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(71) Applicant: ARIZONA BOARD OF REGENTS ON BEHALF OF ARIZONA STATE UNIVERSITY [US/US];
1475 North Scottsdale Road, Suite 200, Scottsdale, Arizona
85257 (US).

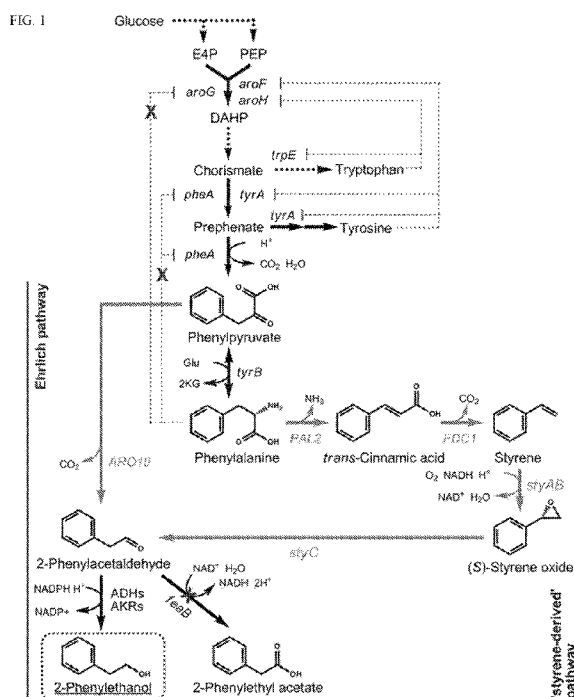
(72) Inventors: NIELSEN, David; ECG 301, 501 E. Tyler
Mall, Tempe, AZ 85287-6106 (US). MACHAS, Michael;
4410 East Vista Drive, Phoenix, AZ 85032 (US). MCKEN-
NA, Rebekah; 5120 E. Hampton Ave # 1010, Mesa, AZ
85206 (US).

(74) Agent: LEWIS, Jessica L.; Quarles & Brady LLP, 33 East
Main Street, Suite 900, Madison, WI 53703-3095 (US).

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(54) Title: MICROBIAL PRODUCTION OF 2-PHENYLETHANOL FROM RENEWABLE SUBSTRATES



(57) Abstract: Described herein are engineered metabolic pathways in recombinant microorganism host cells which result in the production of 2-phenylethanol or 2-phenylacetic acid. Also described herein are methods of using the recombinant microorganisms for the production of 2-phenylethanol or 2-phenylacetic acid.

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Microbial production of 2-phenylethanol from renewable substrates

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Patent Application No. 62/536,666, filed July 25, 2017, which is incorporated herein by reference as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

BACKGROUND

[0003] With a ‘rose-like’ aroma, 2-phenylethanol (2PE) is an important molecule in the flavor and fragrance industries. More specifically, 2PE is used in the production of various foods and beverages and, most notably, remains the most used fragrance compound in the cosmetics and perfume industries. Meanwhile, in addition to its traditional usages as a specialty chemical, 2PE has also garnered recent interest as a potential biofuel molecule due to its low volatility, high energy density and non-hygroscopic properties, or alternatively as a fuel additive helpful for preventing knocking as a result of its high octane number and reduced gas-phase reactivity. Altogether, annual global demand for 2PE exceeds 10,000 tons, with a market size expected to reach \$700 million by 2019. Traditional 2PE production methods involve its extraction from the essential oils of many flowering plant species – most notably, rose oil, which contains up to 60% 2PE. Although extraction is still practiced to obtain the natural product, as said process is expensive and poorly scalable, the bulk of 2PE production now instead occurs via its chemical synthesis from petrochemical feedstocks. Though cheaper, 2PE production in such manner is both non-renewable and unsustainable, and furthermore employs carcinogenic precursors (i.e., benzene) as feedstocks; undesirable from a ‘green chemistry’ perspective and a feature that imposes usage restrictions, especially in flavor/fragrance applications.

[0004] In light of the above limitations, microbiological production of 2PE via a variety of synthesis routes has recently been explored as a more sustainable alternative. A natural fermentation product of several yeast strains (albeit typically at only trace levels), 2PE is in large part responsible for the ‘floral’ aromas present in many fermented foods and beverages (Kieser et

al., 1964; Lee and Richard, 1984). In yeast, 2PE is produced via the Ehrlich pathway (Ehrlich, *Berichte der Dtsch. Chem. Gesellschaft* 40:1027–1047, 1907; Hazelwood et al., *Appl. Environ. Microbiol.* 74:2259–2266 (2008)); a two-step pathway stemming from phenylpyruvate, an intermediate of the shikimic acid (SA) pathway and precursor to L-phenylalanine (Phe). First, phenylpyruvate decarboxylase (PPDC) serves to convert phenylpyruvate to 2-phenylacetaldehyde which is subsequently reduced to 2PE by an alcohol dehydrogenase (FIG. 1). In *Saccharomyces cerevisiae*, for example, ARO10 catalyzes the first step whereas reduction of 2-phenylacetaldehyde to 2PE occurs by the aid of one or more native dehydrogenases (including ADH1-5) (Dickinson et al., *J. Biol. Chem.* 278:8028–34 (2003)). Achieving high levels of 2PE via their native Ehrlich pathway, however, typically requires select yeast strains (e.g., *S. cerevisiae*, *Kluyveromyces marxianus*) to be cultured under nitrogen limited conditions while supplementing the medium with excess exogenous phenylalanine (note: phenylalanine transaminase (e.g., ARO9 in *S. cerevisiae*) converts phenylalanine and 2-ketoglutarate to phenylpyruvate and L-glutamate, the latter then being degraded to provide nitrogen for growth). However, as phenylalanine is an expensive and poorly scalable feedstock, 2PE production directly from renewable biomass sugars represents a more promising approach.

[0005] To date, microbial 2PE production from glucose has focused predominantly on expanded applications of the Ehrlich pathway, most commonly via its functional reconstruction in other, heterologous microbes. For example, Atsumi *et al.* (*Nature* 451:86–9 (2008)) first reported the functional reconstruction of the Ehrlich pathway in *Escherichia coli* (comprised of *kivd* from *Lactococcus lactis* and *ADH2* from *S. cerevisiae*), demonstrating production of 57.3 mg/L 2PE from 36 g/L glucose (a yield of 1.59 mg/g) using a wild-type background (Atsumi et al., 2008). Kang *et al.* (*Appl. Biochem. Biotechnol.* 172:2012–21 (2014)) later also reconstructed the Ehrlich pathway in *E. coli* (in this case instead using *kdc* and *ADH1* from *Pichia pastoris* and *S. cerevisiae*, respectively) and, following deregulation of metabolite flux through the SA pathway, reported 2PE titers as high as 285 mg/L (Kang et al., 2014). Finally, expressing the Ehrlich pathway composed instead of *ipdC* from *Azospirillum brasilense* and *yahK* from *E. coli* in a phenylalanine over-producing host, Koma *et al.* (*Appl. Environ. Microbiol.* 78:6203–6216 (2012)) engineered *E. coli* for direct 2PE production from glucose at titers reaching 940.6 mg/L and a yield of 94.06 mg/g (Koma et al., 2012). However, functional reconstruction of the Ehrlich pathway in *E. coli* has its limitations and further work is needed to improve biosynthetic production of 2PE.

SUMMARY

[0006] In a first aspect, provided herein is a recombinant organism comprising (i) at least one heterologous gene encoding an enzyme having phenylalanine ammonia lyase (PAL) activity, (ii) at least one heterologous gene encoding an enzyme having trans-cinnamic acid decarboxylase (CADC) activity, (iii) at least one heterologous gene encoding an enzyme having styrene monooxygenase (SMO) activity, (iv) at least one heterologous gene encoding an enzyme having styrene oxide isomerase (SOI) activity, and (v) at least one gene encoding an enzyme having 2-phenylacetaldehyde reductase (PAR) activity, wherein the recombinant microorganism is capable of producing 2-phenylethanol from a fermentable carbon substrate. The organism can be *Escherichia coli*. The organism can be a phenylalanine overproducing strain of *E. coli*. The gene encoding a polypeptide having phenylalanine ammonia lyase activity can be derived from *Arabidopsis thaliana*. The gene encoding polypeptides having trans-cinnamic acid decarboxylase activity can be derived from *Saccharomyces cerevisiae*. The gene encoding a polypeptide having styrene monooxygenase activity can be derived from *Pseudomonas putida*. The gene encoding a polypeptide having styrene oxide isomerase activity can be derived from *Pseudomonas putida*.

[0007] In another aspect, provided herein is a method of producing 2-phenylethanol comprising the steps of (i) contacting a recombinant organism engineered to produce 2-phenylethanol with a fermentable carbon substrate, and (ii) growing the recombinant organism for a time sufficient to produce 2-phenylethanol. The fermentable carbon substrate can be selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, methane, formaldehyde, formate, amino acids, and carbon-containing amines. The fermentable carbon source can be glucose, xylose, or glycerol. The fermentable carbon substrate is selected from the group consisting of lignin-derived aromatic monomers, lignin-derived aromatic oligomers, and combinations thereof. The fermentable carbon source can be a biomass hydrolysate.

[0008] In a further aspect, provided herein is a recombinant organism comprising, (i) at least one heterologous gene encoding an enzyme having phenylalanine ammonia lyase (PAL) activity, (ii) at least one heterologous gene encoding an enzyme having trans-cinnamic acid decarboxylase (CADC) activity, (iii) at least one heterologous gene encoding an enzyme having styrene

monooxygenase (SMO) activity, (iv) at least one heterologous gene encoding an enzyme having styrene oxide isomerase (SOI) activity, and (v) at least one gene encoding an enzyme having 2-phenylacetaldehyde dehydrogenase (PADH) activity, wherein the engineered microorganism is capable of producing 2-phenylacetic acid from a fermentable carbon substrate. The organism can be *Escherichia coli*. The organism can be a phenylalanine overproducing strain of *E. coli*. The gene encoding a polypeptide having phenylalanine ammonia lyase activity can be derived from *Arabidopsis thaliana*. The gene encoding polypeptides having trans-cinnamic acid decarboxylase activity can be derived from *Saccharomyces cerevisiae*. The gene encoding a polypeptide having styrene monooxygenase activity can be derived from *Pseudomonas putida*. The gene encoding a polypeptide having styrene oxide isomerase activity can be derived from *Pseudomonas putida*.

[0009] In another aspect, provided herein is a method of producing 2-phenylacetic acid comprising the steps of (i) contacting a recombinant organism engineered to produce 2-phenylacetic acid with a fermentable carbon substrate, and (ii) growing the recombinant organism for a time sufficient to produce 2-phenylacetic acid. The fermentable carbon substrate can be selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, methane, formaldehyde, formate, amino acids, carbon-containing amines. The fermentable carbon source can be selected from the group consisting of glucose, xylose, or glycerol. The fermentable carbon substrate is selected from the group consisting of lignin-derived aromatic monomers, lignin-derived aromatic oligomers, and combinations thereof. The fermentable carbon source can be a biomass hydrolysate.

[0010] Although the following description refers to certain aspects or embodiments, such aspects or embodiments are illustrative and non-exhaustive in nature. Having reviewed the present disclosure, persons of ordinary skill in the art will readily recognize and appreciate that numerous other possible variations or alternative configurations or aspects are possible and were contemplated within the scope of the present disclosure. Accordingly, the detailed descriptions are to be regarded as illustrative in nature and not restrictive.

BRIEF DESCRIPTION OF DRAWINGS

[0011] FIG. 1 shows a comparison of 2PE biosynthesis via the established Ehrlich and proposed styrene-derived pathways. Endogenous pathway steps shown with black arrows whereas heterologous steps are shown in gray.

[0012] FIG. 2 shows a comparison in the change in Gibbs free energy due to reaction ($\Delta G'^{\circ}$) with progress through each of the two pathways (Ehrlich pathway, open squares-dotted line; styrene pathway, open circles-solid line) from phenylpyruvate to 2PE. $\Delta G'^{\circ}$ was determined for each reaction using the eQuilibrator online tool at a reference state of 25°C, pH 7, and ionic strength of 0.1 M.

[0013] FIG. 3 shows screening styrene oxide isomerase enzyme activity using *E. coli* BW25113 pColaK-styC whole resting cells. Conversion of (*S*)-styrene oxide (open shapes, dotted line) to 2-phenylacetaldehyde (solid shapes, solid line) by *StyC* using three different cell densities ($OD_{600} \sim 0.01, 0.03$, and 0.07 are squares, circles, and diamonds, respectively). Error bars reported at one standard deviation from triplicate experiments.

[0014] FIG. 4 shows the growth response of *E. coli* NST74 following exogenous 2PE addition at final concentrations of 0 g/L (control; square), 1 g/L (circle), 1.25 g/L (upright triangle), 1.5 g/L (diamond), 1.75 g/L (inverted triangle) and 2 g/L (right triangle). Error bars reported at one standard deviation from triplicate experiments.

[0015] FIG. 5 shows the final titers of 2PE after 72 hours (h) of culturing for the Ehrlich (left) and styrene-derived (center) pathways in *E. coli* NST74 strains harboring various deletions of genes *feaB*, *crr*, *pykA* and *pykF*. Also shown for comparison are final Phe titers by the same host strain in the absence of either pathway (right). Final acetate concentrations are also shown (n.d. indicates not detected). Error bars reported at one standard deviation from duplicate experiments.

[0016] FIG. 6 shows the effect of induction timing on production of 2PE and Phe as well as growth after 72 hours by both pathways when expressed in *E. coli* NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$. Upper panels: final OD_{600} (dark, solid) and OD_{600} at time of induction for the Ehrlich (left; light, striped) and styrene-derived (right; light, striped) pathways. Lower panels: Final concentrations of 2PE (dark, solid) and Phe (striped, gold) for the Ehrlich (left) and styrene-derived (right) pathways. Error bars reported at one standard deviation from duplicate experiments.

[0017] FIG. 7 shows acetate accumulation after 72 h by *E. coli* NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ expressing the Ehrlich pathway, as a function of induction timing. Error bars reported at one standard deviation from duplicate experiments.

[0018] FIG. 8 shows the effect of various initial concentrations of glucose (ranging from 5 to 50 g/L) for 2PE production of *E. coli* NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ induced at inoculation. Left: Glucose consumed as a percentage of glucose fed (light, striped) and mass yield of 2PE from

glucose (dark, solid) after 96 h of culturing for the Ehrlich (left) and styrene-derived (center) pathways. Right: 2PE titers for the Ehrlich (dark, solid) and styrene-derived (light, striped) pathways at the 96 h mark are shown for various concentrations of initial glucose. Error bars reported at one standard deviation from duplicate experiments.

[0019] FIG. 9 shows the time course analysis of 2PE production metrics over 87 h in the Ehrlich (squares) and styrene-derived (circles) pathways with 30 g/L initial glucose in *E. coli* NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ induced at inoculation. Upper: glucose consumption. Middle: 2PE production. Lower: OD_{600} . Error bars reported at one standard deviation from duplicate experiments.

[0020] FIGS. 10A-10B show the enzymatic pathway for the biosynthesis of (A) 2-phenylethanol composed of i) phenylalanine ammonia lyase (PAL), ii) trans-cinnamic acid decarboxylase (CADC), iii) styrene monooxygenase (SMO), iv) styrene oxide isomerase (SOI), and v) 2-phenylacetaldehyde reductase (PAR); and, (B) 2-phenylacetic acid composed of i) phenylalanine ammonia lyase (PAL), ii) trans-cinnamic acid decarboxylase (CADC), iii) styrene monooxygenase (SMO), iv) styrene oxide isomerase (SOI), and v) 2-phenylacetaldehyde dehydrogenase (PADH).

INCORPORATION BY REFERENCE

[0021] All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though set forth in their entirety in the present application.

DETAILED DESCRIPTION

[0022] The present disclosure describes microorganisms engineered to produce 2-phenylethanol and/or 2-phenylacetic acid from renewable carbon sources. The recombinant microorganisms described herein are based at least in part on the inventors' development of engineered enzyme pathways for the microbial biosynthesis of 2-phenylethanol and 2-phenylacetic acid from renewable biomass resources in the bacterium *Escherichia coli* (*E. coli*). The pathways uniquely proceed from L-phenylalanine as the immediate endogenous precursor. The pathways uniquely include trans-cinnamate, styrene, and/or (S)-styrene oxide as intermediate precursors.

[0023] Recombinant host microorganisms are engineered to produce 2-phenylethanol and/or 2-phenylacetic acid from L-phenylalanine via an enzymatic pathway comprising heterologous enzymes with phenylalanine ammonia lyase, trans-cinnamic acid decarboxylase, styrene monooxygenase, and styrene oxide isomerase activity and at least one enzyme with 2-phenylacetaldehyde reductase activity or 2-phenylacetaldehyde dehydrogenase activity. In another aspect, the present invention describes methods of producing 2-phenylethanol or 2-phenylacetic acid using the engineered microorganisms described herein.

[0024] In some aspects, an engineered microorganism provided herein will comprise the complete biosynthetic pathway required for the conversion of L-phenylalanine to 2-phenylethanol. The host microorganism will comprise an enzyme having phenylalanine ammonia lyase activity, an enzyme having trans-cinnamic acid decarboxylase activity, an enzyme having styrene monooxygenase activity, an enzyme having styrene oxide isomerase activity and an enzyme with 2-phenylacetaldehyde reductase activity. Portions of the pathway have previously been described in U.S. Patent 9,150,884, which is incorporated herein by reference.

[0025] In some aspects, an engineered microorganism provided herein will comprise the complete biosynthetic pathway required for the conversion of L-phenylalanine to 2-phenylacetic acid. The host microorganism will comprise an enzyme having phenylalanine ammonia lyase activity, an enzyme having trans-cinnamic acid decarboxylase activity, an enzyme having styrene monooxygenase activity, an enzyme having styrene oxide isomerase activity and an enzyme with 2-phenylacetaldehyde dehydrogenase activity. Portions of the pathway have previously been described in U.S. Patent 9,150,884, which is incorporated herein by reference.

[0026] The term “host” refers to a suitable organism or cell line such as a strain of bacteria, for example, into which genes can be transferred to impart desired genetic attributes and functions. The host organisms of the present invention will include any organism capable of expressing the genes required for 2-phenylethanol or 2-phenylacetic acid production. Typically, the host 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*, *Sphingomonas*, *Clostridium*, *Acinetobacter*, *Actinomyces* such as *Streptomyces*, *Corynebacterium*; methanotrophs such as *Methylosinus*, *Methylomonas*, *Rhodococcus* and *Pseudomonas*; cyanobacteria, such as *Synechococcus* and *Synechocystis*; yeasts, such as *Saccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*,

Mucor, *Pichia*, *Yarrowia*, and *Torulopsis*; filamentous fungi, such as *Aspergillus* and *Arthrotrichum*; and algae, such as *Chlamydomonas*, for example. The genes encoding polypeptides with the PAL, CAD, SMO, SOI, PAR and PADH activities used in the present invention may be native to or introduced in these and other microbial hosts and expressed or over-expressed to prepare large quantities of 2-phenylethanol or 2-phenylacetic acid.

[0027] Microbial expression systems and expression vectors containing regulatory sequences that direct high-level expression of foreign proteins and overexpression 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 2-phenylethanol or 2-phenylacetic acid. These chimeric genes could then be introduced into appropriate microorganisms via transformation to allow for expression of high levels of the enzymes.

[0028] Although any of the above mentioned microorganisms would be useful for the production of 2-phenylethanol or 2-phenylacetic acid, 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 *Brevibacterium* sp. Particularly useful phenylalanine over-producing strains include, but are not limited to, *Microbacterium ammoniophilum* ATCC 10155, *Corynebacterium lilium* 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, at least one gene encoding a polypeptide having CAD activity, at least one gene encoding a polypeptide having SMO activity, at least one gene encoding a polypeptide having SOI activity, and at least one gene encoding a polypeptide having PAR or PADH activity.

[0029] 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 *E. coli* phenylalanine over-producing strains are NST74 and NST37 (U.S. Pat. No. 4,681,852). Meanwhile, still others may include specific strains of *Corynebacterium glutamicum* (U.S. Pat. No. 3,660,235).

[0030] As used herein, "phenylalanine ammonia lyase (PAL)" refers to an enzyme that catalyzes the conversion of L-phenylalanine to trans-cinnamic acid. The term encompasses wild

type or naturally occurring phenylalanine ammonia lyase, as well as functional fragments or variants of a wild type phenylalanine ammonia lyase. 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 [*Arabidopsis 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., *Rhodospiridium* 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. In one embodiment, the phenylalanine ammonia lyase is *PAL2* from *Arabidopsis thaliana* (SEQ ID NO:1).

[0031] As used herein, "trans-cinnamic acid decarboxylase (CADC)" refers to an enzyme that catalyzes the conversion of trans-cinnamic acid to styrene. The term encompasses wild type or naturally occurring trans-cinnamic acid decarboxylase, as well as functional fragments or variants of a wild type trans-cinnamic acid decarboxylase. Genes which 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). 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 *PADI* (L09263.1 GI:393284) and *FDCI* (NP-010828.1 GI:6320748) both be present and undisturbed in the genome. Genomic disruption of either *PADI* or *FDCI* resulted in the loss of CADC activity upon exogenously supplied trans-cinnamic acid. In *E. coli*, expression of *FDCI* alone may be sufficient for conferring trans-cinnamic acid decarboxylase (CADC) activity. Without being bound to any particular theory, this is believed to be due to the complementary function of native *ubiX*, a known homolog of *PADI*. Considering the structural similarity between ferulic acid and

trans-cinnamic acid, we expect 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. In one embodiment, the trans-cinnamic acid decarboxylase is *FDC1* from *S. cerevisiae* (SEQ ID NO:2).

[0032] As used herein, "styrene monooxygenase (SMO)" refers to an enzyme that catalyzes the conversion of styrene to (S)-styrene oxide. The term encompasses wild type or naturally occurring styrene monooxygenase, as well as functional fragments or variants of a wild type styrene monooxygenase. Genes which encode styrene monooxygenase activity have been identified in the literature. In addition, enzymes which have been classified as an alkene monooxygenase may also display the necessary SMO activity. Genes encoding SMO activity, for example, have been isolated from *Pseudomonas fluorescens* (Z92524.1 GI:2154926). Furthermore, SMO activity has been reported in *Rhodococcus opacus* ADP1, *Rhodococcus opacus* 1CP, *Rhodococcus* sp. AD45, and *Pseudomonas* sp. strain VLB120. In some embodiments, styrene monooxygenase may be composed of a single protein subunit. In some embodiments, styrene monooxygenase may be a multi-subunit protein. In one embodiment, the styrene monooxygenase is comprised of both monooxygenase and reductase subunits, encoded by the individual genes *styA* (SEQ ID NO:3) and *styB* (SEQ ID NO:4) from *Pseudomonas putida* S12, respectively. In one embodiment, the styrene monooxygenase is comprised of the native gene cluster *styAB* from *P. putida* S12 (SEQ ID NO:5).

[0033] As used herein, "styrene oxide isomerase (SOI)" refers to an enzyme that catalyzes the conversion of (S)-styrene oxide to 2-phenylacetaldehyde. The term encompasses wild type or naturally occurring styrene oxide isomerase, as well as functional fragments or variants of a wild type styrene oxide isomerase. In some embodiments the styrene oxide isomerase is heterologous to the host microorganism. Genes which encode styrene oxide isomerase activity have been identified in the literature. Genes encoding SOI activity, for example, have been isolated from *Metarhizium majus* (MAJ_11235 GI:26280817) and *Pseudomonas fluorescens* (Z92524.1 GI:2154926). Furthermore, SOI activity has been reported in *Rhodococcus opacus*, *Rhodococcus opacus* 1CP, *Corynebacterium* sp., *Xanthobacter* sp., *Pseudomonas* sp. strain VLB120, and *Pseudomonas putida* CA-3. In one embodiment, the styrene oxide isomerase is *styC* from *P. putida* S12 (SEQ ID NO:6). In one embodiment, the styrene monooxygenase and styrene oxide isomerase are comprised of the native operon *styABC* from *P. putida* S12 (SEQ ID NO:7).

[0034] As used herein, “2-phenylacetaldehyde reductase (PAR)” refers to an enzyme that catalyzes the conversion of 2-phenylacetaldehyde to 2-phenylethanol. The term encompasses wild type or naturally occurring 2-phenylacetaldehyde reductase, as well as functional fragments or variants of a wild type 2-phenylacetaldehyde reductase. Genes which encode 2-phenylacetaldehyde reductase activity have been identified in the literature. In addition, enzymes which have been classified as aldo-keto reductases and alcohol dehydrogenases may also display the necessary PAR activity. Genes encoding PAR activity, for example, have been isolated from *Solanum lycopersicum* (NC_015438.2 GI:100134901). Furthermore, PAR activity has been reported in *S. cerevisiae*, *Rosa hybrid cultivar*, *Petunia x hybrid*. In some embodiments, an enzyme or a gene encoding an enzyme with PAR activity is native to the host organism. In some embodiments, the PAR enzyme is heterologous to the host organism. In some embodiments, the PAR is selected from the group consisting of *dkgA* (NC_000913.3 GI:948543), *dkgB* (NC_000913.3 GI:944901), and *yeaE* (NC_000913.3 GI:946302) from *E. coli*.

[0035] As used herein, “2-phenylacetaldehyde dehydrogenase (PADH)” refers to an enzyme that catalyzes the conversion of 2-phenylacetaldehyde to 2-phenylacetic acid. The term encompasses wild type or naturally occurring 2-phenylacetaldehyde dehydrogenase, as well as functional fragments or variants of a wild type 2-phenylacetaldehyde dehydrogenase. Genes which encode 2-phenylacetaldehyde dehydrogenase activity have been identified in the literature. In addition, enzymes which have been classified as aldehyde dehydrogenase may also display the necessary PADH activity. Genes encoding PADH activity, for example, have been isolated from *Pseudomonas putida* KT2440 (NC_002947.4 GI:1046275), *Salmonella enterica* (NC_003197.2 GI:1254652), *Acinetobacter pittii* (NC_016603.1 GI:11639512), *Klebsiella pneumoniae* (NC_016845.1 GI:11847382), and *Pseudomonas fluorescens* (Z92524.1 GI:2154926). Furthermore, PADH activity has been reported in *Brevibacterium* sp., *Xanthobacter* sp., and *Flavobacterium* sp.. In some embodiments, an enzyme or a gene encoding an enzyme with PADH activity is native to the host organism. In some embodiments, the PADH enzyme is heterologous to the host organism. In one embodiment, the PADH enzyme is encoded by *feaB* (NC_000913.3 GI:945933) from *E. coli*.

[0036] It is also envisioned that native genes encoding enzymes with PAR or PADH activity may be downregulated, silenced, eliminated or mutated in the host organism to reduce or eliminate their inherent net and/or specific activity and direct the products of the biosynthetic pathway to

either 2-phenylethanol or 2-phenylacetic acid, as appropriate. As 2-phenylethanol is produced from 2-phenylacetaldehyde by an enzyme with PAR activity or 2-phenylacetic acid is produced from 2-phenylacetaldehyde by an enzyme with PADH activity, deletion of one or more genes encoding enzymes with PAR activity will push the products of the reaction to 2-phenylacetic acid and deletion of one or more genes encoding enzymes with PADH activity will push the products of the reaction to 2-phenylethanol. In some embodiment, one or more genes encoding enzymes with PADH activity are deleted from the host organism. In some embodiments, a host organism lacking a gene encoding an enzyme with PADH activity is selected. In some embodiments, one or more genes encoding enzymes with PAR activity are deleted from the host organism. In some embodiments, a host organism lacking a gene encoding an enzyme with PAR activity is selected.

[0037] It is also envisioned that native genes encoding enzymes with PAR or PADH activity may be upregulated or mutated in the host organism to increase their inherent net and/or specific activity and direct the products of the biosynthetic pathway to either 2-phenylethanol or 2-phenylacetic acid, as appropriate. As 2-phenylethanol is produced from 2-phenylacetaldehyde by an enzyme with PAR activity or 2-phenylacetic acid is produced from 2-phenylacetaldehyde by an enzyme with PADH activity, upregulation of one or more genes encoding native enzymes with PAR activity will push the products of the reaction to 2-phenylethanol and upregulation of one or more native genes encoding enzymes with PADH activity will push the products of the reaction to 2-phenylacetic acid. In some embodiments, one or more native genes encoding enzymes with PAR activity are upregulated in the host organism. In some embodiments, one or more native genes encoding enzymes with PADH activity are upregulated in the host organism.

[0038] As used herein, the term “heterologous” refers to any biological entity such as, but not limited to, DNA, RNA, proteins, enzymes, polypeptides, antibodies, and the like, that are not naturally occurring in the host cell or host organism. Heterologous genes or proteins are those that have been derived from a different organism or species than the host organism into which they are introduced.

[0039] It will be appreciated that the present disclosure 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)).

[0040] 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.

[0041] 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.

[0042] 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. “Over-expression” 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.

[0043] “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. As used herein, the term “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.

[0044] As used herein, the term "recombinant" refers to a biomolecule that has been manipulated *in vitro*, e.g., using recombinant DNA technology to introduce changes to a genome. Introducing such changes to a genome can be achieved by transformation. As used herein, the term “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.

[0045] 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.

[0046] The methods of producing 2-phenylethanol and 2-phenylacetic acid described herein involves the incorporation of genes encoding polypeptides displaying PAL, CADC, SMO, SOI, and PAR or PADH 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 2-phenylethanol or 2-phenylacetic acid.

[0047] In some aspects, the present invention comprises an *in vivo* method for the production of 2-phenylethanol via a recombinant organism co-expressing at least one gene encoding a polypeptide having phenylalanine ammonia lyase (PAL) activity to convert endogenously-synthesized L-phenylalanine to trans-cinnamic acid, at least one gene encoding a polypeptide having trans-cinnamic acid decarboxylase (CADC) activity to convert trans-cinnamic acid to styrene, at least one gene encoding a polypeptide having styrene monooxygenase (SMO) activity to convert styrene to (S)-styrene oxide, at least one gene encoding a polypeptide having styrene oxide isomerase (SOI) activity to convert (S)-styrene oxide to 2-phenylacetaldehyde, and at least one gene encoding a polypeptide having 2-phenylacetaldehyde reductase (PAR) activity to convert

2-phenylacetaldehyde to 2-phenylethanol. The reaction schemes are illustrated in FIG. 10A. The recombinant organism is grown on a fermentable carbon substrate under conditions and for a time suitable to produce 2-phenylethanol.

[0048] In some aspects, provided herein is an *in vivo* method for the production of 2-phenylacetic acid via a recombinant organism co-expressing at least one gene encoding a polypeptide having phenylalanine ammonia lyase (PAL) activity to convert endogenously-synthesized L-phenylalanine to trans-cinnamic acid, at least one gene encoding a polypeptide having trans-cinnamic acid decarboxylase (CADC) activity to convert trans-cinnamic acid to styrene, at least one gene encoding a polypeptide having styrene monooxygenase (SMO) activity to convert styrene to (S)-styrene oxide, at least one gene encoding a polypeptide having styrene oxide isomerase (SOI) activity to convert (S)-styrene oxide to 2-phenylacetaldehyde, and at least one gene encoding a polypeptide having 2-phenylacetaldehyde dehydrogenase (PADH) activity to convert 2-phenylacetaldehyde to 2-phenylacetic acid. This reaction scheme is illustrated in FIG. 10B. The recombinant organism is grown on a fermentable carbon substrate under conditions and for a time suitable to produce 2-phenylacetic acid.

[0049] Growth of the recombinant organism can be carried out in suitable medium and for a suitable time to produce the desired products. For example, seed cultures may be grown in 3 mL LB broth supplemented with appropriate antibiotics at 32°C for 12 – 16 h. Next, 0.5 mL of seed culture may be used to inoculate 50 mL (in 250 mL shake flasks) of pH 6.8 MM1 media, with the following recipe (in g/L): glucose (20), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), $(\text{NH}_4)_2\text{SO}_4$ (4.0), MOPS (24.7), KH_2PO_4 (0.3), and K_2HPO_4 (1.0), as well as 1 mL/L of a trace mineral solution containing (in g/L): Thiamine HCl (0.101), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.584), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.288), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.714), CuSO_4 (0.1596), H_3BO_3 (2.48), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.370), and FeCl_3 (0.050). Once inoculated, cultures may be grown at 32°C while shaking at 200 RPM until reaching an OD_{600} of 0.8 (~8 h), at which time they may be induced by addition of IPTG at a final concentration of 0.2 mM. Following induction, strains may be cultured for a total of 72 h. Intermittently throughout each culture, pH may be increased back to its initial value by adding a minimal volume (typically ~0.2-0.4 mL) of 0.4 g/L K_2HPO_4 solution.

[0050] The recombinant organism may be grown in the any system known in the art suitable for the grown and propagation of the host organism. Suitable growth systems include, but are not

limited to, shaker flasks, incubators, fermenters, bioreactors, batch bioreactors, fed-batch bioreactors, continuous bioreactors, immobilized cell bioreactors, airlift bioreactors, and the like.

[0051] 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. In some embodiment, the fermentable carbon substrate is derived from a renewable biomass feedstock.

[0052] As used herein, the term “renewable biomass feedstock” refers to any renewable biological material, living or recently dead and any byproduct of those organisms, plant or animal. As used herein, the term “biomass” refers to, without limitation, organic materials produced by plants (such as leaves, roots, seeds and stalks), and microbial and animal metabolic wastes. Common biomass sources include: (1) agricultural residues, such as corn stalks, straw, seed hulls, sugarcane leavings, bagasse, nutshells, and manure from cattle, poultry, and hogs; (2) wood materials, such as wood or bark, sawdust, timber slash, and mill scrap; (3) municipal waste, such as waste paper and yard clippings; (4) algae-derived biomass, including carbohydrates and lipids from microalgae (e.g., *Botryococcus braunii*, *Chlorella*, *Dunaliella tertiolecta*, *Gracilaria*, *Pleurochrysis carterae*, and *Sargassum*) and macroalgae (e.g., seaweed); and (5) energy crops, such as poplars, willows, switch grass, miscanthus, sorghum, alfalfa, prairie bluestem, corn, soybean, and the like. The term also refers to the primary building blocks of the above—lignin, cellulose, hemicellulose, carbohydrates, etc. Prior to use in the methods of the present invention biomass may be processed by any means known in the art to produce a fermentable carbon source suitable for use in the present invention. In some embodiments, the fermentable carbon source is a biomass hydrolysate. The term “biomass hydrolysate” refers to the product resulting from saccharification of biomass such as lignocellulosic biomass. In some cases, the biomass is pretreated or pre-processed prior to saccharification, and saccharified enzymatically. In some embodiments, the fermentable carbon source is derived from lignin. In some embodiments, the fermentable carbon source comprises mixtures of lignin-derived aromatic monomers and/or lignin derived aromatic oligomers.

[0053] The 2-phenylethanol and 2-phenylacetic acid produced by the methods described herein can be recovered by any suitable means known in the art. Examples of separation methods that can be used to separate 2-phenylethanol and/or 2-phenylacetic acid from culture media include but are

not limited to solvent extraction, perstraction, gas stripping, vacuum stripping, pervaporation, adsorption, ion exchange adsorption, precipitation, and the like.

[0054] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, such as, MOLECULAR CLONING: A LABORATORY MANUAL, second edition (Sambrook et al., 1989) Cold Spring Harbor Laboratory Press; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel et al., eds., 1987 and annual updates); OLIGONUCLEOTIDE SYNTHESIS (M. J. Gait, ed., 1984); PCR: THE POLYMERASE CHAIN REACTION, (Mullis et al., eds., 1994); and MANUAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, Second Edition (A. L. Demain, et al., eds. 1999).

[0055] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

[0056] The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements, or method steps. The phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," "having," "containing," "involving," and variations thereof, is meant to encompass the items listed thereafter and additional items. Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed. Ordinal terms are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term), to distinguish the claim elements.

[0057] The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

[0058] As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

[0059] As used herein, the terms “approximately” or “about” in reference to a number are generally taken to include numbers that fall within a range of 5% in either direction (greater than or less than) the number unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value). Where ranges are stated, the endpoints are included within the range unless otherwise stated or otherwise evident from the context.

[0060] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

[0061] The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

EXAMPLES

[0062] Example 1

[0063] The embodiments described here demonstrate the production of 2-phenylethanol and 2-phenylacetic acid in engineered microorganisms via the heterologous metabolic pathways described herein.

[0064] **Materials and Methods**

[0065] Microorganisms. All strains used in this study are listed in Table 1. *E. coli* NEB 10-beta was obtained from New England Biolabs (NEB; Ipswich, MA) and was used for cloning and the

propagation of all plasmids. *E. coli* NST74 (ATCC 31884), a feedback resistant mutant of *E. coli* which overproduces Phe (Tribe, 1987), *P. putida* S12 (ATCC 700801), which served as the genetic source of *styAB*, *styC*, and *styABC*, and *S. cerevisiae* W303 (ATCC 200060), which served as the genetic source of *ARO10* were all purchased from the American Type Culture Collection (ATCC; Manassas, VA). *E. coli* strains JW1380-1, JW1843-2, JW1666-3, and JW2410-1 were obtained from the Coli Genetic Stock Center (CGSC; New Haven, CT) and served as the genetic source for the *feaB::FRT-kan^R-FRT*, *pykA::FRT-kan^R-FRT*, *pykF::FRT-kan^R-FRT*, and *crr::FRT-kan^R-FRT* deletion cassettes, respectively, along with wild-type *E. coli* BW25113. Chromosomal in-frame gene deletions in *E. coli* and subsequent *kan^R* marker removal were accomplished via a method modified from that of Datsenko and Wanner (Datsenko and Wanner, 2000), as previously described (Pugh et al., 2014).

[0066] Plasmid Construction. All plasmids constructed and used in this study are listed in Table 1. Custom DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Genomic DNA (gDNA) was prepared from cell cultures using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA) according to vendor protocols. All genes were PCR amplified with Q5 High-Fidelity DNA Polymerase (New England Biolabs (NEB)) using standard protocols. Amplified linear DNA fragments were purified using the Zymo Research DNA Clean & Concentrator Kit (Zymo Research) according to manufacturer protocols. Once purified, DNA fragments were then digested with appropriate restriction endonuclease enzymes at 37°C for > 6 hours (h). Digested fragments were gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) and ligated at room temperature for >1 h using T4 DNA ligase (NEB). Ligation reactions were transformed into chemically-competent *E. coli* NEB 10-beta (NEB) and selected by plating on Luria-Bertani (LB) solid agar containing appropriate antibiotics. Transformant pools were subsequently screened by colony PCR and restriction digest mapping. To enable high expression, the backbone of plasmid pY3 (Addgene plasmid #50606; a gift from Prof. Jay Keasling and originally derived from pBbA5a) (Juminaga et al., *Appl. Environ. Microbiol.* 78:89–98 (2012)), was used for expression of *PAL2* and *FDC1*, as well as *ARO10*. Plasmid pY-PAL2FDC1 was constructed by cloning a previously assembled operon composed of *PAL2* from *Arabidopsis thaliana* and *FDC1* from *S. cerevisiae* from pTpal-fdc (McKenna et al., *Biotechnol. J.* 8:1465–75 (2013)) using the primer pair described by SEQ ID NO:8 and SEQ ID NO:9 and inserting it into the pY3-derived backbone. Plasmid pTrcColaK-styABC was

constructed by cloning the native *styABC* operon from *P. putida* S12 using the primer pair described by SEQ ID NO:10 and SEQ ID NO:11 and inserting it into pTrcColaK.

[0067] Assaying SOI activity in whole resting cells. SOI activity was assayed in whole resting cells engineered to express *styC* from *P. putida* S12. More specifically, *E. coli* BW25113 was first transformed with either pTrcColaK-*styC* or pTrcColaK (as control). Seed cultures were prepared by growing individual colonies from LB-agar plates in 3 mL of LB for ~12 h at 32°C. Seed cultures were used to inoculate 50 mL of LB broth supplemented with 35 mg/L kanamycin in a 250 mL shake flask. Flasks were cultured at 32°C with shaking for ~8 h, at which time they were induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM. Following induction, cultures were incubated at 32°C overnight, after which cells were then harvested by centrifugation at 3,000 x g. The cell pellet was washed twice with pH 7.4 phosphate buffered saline (PBS) solution, and resuspended in 50 mL pH 7.4 PBS solution to a final cell density determined as an optical density at 600 nm (OD_{600}) of ~4. For the assay, a series of resting cell suspensions, each with a total volume of 50 mL in a 250 mL shake flask, were prepared at final cell densities of OD_{600} ~0.01, 0.03, and 0.07 (i.e., by resuspending an appropriate volume of the above stock suspension) in fresh pH 7.4 PBS solution supplemented with (*S*)-styrene oxide at an initial concentration of 1300 mg/L. Over the course of 6.5 hours, flasks were incubated at 32°C with shaking at 200 RPM while samples (each 0.5 mL) were periodically taken for HPLC analysis to determine concentrations of residual (*S*)-styrene oxide and produced 2-phenylacetaldehyde, as described below.

[0068] Assaying 2PE toxicity. The effects of 2PE on *E. coli* growth rate and yield was determined by monitoring the impacts of its exogenous addition at increasing final concentrations on growing cultures. Approximately 1 mL of an *E. coli* NST74 seed culture was used to inoculate 50 mL of LB broth in a 250 mL shake flask. When cultures reached OD_{600} ~0.6, 2PE was added to the flasks at an array of final concentrations ranging from 0 to 2 g/L. Culturing then resumed for an additional 6 h with periodic monitoring of OD_{600} .

[0069] Production of 2PE from glucose by engineered *E. coli*. Seed cultures were grown in 3 mL LB broth supplemented with appropriate antibiotics at 32°C for 12 – 16 h. Next, 0.5 mL of seed culture was used to inoculate 50 mL (in 250 mL shake flasks) of pH 6.8 MM1 – a phosphate-limited minimal media adapted from McKenna and Nielsen (McKenna and Nielsen, *Metab. Eng.* 13:544–54 (2011)), with the following recipe (in g/L): glucose (20), $MgSO_4 \cdot 7H_2O$ (0.5),

(NH₄)₂SO₄ (4.0), MOPS (24.7), KH₂PO₄ (0.3), and K₂HPO₄ (1.0), as well as 1 mL/L of a trace mineral solution containing (in g/L): Thiamine HCl (0.101), MnCl₂·4H₂O (1.584), ZnSO₄·7H₂O (0.288), CoCl₂·6H₂O (0.714), CuSO₄ (0.1596), H₃BO₃ (2.48), (NH₄)₆Mo₇O₂₄·4H₂O (0.370), and FeCl₃ (0.050). Once inoculated, cultures were grown at 32°C while shaking at 200 RPM until reaching an *OD*₆₀₀ of 0.8 (~8 h), at which time they were induced by addition of IPTG at a final concentration of 0.2 mM unless otherwise stated. Following induction, strains were cultured for a total of 72 hours (unless otherwise stated), during which time samples were periodically withdrawn for cell growth and metabolite analysis. Meanwhile, intermittently throughout each culture, pH was increased back to its initial value by adding a minimal volume (typically ~0.2-0.4 mL) of 0.4 g/L K₂HPO₄ solution.

[0070] Analytical Methods. Cell growth was measured as *OD*₆₀₀ using a UV/Vis spectrophotometer (Beckman Coulter DU800, Brea, CA). Culture samples were centrifuged at 11,000 x g for 4 min to pellet cells, after which 0.25 mL of the resulting supernatant was then transferred to a glass HPLC vial containing an equal volume of 1 N HCl before being sealed with a Teflon-lined cap. Analysis of all aromatic metabolites was performed via high performance liquid chromatography (HPLC; Agilent 1100 series HPLC, Santa Clara, CA) using a diode array (UV/Vis) detector. Separation was achieved on a reverse-phase 5µm Hypersil Gold C18 column (4.6 mm x 100 mm; Thermo Fisher, USA) operated at 45°C using mobile phase consisting of water with 0.1% formic acid (A) and methanol (B), flowing at a constant rate of 0.75 mL/min according to the following gradient: 5% B at 0 min, 5% to 80% B from 0 to 10.67 min, 80% B from 10.67 to 13.33 min, 80% to 5% B from 13.33 to 18.67 min, and 5% B from 18.67 to 20 min. The eluent was monitored using a diode array detector (DAD) set at 215 nm for detection of Phe, trans-cinnamic acid, styrene and (*S*)-styrene oxide, and 258 nm for detection of 2-phenylacetaldehyde, 2-phenylacetic acid, and 2PE. Glucose and acetate analysis, meanwhile, was performed using the same HPLC system equipped with a refractive index detector (RID) and an Aminex HPX-87H column (BioRad, Hercules, CA) operated at 35°C. The column was eluted using 5 mM H₂SO₄ as the mobile phase at a constant flow rate of 0.55 mL/min for 20 min. External calibrations were prepared and used to quantify each species of interest.

[0071] Results

[0072] Comparative Assessment of Alternative 2PE Pathways. As seen in FIG. 1, both the proposed, styrene-derived, and established, Ehrlich, pathways stem from precursors in the SA

pathway, namely Phe and phenylpyruvate, respectively. As such, both pathways share the same theoretical yield, estimated as 0.36 g/g on glucose (with functional PTS, based on estimates derived from Varma et al. (1993)). Moreover, both pathways converge at 2-phenylacetaldehyde before its reduction to 2PE, as has been reported to readily occur in *E. coli* via one or more native, NADPH-dependent alcohol dehydrogenases (ADHs; e.g., *yqhD*, *yahK*, *yjgB*) and/or aldo-keto reductases (AKRs; e.g., *dkgA*, *dkgB*, *yeaE*) (Kunjapur et al., *J. Am. Chem. Soc.* 136:11644–11654 (2014); Rodriguez and Atsumi, *Metab. Eng.* 25:227–37 (2014)). However, between the last common precursor (i.e., phenylpyruvate) and 2PE, the two pathways differ greatly and in several important ways. For instance, unlike the Ehrlich pathway, which employs only one foreign enzyme, the styrene-derived pathway is instead composed of four heterologous steps. However, despite its length, the thermodynamic driving force associated with the styrene-derived pathway is nearly 10-fold greater than that of the Ehrlich pathway. More specifically, when compared from phenylpyruvate to 2PE, the net change in Gibbs free energy of reaction ($\Delta_r G'^{\circ}$) for the Ehrlich pathway is -50.9 kJ/mol compared to -474.4 kJ/mol for the styrene-derived pathway (FIG. 2); the bulk of the difference being due to the highly favorable conversion of styrene to (*S*)-styrene oxide via styrene monooxygenase (NADH-dependent, encoded by *styAB*), which contributes -419.4 kJ/mol (or 88%) to the total $\Delta_r G'^{\circ}$ of the pathway (Flamholz et al., 2012). As a consequence, however, the styrene-derived pathway consumes twice as many reducing equivalents (1 NADH and 1 NADPH per molecule of 2PE produced) than the Ehrlich pathway (only 1 NADPH). Accordingly, whereas similarities certainly exist, both 2PE pathways appear to possess their own unique and inherent merits and limitations, the likes of which were next experimentally investigated.

[0073] Engineering 2PE Pathways. Construction of the styrene-derived 2PE pathway began from our previously-engineered (*S*)-styrene oxide pathway, comprised of *PAL2* from *A. thaliana*, *FDC1* from *S. cerevisiae*, and *styAB* from *P. putida* S12 (McKenna and Nielsen, 2011; McKenna et al., 2013). To then convert (*S*)-styrene oxide to 2-phenylacetaldehyde (before its reduction to 2PE by native ADHs/AKRs), however, it was first necessary to identify a suitable gene encoding SOI activity. Of particular interest was *styC* from *P. putida* S12 (Panke et al., *Appl. Environ. Microbiol.* 64:2032–43 (1998)), which together with *styAB*, functions as part of its native styrene degradation pathway (O'Connor et al., *Appl. Environ. Microbiol.* 61:544–8 (1995); Warhurst and Fewson, *J. Appl. Bacteriol.* 77:597–606 (1994)). Following the cloning and subsequent expression

of *styC* in *E. coli* BW25113 pTrcColaK-*styC*, a whole resting cell assay was performed wherein, as seen in FIG. 3, recombinant SOI activity was demonstrated via the conversion of exogenous (*S*)-styrene oxide to 2-phenylacetaldehyde (note: control experiments using *E. coli* BW25113 pTrcColaK showed no conversion of (*S*)-styrene oxide; data not shown). Initially, the assay was performed at a high cell density (i.e., $OD_{600} \sim 4$; representing that of a typical culture), however, under such conditions 100% conversion was achieved in <10 min with stoichiometric yield (data not shown). To slow the net reaction rate and allow for improved monitoring, the experiment was repeated at lower cell densities (specifically, OD_{600} of 0.01, 0.03, and 0.07). In this case, increasing cell density resulted in faster rates of (*S*)-styrene oxide consumption and 2-phenylacetaldehyde production, with the former reaching as high as 5.6 g/L-h. For comparison, when previously assayed under analogous conditions, *styAB*-expressing *E. coli* resting cells produced (*S*)-styrene oxide from exogenous styrene at rates reaching only as high as ~ 0.1 g/L-h; albeit at much higher cell densities ($OD_{600} \sim 1$).

[0074] Based on this result, *styC* was cloned for expression as part of the full, styrene-derived pathway, in this case as part of the natural *styABC* operon (encoding both SMO and SOI) and expressed via a P_{trc} promoter as plasmid pTrcColaK-*styABC*. To complete the pathway, *PAL2* and *FDC1* were cloned in a synthetic operon for expression via P_{lacUV5} on plasmid pY-PAL2FDC1. In the case of the Ehrlich pathway, meanwhile, PPDC plays a key role as the first committed pathway step. Previously, Atsumi *et al.* evaluated 5 different PPDC isozymes (namely those encoded by *ARO10*, *PDC6* and *THI3* from *S. cerevisiae*, *kivd* from *L. lactis*, and *pdc* from *C. acetobutylicum*) in *E. coli*, ultimately finding *ARO10* to support the greatest 2PE production from glucose (Atsumi *et al.*, 2008). Accordingly, *ARO10* was fused to a P_{lacUV5} promoter for its expression from pY-*ARO10*.

[0075] Demonstrating and Comparing 2PE Production via Alternative Pathways. The Ehrlich and styrene-derived pathways were both constructed as described in Table 1 and first introduced and expressed in *E. coli* NST74 (a previously-engineered, Phe-overproducing strain (Tribe, US Patent No. 4681852)), with the resulting strains producing 158 ± 12 and 182 ± 4 mg/L of 2PE, respectively. However, in addition to 2PE, both strains also co-produced 2-phenylacetic acid as a major byproduct, whose final titers reached 352 ± 12 and 503 ± 21 mg/L, respectively. In *E. coli*, 2-phenylacetaldehyde is converted to 2-phenylacetic acid via its native, NAD^+ -dependent 2-phenylacetaldehyde dehydrogenase, encoded by *feaB* (FIG. 1) (Parrott *et al.*, 1987). In this case,

the ~1.5-fold greater 2-phenylacetic acid production accompanying the styrene-derived pathway was likely due to its aforementioned increased redox requirement, which would be partially balanced via oxidation of 2-phenylacetaldehyde to 2-phenylacetic acid (regenerating 1 NADH; FIG. 1). To eliminate undesirable accumulation of 2-phenylacetic acid, *feaB* was next deleted from NST74. When introduced and expressed in NST74 Δ *feaB*, 2-phenylacetic acid production was no longer detected for either the Ehrlich or styrene-derived pathway and, after 72 hours, 2PE titers now reached 552 ± 14 and 643 ± 29 mg/L, respectively; in both cases at similar glucose yields (35.1 ± 0.5 and 37.7 ± 1.2 mg/g, or 9.7 and 10.5% of the theoretical maximum).

[0076] To assess if 2PE production in these initial strains was perhaps limited by end-product inhibition, a growth challenge study was performed determine to the response of *E. coli* growth to the addition of exogenous 2PE at a range of increasing final concentrations (FIG. 4). While growth rate and yield were reduced in the presence of as little as 1 g/L 2PE, severe growth inhibition did not occur until reaching about 2 g/L 2PE. This compares well with prior reports wherein 2PE was reported to inhibit *E. coli* at levels of ~1 g/L (Kang et al., 2014), and suggests that, at least in these initial strains, 2PE production by either pathway was likely not yet limited by end-product inhibition.

[0077] Host Strain Engineering to Increase Precursor Availability. Robust 2PE production by either pathway depends on ample production of SA pathway precursors (FIG. 1), which in turn is known to benefit from increased availability of phosphoenolpyruvate (PEP). Noda et al. previously reported deletion of *pykF* and *pykA* (encoding pyruvate kinase isozymes I and II, respectively, which convert PEP to pyruvate, producing ATP) as an effective strategy for promoting PEP availability, in their case also enhancing the production of various chorismate-derived aromatic products (Noda et al., 2016). Meanwhile, it has been further demonstrated that PEP availability can be improved via the partial inactivation (i.e., by deleting *crr*, encoding IIA^{Glc}) of the glucose-specific phosphotransferase system (PTS; which facilitates glucose uptake via its phosphorylation at the expense of PEP) (Gosset, 2005). Said mutation also further benefits the culture by reducing rates of glucose uptake which, in turn, also decreases overflow metabolism and the associated production of unwanted (and potentially inhibitory) acetate (Gosset, 2005; Liu et al., 2014). Accordingly, NST74 Δ *feaB* was further engineered to systematically introduce Δ *pykA*, Δ *pykF*, and Δ *crr* mutations, upon which the resulting strains were tested for their relative ability to support 2PE production via the two pathways. The resulting 2PE titers are compared in FIG. 5, along with

the relative effects of the same mutations on Phe production by each host strain (i.e., in the absence of either pathway) for comparison. As can be seen, compared to the above results using NST74 $\Delta feaB$ as host, deletion of *crr* had a significant effect on 2PE production by both the Ehrlich and styrene-derived pathways, improving final titers by 77% and 67%, respectively. Deletion of *crr* also resulted in reductions in acetate accumulation, in each case by 45-60%. Meanwhile, the additional, combined deletion of both *pykA* and *pykF* led to even further improvements in 2PE production by both the Ehrlich and styrene-derived pathways, reaching 1163 ± 3 and 1468 ± 47 mg/L (or 9.52 ± 0.02 and 12.02 ± 0.38 mM), respectively, after 72 hours (increases of 19% and 37% relative to using NST74 $\Delta feaB \Delta crr$ as host). Interestingly, as is most prominent in the case of the styrene-derived pathway, individual deletion of just *pykA* or *pykF* alone gave little or no improvement, suggesting that full inactivation of pyruvate kinase activity is necessary to realize the beneficial effects of this strategy. That said, analogous experiments in the absence of the pathway (i.e., for Phe production) suggest the $\Delta pykA$ mutation to perhaps be most important (FIG. 5). For comparison, in the absence of either pathway, NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ produced a total of 2076 ± 19 mg/L (12.57 ± 0.11 mM) Phe. Accordingly, and assuming constant flux through the SA pathway in each case, this suggests that the styrene-derived pathway was more efficient than the Ehrlich pathway (96 vs. 76%) at assimilating and ultimately converting their corresponding endogenous precursor to 2PE.

[0078] Interestingly, acetate production via the styrene-derived pathway was minimal (i.e., 0.5-0.44 g/L) regardless of which host background was used and, in all cases, was 14- to 71-fold lower than when expressing the Ehrlich pathway. Most strikingly, although in the absence of either pathway acetate accumulation was undetected with NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$, upon introduction of the Ehrlich pathway, acetate levels rose back up to 5.23 ± 0.06 g/L. As said behavior was unique to the Ehrlich pathway, we hypothesized that acetate production could perhaps be occurring as a result of ARO10 promiscuity. Decarboxylation of pyruvate, for example, yields acetaldehyde which, in turn, could be oxidized to acetate via *E. coli*'s NADP⁺-dependent aldehyde dehydrogenase (encoded by *aldB*) (Ho and Weiner, 2005). To provide an initial assessment of this proposed phenomena, control cultures were prepared of *E. coli* BW25113 pY-ARO10 which were then grown in the absence or presence of 6 g/L exogenously-supplied sodium pyruvate. After 48 hours, as seen in Table 2, accumulated acetate levels were 3.8-fold higher following sodium pyruvate addition (5.41 vs. 1.41 g/L). While more detailed characterizations are

needed, these findings certainly support the proposed, ARO10-associated mechanism of acetate accumulation.

[0079] Culture Condition Manipulation to Further Improve 2PE Production. Induction timing and initial substrate concentration were next manipulated to further improve 2PE production. In the first case, the timing of IPTG-induced expression of the Ehrlich and styrene-derived pathways in NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ was investigated at six different points (from inoculation to late exponential phase), the results of which are compared in FIG. 6. In both cases, induction at inoculation gave the greatest final 2PE titers, suggesting greater net flux through each pathway was realized when each was given maximal time to compete for endogenous precursors (consistent with observations of reduced biomass production at earlier inductions; FIG. 6). In contrast, when induced too late (i.e., at 19 hours or beyond), neither pathway effectively competed for its requisite precursor, which instead was then assimilated into additional biomass and/or accumulated Phe. In the case of the Ehrlich pathway, net acetate accumulation followed a similar pattern to that of 2PE production, with less build-up occurring for later inductions (FIG. 6 and FIG. 7; reductions in 2PE and acetate production were 66% and 71%, respectively, when cultures were induced at inoculation versus after 29 hours); an observation that further supports the above hypothesis that significant acetate byproduct formation is ARO10-associated, perhaps resulting due to its promiscuity.

[0080] All of the above 2PE production studies were performed by initially supplying each culture with 20 g/L glucose which, in all cases, was fully consumed within 72 hours (data not shown). As this suggests a possible substrate limitation, a series of batch experiments were next performed wherein increasing amounts of initial glucose (5 to 50 g/L) were instead supplied, in all cases using the best-performing host strain (i.e., NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$) and optimal induction timing (i.e., at inoculation). FIG. 8 compares glucose consumption, along with 2PE yield and final titers for both pathways. In all cases, glucose is fully consumed when initially supplied at 5 to 30 g/L, with higher initial glucose levels resulting in increased 2PE titers. At higher initial glucose concentrations (i.e., 40 and 50 g/L), however, clear differences emerge with respect to the two pathways. Though also declining (perhaps due to a nutrient limitation or onset of substrate inhibition), greater conversion at higher glucose loadings and, as a result, increased 2PE titers and yields remain possible via the styrene-derived pathway. Ultimately, when supplied with 50 g/L glucose, 2PE titers via the styrene-derived pathway reached their maximum level of 1941 ± 13

mg/L at a yield of 60.5 ± 0.3 mg/g (16.8% of theoretical); a final 2PE titer ~2-fold greater than the highest value reported to date for *E. coli* expressing the Ehrlich pathway.

[0081] A series of batch cultures were lastly performed to investigate the dynamics of 2PE production via both pathways, in each case utilizing NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$ as host while supplying 30 g/L glucose (to ensure full utilization) and performing induction at inoculation. FIG. 9 compares glucose consumption, 2PE production, and biomass accumulation in each case. Initially, rates of all three are slower for the styrene-derived pathway. Notably, for instance, while expressing the Ehrlich pathway, average volumetric rates of glucose consumption and 2PE production during the first 24 h were 401 ± 13 and 26.8 ± 0.2 mg/L-h, respectively, compared to just 248 ± 7 and 15.3 ± 0.1 mg/L-h for the styrene-derived pathway. However, by ~36 h, 2PE production by the Ehrlich pathway (which occurred coincidentally with cell growth) levels off, whereas production continues for an additional ~30 h via the styrene-derived pathway (during which time cell growth had already entered the stationary phase). Ultimately, after 87 hours, final 2PE titers reached 1823 ± 16 and 1212 ± 17 mg/L for the styrene-derived and Ehrlich pathways, respectively, at yields of 60.8 ± 0.5 and 40.4 ± 0.6 mg/g – both improvements of ~50%. Finally, it should be noted that, by 36 hours, acetate accumulation in cultures expressing the Ehrlich pathway had surpassed 1.4 g/L while remaining under 0.5 g/L for the styrene-derived pathway (data not shown), suggesting that the ability of the styrene-derived pathway to mitigate inhibitory byproduct accumulation might contribute to its capacity to maintain longer periods of productivity and greater net 2PE production.

[0082] Discussion and Conclusion

[0083] A novel route to 2PE has been engineered as a robust alternative to the established, Ehrlich pathway. Ultimately, for example, when compared under otherwise analogous conditions (FIG. 9), 2PE titers and yields were about 50% greater via the styrene-derived versus Ehrlich pathway, with final titers capable of approaching ~2 g/L with additional glucose supplementation. As characterized via both *in silico* analyses and experimental studies, relative to the Ehrlich pathway, the styrene-derived 2PE pathway was found to possess its own unique and notable advantages, as well as certain caveats. For example, as has been previously characterized with respect to (*S*)-styrene oxide production (also produced via styrene, as in FIG. 1) (McKenna et al., 2013), the highly favorable SMO reaction (which is largely responsible for the ~10-fold greater thermodynamic driving force of the styrene-derived pathway), serves to effectively ‘pull’ more

precursor (i.e., Phe) into the pathway. This phenomenon is further supported in the case of 2PE production, noting that conversion of the endogenous precursors via the styrene-derived pathway was 96% versus just 76% by the Ehrlich pathway. Additionally, and in contrast to the Ehrlich pathway, which branches off from native metabolism (i.e., at phenylpyruvate), the styrene-derived pathway instead extends from a terminal pathway metabolite (i.e., Phe; FIG. 1). In this way, the styrene-derived pathway also importantly avoids introducing a competitive ‘branch point’. In the Ehrlich pathway, for example, as PPDC (e.g., ARO10, $K_m = 100 \mu\text{M}$ (Kneen et al., 2011)) must directly compete against native Phe aminotransferase (i.e., TyrB, $K_m = 12 \mu\text{M}$ (Gelfand and Steinberg, 1977)) for available phenylpyruvate, kinetic limitations can in turn reduce the flux of metabolites that enter the pathway at its first committed step. Of course, deletion of *tyrB* eliminates such competition, preserving phenylpyruvate for the Ehrlich pathway, however, said mutation comes at the cost of a Phe auxotrophy, thereby necessitating its supplementation and leading to increased media costs and reduced scalability. Viewed in this way, the styrene-derived pathway more broadly provides improved compatibility with the host background.

[0084] One of the most significant differences between employing the two 2PE pathways concerns not the product, but rather a byproduct, namely acetate. Previously, as further demonstrated here, improvements in the production of Phe and/or other aromatic derivatives can be realized by inactivation of *crr*, *pykA*, and *pykF*. Whereas deletion of *crr* reduces rates of glucose consumption and thus acetate production (Liu et al., 2014), Noda *et al.* reported a 4.5-fold decrease in acetate yield by deleting both *pykA* and *pykF* (Noda *et al.*, 2016). However, as evidenced by the results of FIG. 5, even when using a host background virtually deficient in acetate accumulation (i.e., NST74 $\Delta feaB \Delta crr \Delta pykA \Delta pykF$), acetate production reemerged upon introduction of the Ehrlich pathway, reaching final concentrations as high as $5.23 \pm 0.06 \text{ g/L}$. Previous studies have found that acetate concentrations above 1 g/L can deter biomass and protein production, reduce protein stability, and lower pH, causing cell lysis (De Mey et al., 2007). Accordingly, and regardless of the specific mechanism, the ability to avoid acetate byproduct accumulation when employing the styrene-derived pathway is postulated as a significant reason for the ability of this pathway to support superior 2PE production metrics. Though still warranting further investigation, acetate byproduct accumulation when expressing the Ehrlich pathway is thought to be a result of ARO10 promiscuity. Although prior reports suggest that, at least with respect to its native expression in *S. cerevisiae*, ARO10 displays minimal activity on pyruvate (with *in vitro* assays

reporting $k_{cat}/K_m = 200$ and $0.035 \text{ mM}^{-1} \cdot \text{s}^{-1}$ for phenylpyruvate and pyruvate, respectively (Kneen et al., 2011)), here, the experimental evidence presented suggests otherwise, that is at least with respect to its recombinant *in vivo* function in *E. coli*. That said, whereas such effects might be avoided by constructing the Ehrlich pathway using a PPDC with greater recombinant specificity, to the best of our knowledge, such an isozyme has so far not been identified/reported. Thus, for now at least, an additional advantage of the styrene-derived pathway appears to the greater substrate specificity of its associated enzymes. And, as high acetate accumulation can be a substantial hurdle in scale-up, especially with high glucose levels (Xu et al., 1999), such prospects might be improved by the alternative application of this novel pathway.

[0085] With final 2PE titers via the styrene-derived pathway ultimately approaching $\sim 2 \text{ g/L}$ (at high glucose loading), and in contrast to preliminary cultures, said output now approaches the toxicity limit of 2PE. As the mode of aromatic toxicity against bacteria has most commonly been reported to be associated with their accumulation within and disruption of the cytoplasmic membrane (Sikkema et al., 1994), a similar phenomenon was also anticipated here. In fact, with a toxicity threshold determined as $\sim 2 \text{ g/L}$, the present observations of 2PE toxicity agree well with previously-reported model used to predict the toxicity of various aromatic bioproducts (e.g., styrene, (*S*)-styrene oxide, and various phenolics) based on estimates of the membrane-water partitioning coefficient ($K_{M/W}$) (McKenna et al., 2013). Meanwhile, various strategies for *in situ* 2PE removal have also been investigated, including, for example, via its extraction in a biphasic ionic liquid system which gave 3- to 5-fold increases in 2PE production by *S. cerevisiae* (Sendovski et al., 2010). Other approaches, meanwhile, including pervaporation (Etschmann et al., 2005) and solid-phase extraction (i.e., using hydrophobic resins) (Achmon et al., 2011) have shown as high as 10-fold improvements in 2PE productivity, and would likely provide similar benefits to the strains developed here.

Table 1. Strains, plasmids, and pathways constructed and/or used in this Example.

Strains	Description	Source
<i>E. coli</i> NST74	<i>aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr), pheO352, aroG397(fbr)</i>	ATCC 31884

<i>E. coli</i> BW25113	$\Delta(araD-araB)567$, $\Delta lacZ4787::rrnB-3$, λ^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	CGSC
<i>E. coli</i> NEB-10 beta	<i>araD139</i> $\Delta(ara, leu)7697$ <i>fhuA lacX74 galK16 galE15</i> <i>mcrA f80d(lacZΔM15)recA1 relA1 endA1 nupG rpsL</i> <i>rph spoT1Δ(mrr-hsdRMS-mcrBC)</i>	NEB
<i>S. cerevisiae</i> W303	Source of <i>ARO10</i> , <i>FDC1</i>	ATCC 200060
<i>P. putida</i> S12	Source of <i>styABC</i>	ATCC 700801
<i>E. coli</i> NST74 $\Delta feaB$	$\Delta feaB$ mutation in <i>E. coli</i> NST74	This Example
<i>E. coli</i> NST74 $\Delta feaB \Delta crr$	Δcrr mutation in <i>E. coli</i> NST74 $\Delta feaB$	This Example
<i>E. coli</i> NST74 $\Delta feaB \Delta crr$ $\Delta pykA$	$\Delta pykA$ mutation in <i>E. coli</i> NST74 $\Delta feaB \Delta crr$	This Example
<i>E. coli</i> NST74 $\Delta feaB \Delta crr$ $\Delta pykF$	$\Delta pykF$ mutation in <i>E. coli</i> NST74 $\Delta feaB \Delta crr$	This Example
<i>E. coli</i> NST74 $\Delta feaB \Delta crr$ $\Delta pykA \Delta pykF$	$\Delta pykF$ mutation in <i>E. coli</i> NST74 $\Delta feaB \Delta crr \Delta pykA$	This Example
Plasmids	Features and/or Construction	Source
pTrcColaK	ColA ori, <i>lacI^q</i> , <i>Kan^r</i> , <i>P_{Trc}</i>	McKenna et al. (2013)
pBbA5a (via pY3)	p15A ori, <i>lacI</i> , <i>Amp^r</i> , <i>P_{lacUV5}</i>	Juminaga et al. (2012)
pCP20	FLP, ts-rep, [cI857](λ)(ts), <i>Amp^r</i>	CGSC
pKD46	repA101(ts) and R101 ori, <i>Amp^r</i> , <i>araC</i> , <i>araBp</i>	CGSC

pTpal-fdc	<i>PAL2</i> from <i>A. thaliana</i> and <i>FDC1</i> of <i>S. cerevisiae</i> inserted into the <i>NcoI</i> and <i>XbaI</i> and <i>SbfI</i> and <i>HindIII</i> sites of pTrc99A	McKenna et al. (2013)
pY-PAL2FDC1	<i>PAL2-FDC1</i> operon from pTpal-fdc inserted into the <i>BglIII</i> and <i>XhoI</i> sites of pY3	This Example
pTrcColaK-styC	<i>styC</i> of <i>P. putida</i> S12 inserted into the <i>PstI</i> and <i>HindIII</i> sites of pTrcColaK	This Example
pTrcColaK-styABC	<i>styABC</i> of <i>P. putida</i> S12 inserted into the <i>XbaI</i> and <i>HindIII</i> sites of pTrcColaK	This Example
pY-ARO10	<i>ARO10</i> of <i>S. cerevisiae</i> inserted into the <i>BglIII</i> and <i>XhoI</i> sites of pY3	This Example
Pathways	Description: Composed of plasmids	Source
Ehr	‘Ehrlich’ pathway: pY-ARO10	This Example
Sty	‘Styrene-derived’ pathway: pY-PAL2FDC1 and pTrcColaK-styABC	This Example

[0086] Table 2. Acetate accumulation in cultures of *E. coli* BW25113 pY-aro10 grown in pH 6.8 MM1 media supplemented with sodium pyruvate at a total concentration of 0 or 6 g/L (note: sodium pyruvate was added periodically through the cultures, at each of 8, 18 and 27 h, in each case being added at a final concentration of 2 g/L).

Hours after inoculation [h]	0 g/L sodium pyruvate fed	6 g/L sodium pyruvate fed
	Acetate [g/L]	
18	0.09	0.54
27	0.49	0.95
48	1.41	5.41

[0087] Example 2

[0088] The embodiments described here demonstrate the production of 2-phenylethanol (2PE) in engineered microorganisms via the heterologous metabolic pathways described herein.

[0089] *Materials and Methods*

[0090] All genes were PCR amplified using a BioRad iCycler system, Phusion DNA Polymerase (New England Biolabs, Ipswich, MA, USA), and custom oligonucleotide primers. PCR cycling and reaction conditions were standardized according to manufacturer instructions. All PCR amplified DNA fragments were purified using the Zyppy DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA). Gene fragments and plasmids were treated by endonuclease digestion according to manufacturer's protocols. All digested fragments were first gel purified using the Zyppy DNA purification kit (Zymo Research, Irvine, CA, USA) and then ligated with T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA) at 4°C overnight before the mixture was then transformed into chemically competent *E. coli* NEB10-Beta. Transformants were selected on LB solid agar with appropriate antibiotics and cultured at 37°C overnight. Transformant pools were screened using colony PCR with final confirmation by gene sequencing. *PAL2* was amplified from cDNA of clone U12256 from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) using primers given by SEQ ID NO: 12 and SEQ ID NO: 13 and cloned into pTrc99a, resulting in construction of the plasmid pTpal. *FDC1* was amplified from gDNA of *Saccharomyces cerevisiae* using primers given by SEQ ID NO: 15 and SEQ ID NO: 15 and cloned into pTpal, resulting in construction of the plasmid pTpal-fdc.

[0091] Seed cultures were grown in 3mL LB broth supplemented with appropriate antibiotics at 32°C for 12–16 h. Next, 0.5mL of seed culture was used to inoculate 50mL (in 250mL shake flasks) of pH 6.8 MM1 – a phosphate-limited minimal media adapted from McKenna and Nielsen, with the following recipe (in g/L): glucose (20), MgSO₄·7H₂O (0.5), (NH₄)₂SO₄ (4.0), MOPS (24.7), KH₂PO₄ (0.3), and K₂HPO₄ (1.0), as well as 1mL/L of a trace mineral solution containing (in g/L): MnCl₂·4H₂O (1.584), ZnSO₄·7H₂O (0.288), CoCl₂·6H₂O (0.714), CuSO₄ (0.1596), H₃BO₃ (2.48), (NH₄)₆Mo₇O₂₄·4H₂O (0.370), and FeCl₃ (0.050). Once inoculated, cultures were grown at 32°C while shaking at 200 RPM until reaching an OD₆₀₀ of 0.8 (~8 h), at which time they were induced by addition of IPTG at a final concentration of 0.2 mM. Following induction, strains were cultured for a total of 72 h (unless otherwise stated), during which time samples were periodically withdrawn for cell growth and metabolite analysis. Meanwhile, intermittently

throughout each culture, pH was increased back to its initial value by adding a minimal volume (typically ~0.2–0.4mL) of 0.4 g/L K₂HPO₄ solution.

[0092] Results

[0093] As a proof of concept, pTpal-fdc was co-transformed with pTrcColaK-styABC into *E. coli* NST74 with a *feaB* deletion (NST74Δ*feaB*). After culturing for 96 h, the cultures grew to an OD₆₀₀ of 11.0 ± 1.0 with 2PE titers final reaching 759.8 ± 3.0 mg/L with no accumulation of intermediates (including L-phenylalanine). Additionally, the same two plasmids were also co-transformed into NST74Δ*feaB* with various additional deletions shown to improve production of L-phenylalanine (i.e., deletion of *crr*, *pykA*, *pykF*).

[0094] Table 3 (below) demonstrates production of 2PE after 72 hours of culturing in MM1 media by *E. coli* NST74Δ*feaB* with engineered with additional deletions to the genes *crr*, *pykA*, and/or *pykF*. Each strain was co-transformed with pTpal-fdc and pTrcColaK-styABC. In this case, it can be seen that NST74Δ*feaB* was the host strain that supported the highest 2PE production using these plasmids.

Table 3. 2PE Production

Host Strain	2PE Titer (mg/L)
NST74Δ <i>feaB</i> Δ <i>crr</i>	507 ± 159
NST74Δ <i>feaB</i> Δ <i>pykA</i>	688 ± 354
NST74Δ <i>feaB</i> Δ <i>crr</i> Δ <i>pykA</i>	605 ± 28
NST74Δ <i>feaB</i> Δ <i>crr</i> Δ <i>pykF</i>	704 ± 348
NST74Δ <i>feaB</i> Δ <i>crr</i> Δ <i>pykA</i> Δ <i>pykF</i>	531 ± 158

Sequence Listing

SEQ ID NO:1 is the nucleotide sequence of a gene from *A. thaliana* encoding an phenylalanine ammonia-lyase (*PAL2*).

SEQ ID NO:2 is the nucleotide sequence of a gene from *S. cerevisiae* encoding a ferulate decarboxylase (*FDC1*).

SEQ ID NO:3 is the nucleotide sequence of a gene from *P. putida* S12 encoding subunit A of a styrene monooxygenase (*styA*).

SEQ ID NO:4 is the nucleotide sequence of a gene from *P. putida* S12 encoding subunit B of a styrene monooxygenase (*styB*).

SEQ ID NO:5 is the nucleotide sequence of a native gene cluster from *P. putida* S12 encoding both A and B subunits of a styrene oxide isomerase (*styAB*).

SEQ ID NO:6 is the nucleotide sequence of a gene from *P. putida* S12 encoding a styrene oxide isomerase (*styC*).

SEQ ID NO:7 is the nucleotide sequence of a native operon from *P. putida* S12 encoding both A and B subunits of a styrene oxide isomerase as well as a styrene oxide isomerase (*styABC*).

SEQ ID NO:8 is a primer used to amplify *PAL2* and *FDC1* from pTpal-fdc (McKenna, R., Pugh, S., Thompson, B. and Nielsen, D. R. (2013), Microbial production of the aromatic building-blocks (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol from renewable resources. Biotechnology Journal, 8: 1465–1475. doi:10.1002/biot.201300035).

SEQ ID NO:9 is a primer used to amplify *PAL2* and *FDC1* from pTpal-fdc (McKenna, R., Pugh, S., Thompson, B. and Nielsen, D. R. (2013), Microbial production of the aromatic building-blocks (*S*)-styrene oxide and (*R*)-1,2-phenylethanediol from renewable resources. Biotechnology Journal, 8: 1465–1475. doi:10.1002/biot.201300035).

SEQ ID NO:10 is a primer used to amplify *styABC* from *P. putida* S12.

SEQ ID NO:11 is a primer used to amplify *styABC* from *P. putida* S12.

SEQ ID NO:12 is a primer used to amplify *PAL2* from *A. thaliana* cDNA from clone U12256.

SEQ ID NO:13 is a primer used to amplify *PAL2* from *A. thaliana* cDNA from clone U12256.

SEQ ID NO:14 is a primer used to amplify *FDC1* from *S. cerevisiae*.

SEQ ID NO:15 is a primer used to amplify *FDC1* from *S. cerevisiae*.

SEQ ID NO:1

LENGTH: 2154

TYPE: DNA

ORGANISM: *Arabidopsis thaliana*

SEQUENCE: 1

ATGGATCAAATCGAAGCAATGTTGTGCGGCGGAGGAGAGAAGACAAAAGTGGCGG
TTACTACGAAGACTTTGGCAGATCCATTGAATTGGGGTTTAGCAGCGGATCAAATGA
AAGGAAGTCATTTAGATGAAGTGAAGAAGATGGTCGAAGAGTATCGTAGACCAGTC
GTGAATCTTGGCGGAGAAACACTGACGATCGGACAAGTTGCTGCCATCTCCACCGTA
GGAGGCAGCGTTAAGGTTGAGTTAGCGGAGACTTCAAGAGCCGGTGTGAAAGCTAG
CAGTGATTGGGTTATGGAGAGCATGAACAAAGGTACTGACAGTTACGGAGTCACCA
CCGGCTTTGGTGCTACTTCTCACCGGAGAACCACAAAACGGCACCGCATTACAAACA
GAACTCATTAGATTTTTGAACGCCGGAATATTCGGAAACACGAAGGAGACATGTCA
CACACTGCCGCAATCCGCCACAAGAGCCGCCATGCTCGTCAGAGTCAACACTCTTCT
CCAAGGATACTCCGGGATCCGATTCGAGATCCTCGAAGCGATTACAAGTCTCCTCAA
CCACAACATCTCTCCGTCACCTCTCCGTGGAACCATTACCGCCTCCGGCGATCT
CGTTCCTCTCTCTTACATCGCCGGAATCTCACCGGCCGTCTTAATTCAAAGCCACC
GGTCCCGACGGTGAATCGCTAACC CGCGAAAGAAGCTTTTGAGAAAGCCGGAATCAG
TACTGGATTCTTCGATTTACAACCTAAGGAAGGTTTAGCTCTCGTTAATGGCACGGC
GGTTGGATCTGGAATGGCGTCGATGGTTCTATTCTGAAGCGAATGTCCAAGCGGTGTT
AGCGGAGGTTTTATCAGCGATCTTCGCGGAGGTTATGAGCGGGAAACCTGAGTTTAC
CGATCATCTGACTCATCGTTTAAAACATCATCCCGGACAAATCGAAGCGGCGGCGAT
AATGGAGCACATACTCGACGGAAGCTCATACATGAAATTAGCTCAAAAGGTTACG
AGATGGATCCATTGCAGAAACCAAAACAAGATCGTTACGCTCTTCGTACATCTCCTC
AATGGCTAGGTCCTCAAATTGAAGTAATCCGTCAAGCTACGAAATCGATAGAGCGT
GAAATCAACTCCGTTAACGATAATCCGTTGATCGATGTTTCGAGGAACAAGGCGATT
CACGGTGGTAACTTCCAAGGAACACCAATCGGAGTTTCTATGGATAACACGAGATT
GGCGATTGCTGCGATTGGGAAGCTAATGTTTGCTCAATTCTCTGAGCTTGTTAATGA
TTTCTACAACAATGGACTTCCTTCGAATCTAACTGCTTCGAGTAATCCAAGTTTGGAT
TATGGATTCAAAGGAGCAGAGATTGCTATGGCTTCTTATTGTTCTGAGCTTCAATAC
TTGGCTAATCCAGTCACAAGCCATGTTCAATCAGCTGAGCAACATAATCAAGATGTG
AACTCTCTTGGTTTGATCTCGTCTCGTAAAACATCTGAAGCTGTGGATATTCTTAAGC
TAATGTCAACAACGTTCTTGTGGGGATATGTCAAGCTGTTGATTTGAGACATTTGG
AGGAGAATCTGAGACAAACTGTGAAGAACACAGTTTCTCAAGTTGCTAAGAAAGTG
TTAACC ACTGGAATCAACGGTGAGTTACATCCGTCAAGGTTTTGCGAGAAGGACTTG
CTTAAGGTTGTTGATCGTGAGCAAGTGTTACGTATGTGGATGATCCTTGTAGCGCT
ACGTACCCGTTGATGCAGAGACTAAGACAAGTTATTGTTGATCACGCTTTGTCCAAC
GGTGAGACTGAGAAGAATGCAGTGACTTCGATCTTTCAAAGATTGGAGCTTTTGAA
GAGGAGCTTAAGGCTGTGCTTCAAAGGAAGTTGAAGCGGCTAGAGCGGCTTATGG
GAATGGA ACTGCGCCGATTCTTAACCGGATTAAGGAATGTAGGTCGTATCCGTTGTA
TAGGTTCTGTAGGGAAGAGCTTGGAACGAAGTTGTTGACTGGAGAAAAGGTTGTGT
CTCCGGGAGAGGAGTTTGATAAGGTCTTCACTGCTATGTGTGAAGGTAACTTATTG
ATCCGTTGATGGATTGTCTCAAGGAATGGAACGGAGCTCCGATTCCGATTGCTAA

SEQ ID NO:2

LENGTH: 1512

TYPE: DNA

ORGANISM: *Saccharomyces cerevisiae*

SEQUENCE: 2

ATGAGGAAGCTAAATCCAGCTTTAGAATTTAGAGACTTTATCCAGGTCTTAAAAGAT
GAAGATGACTTAATCGAAATTACCGAAGAGATTGATCCAAATCTCGAAGTAGGTGC
AATTATGAGGAAGGCCTATGAATCCCACCTTACCAGCCCCGTTATTTAAAAATCTCAA
AGGTGCTTCGAAGGATCTTTTCAGCATTTTAGGTTGCCAGCCGGTTTGAGAAGTAA
GGAGAAAGGAGATCATGGTAGAATTGCCCATCATCTGGGGCTCGACCCAAAAACAA
CTATCAAGGAAATCATAGATTATTTGCTGGAGTGTAAGGAGAAGGAACCTCTCCCCC
CAATCACTGTTTCTGTGTCATCTGCACCTTGTAACACATATACTTTCTGAAGAAA
AAATACATCTACAAAGCCTGCCAACACCATATCTACATGTTTCAGACGGTGGCAAGT
ACTTACAAACGTACGGAATGTGGATTCTTCAAACCTCCAGATAAAAAATGGACTAATT
GGTCAATTGCTAGAGGTATGGTTGTAGATGACAAGCATATCACTGGTCTGGTAATTA
AACCACAACATATTAGACAAATTGCTGACTCTTGGGCAGCAATTGGAAAAGCAAAT
GAAATTCCTTTCGCGTTATGTTTTGGCGTTCCCCCAGCAGCTATTTAGTTAGTTCCA
TGCCAATTCCTGAAGGTGTTTCTGAATCGGATTATGTTGGCGCAATCTTGGGTGAGT
CGTTTCCAGTAGTAAAATGTGAGACCAACGATTTAATGGTTCCTGCAACGAGTGAG
ATGGTATTTGAGGGTACTTTGTCCTTAACAGATACACATCTGGAAGGCCCATTTGGT
GAGATGCATGGATATGTTTTCAAAGCCAAGGTCATCCTTGTCCATTGTACACTGTC
AAGGCTATGAGTTACAGAGACAATGCTATTCTACCTGTTTCGAACCCCGGTCTTTGT
ACGGATGAGACACATACCTTGATTGGTTCCTAGTGGCTACTGAGGCCAAGGAGCT
GGCTATTGAATCTGGCTTGCCAATTCTGGATGCCTTTATGCCTTATGAGGCTCAGGCT
CTTTGGCTTATCTTAAAGGTGGATTTGAAAGGGCTGCAAGCATTGAAGACAACGCCT
GAAGAATTTTGTAAAGAGGTAGGTGATATTTACTTTAGGACAAAAGTTGGTTTTATA
GTCCATGAAATAATTTTGGTGGCAGATGATATCGACATATTTAACTTCAAAGAAGTC
ATCTGGGCCTACGTTACAAGACATACACCTGTTGCAGATCAGATGGCTTTTGATGAT
GTCACTTCTTTTCCTTTGGCTCCCTTTGTTTCGCAGTCATCCAGAAGTAAGACTATGA
AAGGTGGAAAGTGCGTTACTAATTGCATATTTAGACAGCAATATGAGCGCAGTTTTG
ACTACATAACTTGTAATTTTGAAGGGATATCCAAAAGGATTAGTTGACAAAGTA
AATGAAAATTGGAAAAGGTACGGATATAAATAA

SEQ ID NO:3

LENGTH: 1248

TYPE: DNA

ORGANISM: *Pseudomonas putida* S12

SEQUENCE: 3

ATGAAAAAGCGTATCGGTATTGTTGGTGCAGGCACTGCCGGCCTCCATCTTGGTCTC
TTCCTTCGTCAGCATGACGTCGACGTCCTGTGTACACTGATCGTAAGCCCGATGAG
TACAGCGGACTGCGTCTCCTGAATAACCGTTGCTCACAACGCGGTGACGGTGCAGCG
GGAGGTTGCCCTCGACGTCAATGAGTGGCCGTCTGAGGAGTTTGGTTATTTTCGGCCA
CTACTACTACGTAGGTGGGCCGACGCCATGCGTTTCTACGGTGATCTCAAGGCTCC
CAGCCGTGCAGTGGACTACCGTCTCTACCAGCCGATGCTGATGCGTGCCTGGAAGC
CAGGGGCGGCAAGTTCTGCTACGACGCGGTGTCTGCCGAAGATCTGGAAGGGCTGT

CGGAGCAGTACGATCTGCTGGTTGTGTGCACTGGTAAATACGCCCTCGGCAAGGTGT
TCGAGAAGCAGTCCGAAACTCGCCCTTCGAGAAGCCGCAACGGGCACTGTGCGTT
GGTCTCTTCAAGGGCATCAAGGAAGCACCGATTTCGCGCGGTGACTATGTCCTTCTCG
CCAGGGCATGGCGAGCTGATTGAGATTCCAACCCTGTCGTTCAATGGCATGAGCACA
GCGCTGGTGCTCGAAAACCATATTGGTAGCGATCTGGAAGTTCTCGCCCACACCAAG
TATGACGATGACCCGCGTGCGTTCTTCGATCTGATGCTGGAGAAGCTGGGTAAGCAT
CATCCTTCCGTTGCCGAGCGCATCGATCCGGCTGAGTTCGACCTTGCCAACAGTTCT
CTGGACATCCTCCAGGGTGGTGTGTGCCGGCATTCCGCGACGGTCATGCGACCCTC
AATAACGGCAAAACCATCATTGGGCTGGGCGACATCCAGGCAACTGTCGATCCGGT
CTTGGGCCAGGGCGCGAACATGGCGTCCTATGCGGCATGGATTCTGGGCGAGGAAA
TCCTTGCGCACTCTGTCTACGACCTGCGCTTCAGCGAACACCTGGAGCGTCGCCGCC
AGGATCGCGTGCTGTGTGCCACGCGATGGACCAACTTCACTCTGAGCGCTCTCTCGG
CACTTCCGCCGGAGTTCCTCGCCTTCCTTCAGATCCTGAGCCAGAGCCGTGAAATGG
CTGATGAGTTCACGGACAACCTTCAACTACCCGGAACGTCAGTGGGATCGCTTCTCCA
GCCCAGAACGTATCGGACAGTGGTGCAGTCAGTTCGCACCCACTATCGCGGCCTGA

SEQ ID NO:4

LENGTH: 513

TYPE: DNA

ORGANISM: *Pseudomonas putida* S12

SEQUENCE: 4

ATGACGTTAAAAAAGATATGGCGGTGGATATCGACTCCACCAACTTCCGCCAGGC
GGTTGCATTGTTTCGCGACGGGAATTGCGGTTCTCAGCGCGGAGACTGAAGAGGGCG
ATGTGCACGGCATGACCGTGAACAGTTTCACCTCCATCAGTCTGGATCCGCCGACTG
TGATGGTTTCCCTGAAATCGGGCCGTATGCATGAGTTGCTGACTCAAGGCGGACGCT
TCGGAGTTAGCCTCTTGGGTGAAAGCCAGAAGGTGTTCTCGGCATTCTTCAGCAAGC
GCGCGATGGATGACACGCCTCCCCCGCCTTCACCATTACAGGCCGGCCTTCCCACTC
TGCAGGGCGCCATGGCCTGGTTTGAATGCGAGGTGGAGAGCACGGTTCAAGTACAC
GACCACACGCTCTTCATTGCGCGCGTTAGCGCCTGTGGAACGCCTGAGGCGAATACC
CCCCAGCCGCTGCTGTTCTTTGCCAGCCGTTATCACGGCAACCCGTTGCCACTGAAT
TGA

SEQ ID NO:5

LENGTH: 1815

TYPE: DNA

ORGANISM: *Pseudomonas putida* S12

SEQUENCE: 5

ATGAAAAAGCGTATCGGTATTGTTGGTGCAGGCACTGCCGGCCTCCATCTTGGTCTC
TTCCTTCGTCAGCATGACGTCGACGTCAGTGTGTACACTGATCGTAAGCCCGATGAG
TACAGCGGACTGCGTCTCCTGAATAACCGTTGCTCACAACGCGGTGACGGTGCAGCG
GGAGGTTGCCCTCGACGTCAATGAGTGGCCGTCTGAGGAGTTTGGTTATTTTCGGCCA
CTACTACTACGTAGGTGGGCGCAGCCCATGCGTTTCTACGGTGATCTCAAGGCTCC
CAGCCGTGCAGTGGACTACCGTCTCTACCAGCCGATGCTGATGCGTGCAGTGGAAAGC
CAGGGGCGGCAAGTTCTGCTACGACGCGGTGTCTGCCGAAGATCTGGAAGGGCTGT

CGGAGCAGTACGATCTGCTGGTTGTGTGCACTGGTAAATACGCCCTCGGCAAGGTGT
TCGAGAAGCAGTCCGAAACTCGCCCTTCGAGAAGCCGCAACGGGCACTGTGCGTT
GGTCTCTTCAAGGGCATCAAGGAAGCACCGATTTCGCGCGGTGACTATGTCCTTCTCG
CCAGGGCATGGCGAGCTGATTGAGATTCCAACCCTGTCGTTCAATGGCATGAGCACA
GCGCTGGTGCTCGAAAACCATATTGGTAGCGATCTGGAAGTTCTCGCCCACACCAAG
TATGACGATGACCCGCGTGCGTTCCTCGATCTGATGCTGGAGAAGCTGGGTAAGCAT
CATCCTTCCGTTGCCGAGCGCATCGATCCGGCTGAGTTCGACCTTGCCAACAGTTCT
CTGGACATCCTCCAGGGTGGTGTGTGCCGGCATTCCGCGACGGTCATGCGACCCTC
AATAACGGCAAAACCATCATTGGGCTGGGCGACATCCAGGCAACTGTCGATCCGGT
CTTGGGCCAGGGCGCGAACATGGCGTCTTATGCGGCATGGATTCTGGGCGAGGAAA
TCCTTGCGCACTCTGTCTACGACCTGCGCTTCAGCGAACACCTGGAGCGTCGCCGCC
AGGATCGCGTGCTGTGTGCCACGCGATGGACCAACTTCACTCTGAGCGCTCTCTCGG
CACTTCCGCCGGAGTTCCTCGCCTTCCTTCAGATCCTGAGCCAGAGCCGTGAAATGG
CTGATGAGTTCACGGACAACCTTCAACTACCCGGAACGTCAGTGGGATCGCTTCTCCA
GCCCGGAACGTATCGGACAGTGGTGCAGTCAGTTCGCACCCACTATCGCGGCCTGA
CGCTATTGCTCCGCTGGTCAAGGCCAGCGGAGCCCTAACTCCTGGGTGATTCAAATG
ACGTTAAAAAAGATATGGCGGTGGATATCGACTCCACCAACTTCCGCCAGGCGGT
TGCATTGTTTCGCGACGGGAATTGCGGTTCTCAGCGCGGAGACTGAAGAGGGCGATG
TGCACGGCATGACCGTGAACAGTTTACCTCCATCAGTCTGGATCCGCCGACTGTGA
TGGTTTCCCTGAAATCGGGCCGTATGCATGAGTTGCTGACTCAAGGCGGACGCTTCG
GAGTTAGCCTCTTGGGTGAAAGCCAGAAGGTGTTCTCGGCATTCTTCAGCAAGCGCG
CGATGGATGACACGCCTCCCCCGCCTTCACCATTACAGGCCGGCCTTCCCCTCTGC
AGGGCGCCATGGCCTGGTTCGAATGCGAGGTGGAGAGCACGGTTCAAGTACACGAC
CACACGCTCTTCATTGCGCGCGTTAGCGCCTGTGGAACGCCTGAGGCGAATACCCCC
CAGCCGCTGCTGTTCTTTGCCAGCCGTTATCACGGCAACCCGTTGCCACTGAATTGA

SEQ ID NO:6

LENGTH: 510

TYPE: DNA

ORGANISM: *Pseudomonas putida* S12

SEQUENCE: 6

ATGCTTCATGCCTTCGAACGCAAAATGGCCGGCCACGGCATCCTGATGATCTTCTGC
ACCCTTCTATTTGGTGTGTTGGTCTTTGGATGAACTTGGTTGGCGGCTTTGAAATCATCC
CGGGATACATCATCGAGTTTCATGTCCCAGGTTCCCTGAGGGCTGGGCGAGGGCTC
ATTCCGGCCCCGCACTGAATGGAATGATGGTGATAGCAGTGGCATTCTGTTTTGCCCA
GCCTTGGCTTCGCCGATAAGACGGCGCGCTTGCTGGGCAGCATTATCGTCCTGGACG
GTTGGTTCGAACGTCGGTTTCTACCTTTTCTCCAACCTTCTCTCCCAATCGTGGCCTGAC
CTTCGGCCCCAACCAATTTGGGCCTGGCGATATCTTCAGCTTCTCGCCCTGGCTCCC
GCCTATCTGTTTGGTGTCTCGCAATGGGGGCGCTCGCAGTGATCGGCTACCAGGCA
TTGAAGAGCACCCGTTCTCGTAAAGCTGTTCCGCACGCTGCTGCGGAATGA

SEQ ID NO:7

LENGTH: 2394

TYPE: DNA

ORGANISM: *Pseudomonas putida* S12

SEQUENCE: 7

ATGAAAAAGCGTATCGGTATTGTTGGTGCAGGCACTGCCGGCCTCCATCTTGGTCTC
TTCCTTCGTCAGCATGACGTCGACGTCAGTGTGTACACTGATCGTAAGCCCGATGAG
TACAGCGGACTGCGTCTCCTGAATACCGTTGCTCACAACGCGGTGACGGTGCAGCG
GGAGGTTGCCCTCGACGTCAATGAGTGGCCGTCTGAGGAGTTTGGTTATTTTCGGCCA
CTACTACTACGTAGGTGGGCCGCAGCCCATGCGTTTCTACGGTGATCTCAAGGCTCC
CAGCCGTGCAGTGGACTACCGTCTCTACCAGCCGATGCTGATGCGTGCAGTGGAAAGC
CAGGGGCGGCAAGTTCTGCTACGACGCGGTGTCTGCCGAAGATCTGGAAGGGCTGT
CGGAGCAGTACGATCTGCTGGTTGTGTGCACTGGTAAATACGCCCTCGGCAAGGTGT
TCGAGAAGCAGTCCGAAAACCTCGCCCTTCGAGAAGCCGCAACGGGCACTGTGCGTT
GGTCTCTTCAAGGGCATCAAGGAAGCACCGATTTCGCGCGGTGACTATGTCCTTCTCG
CCAGGGCATGGCGAGCTGATTGAGATTCCAACCCTGTCGTTCAATGGCATGAGCACA
GCGCTGGTGCTCGAAAACCATATTGGTAGCGATCTGGAAGTTCTCGCCCACACCAAG
TATGACGATGACCCGCGTGCGTTCCTCGATCTGATGCTGGAGAAGCTGGGTAAGCAT
CATCCTTCCGTTGCCGAGCGCATCGATCCGGCTGAGTTCGACCTTGCCAACAGTTCT
CTGGACATCCTCCAGGGTGGTGTGTGCCGGCATTCCGCGACGGTCATGCGACCCTC
AATAACGGCAAAACCATCATTGGGCTGGGCGACATCCAGGCAACTGTGATCCGGT
CTTGGGCGAGGGCGCAACATGGCGTCTATGCGGCATGGATTCTGGGCGAGGAAA
TCCTTGCGCACTCTGTCTACGACCTGCGCTTCAGCGAACACCTGGAGCGTCGCCGCC
AGGATCGCGTGCTGTGTGCCACGCGATGGACCAACTTCACTCTGAGCGCTCTCTCGG
CACTTCCGCCGGAGTTCCTCGCCTTCCTTCAGATCCTGAGCCAGAGCCGTGAAATGG
CTGATGAGTTCACGGACAACCTCAACTACCCGGAACGTCAGTGGGATCGCTTCTCCA
GCCCGGAACGTATCGGACAGTGGTGCAGTCAGTTCGCACCCACTATCGCGGCCTGA
CGCTATTGCTCCGCTGGTCAAGGCCAGCGGAGCCCTAACTCCTGGGTGATTCAAATG
ACGTTAAAAAAAGATATGGCGGTGGATATCGACTCCACCAACTTCCGCCAGGCGGT
TGCATTGTTTCGCGACGGGAATTGCGGTTCTCAGCGCGGAGACTGAAGAGGGCGATG
TGCACGGCATGACCGTGAACAGTTTCACCTCCATCAGTCTGGATCCGCCGACTGTGA
TGGTTTCCCTGAAATCGGGCCGTATGCATGAGTTGCTGACTCAAGGCGGACGCTTCG
GAGTTAGCCTCTTGGGTGAAAGCCAGAAGGTGTTCTCGGCATTCTTCAGCAAGCGCG
CGATGGATGACACGCCTCCCCCGCCTTCACCATTACAGGCCGGCCTTCCCCTCTGC
AGGGCGCCATGGCCTGGTTCGAATGCGAGGTGGAGAGCACGGTTCAAGTACACGAC
CACACGCTCTTCATTGCGCGCGTTAGCGCCTGTGGAACGCCTGAGGCGAATACCCCC
CAGCCGCTGCTGTTCTTTGCCAGCCGTTATCACGGCAACCCGTTGCCACTGAATTGA
TTGCGCACGAACAAAACAACAAAAACCGGTGAGGCCTTTCTGTGCCGATCACCGGA
AGAGGAGATAGCCATGCTTCATGCCTTCGAACGCAAAATGGCCGGCCACGGCATCC
TGATGATCTTCTGCACCTTCTATTTGGTGTGTTGTTCTTGGATGAACTTGGTTGGCGG
CTTTGAAATCATCCCGGGATACATCATCGAGTTTCATGTCCCGGGTTCCCCTGAGGG
CTGGGCGAGGGCTCATTCCGGCCCCGCACTGAATGGAATGATGGTGATAGCAGTGG
CATTCGTTTTTGGCCAGCCTTGGCTTCGCCGATAAGACGGCGCGCTTGCTGGGCAGCA
TTATCGTCTTGACGGTTGGTTCGAACGTCGGTTTCTACCTTTTCTCCAACCTTCTCTCC
CAATCGTGGCCTGACCTTCGGCCCCAACCAATTTGGGCCTGGCGATATCTTCAGCTT
CCTCGCCCTGGCTCCCGCCTATCTGTTTGGTGTCTCGCAATGGGGGCGCTCGCAGT
GATCGGCTACCAGGCATTGAAGAGCACCCGTTCTCGTAAAGCTGTTCCGCACGCTGC
TGCGGAATGA

SEQ ID NO:8
LENGTH: 38
TYPE: DNA
ORGANISM: artificial sequence
FEATURE:
OTHER INFORMATION: Primer

SEQUENCE: 8
GGAAGATCTAGGAGGTAACCAATGGATCAAATCGAAGC

SEQ ID NO:9
LENGTH: 33
TYPE: DNA
ORGANISM: artificial sequence
FEATURE:
OTHER INFORMATION: Primer

SEQUENCE: 9
TTCCTCGAGCTTCTCTCATCCGCCAAAACAGCC

SEQ ID NO:10
LENGTH: 41
TYPE: DNA
ORGANISM: artificial sequence
FEATURE:
OTHER INFORMATION: Primer

SEQUENCE: 10
ATATCTAGACTAGGAGGCAGAACATGAAAAAGCGTATCGGT

SEQ ID NO: 11
LENGTH: 27
TYPE: DNA
ORGANISM: artificial sequence
FEATURE:
OTHER INFORMATION: Primer

SEQUENCE: 11
ACTAAGCTTTTCATTCCGCAGCAGCGTG

SEQ ID NO:12
LENGTH: 37
TYPE: DNA
ORGANISM: artificial sequence

FEATURE:

OTHER INFORMATION: Primer

SEQUENCE: 12

TATCCATGGGCGGGAGGTAACCAATGGATCAAATCGA

SEQ ID NO: 13

LENGTH: 27

TYPE: DNA

ORGANISM: artificial sequence

FEATURE:

OTHER INFORMATION: Primer

SEQUENCE: 13

ATTTCTAGATTAGCAAATCGGAATCGG

SEQ ID NO: 14

LENGTH: 43

TYPE: DNA

ORGANISM: artificial sequence

FEATURE:

OTHER INFORMATION: Primer

SEQUENCE: 14

ATACCTGCAGGGGGAGGAATTATATGAGGAAGCTAAATCCAGC

SEQ ID NO: 15

LENGTH: 35

TYPE: DNA

ORGANISM: artificial sequence

FEATURE:

OTHER INFORMATION: Primer

SEQUENCE: 15

ATTAAGCTTTTATTTATATCCGTACCTTTTCCAAT

CLAIMS

We claim:

1. A recombinant organism comprising,
 - (i) at least one heterologous gene encoding an enzyme having phenylalanine ammonia lyase (PAL) activity,
 - (ii) at least one heterologous gene encoding an enzyme having trans-cinnamic acid decarboxylase (CADC) activity,
 - (iii) at least one heterologous gene encoding an enzyme having styrene monooxygenase (SMO) activity,
 - (iv) at least one heterologous gene encoding an enzyme having styrene oxide isomerase (SOI) activity, and
 - (v) at least one gene encoding an enzyme having 2-phenylacetaldehyde reductase (PAR) activity,wherein the recombinant microorganism is capable of producing 2-phenylethanol from a fermentable carbon substrate.
2. The organism of claim 1, wherein the organism is *Escherichia coli*.
3. The organism of claim 2, wherein the organism is a phenylalanine overproducing strain of *E. coli*.
4. The organism of claim 1, wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Arabidopsis thaliana*.
5. The organism of claim 1, wherein the gene encoding polypeptides having trans-cinnamic acid decarboxylase activity is derived from *Saccharomyces cerevisiae*.
6. The organism of claim 1, wherein the gene encoding a polypeptide having styrene monooxygenase activity is derived from *Pseudomonas putida*.

7. The organism of claim 1, wherein the gene encoding a polypeptide having styrene oxide isomerase activity is derived from *Pseudomonas putida*.
8. A method of producing 2-phenylethanol comprising the steps of,
(i) contacting the recombinant organism of claim 1 with a fermentable carbon substrate, and
(ii) growing the recombinant organism for a time sufficient to produce 2-phenylethanol.
9. The method of claim 8, wherein the fermentable carbon substrate is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, methane, formaldehyde, formate, amino acids, carbon-containing amines.
10. The method of claim 8, wherein the fermentable carbon substrate is selected from the group consisting of glucose, xylose, or glycerol.
11. The method of claim 8, wherein the fermentable carbon substrate is selected from the group consisting of lignin-derived aromatic monomers, lignin-derived aromatic oligomers and combinations thereof.
12. The method of claim 8, wherein the fermentable carbon substrate is a biomass hydrolysate.
13. A recombinant organism comprising,
(i) at least one heterologous gene encoding an enzyme having phenylalanine ammonia lyase (PAL) activity,
(ii) at least one heterologous gene encoding an enzyme having trans-cinnamic acid decarboxylase (CADC) activity,
(iii) at least one heterologous gene encoding an enzyme having styrene monooxygenase (SMO) activity,
(iv) at least one heterologous gene encoding an enzyme having styrene oxide isomerase (SOI) activity, and

(v) at least one gene encoding an enzyme having 2-phenylacetaldehyde dehydrogenase (PADH) activity,

wherein the engineered microorganism is capable of producing 2-phenylacetic acid from a fermentable carbon substrate.

14. The organism of claim 13, wherein the organism is *Escherichia coli*.

15. The organism of claim 14, wherein the organism is a phenylalanine overproducing strain of *E. coli*.

16. The organism of claim 13, wherein the gene encoding a polypeptide having phenylalanine ammonia lyase activity is derived from *Arabidopsis thaliana*.

17. The organism of claim 13, wherein the gene encoding polypeptides having trans-cinnamic acid decarboxylase activity is derived from *Saccharomyces cerevisiae*.

18. The organism of claim 13, wherein the gene encoding a polypeptide having styrene monooxygenase activity is derived from *Pseudomonas putida*.

19. The organism of claim 13, wherein the gene encoding a polypeptide having styrene oxide isomerase activity is derived from *Pseudomonas putida*.

20. A method of producing 2-phenylacetic acid comprising the steps of,
(i) contacting the recombinant organism of claim 13 with a fermentable carbon source,
and
(ii) growing the recombinant organism for a time sufficient to produce 2-phenylacetic acid.

21. The method of claim 20, wherein the fermentable carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, glycerol, carbon dioxide, methanol, methane, formaldehyde, formate, amino acids, carbon-containing amines.

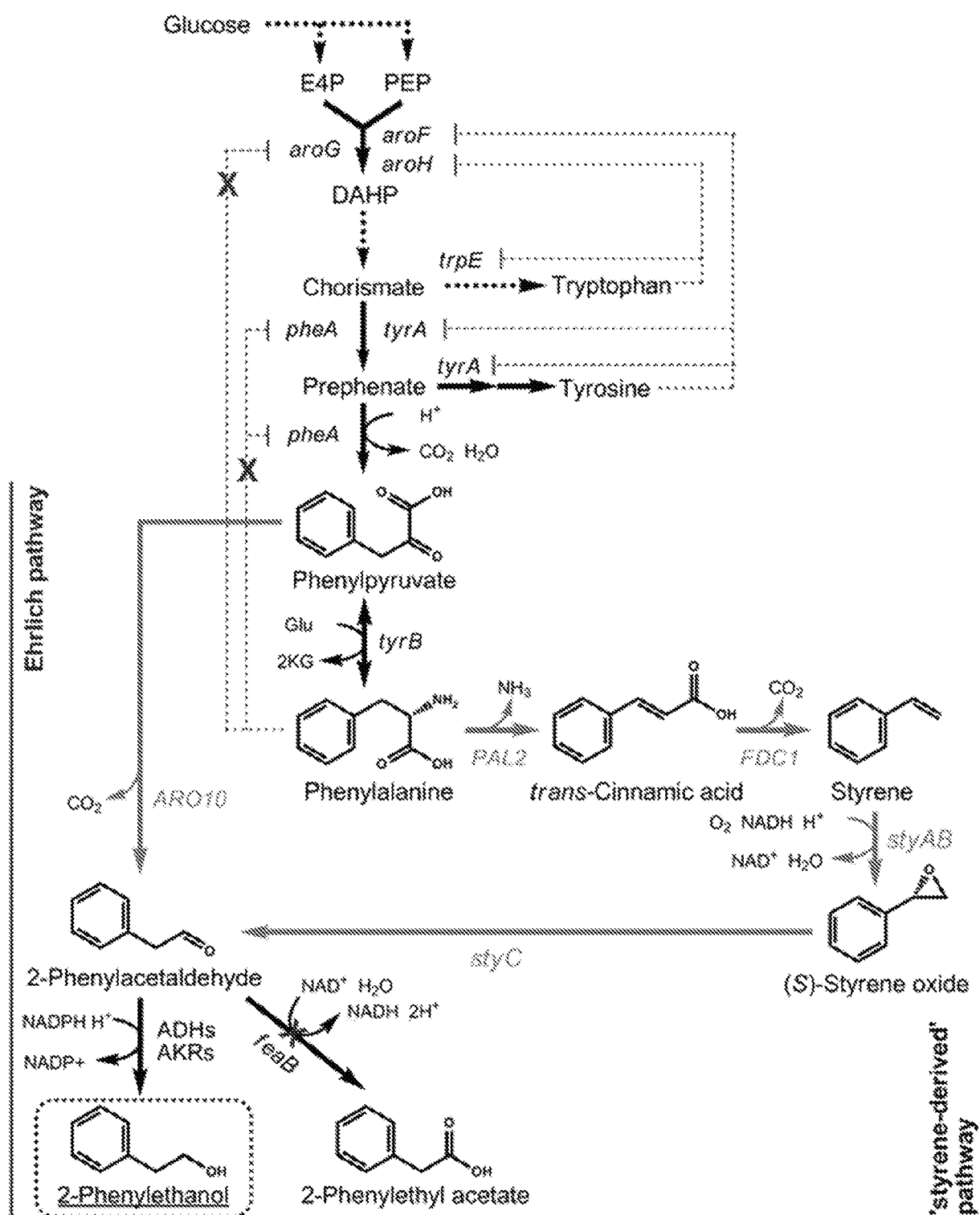
22. The method of claim 20, wherein the fermentable carbon source is selected from the group consisting of glucose, xylose, or glycerol.

23. The method of claim 20, wherein the fermentable carbon substrate is selected from the group consisting of lignin-derived aromatic monomers, lignin-derived aromatic oligomers, and combinations thereof.

24. The method of claim 20, wherein the fermentable carbon substrate is a biomass hydrolysate.

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FIG. 1



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FIG. 2

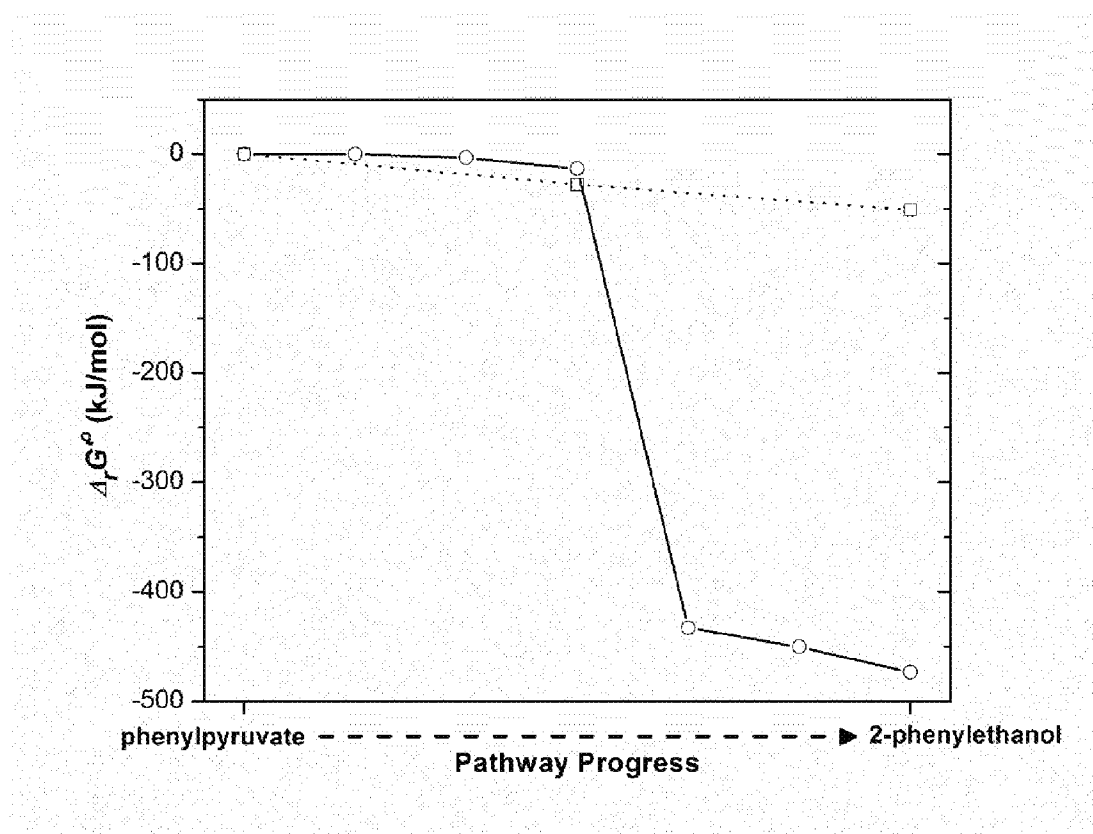
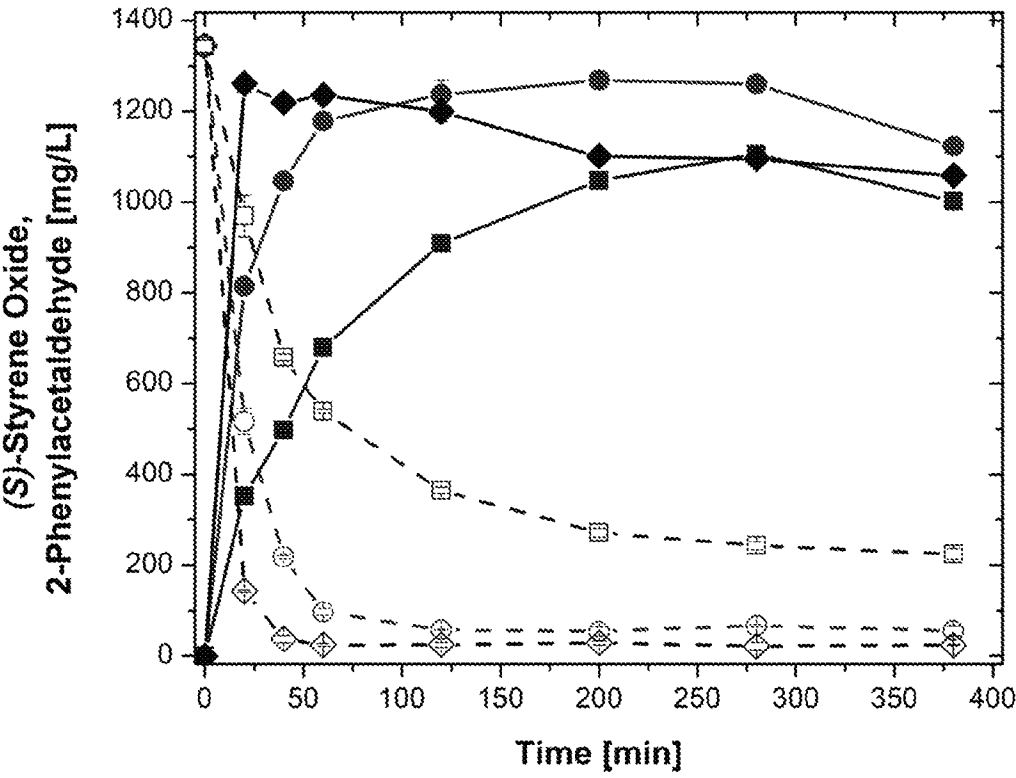


FIG. 3



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FIG. 4

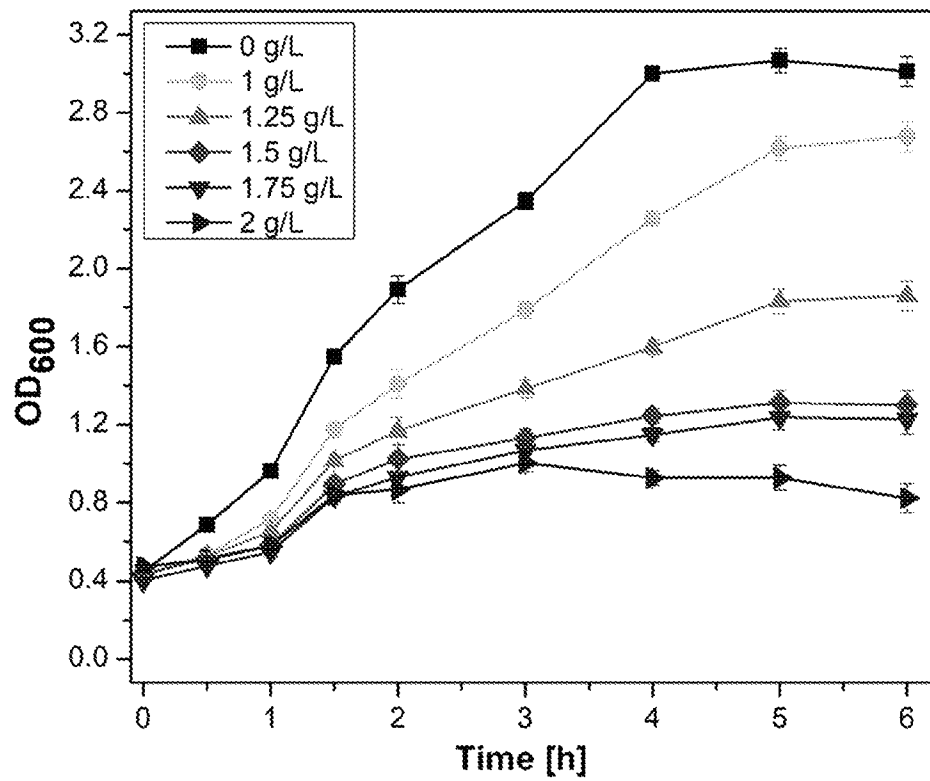


FIG. 5

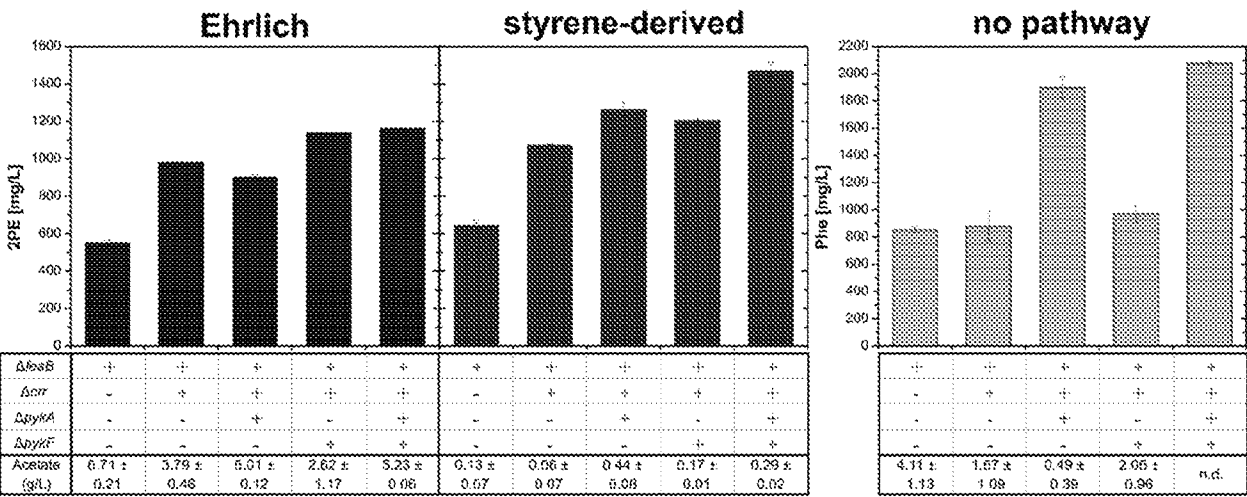


FIG. 6

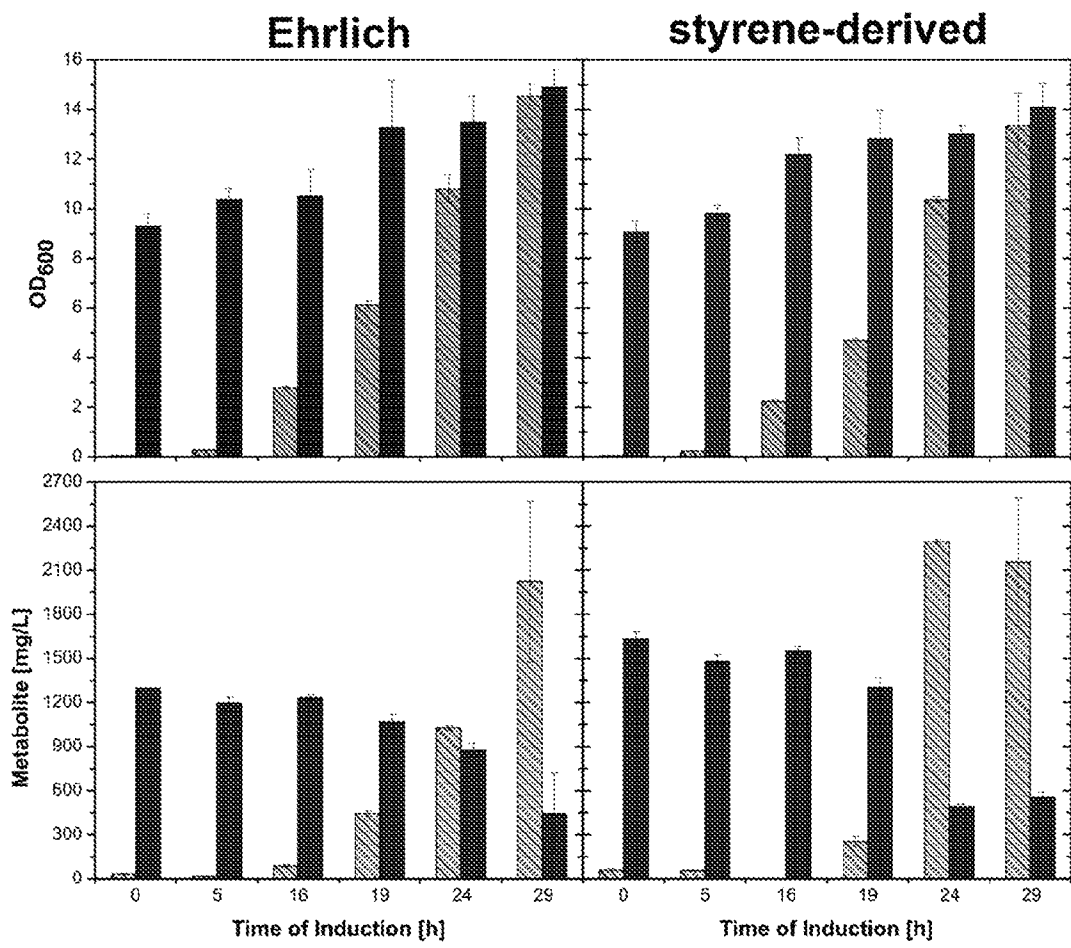


FIG. 7

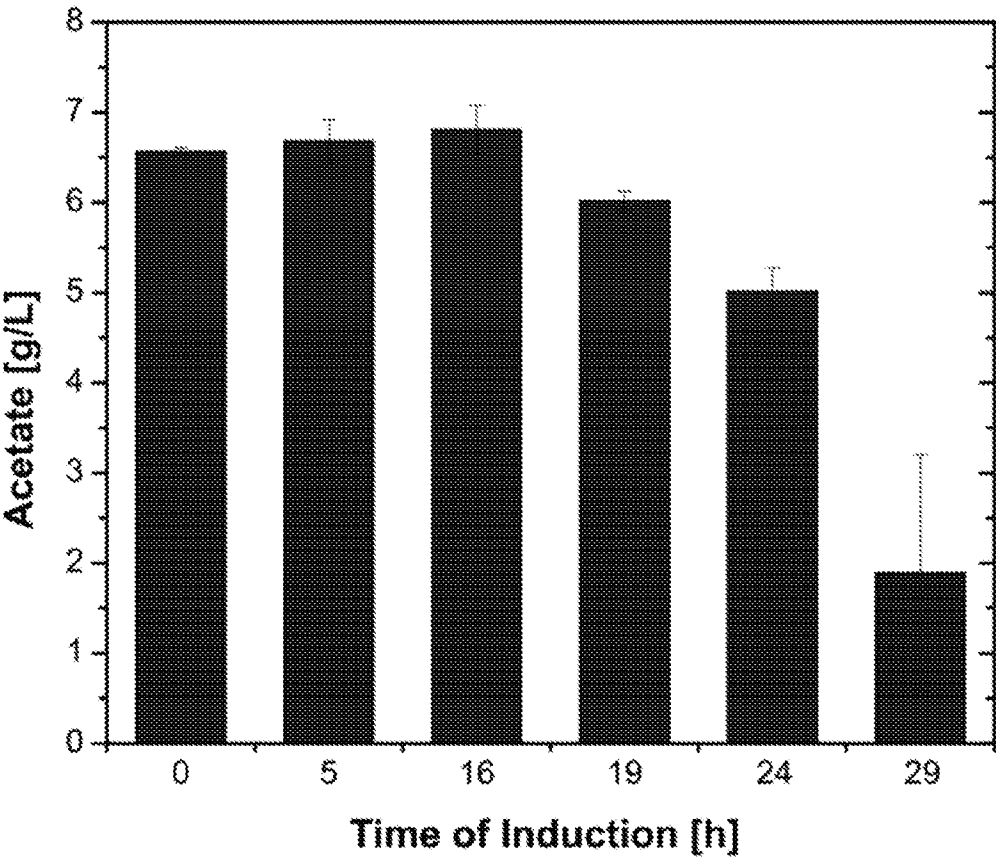


FIG. 8

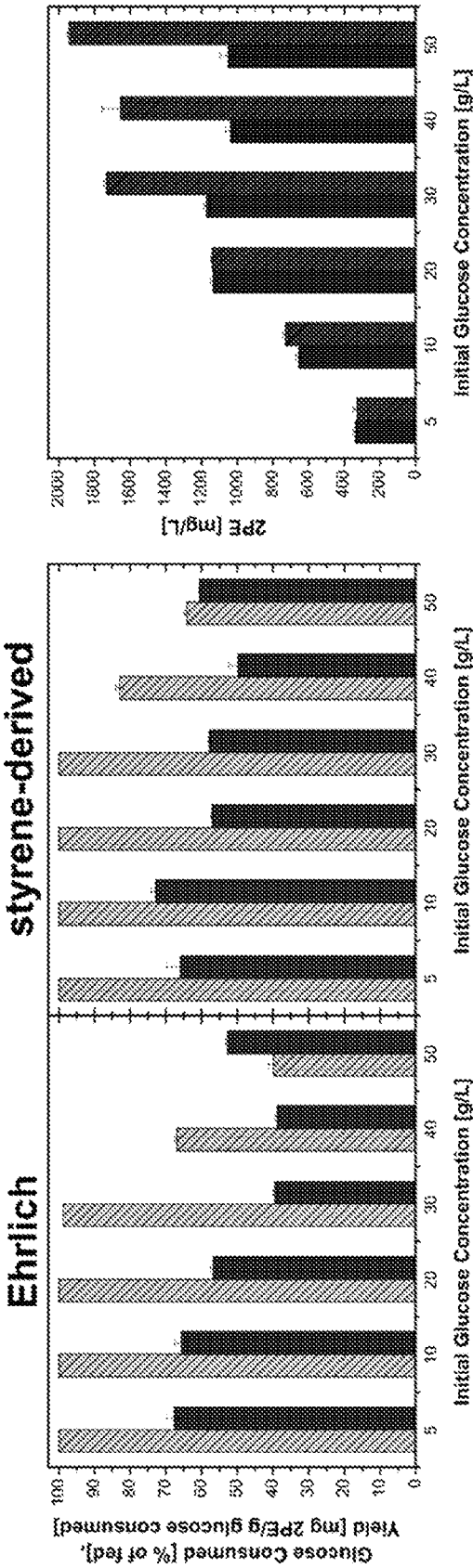
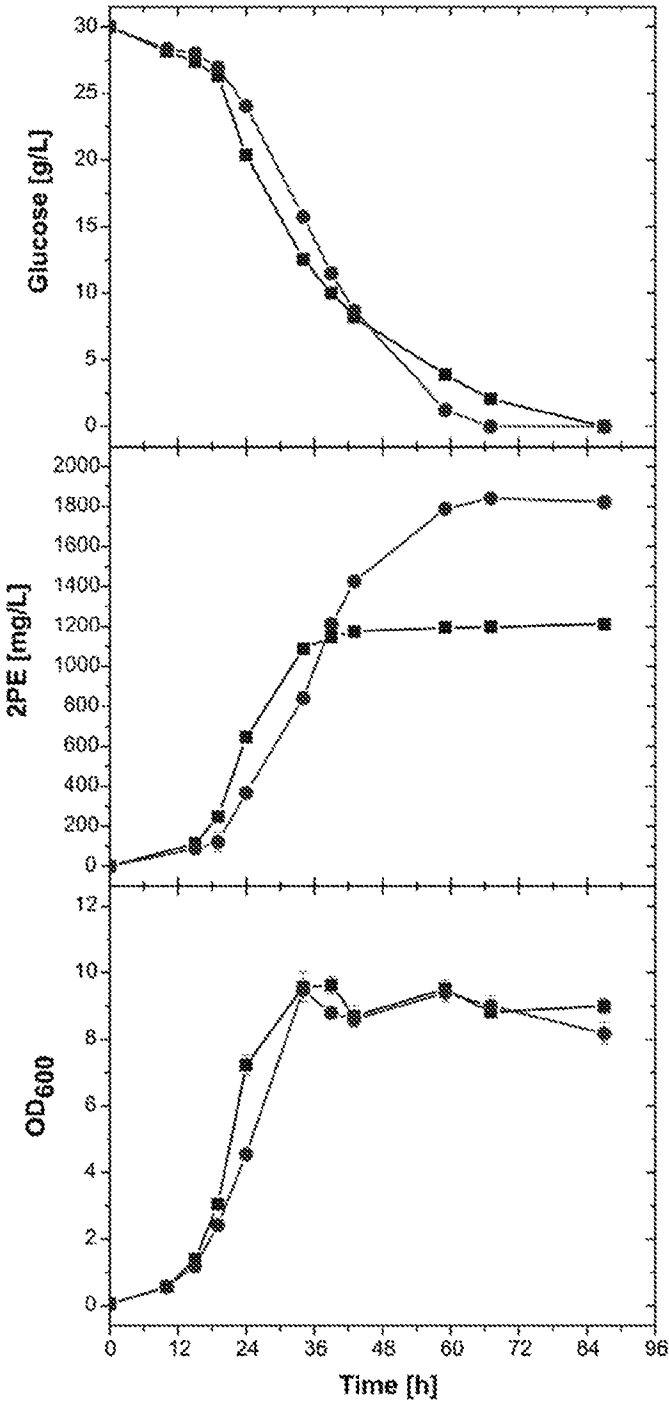
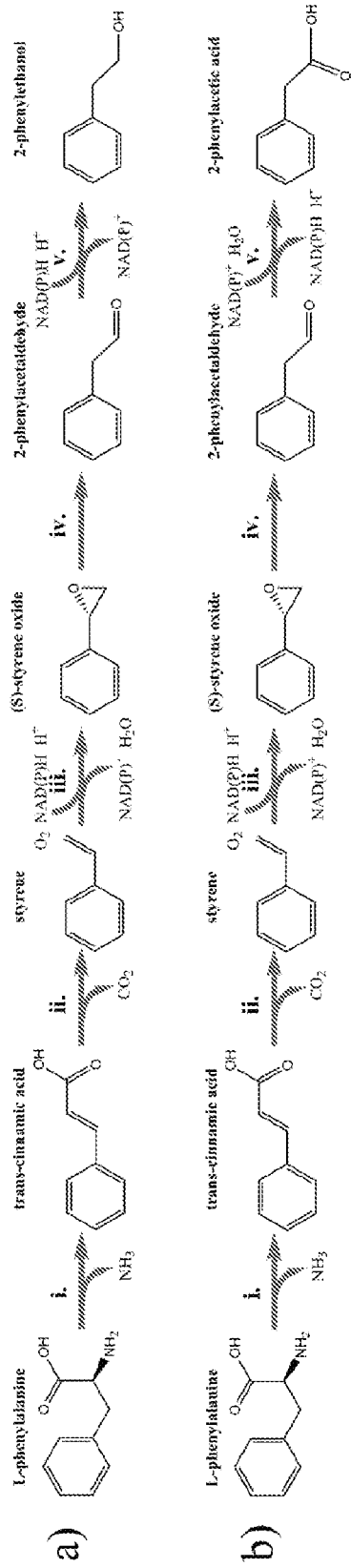


FIG. 9



FIGS. 10A-10B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/42687

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - C12N 1/21, C12N 9/02, C12N 9/14 (2018.01)
 CPC - C12P 7/18, C12P 7/26, C12N 1/20

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)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0256595 A1 (YOSHINKUNI et al.) 20 October 2011 (20.10.2011); abstract; para [0069], [0080]	1-7, 13-19
Y	US 2014/0057325 A1 (NIELSEN et al.) 27 February 2014 (27.02.2014); para [0011]; [0146]-[0147]; claims 9-10, 17	1-7, 13-19
Y	US 2017/0067084 A1 (NATIONAL UNIVERSITY OF SINGAPORE) 9 March 2017 (09.03.2017); para [0314]-[0315]; Table 1	13-19

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

☐ See patent family annex.

* 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

22 October 2018

Date of mailing of the international search report

09 NOV 2018

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. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/42687

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.:
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-7 and 13-19, directed to a recombinant organism.

Group II, claims 8-12 and 20-24, directed to a method of producing 2-phenylethanol or 2-phenylacetic acid.

The inventions listed as Groups I-II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

*****Continued in Supplemental Box*****

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.:
1-7, 13-19

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.

Continuation of Box No. III (Observations where unity of invention is lacking):

Special technical features:

Group I has the special technical feature of a recombinant organism at least one heterologous gene encoding an enzyme, that is not required by Group II.

Group II has the special technical feature of contacting a recombinant organism with a fermentable carbon substrate, and growing the recombinant organism for a time, that is not required by Group I.

Common technical features:

Groups I-II share the common technical feature of a recombinant microorganism capable of producing 2-phenylethanol from a fermentable carbon substrate, and an engineered microorganism capable of producing 2-phenylacetic acid from a fermentable carbon substrate. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is taught by US 2011/0256595 A1 to Yoshinkuni et al. (hereinafter Yoshinkuni) and US 2017/0067084 A1 to the National University of Singapore (hereinafter Univ Singapore).

Yoshinkuni teaches a recombinant engineered microorganism capable of producing 2-phenylethanol from a fermentable carbon substrate (Abstract "recombinant microorganism, and microbial systems are provided for...converting suitable monosaccharides or oligosaccharides into commodity chemicals, such as biofuels. Commodity chemicals produced by the methods described herein are also provided. Commodity chemical enriched, refinery-produced petroleum products are also provided, as well as methods for producing the same"; para [0066] "the recombinant microorganism comprises one or exogenous genes that allow it to grow on the polysaccharide as a sole source of carbon"; [0069] "In certain aspects, the commodity chemical is ... 2-phenylethanol").

Univ Singapore teaches an engineered microorganism capable of producing 2-phenylacetic acid from a fermentable carbon substrate (para [0314]-[0315] "Preparation of (S)-7 from biotransformation of (S)-1 with E. coli (LZ37) ... Newly prepared E. coli (LZ37) cells were suspended in KP buffer (200 mM, pH 8) containing glucose (0.5%, w/v) to form a 100 mL suspension (20 g cdw L.sup.-1) in a tri-baffled flask (500 mL). 1.652 g of (S)-1 (solid) and 10 mL of ethyl oleate was added into the flask to start the reaction at 250 rpm and 30.degree. C. 100 .mu.L aliquots of the aqueous phases were taken out at different time points to follow the reaction ... The collected crystals were combined and dried overnight under vacuum ... (S)-2-Hydroxy-2-phenylacetic acid 7 was obtained"; TABLE 1 "List of recombinant strains ... LZ37").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.