and ligase were purchased from New England Biolabs (Ipswich, MA). Nutrients and chemicals used for the growth and maintenance of cells were purchased from DIFCO Laboratories (Detroit, MI).

[00153] **General Methods**

[00154] PCR reactions were performed using a BioRad iCycler system with Phusion DNA Polymerase (Finnzymes, Espoo, Finland). Custom DNA oligonucleotide primers were synthesized by and purchased from Integrated DNA Technologies (Coralville, IA). PCR cycling and reaction conditions were standardized according to manufacturer instructions.

[00155] An HPLC assay was developed to simultaneously separate and measure aqueous levels of phenylalanine, CA, and styrene in microbial cultures. For a typical assay, 1 mL culture was removed from shake flask culture and centrifuged to pellet cells. 0.75 mL of supernatant was then transferred to a sealed HPLC vial. A Hewlett Packard 1100 series HPLC system with an auto sampler and a diode array UV/Vis detector with a reverse-phase Hypersil Gold SBC18 column (4.6mm x 150 mm; Thermo Fisher, USA) was used to achieve separation and detection of the species. 5 microliters of sample was injected for analysis according to the following methodology. A total flow rate of 1.0 ml/min and column temperature of 45°C were held constant throughout. The column was eluted with solvent A containing double-distilled water and solvent B containing methanol plus 0.1% trifluoroacetic acid (TFA). The eluent initially consisted

of 95% solvent A and 5% solvent B and then, over the course of the first 8 min, a linear gradient was applied to eventually reach 80% solvent B and 20% solvent A. These conditions were then held for 2 min before a linear gradient returning to the final conditions of 95% solvent A and 5% solvent B was applied over the course of 4 min. The UV detector was used to monitor the eluent at 215 nm (for L-phenylalanine and L-tyrosine), and 258 nm (for *trans*-cinnamic acid, *p*-coumaric acid, styrene). Under these conditions L-phenylalanine, L-tyrosine, *p*-coumaric acid, *trans*-cinnamic acid, and styrene were read at 4.5, 6.7, 8.67, 8.78, and 10.4 min respectively.

[00156] All gas chromatography (GC) analysis was performed on a Hewlett Packard 5890 Series II gas chromatograph with a flame ionizing detector (FID) and Agilent DB-5 (30 m x 0.25 mm ID) fused-silica capillary column using helium as the carrier gas. A GC-FID method was developed to separate and measure styrene concentrations in off gas and headspace samples. In this case, the injector, column, and detector temperatures were set to 180, 150, and 280 °C, respectively, and remained constant throughout the method. A GC-FID assay was developed to separate and measure styrene concentrations in n-dodecane solvent samples. In this case, the column temperature began at 60 °C and increased linearly at a rate of 45 °C/min until reaching a final temperature of 280 °C. The injector and detector temperatures were set to 180 and 280 °C, respectively, and remained constant throughout the method.

[00157] For all culture experiments, seeds cultures were first grown in Luria Broth (LB) media overnight. Minimal media 1 (herein referred to as "MM1") was used for fermentations which contained glucose (nominally 15 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), NH_4SO_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 (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)).

[00158] Cloning of candidate genes encoding PAL activity from *A. variabilis*, *N. punctiforme*, *S. maritimus,* and *A. thaliana*

[00159] Candidate PAL encoding genes, namely SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, were amplified via PCR using genomic DNA templates derived from *A. variabilis*, *N. punctiforme*, and *S. maritimus*, respectively. The oligonucleotides primers used to amplify *pal* from *A. variabilis* (SEQ ID NO:1) are given as SEQ ID NO:12 and SEQ ID NO:13. The oligonucleotides primers used to amplify *pal* from *N. punctiforme* (SEQ ID NO:2) are given as SEQ ID NO:14 and SEQ ID NO:15. The oligonucleotides primers used to amplify *encP* from *S. maritimus* (SEQ ID NO:3) are given as SEQ ID NO:16 and SEQ ID NO:17. The oligonucleotides primers used to amplify *PAL1* and *PAL2* from *A. thaliana* (SEQ ID NO:4 and SEQ ID NO:5, respectively) are given as SEQ ID NO:26 and SEQ ID NO:27 and SEQ ID NO:28 and SEQ ID NO:29, respectively. In all cases, amplified linear DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, CA). Amplified fragments from *A. variabilis*, *N. punctiforme*, and *S. maritimus* were then treated by restriction endonuclease digestion with the enzymes *BamHI* and *EcoRI* while fragments from *A. thaliana* were treated with *EcoRI* and *SphI* with appropriate digestion buffer for 3 h at 37°C. Samples of the expression vector pSTV28 were similarly digested with either *BamHI* and *EcoRI* or *EcoRI* and *SphI*. All digested fragments were subsequently purified using the Zyppy Gel DNA recovery kit (Zymo Research, Orange, CA) per manufacturer's instruction. Gene inserts and linearized plasmid DNA were then appropriately ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, MA) at 4°C overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Selection of transformants was achieved by plating transformed cells on LB solid agar media containing 34 mg/L chloramphenicol and culturing overnight at 37°C. Vectors with correct gene insert for all PAL encoding genes were confirmed by mapping the recombinant plasmid by digestion with appropriate restriction enzymes. These cloning works resulted in the successful generation of the plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At.

[00160] Cloning of candidate genes encoding CADC activity from *L. plantarum*, *B. subtilis*, and *S. cerevisiae*.

[00161] Candidate CADC encoding genes, namely SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, were amplified via PCR using genomic DNA templates derived from *L.*

plantarum, *B. subtilis*, and *S. cerevisiae*, respectively. The oligonucleotides primers used to amplify *pdc* from *L. plantarum* (SEQ ID NO:8) are given as SEQ ID NO:18 and SEQ ID NO:19. The oligonucleotides primers used to amplify *padC* from *B. subtilis* (SEQ ID NO:9) are given as SEQ ID NO:20 and SEQ ID NO:21. The oligonucleotides primers used to amplify *PAD1* from *S. cerevisiae* (SEQ ID NO:10) are given as SEQ ID NO:22 and SEQ ID NO:23. The oligonucleotides primers used to amplify *FDC1* from *S. cerevisiae* (SEQ ID NO:11) are given as SEQ ID NO:24 and SEQ ID NO:25. In all cases, amplified DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, CA). Fragments were then treated by restriction enzyme digestion with appropriate enzymes and buffer for 3 h at 37°C. Amplified fragments of *padc* and *pdc* were digested with *BamHI* and *SbfI* for which the *E. coli* expression vector pTrc99A was also digested with *BamHI* and *SbfI*. Amplified DNA fragments of *PAD1* was digested with *NcoI* and *XbaI* and DNA fragments of *FDC1* were digested with *SalI* and *HindIII*. Meanwhile, the *E. coli* expression vector pTrc99A was similarly digested with either *NcoI* and *XbaI* for the insertion of *PAD1* or with *SalI* and *HindIII* for the insertion of *FDC1*. All digested fragments were subsequently purified using the Zyppy Gel DNA recovery kit (Zymo Research, Orange, CA) per manufacturer's instruction. Gene inserts and linearized plasmid DNA were then appropriately ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, MA) at 4°C overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Selection of transformants was achieved by plating transformed cells on LB solid agar media containing 100 mg/L ampicillin and culturing overnight at 37°C. Among the resultant transformants, the vectors with the correct insertion of the genes *padC*, *pdc*, and *PAD1* were confirmed among clones by digestion with restriction enzyme *SphI*. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 2.5 kb and 2 kb (*pdc*), 2.5kb, 1.75kb and 0.25kb (*padc*), and 3 kb and 1.8 kb (*PAD1*) when separated on a 0.7% w./v. agarose gel at 90V for 60 min. The vector with the correct gene insert for *FDC1* was confirmed among clones by digestion with restriction enzymes *HindIII* and *NdeI*. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 3.3 kb and 2.4 kb when separated on a 0.7%

w./v. agarose gel at 90V for 60 min. These cloning works resulted in the successful generation of the plasmids pTpadCBs, pTpdcLp, pTpad1Sc, and pTfdc1Sc. The newly generated plasmid pTpad1Sc was then digested with *SalI* and *HindIII* and cleaned using Zyppy Gel DNA recovery kit (Zymo Research, Orange, CA). The *SalI* and *HindIII* digested *FDC1* fragment was then ligated with pTpad1Sc by treatment with T4 DNA ligase (New England Biolabs, Ipswich, MA) at 4°C overnight. Ligase reaction mixtures were then transformed into chemically competent *E. coli* NEB10-Beta. Transformants were then plated on LB agar containing 100 mg/L ampicillin and incubated at 37°C overnight. The successfully generated plasmid pTpad1-fdc1 was then confirmed among clones by digestion with restriction enzymes *BamHI* and *HindIII*. Under these conditions, vectors containing the correct gene insert were identified as those which displayed fragments of 4.8 kb and 1.6 kb when separated on a 0.7% w./v. agarose gel at 90V for 60 min.

[00162] Cloning of candidate genes encoding styrene oxygenase activity from *P. putida* and *R. opacus*

[00163] Candidate styrene oxygenase encoding genes, namely SEQ ID NO:6 and SEQ ID NO:7, were amplified via PCR using genomic DNA templates derived from *P.putida and R. opacus*, respectively. The oligonucleotides primers used to amplify *styAB* from *P. putida* (SEQ ID NO:6) are given as SEQ ID NO:30 and SEQ ID NO:31. The oligonucleotides primers used to amplify *styA2B* from *R. opacus* (SEQ ID NO:7) are given as SEQ ID NO:32 and SEQ ID NO:33. In all cases, amplified DNA fragments were subsequently cleaned using Zyppy Clean and Concentrator kit (Zymo Research, Orange, CA). Potential styrene monooxygenases were coexpressed on the plasmid pTfdc1Sc. Fragments and the vector were then treated by restriction enzyme digestion with appropriate enzymes and buffer for 3 h at 37°C. Amplified DNA fragments and pTfdc1Sc of *styAB* and *styA2B* were digested with *BamHI* and *XbaI* and *NcoI* and *XbaI*, respectively. Digested fragments were subsequently cleaned using Zyppy Gel DNA recovery kit (Zymo Research, Orange, CA). Gene inserts and linearized plasmid DNA were ligated together by treatment with T4 DNA ligase (New England Biolabs, Ipswich, MA) at 4°C overnight. Ligation mixtures were subsequently transformed into chemically competent *E. coli* NEB10-beta (New England Biolabs, Ipswich, MA). Selection of

transformants was achieved by plating transformed cells on LB solid agar media containing 100 mg/L ampicillin and culturing overnight at 37°C. Vectors with correct gene insert were then identified and confirmed by mapping the recombinant plasmid by digestion with appropriate restriction enzymes. These works resulted in the generation of plasmids pTfdc1Sc-styABPp and pTfdc1Sc-styA2BRo.

[00164] Example 1.

[00165] Assaying PAL/TAL activities in recombinant *E. coli*.

[00166] *E. coli* BL21(DE3) was individually transformed with each of the plasmids pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At. Seeds cultures of each strain were first grown in LB broth supplemented with 34 mg/L chloramphenicol at 30 °C while shaking at 250 rpm overnight. 50 µl of seed culture was then used to inoculate 5 ml of LB broth supplemented with 34 mg/L chloramphenicol. Cultures were grown at 30 °C to an OD_{600} of approximately 0.6. Cultures were then induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then allowed to grow for an additional 6 h. Cultures were harvested by first centrifuging for 4 minutes at 1700 x g before resuspending the cell pellet in 900 µL distilled water. Cells were then lysed using FastBreak Cell Lysis Reagent kit (Promega, Madison, WI) according to manufacturer's specifications and the supernatant collected. PAL activity assays were then performed at room temperature in 50 mM Tris-HCl buffer containing 100 mM L-phenylalanine. The reaction was initiated by the addition of 5 µL of crude cell lysate and followed for 30 min at 20 sec intervals. A molar extinction coefficient of 9,000 M^{-1} cm^{-1} and a 1 cm path length were then used to establish concentration calibrations. The total protein content in each crude lysate was determined using a Bradford protein assay with external standards as a calibration. The enzyme activity was expressed in terms of U mg^{-1} total protein. The obtained results are listed in Table 1.

[00167] Table 1. Specific activity of PAL isoenzymes from *A. variabilis*, *N. punctiforme*, *S. maritimus* and *A. thaliana* on L-phenylalanine and L-tyrosine when expressed in recombinant *E. coli*.

E. coli Strain	Substrate	Specific Activity (U mg^{-1} total protein)
BL21(DE3)	L-phenylalanine	Not detected

	L-tyrosine	Not detected
BL21(DE3) pSencPSm	L-phenylalanine	Not detected
	L-tyrosine	Not detected
BL21(DE3) pSpalAv	L-phenylalanine	0.018 ± 0.006
	L-tyrosine	Not detected
BL21(DE3) pSpalNp	L-phenylalanine	0.006 ± 0.003
	L-tyrosine	Not detected
BL21(DE3) pSpal1At	L-phenylalanine	0.026 ± 0.010
	L-tyrosine	Not detected
BL21(DE3) pSpal2At	L-phenylalanine	0.038 ± 0.001
	L-tyrosine	Not detected

[00168] Both PAL and TAL activities were also investigated according to whole cell assays. Seed cultures consisting of 5 ml of LB broth containing 34 mg/L chloramphenicol were prepared of *E. coli* BL21(DE3) strains that were individually transformed with each of pSpalAv, pSpalNp, pSencPSm, pSpal1At, and pSpal2At. These cultures were grown for 12 hours at 30°C while agitating at 250 rpm. 1 mL of each culture was then used to inoculate 3 x 250 mL cultures flasks containing 50 mL of LB supplemented with 34 mg/L chloramphenicol. All cultures were then grown at 30°C while agitating at 250 rpm until an OD_{600} of 0.6 was reached, at which point the cultures were induced by IPTG addition to a final concentration of 0.2 mM. Cultures were then grown for an additional 6 h before the cells were then collected by centrifugation in 50 ml Falcon tubes for 5 min at 1400 x g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was then resuspended in 10 ml pH7 PBS buffer before the appropriate substrate, L-phenylalanine or L-tyrosine, was added at a final concentration of 1 g/L. The results for L-phenylalanine addition are shown in Figure 2. For all strains tested the addition of L-tyrosine did not result in the formation of *p*-coumaric acid, indicating that no TAL activity was displayed by these recombinant strains.

[00169] These results demonstrate how PAL activity can be attained in recombinant *E. coli* by the expression of *pal* from either *A. variabilis, N. punctiforme,* or *A. thaliana*. Importantly, the selected isoenzymes show high substrate specificity such that no TAL activity was also observed. These results further establish the generation of recombinant *E. coli* strains that are specifically capable of converting phenylalanine to *trans*-cinnamic acid.

[00170] Example 2.

[00171] Assaying PADC activities in recombinant *E. coli*.

[00172] Seed cultures consisting of 5 ml of LB broth containing 100 mg/L ampicillin were prepared of *E. coli* BL21(DE3) strains that were individually transformed with each of pTpad1-fdc1, pTpad1Sc, pTfdc1Sc, pTpdcLp, and pTpadCBs. These cultures were grown for 12 hours at 30°C while agitating at 250 rpm. 1 mL of each culture was then used to inoculate 3 x 250 mL cultures flasks containing 50 mL of LB supplemented with 100 mg/L ampicillin. The culture was then grown at 30°C while agitating at 250 rpm until it reached an OD_{600} of 0.6, at which point the cultures were induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then grown for an additional 6 h. Cells were then collected by centrifuging in 50 ml Falcon tubes for 5 min at 1400 x g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was resuspended in 10 ml pH7 PBS buffer and the appropriate substrate (*trans*-cinnamic acid or *p*-coumaric acid) added at a final concentration of 1 g/L. Enzyme activity was monitored by taking 1 mL samples from the culture at both the time of initiation as well as after 12 h of culture. All samples were then analyzed by HPLC using the methods described herein. The results are shown in Figure 3.

[00173] These results demonstrate that PADC candidate isoenzymes from *L. plantarum and B. subtilis* display activity on *p*-coumaric acid alone. Meanwhile, *FDC1* from *S. cerevisiae* demonstrates broad substrate specificity with activities on both p-coumaric and *trans*-cinnamic acid. It is important to note that *FDC1* is unique in that only its expression resulted in CADC activity in recombinant *E. coli*. Furthermore, our experiments demonstrate that the expression of *FDC1* alone is sufficient for achieving CADC activity in recombinant *E. coli* and is not dependent upon the co-expression of *PAD1*. Finally, these results also indicate that *FDC1* shows a preference for *trans*-cinnamic acid over *p*-coumaric acid as substrates since higher product yields can be obtained on the former substrate.

[00174] Example 3.

[00175] PAL and CADC co-expression in *E. coli* NST74 to convert glucose to styrene in shake flask cultures.

[00176] The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSpalAv and pTfdc1Sc resulting in the construction of *E. coli* NST74 pSpalAv pTfdc1Sc. Similarly, *E. coli* NST74 was co-transformed with the following combinations of plasmids: pSpalNp together with pTfdc1Sc, pSpal1At together with pTfdc1Sc, and pSpal2At together with pTfdc1Sc. All strains were selected on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol and screened for said resistances. These transformations resulted in the generation of *E. coli* NST74 pSpalNp pTfdc1Sc, *E. coli* NST74 pSpal1At pTfdc1Sc, and *E. coli* NST74 pSpal2At pTfdc1Sc. Single colonies of each strain were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. Seed cultures were grown for 12 hours at 32°C with shaking at 250 rpm. 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These cultures were performed in 100 mL serum bottles outfitted with septa caps that were sealed upon inoculation. A closed system was used in this example to avoid volatile product losses. The cells were then cultivated for 10 h at 30°C with shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM. 1 ml samples were taken from each culture at the time of induction and 29 h post induction and analyzed for metabolite content via HPLC using methods described herein. The strains *E. coli* NST74 pSpalAv pTfdc1Sc, *E. coli* NST74 pSpalNp pTfdc1Sc, *E. coli* NST74 pSpal1At pTfdc1Sc, and *E. coli* NST74 pSpal2At pTfdc1Sc resulted in final styrene titers of 210 mg/L, 185 mg/L, 188 mg/L, and 245 mg/L, respectively.

[00177] These results illustrate how a strain of *E. coli* can be constructed to synthesize styrene as a dominant product when supplied with glucose as a sole carbon and energy source. The inventions describes a process in which recombinant *E. coli* has been engineered to co-express enzymes which display both PAL and CADC

activities. In the preferred embodiments, the *E. coli* host strain will be a phenylalanine over-producing strain, PAL activity will be encoded by *pal* from *N. punctiforme*, and CADC activity will be encoded by *FDC1* from *S. cerevisiae*.

[00178] Example 4.

[00179] Whole cell production of styrene upon L-phenylalanine supplementation.

[00180] The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSpalAt1 and pTfdc1Sc resulting the generation of *E. coli* NST74 pSpalAt1 pTfdc1Sc. This transformation resulted in the generation of *E. coli* NST74 pSpal1At pTfdc1Sc. Strains were selected on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol and screened for said resistances. Single colonies were then selected from the resultant transformants and were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32°C while shaking at 250 rpm. After which 1 ml of each seed culture was then used to inoculate 50 mL LB supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Culture were then grown at 30°C while agitating at 250 rpm until reaching an OD_{600} of 0.6, at which point all cultures were induced by the addition of IPTG to a final concentration of 0.2 mM. Cultures were then incubated for an additional 12 h. Cells were then harvested and collected by centrifugation in 50 ml conical tubes for 5 min at 1400 x g and washed once with pH7 PBS (phosphate buffered saline) buffer. The entire cell pellet was resuspended in 10 ml pH7 PBS buffer and the appropriate substrate (L-phenylalanine) added at a final concentration of either 400 or 950 mg/L. After which 1 mL samples from the culture were taken at both the time of initiation as well as periodically over the course of the following 27 h. All samples were then analyzed by HPLC using the methods described herein. The results are shown in Figure 4.

[00181] These results show the ability of *E. coli* NST74 pSpal1At pTfdc1Sc to achieve even higher final styrene titers upon the exogenous supplementation of the cultures with L-phenylalanine. When 400 mg/L of L-phenylalanine was added to the cell suspensions up to 300 mg/L of styrene was produced. Meanwhile, when 950 mg/L of L-

phenylalanine was added to the cell suspensions up to 500 mg/L of styrene was produced.

[00182] Example 5.

[00183] Continuous recovery of styrene from cultures by gas stripping or vacuum extraction.

[00184] Considering the innate volatility of styrene we propose that styrene could be removed from cultures as vapor on either a continuous or discrete basis. Styrene vapor removal could be accomplished either as it is synthesized or shortly thereafter. Styrene vapor removal could be accomplished either by gas stripping by culture aeration or by vacuum application upon the headspace. Styrene vapors could then be collected, for example, by condensation.

[00185] The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and pTrc99A-fdc1Sc resulting in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc. The strain was selected via its ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32 °C with shaking at 250 rpm. After that 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These flask cultures were grown for 12 hours at 32 °C with shaking at 250 rpm. Then 20 mL of these flask cultures were then used to inoculate a 2 L bioreactor containing 1 L MM1 supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. The bioreactor culture was then grown at 30 °C with agitation at 250 rpm and aeration at 0.42 L/min. Upon reaching an OD_{600} of 0.5 the culture was induced by the addition of IPTG to a final concentration of 0.2 mM. The culture was then grown at these conditions for an additional 33 hours. The outlet gas stream was routinely monitored for styrene content by sampling the outlet gas with a 200 µL gas tight syringe and analyzing those samples on the GC-FID. In addition, 1 mL of the aqueous media was also sampled and analyzed by HPLC, and the results are shown in

Figure 5. Figure 6 shows the net equivalent styrene titer achieved as a function of culture time, accounting for both the styrene stripped from the bioreactor as well as residual amount that remains in the aqueous media. After 33 hours, the net equivalent styrene titer achieved in the bioreactor was equal to 523 mg/L.

[00186] Example 6.

[00187] Continuous recovery of styrene from cultures by solvent extraction.

[00188] The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and pTrc99A-fdc1Sc resulting in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc. The strain was selected via its ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32 °C with shaking at 250 rpm. After which 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol in a 250 mL baffled flasks. An additional 10 mL of n-dodecane was added to the flask as an insoluble solvent phase. Cultures were then grown for 8 h at 30 °C while shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM. Growth continued at these conditions for the next 48 hours. After 48 hours, the n-dodecane phase was separated from the aqueous media phase by gravity, and 1 mL was analyzed for metabolite content via GC-FID. The styrene concentration in the n-dodecane phase was 2.72 g/L. This level of production would be equivalent to 544 mg/L of styrene produced in the aqueous culture media.

[00189] Example 7.

[00190] Co-expression of PAL, CADC, and styrene monooxygenase encoding isoenzymes in *E. coli* NST74 to convert glucose to styrene oxide in batch cultures.

[00191] The phenylalanine over-producing strain *E. coli* NST74 was co-transformed with the plasmids pSTV28-pal2At and pTrc99A-fdc1Sc-styABPp resulting

in the construction of *E. coli* NST74 pSTV28-pal2At pTrc99A-fdc1Sc-styABPp. The strain was selected via their ability to survive and grow on LB agar supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Single colonies were then selected from the resultant transformants and those strains were grown in 5 mL LB broth supplemented with both 100 mg/L ampicillin and 34 mg/L chloramphenicol. These seed cultures were grown for 12 hours at 32 °C with shaking at 250 rpm. 1 ml of each seed culture was then used to inoculate 50 mL MM1 supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol. These cultures were grown in 250 mL baffled flasks. The cells were then grown for 8 h at 30 °C with shaking at 250 rpm prior to being induced by the addition of IPTG to a final concentration of 0.2 mM and grown at these conditions for the next 48 hours. 1 ml samples were taken from each culture at intervals of 24 and 48 hours post induction and analyzed for metabolite content via HPLC using methods described herein. Figure 5 shows the results obtained with the strain NST74 pSTV28-pal2At pTrc99a-fdc1Sc-styABPp for three identical trials run in parallel.

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Claims

1. A method for the production of styrene comprising:

(i) contacting a recombinant host cell with a fermentable carbon substrate, said recombinant host comprising:

a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity

b) a t least one gene encoding a polypeptide having *trans*-cinnamic acid decarboxylase activity

(ii) growing said recombinant cell for a time sufficient to produce styrene; and

(iii) optionally recovering said styrene.

2. A method for the production of styrene oxide comprising:

(i) contacting a recombinant host cell with a fermentable carbon substrate, said recombinant host comprising:

a) at least one gene encoding a polypeptide having phenylalanine ammonia lyase activity

b) at least one gene encoding a polypeptide having *trans*-cinnamic acid decarboxylase activity

c) at least one gene encoding a polypeptide having styrene oxygenase activity

(ii) growing said recombinant cell for a time sufficient to produce styrene oxide.

3. A method according to claims 1 and 2 wherein the sequence of gene encoding a polypeptide having phenylalanine ammonia lyase activity is as set forth in SEQ ID NO:1, 2, 4, or 5.

4. A method according to claims 1 and 2 wherein the sequence of gene encoding a polypeptide having *trans*-cinnamic acid decarboxylase activity is as set forth in SEQ ID NO:11.

5. A method according to claims 2 wherein the sequence of gene encoding a polypeptide having *trans*-cinnamic acid decarboxylase activity is as set forth in SEQ ID NO:6 or 7.

6. A method according to claims 1 and 2 wherein said fermentable carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, formaldehyde, formate, amino acids, and carbon-containing amines.

7. A method according to claim 4 wherein said fermentable carbon source is selected from the group consisting of glucose or glycerol.

8. A method according to claim 1 and 2 wherein said recombinant host cell is selected from the group consisting of bacteria, yeast, filamentous fungi, cyanobacteria, algae, and plant cells.

9. A method according to claim 8 wherein said recombinant host cell is selected from the group consisting of *Escherichia, Salmonella, Bacillus, Acinetobacter, Streptomyces, Corynebacterium, Methylosinus, Methylomonas, Rhodococcus, Pseudomonas, Rhodobacter, Synechocystis, Saccharomyces, Klebsiella, Zygosaccharomyces, Kluyveromyces, Candida, Hansenula, Debaryomyces, Mucor, Pichia, Torulopsis, Aspergillus, Arthrobotrys, Brevibacterium, Microbacterium, Arthrobacter, Ctirobacter, Chlamydomonas,* and *Zymomonas.*

10. A method according to claim 8 wherein said recombinant host cell is a phenylalanine overproducing strain.

11. A method according to claims 1 and 2 wherein said recombinant host cell is a cell isolated from plants selected from the group consisting of soybean, rapeseed, sunflower, cotton, corn, tobacco, alfalfa, wheat, barley, oats, sorghum, rice, broccoli, cauliflower, cabbage, parsnips, melons, carrots, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses.

12. A method according to claims 1 and 2 wherein said gene encoding a polypeptide having

13. A method according to claims 1 and 2 wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Anabaena variabilis*.

14. A method according to claims 1 and 2 wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Nostoc punctiforme*.

15. A method according to claims 1 and 2 wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Arabadopsis thaliana*.

16. A method according to claims 1 and 2 wherein the genes encoding polypeptides having *trans*-cinnamic acid decarboxylase activity are derived from *Saccharomyces cerevisiae*.

17. A method according to claim 2 wherein the gene encoding a polypeptide having styrene oxygenase activity is derived from *Pseudomonas putida*.

18. A method according to claim 2 wherein the gene encoding a polypeptide having styrene oxygenase activity is derived from *Rhodococcus opacus*.

19. A method according to claim 1 wherein styrene is optionally recovered from the cultures.

20. A method according to claim 19 wherein styrene recovery is performed on either a discrete or continuous basis.

21. A method according to claim 19 wherein styrene recovery is performed by extraction with a biocompatible organic solvent.

22. A method according to claim 19 wherein styrene recovery is performed by gas stripping via bubbling with air or other gases.

23. A method according to claim 19 wherein styrene recovery is performed by vacuum application upon the head space.

24. A method according to claim 22 wherein styrene vapors are collected via condensation.

25. A method according to claim 23 wherein styrene vapors are collected via condensation.

FIGURE 1

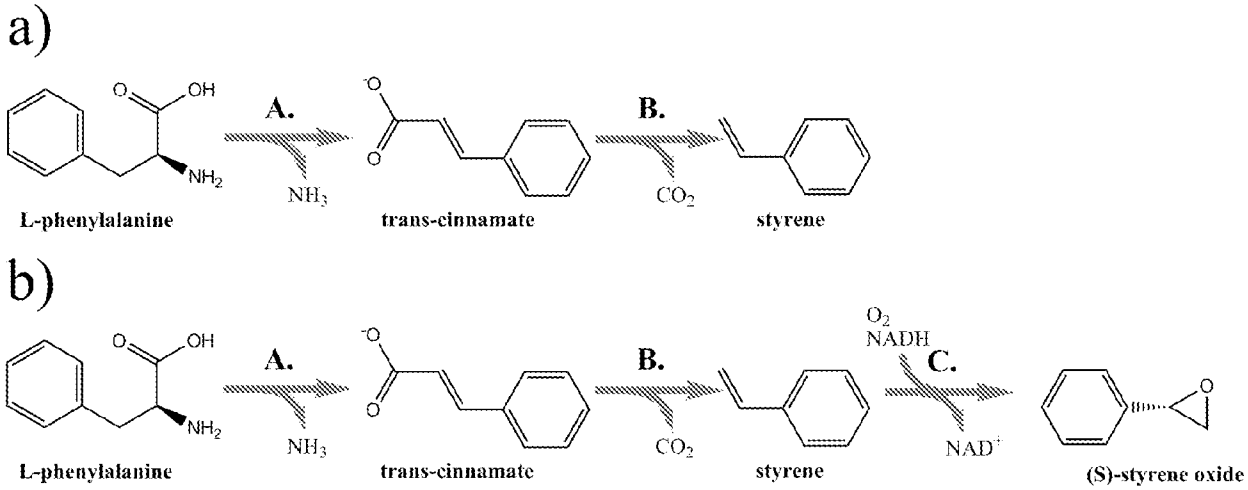


FIGURE 2

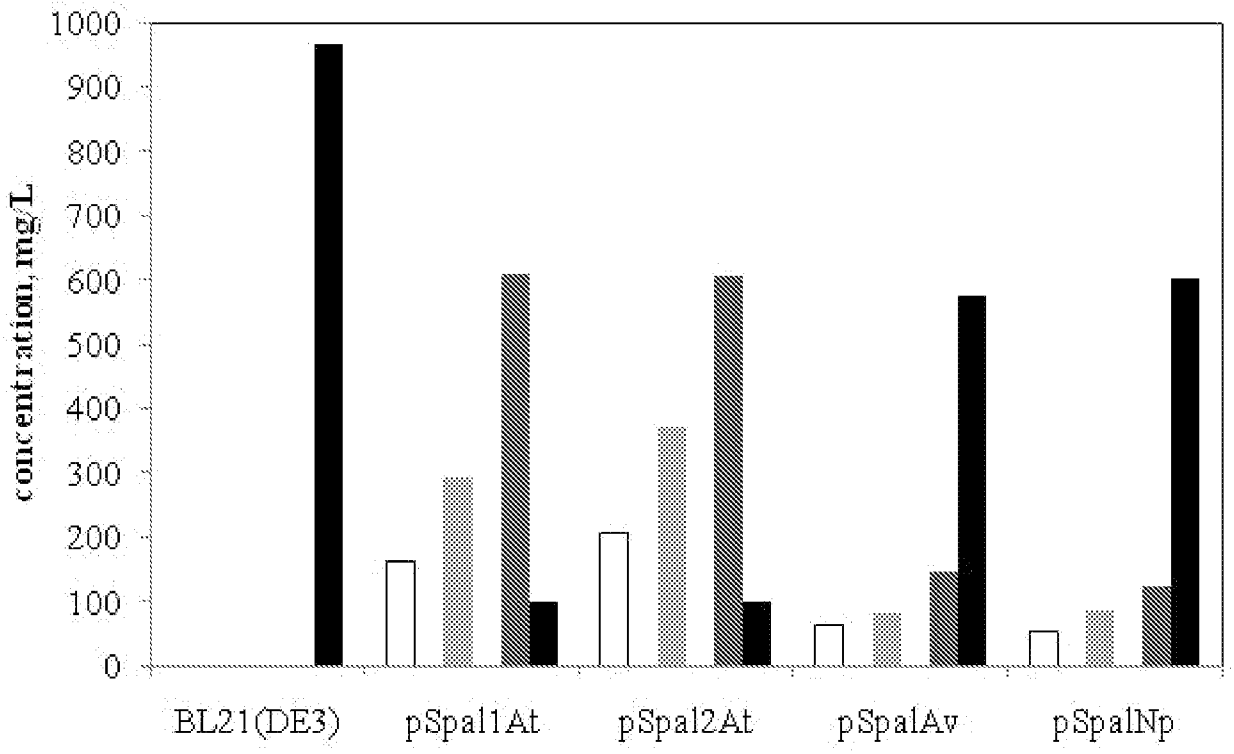


FIGURE 3A

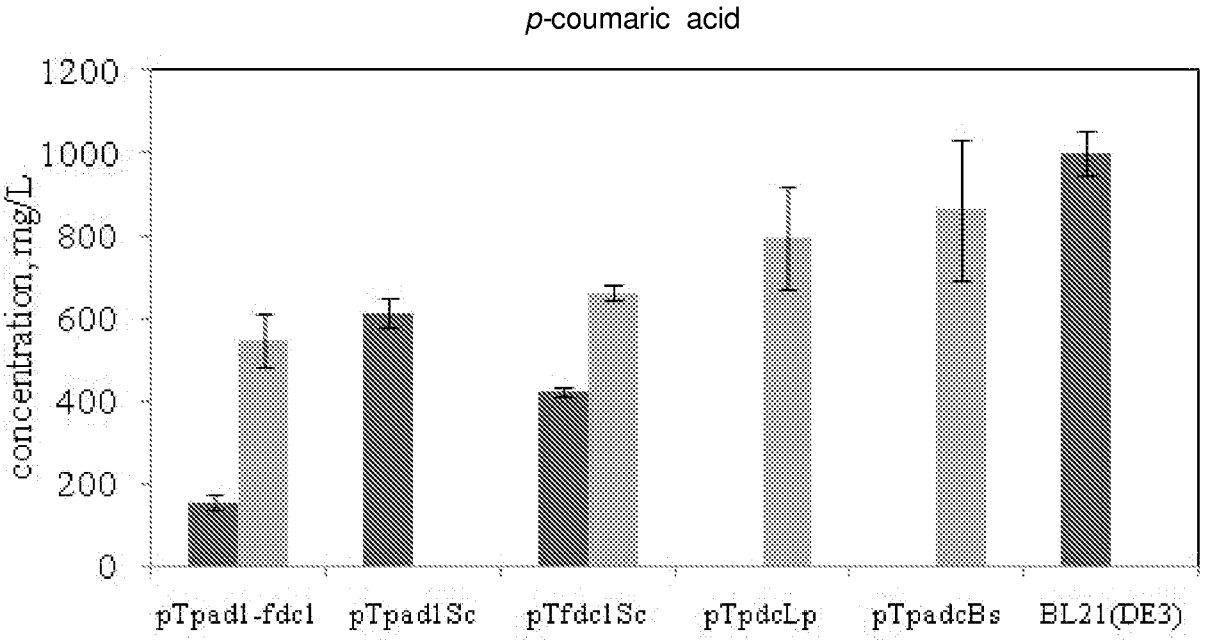


FIGURE 3B

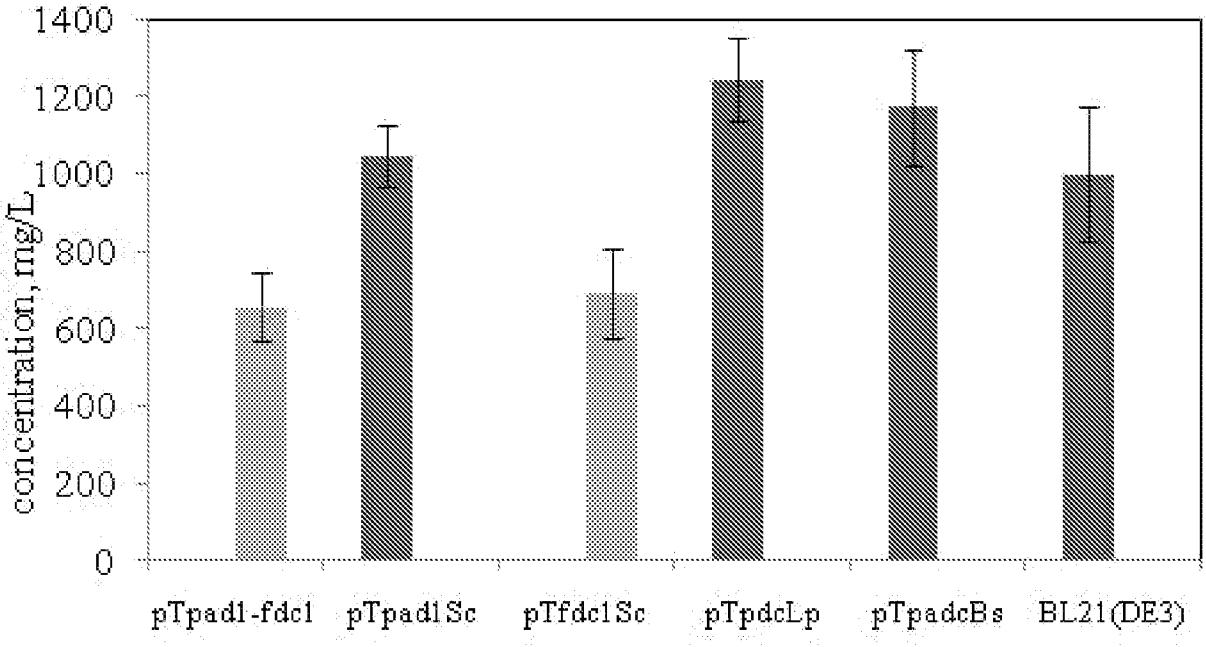


FIGURE 4

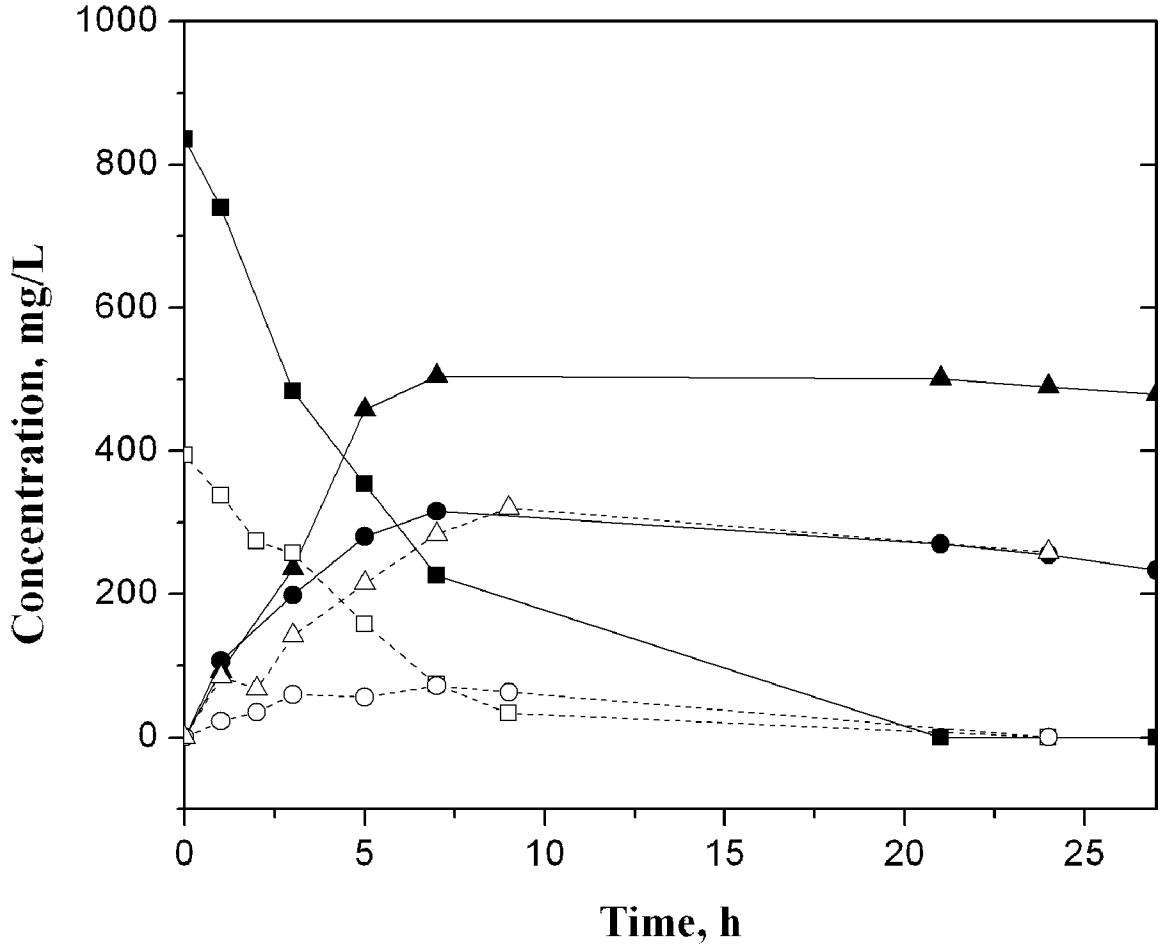


Figure 5

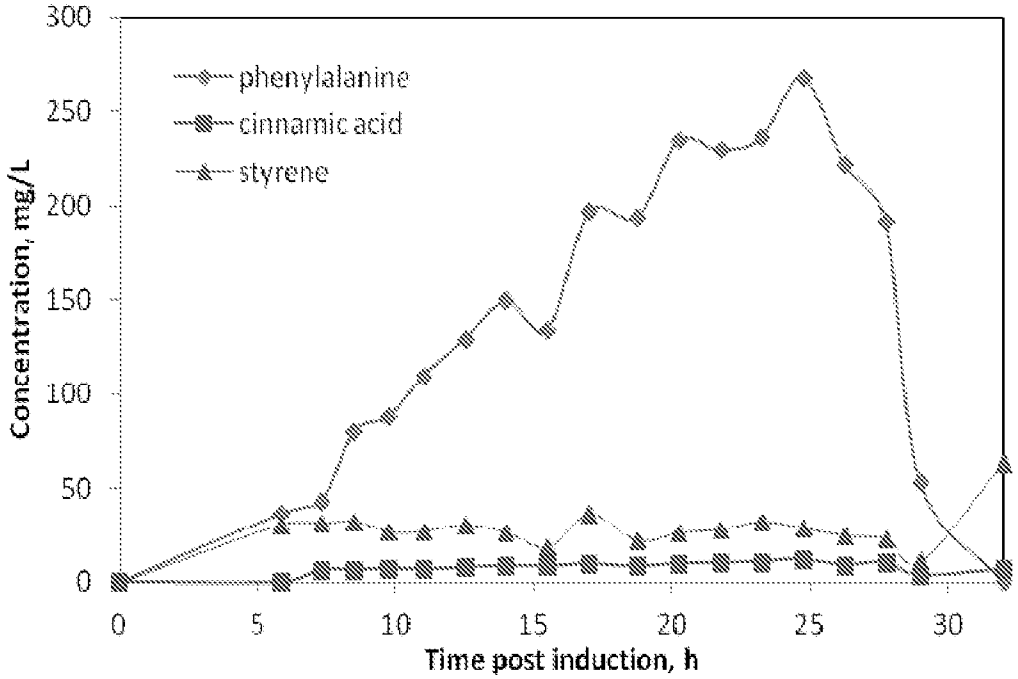


Figure 6

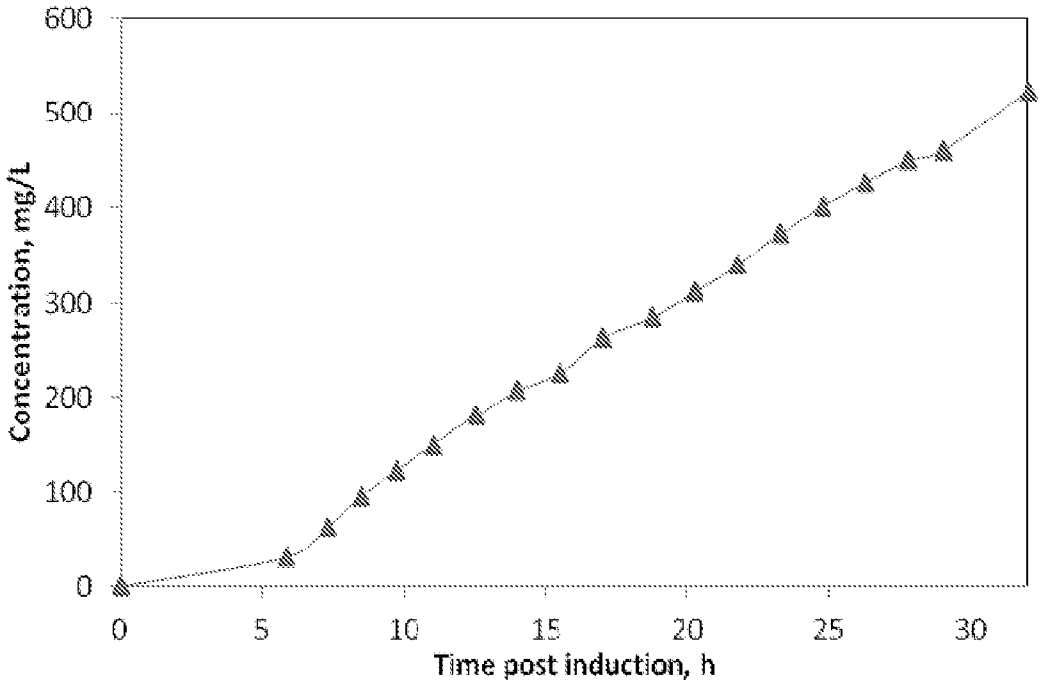
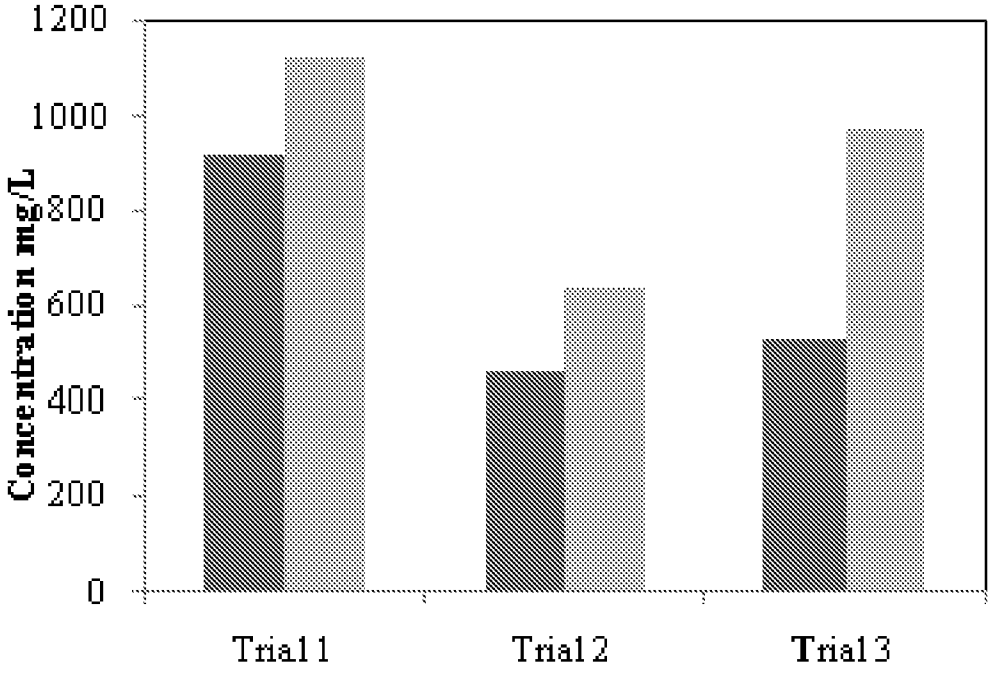


Figure 7


1200
1000
800
600
400
200
0
Concentration mg/L
Trial 1
Trial 2
Trial 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/28191

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/02; C12P 5/00; C12N 1/21 (2012.01)
USPC - 435/189, 435/166, 435/252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/189, 435/166, 435/252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/189, 183, 166, 252,3, 243, 41 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PubWEST (USPT, EPAB, JPAB, PGPB); Google Scholar, GenCore sequence search (NT)
Search terms: styrene production, recombinant microorganism, phenylalanine ammonia lyase, trans-cinnamic acid, ferulic acid decarboxylase (FDC1), phenylacrylic acid decarboxylase (PAD1),styrene monooxygenase (styA, styB), styrene oxide production

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 7,229,806 B2 (BEN-BASSAT et al.) 12 June 2007 (12.06.2007). Especially col 2 ln 37-47, col 18 ln 1-50.	1,2,5,17-25
Y	GOODEY et al. Genetic and Biochemical Analysis of the Ability of Saccharomyces cerevisiae to Decarboxylate Cinnamic Acids. J Gen Microbiol 1982 Vol 128 Pages 2615-2620. Especially pg 2617 para 4, pg 2618 table 3.	1,2,5,17-25
Y	PANKE et al. Pilot-scale production of (S)-styrene oxide from styrene by recombinant Escherichia coli synthesizing styrene monooxygenase. Biotechnol Bioeng 5 October 2002 Vol 80 No 1 Pages 33-41. Especially abstract.	2,5,17,18
Y	GenBank. DQ177365.1 [online] 1 September 2006 [retrieved 24 May 2012] Available on the internet: URL:http://www.ncbi.nlm.nih.gov/nuccore/DQ177365>. Especially pg 1-2.	5,17
Y	GenBank: FJ403049.1. [online] 15 July 2009 [retrieved 24 May 2012]. Available on the internet: <ULR: http://www.ncbi.nlm.nih.gov/nuccore/238516676?sat=15&satkey=2282228>. Especially pg 1.	18
Y	WO 2010/119339 A2 (GRADY et al.) 21 October 2010 (21.10.2010). Especially pg 43 ln 22-29.	22,24
Y	US 2010/0143996 A1 (BUTLER et al.) 10 June 2010 (10.06.2010). Especially para [0055]).	23,25
A	UNIPROT. PAD1_YEAST [online] 11 January 2011[retrieved 24 May 2011]. Available on the internet: <URL: http://www.uniprot.org/uniprot/P33751.txt?version=87>. Especiall pg 1.	1,2

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 2012 (24.05.2012)	22 JUN 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/28191

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 3,4,6-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/28191

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUKAI et al. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in Saccharomyces cerevisiae. J Bioschi Bioeng June 2010 Vol 109 No 6 Pages 564-569. Especially pg 566 col 1 para 2-3, fig 2A.	1,2