

BIOPRODUCTION OF ENANTIOPURE (*R*)- AND (*S*)-2-PHENYLGLYCINOL FROM STYRENE AND RENEWABLE FEEDSTOCKS VIA ARTIFICIAL ENZYME CASCADE

Field of Invention

5

This invention relates to the bioproduction of useful and valuable enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof. More particularly, the present invention provides methods of bioproduction of enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof by one or more recombinant
10 microbial cells genetically engineered to overexpress, relative to a wild type cell, at least one enzyme, which method comprises subjecting a starting material to a plurality of enzyme-catalyzed chemical transformations in a one-pot reaction system, wherein the starting material is selected from a group comprising glucose, *L*-phenylalanine or substituted *L*-phenylalanine, styrene or substituted styrene.

15

Background

The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art
20 or is common general knowledge.

Enantiopure β -amino alcohols are important chiral chemicals used as building blocks in the synthesis of several pharmaceutical compounds. Specifically, (*R*)-2-phenylglycinol **1a** is used in the synthesis of novel antibiotic pyrazolopyrimidines, EGFR inhibitor thienopyrimidines, and
25 3-phosphoinositide-dependent protein kinase-1 (PDK1) inhibitor. (*S*)-2-phenylglycinol **1a** can be used for the synthesis of (4*S*,5*S*)-2-phenyl-4-(methoxycarbonyl)-5-isopropylloxazoline, which is used for the synthesis of a neurotrophic agent. It is also used as a key precursor for the synthesis of chiral ligands for asymmetric annulation or cyclization, potent histone deacetylase inhibitors, and novel anti-cancer asymmetric triplex metallohelices. Due to its
30 wide applications, enantiopure (*R*)- and (*S*)-2-phenylglycinol are high value compounds with the price of \$40-\$100 per kg, and a market demand of more than 1000 tons per year. Moreover, the substituted (*R*)-2-phenylglycinols **1b-1k** (FIG. 1a) are also useful pharmaceutical intermediates. For instance, (*R*)-2-amino-2-(4-methoxyphenyl)ethanol **1k** and (1*R*,2*R*)-1-amino-2-indanol **1l** are used in the synthesis of agonists and Alk5 inhibitors to treat cancers.

35

Several chemical methods were developed for the synthesis of (*R*) and (*S*)-**1a**. However, these methods have one or more disadvantages. For instance, methods for the reduction of *D*- or *L*-

phenylglycine to (*R*)- or (*S*)-**1a** require expensive enantiopure substrates and utilize hazardous chemicals. In addition, the product yield in these methods is low with poor regio- and enantio-selectivity. Furthermore, kinetic resolution is required to obtain an enantiopure product but the kinetic resolution of racemic **1a** to obtain enantiopure (*R*)- or (*S*)-**1a** suffers from low theoretical
5 yield (50%, M. Periasamy, S. Sivakumar & M. N. Reddy, *Synthesis* **2003**, 13, 1965-1967). The use of petroleum-derived chemicals, and energy-intensive multi-step process, further adds to the disadvantages. As the society is moving towards green and sustainable processes, unsustainable chemical-based methods are not preferred.

10 Enzyme catalysis is attractive due to high regio- and stereo-selectivity, mild reaction conditions, and environmental friendliness. The well-studied methods for the synthesis of chiral 2-phenylglycinol are asymmetric reduction of chiral amino ketones by ketone reductase, kinetic resolution of racemic amino alcohols by lipases, and amination of hydroxy ketones using transaminases. However, the enzymatic approaches developed for the synthesis of (*R*)- and
15 (*S*)-**1a** have drawbacks. They require special substrates and the substrates for these reactions are very expensive and commercially unavailable, which makes it difficult to scale up the process. In addition, the substrates are mainly derived from petroleum. Therefore, the process is unsustainable. Further, the kinetic resolution of racemic **1a** suffers from low theoretical yield (50%, J.-D. Zhang *et al.*, *J. Biotechnol.* **2019**, 290, 24-32), thus making the process
20 uneconomical.

Therefore, there is a need to develop green and sustainable methods for the production of enantiopure (*R*) and (*S*)-**1a** from cheap and renewable starting materials.

25 **Summary of Invention**

Aspects and embodiments of the invention will now be discussed by reference to the following numbered clauses.

30 1. A method for producing an enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof using one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes, which method comprises subjecting styrene or a derivative thereof to multiple enzyme-catalyzed chemical transformations in a one-pot reaction system, optionally wherein at least one overexpressed enzyme is located on
35 one or more plasmids or integrated in the chromosome of each of the one or more recombinant microbial cells.

2. The method of Clause 1, wherein the method produces enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof, comprising the steps of:

(a) generating phenylethanediol or a derivative thereof from styrene or a derivative thereof by conducting an epoxidation reaction catalyzed by an epoxidase to form styrene oxide or a derivative thereof and conducting a hydrolysis reaction catalyzed by an epoxide hydrolase on the epoxide, or by a dihydroxylation reaction catalyzed by a dioxygenase;

(b) generating 2-hydroxyacetophenone or a derivative thereof from the phenylethanediol or a derivative thereof by an oxidation reaction catalyzed by an alcohol dehydrogenase or a secondary alcohol oxidase; and

(c) generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof from 2-hydroxyacetophenone or a derivative thereof by a transamination reaction catalyzed by a transaminase or a reductive amination reaction catalyzed by an amine dehydrogenase.

3. The method according to Clause 2, wherein:

(a) the epoxidase is a styrene monooxygenase, optionally wherein the styrene monooxygenase is from *Pseudomonas sp. VLB120*, or its mutants or similar enzymes with more than 50% identity; and/or

(b) the epoxide hydrolase, when used, is from *Solanum tuberosum* or its mutants or similar enzymes with more than 50% identity; and/or

(c) the alcohol dehydrogenase, when used, is from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* or their mutants or similar enzymes with more than 50% identity; and/or

(d) the transaminase is from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Martelella mediterranea*, *Neosartora fischeri*, *Arthrobacter sp.*, or *Aspergillus terreus* or their mutants or similar enzymes with more than 50% identity.

4. The method according to and one of Clause 3, wherein

(i) the epoxide hydrolase, when used, is selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA) from *Bacillus subtilis* or its mutants or similar enzymes with more than 50% identity; and/or

(ii) the transaminase is selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri* or their mutants or similar enzymes with more than 50% identity.

5. The method according to any one of the preceding clauses, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e.

the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is selected from one or more of the group consisting of:

- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
 - (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
 - 5 (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
- 10 6. The method according to Clause 5, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA.
- 15 7. The method according to any one of the preceding clauses, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is selected from one or more of the group consisting of:
- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA;
 - 20 (b) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
 - (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (f) *E. coli* T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
- 25 8. The method according to any one of the preceding clauses, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH.
- 30 9. The method according to any one of the preceding clauses, wherein the method further comprises providing styrene or a derivative thereof by generating *trans*-cinnamic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof by a deamination reaction catalyzed by an ammonia lyase and generating styrene or a derivative thereof from the *trans*-cinnamic acid or a derivative thereof in a decarboxylation reaction catalyzed by a decarboxylase.
- 35

10. The method according to 9, wherein:

(a) the ammonia lyase is phenylalanine ammonia lyase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity; and/or

5 (b) the decarboxylase is phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity.

11. The method according to any one of Clauses 1 to 6 and 9 to 10, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to
10 overexpress multiple enzymes) is *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA.

12. The method according to any one of Clauses 1 to 4 and 7 to 10, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple
15 enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.

13. The method according to any one of Clauses 9 to 12, wherein the method further
20 comprises cells that overexpress the natural L-phenylalanine biosynthetic pathway, which cells convert glucose or glycerol to L-phenylalanine.

14. The method according to Clause 13 wherein the microbial cells producing L-phenylalanine from glucose or glycerol overexpress at least one enzyme is selected from one
25 or more of the group consisting of DAHP synthase (AroG), shikimate kinase (AroK), shikimate dehydrogenase (YdiB), chorismate mutase/prephenate dehydratase (PheA) and tyrosine aminotransferase (TyrB), or their mutants or similar enzymes with more than 50% identity, optionally wherein the microbial cells producing L-phenylalanine from glucose or glycerol overexpress at least one enzyme that is:

30 (a) a feedback inhibition resistant mutant of AroG; and/or

(b) a feedback inhibition resistant mutant of PheA.

15. The method according to Clause 14, wherein the microbial cells producing L-phenylalanine from glucose or glycerol overexpress at least one enzyme is one in which the
35 microbial cells are mutated for deletion or inactivation of *crr* and/or *tyrA* genes.

16. The method according to any one of Clauses 13 to 15, wherein the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme is *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA or *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NfTA.

5

17. The method according to any one of Clauses 13 to 15, wherein the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme is a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA or is a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.

10

18. The method according to any one of the preceding clauses, wherein the one-pot reaction system comprises an aqueous medium.

15

19. The method according to any one of Clauses 1 to 17, wherein the one-pot reaction system comprises use of a bi-phasic medium, optionally wherein the bi-phasic medium consists of an aqueous and solid resin medium, or an aqueous and organic solvent medium (e.g. the organic solvent medium is selected from one or more alkanes solvents and/or one or more ester solvents).

20

20. The method according to any one of the preceding clauses, wherein the one-pot reaction system comprises use of absorbent resins or nanomaterials for in-situ removal of product.

25

21. An isolated nucleic acid molecule encoding at least one heterologous catalytic enzyme selected from the group comprising:

(a) an epoxidase for conducting an epoxidation reaction to form styrene oxide or a derivative thereof from styrene or a derivative thereof and an epoxide hydrolase for conducting a hydrolysis reaction on the epoxide to provide phenylethanediol or a derivative thereof, or an oxygenase for generating phenylethanediol or a derivative thereof from styrene or a derivative thereof by a dihydroxylation reaction;

30

(b) an alcohol dehydrogenase or a secondary alcohol oxidase for generating 2-hydroxyacetophenone or a derivative thereof from phenylethanediol by an oxidation reaction; and

35

(c) a transaminase or an amine dehydrogenase for generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof by a transamination reaction or a reductive amination from 2-hydroxyacetophenone or a derivative thereof; or

(d) an ammonia lyase for generating *trans*-cinnamic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof by a deamination reaction; and

(e) a decarboxylase for generating styrene or a derivative thereof from *trans*-cinnamic acid or a derivative thereof in a decarboxylation reaction.

5

22. The isolated nucleic acid of clause 21, encoding a plurality of said catalytic enzymes.

23. The isolated nucleic acid molecule of clause 22, wherein said plurality of catalytic enzymes is arranged as at least one module selected from the group comprising:

10 i) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms styrene or a derivative thereof to phenylethanol or a derivative thereof;

ii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms phenylethanol or a derivative thereof to a 2-
15 hydroxyacetophenone or a derivative thereof;

iii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms 2-hydroxyacetophenone or a derivative thereof to enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof; and

v) a module comprising heterologous nucleic acid sequences that, when expressed,
20 enzymatically transforms an *L*-phenylalanine or a derivative thereof to styrene or a derivative thereof,

or any combination thereof.

24. An expression construct comprising at least one nucleic acid molecule as described in
25 any one of Clauses 21 to 23.

25. One or more recombinant prokaryotic or eukaryotic cells selected from the group comprising bacterial cells, yeast cells, mammalian cells and insect cells, wherein said cells comprise at least one expression construct as described in Clause 24.

30

26. The one or more recombinant prokaryotic or eukaryotic cells according to Clause 25, wherein said cells are recombinant bacterial cells.

27. The one or more recombinant prokaryotic or eukaryotic cells according to Clause 25
35 or Clause 26, wherein said enzymes have at least 50% amino acid identity with at least one enzyme selected from the group comprising:

(a) a styrene monooxygenase from *Pseudomonas sp. VLB120*;

- (b) an epoxide hydrolase from *Solanum tuberosum*;
 - (c) an alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* (e.g. the epoxide hydrolase is selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA));
 - 5 (d) a transaminase from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Martellella mediterranea*, *Neosartora fischeri*, *Arthrobacter sp.*, or *Aspergillus terreus* (e.g. the transaminase is selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri*);
 - (e) a phenylalanine ammonia lyase from *Arabidopsis thaliana*;
 - 10 (f) a phenylacrylic acid decarboxylase from *Aspergillus niger*.
28. The one or more recombinant cells of clause 27, wherein said cells are selected from:
- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
 - (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
 - 15 (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH;
 - (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
 - (g) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA;
 - 20 (g) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
 - (i) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA;
 - (j) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH;
 - (k) *E. coli* T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (l) *E. coli* T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
 - 25 (m) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA;
 - (n) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NFTA;
 - (o) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA; and
 - (p) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA.
 - 30
29. The one or more recombinant cells of clause 28, wherein said cells are selected from:
- (i) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
 - (ii) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
 - (iii) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA; and
 - 35 (iv) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NFTA.

30. A kit comprising at least one isolated nucleic acid according to any one of Claims 21 to 24.

Drawings

5

FIG. 1 depicts the production of enantiopure (*R*)-2-phenylglycinols **1a-l** and (*S*)-2-phenylglycinol **1a** from styrenes **2a-k**, indene **2l**, bio-based *L*-phenylalanine **6** and renewable feedstocks glucose **8** and glycerol **9**. (a) Artificial enzyme cascade for the conversion of styrene **2a-k**, indene **2l** to (*R*)-**1a-l** and (*S*)-**1a** via one-pot epoxidation-hydrolysis-oxidation-amination reaction; (b) Artificial enzyme cascade for the biotransformation of *L*-phenylalanine **6** to (*R*)- and (*S*)-**1a** via one-pot deamination-decarboxylation-epoxidation-hydrolysis-oxidation-amination reaction; and (c) Conversion of glucose **8** and glycerol **9** to (*R*)- and (*S*)-**1a** via the combination of natural Shikimate pathway for the synthesis of **6** and the artificial enzyme cascade for the conversion of **6** to (*R*)- and (*S*)-**1a**. Enzymes involved are styrene monooxygenase (SMO), epoxide hydrolase (StEH), alcohol dehydrogenase (ADH), transaminase (TA), phenylalanine ammonia lyase (PAL) and phenylacrylic acid decarboxylase (PAD).

FIG. 2 depicts (a) production of hydroxyacetophenone **5a** from 2 and 10 mM of (*R*)-phenylethanediol **4a** by *Escherichia coli* (*E. coli*)- butanediol dehydrogenase (BDHA, 10 g cdw/L) or *E. coli*- secondary alcohol dehydrogenase (CpSADH, 10 g cdw/L); and (b) screening of transaminases for the conversion of **5a** to (*R*)- or (*S*)-2-phenylglycinol **1a** by biotransformation of 10 mM of **5a** with *E. coli* strains expressing transaminases (20 g cdw/L) in phosphate buffer (200 mM, pH 8) containing glucose (0.5%) and amino donor (200 mM). Biotransformations were performed in duplicates with error bars showing $\pm$ SD.

FIG. 3 depicts a schematic representation of recombinant plasmids constructed for the cascade conversion of styrene **2a** to (*R*)- and (*S*)-**1a**.

FIG. 4 depicts cascade biotransformation of styrene **2a** to (*R*)- and (*S*)-**1a** using *E. coli* strains expressing the artificial enzyme cascade shown in FIG. 1a. (a) *E. coli*-SSBB-1-6 harboring three expression plasmids with different copy numbers for the expression of SMO, StEH, BDHA, alanine dehydrogenase (AlaDH) and transaminase from *Bacillus megaterium* (BmTA) at various expression levels for producing (*R*)-**1a**; (b) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude extracts of *E. coli*-SSBB-1-6 showing the expression of enzymes for producing (*R*)-**1a**. Lane M, protein ladder; Lane 1-6, *E. coli*-SSBB-1-6; (c) Synthesis of (*R*)-**1a** from 10 mM of **2a** by *E. coli*-SSBB-1-6 (20 g

cdw/L) at 30 °C for 24 h; (d) *E. coli*-SSBN-1-6 harboring three expression plasmids with different copy numbers to express SMO, StEH, BDHA, AlaDH and transaminase from *Neosartora fischeri* (NfTA) at various expression levels for producing (S)-**1a**; (e) SDS-PAGE analysis of crude extracts of *E. coli*-SSBN-1-6 showing the expression of enzymes for producing (S)-**1a**. Lane M, protein ladder; Lane 7-12, *E. coli*-SSBN-1-6; and (f) Synthesis of (S)-**1a** from 5 mM of **2a** by *E. coli*-SSBN-1-6 (20 g cdw/L) at 30 °C for 48 h. Reaction conditions: Two-phase system of 2 mL of phosphate buffer (200 mM, pH 8) and 2 mL of *n*-hexadecane containing **2a**. Biotransformations were performed in duplicates with error bars showing $\pm$ SD.

10

FIG. 5 depicts cascade biotransformation of L-phenylalanine **6** to (R)- and (S)-**1a** using *E. coli* strains expressing the artificial enzyme cascade shown in FIG. 1b. (a) *E. coli*-PPSSBB and *E. coli*-PPSSBN harboring four expression plasmids for the expression of PAL, PAD, SMO, StEH, BDHA, AlaDH and BmTA or NfTA for the production of (R)- or (S)-**1a**, respectively; (b) SDS-PAGE analysis of crude extracts of *E. coli*-PPSSBB and *E. coli*-PPSSBN showing the expression of enzymes for producing (R)- or (S)-**1a**. Lane M, protein ladder; Lane C, *E. coli* T7 (negative control); Lane 13, *E. coli*-PPSSBB; Lane 14, *E. coli*-PPSSBN; and (c) Synthesis of (R)- and (S)-**1a** from 10 mM of **6** by *E. coli*-PPSSBB and *E. coli*-PPSSBN (15 g cdw/L), respectively in a two-phase system of phosphate buffer (5 mL, 200 mM, pH 8) containing 6 mL and 1 mL of *n*-hexadecane at 30 °C for 24 h. Biotransformations were performed in duplicates with error bars showing $\pm$ SD.

20

FIG. 6 depicts fermentation of glucose **8** or glycerol **9** to (R)- and (S)-**1a** using *E. coli* NST74(DE3) strains expressing the artificial enzyme cascade shown in FIG. 1c. (a) *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN harboring engineered Shikimate pathway and four expression plasmids for the expression of PAL, PAD, SMO, StEH, BDHA, AlaDH and BmTA or NfTA for the production of (R)- or (S)-**1a**, respectively; (b) SDS-PAGE analysis of crude extracts of *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN showing the expression of enzymes for the conversion of **6** to (R)- and (S)-**1a**. Lane M, protein ladder; Lane C, *E. coli* NST74(DE3); Lane 15, *E. coli* NST-PPSSBB; Lane 16, *E. coli* NST-PPSSBN; and (c) Synthesis of (R)- and (S)-**1a** from 20 g/L **8** or **9** by *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN, respectively via fermentation with 50 mL of modified M9 medium containing **8** or **9** and 10 mL of *n*-hexadecane at 25 °C for 24 h. Experiment was performed in duplicates with error bars showing $\pm$ SD.

35

FIG. 7 depicts (a) growing cells fermentation of glucose **8** or glycerol **9** using growing cells of *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN at 25 °C for 24 h for the production of (R)- and

(S)-**1a**, respectively; and (b) coupled fermentation and biotransformation for the production of (R)- and (S)-**1a** from glucose **8** and glycerol **9**. Fermentation was performed using growing cells of *E. coli* NST-Phe at 37 °C for 24-28 h for the production of 67-80 mM of **6** from **8** or **9**. The fermented mixture containing biosynthesized **6** was used for the biotransformation of **6** to (R)- and (S)-**1a** at 30 °C for 24 h using *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively.

FIG. 8 depicts coupled fermentation and biotransformation for the conversion of glucose **8** and glycerol **9** to (R)- and (S)-**1a** from (a) *E. coli* NST-Phe harboring expression plasmid for the expression of key enzymes of Shikimate pathway for improved production of *L*-phenylalanine **6** was coupled with *E. coli*-PPSSBB and *E. coli*-PPSSBN harboring four expression plasmids for the expression of PAL, PAD, SMO, StEH, BDHA, AlaDH and BmTA or NfTA for the production of (R)- or (S)-**1a**, respectively from **8** and **9**; and (b) synthesis of (R)- and (S)-**1a** by *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively from biosynthesized **6** produced by the fermentation of **8** and **9** by *E. coli* NST-Phe. Reaction procedure: Fermentation was performed using growing cells of *E. coli* NST-Phe at 37 °C for 24-28 h for the production of 67-80 mM of **6** from **8** or **9**. Biotransformation was performed by *E. coli*-PPSSBB or *E. coli*-PPSSBN (20 g cdw/L) at 30 °C for 24 h in a two-phase system containing 5 mL of fermented mixture containing biosynthesized **6** (10 mM), phosphate buffer (200 mM, pH 8) and *n*-hexadecane (1 mL). Experiment was performed in duplicates with error bars showing $\pm$ SD.

Description

In a first aspect of the invention, there is provided a method for producing an enantiomerically pure or enantiomerically enriched (R)- or (S)-2-phenylglycinol or a derivative thereof using one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes, which method comprises subjecting styrene or a derivative thereof to multiple enzyme-catalyzed chemical transformations in a one-pot reaction system. In embodiments of this aspect, at least one overexpressed enzyme is located on one or more plasmids or integrated in the chromosome of each of the one or more recombinant microbial cells.

In embodiments herein, the word “comprising” may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word “comprising” may also relate to the situation where only the components/features listed are intended to be present (e.g. the word “comprising” may be replaced by the phrases “consists of” or “consists essentially of”). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the

word “comprising” and synonyms thereof may be replaced by the phrase “consisting of” or the phrase “consists essentially of” or synonyms thereof and *vice versa*.

The phrase, “consists essentially of” and its pseudonyms may be interpreted herein to refer to a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

10 In embodiments of the invention, the styrene itself may be produced from *L*-phenylalanine by a further enzymatic cascade, with the *L*-phenylalanine being obtained either from the cell's natural metabolic processes or an enhanced enzymatic pathway that provides an up-regulated production of *L*-phenylalanine. These multiple reaction steps may be performed simultaneously or sequentially in one reaction vessel, to allow for the green, efficient, and economical production of enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof directly from styrene or a derivative thereof, phenylalanine or a derivative thereof, glucose or glycerol. Such, one-pot cascade reactions may avoid the expensive and energy-consuming isolation and purification of intermediates, minimize waste generation, and overcome the possible thermodynamic hurdles normally encountered in traditional multi-step synthesis. For example, multiple enzymes may be co-expressed inside one recombinant microbe strain, and the whole cells of the strain may be directly applied as catalysts for a series of cascade reactions in one pot. Alternatively, the enzymes could be separately expressed in cells of different strains, purified individually, or immobilized (the purified enzymes or cells containing all or some of said enzymes). In any event, the biocatalysts (enzymes, cells, immobilized enzymes, and immobilized cells) can be mixed together in any suitable combination to effect the one pot transformation discussed herein.

As discussed hereinbefore, there is provided a method for producing an enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof, which method comprises subjecting styrene or a derivative thereof (or other suitable starting materials mentioned herein) to multiple enzyme-catalyzed chemical transformations in a one-pot reaction system.

35 Styrene and derivatives thereof can be manufactured on very large scale in the petrochemical industry (e.g. by hydrocarbon cracking), and so form easily available and cheap starting materials for organic synthesis of the type discussed herein. For example, many aromatic and

aliphatic alkenes are produced in very large amounts and at very low price. As discussed hereinbelow, styrenes and substituted styrenes are very useful substrates to produce various chiral aromatic 1,2-amino alcohols (such as phenylethanol amine, phenylglycinol, nor(pseudo)ephedrine) and α -amino acids (such as phenylglycine) in high enantiomeric purity.

5

While the use of suitable styrene and its derivatives generated from the petrochemical industry is envisaged, the current invention also allows for the conversion of *L*-phenylalanine and derivatives thereof into styrene and derivatives thereof by way of further enzyme-catalyzed transformations which will be discussed hereinbelow. This may enable access to styrene derivatives that may otherwise be difficult to obtain access to and provide a greater pool of possible styrene substrates for use in the enzyme-catalyzed transformations described herein.

It will be understood that the terms "enantiomerically pure" and "enantiomerically enriched" refer to enantiomers of a compound. "Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another. Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., McGraw-Hill Dictionary of Chemical Terms (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., "*Stereochemistry of Organic Compounds*", John Wiley & Sons, Inc., New York, 1994. The compounds of the invention may contain asymmetric or chiral centers, and therefore exist in different stereoisomeric forms. It is intended that all stereoisomeric forms of the compounds of the invention, including but not limited to, diastereomers, enantiomers and atropisomers, as well as mixtures thereof such as racemic mixtures, form part of the present invention. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or *R* and *S*, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes D and L or (+) and (−) are employed to designate the sign of rotation of plane-polarized light by the compound, with (−) or l (L) meaning that the compound is levorotatory. A compound prefixed with (+) or d (D) is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

35

When referred to herein, the term "enantiomerically enriched" may refer to an enantiomeric excess of 50% or more. For example, the methods disclosed herein may provide a final product having an enantiomeric excess of 60%, 70%, 80%, 90%, 95%, 98%, or 99% or more. In embodiments of the invention, only one enantiomer or diastereomer of a chiral compound is provided by the process described herein (i.e. the compound is "enantiomerically pure").

When used herein, the term "derivative thereof" as applied to styrene and *L*-phenylalanine relates to a compound where the benzene ring contains one or more substituents (e.g. 1, 2, 3, 4 or 5, such as 1 to 3, such as 1 or 2 substituents) that are not H. Said substituents may be halo, alkyl, cycloalkyl, aryl, heterocyclic, OH, NH₂, SH and combinations thereof (e.g. alkyl aryl, Oalkyl, N(alkyl)H, N(alkyl)₂, N(alkyl)(aryl) etc).

Unless otherwise stated, the term "alkyl" refers to an unbranched or branched, saturated or unsaturated hydrocarbyl radical (so forming, for example, an alkenyl or alkynyl), which may be substituted or unsubstituted (with, for example, one or more halogen atoms). The alkyl group may be C₁₋₁₀ alkyl and, more preferably, C₁₋₆ alkyl (such as ethyl, propyl, (e.g. *n*-propyl or isopropyl), butyl (e.g. branched or unbranched butyl), pentyl or, more preferably, methyl). The terms "alkenyl" and "alkynyl" are to be interpreted accordingly.

Unless otherwise stated, the term "cycloalkyl" refers to an unbranched or branched, saturated or unsaturated hydrocarbyl radical (so forming, for example, a cycloalkenyl group) that may be substituted or unsubstituted. The cycloalkyl group may be C₃₋₁₂ cycloalkyl and, more preferably, C₅₋₁₀ (e.g. C₅₋₇) cycloalkyl. The term "cycloalkenyl" is to be interpreted accordingly.

The term "halogen", when used herein, includes fluorine, chlorine, bromine and iodine.

The term "aryl" when used herein includes C₆₋₁₄ (such as C₆₋₁₃ (e.g. C₆₋₁₀)) aryl groups that may be substituted or unsubstituted. Such groups may be monocyclic, bicyclic or tricyclic and have between 6 and 14 ring carbon atoms, in which at least one ring is aromatic. The point of attachment of aryl groups may be via any atom of the ring system. However, when aryl groups are bicyclic or tricyclic, they are linked to the rest of the molecule via an aromatic ring. C₆₋₁₄ aryl groups include phenyl, naphthyl and the like, such as 1,2,3,4-tetrahydronaphthyl, indanyl, indenyl and fluorenyl. Most preferred aryl groups include phenyl.

When used herein, the term "aryl alkyl" is to be interpreted in line with the definitions provided hereinbefore for "alkyl" and "aryl", where the point of attachment of the group to the rest of the compound of formula (I) or (II) is through the alkyl portion of the aryl alkyl group.

When used herein, the term "heterocyclic" refers to a fully saturated, partly unsaturated, wholly aromatic or partly aromatic ring system in which one or more (e.g. one to four) of the atoms in the ring system is other than carbon (i.e. a heteroatom, which heteroatom is preferably selected from N, O and S), and in which the total number of atoms in the ring system is between three and twelve (e.g. between five and ten). The heterocyclic groups may be substituted or unsubstituted. Heterocyclic groups that may be mentioned include 7-azabicyclo[2.2.1]heptanyl, 6-azabicyclo[3.1.1]heptanyl, 6-azabicyclo[3.2.1]octanyl, 8-azabicyclo[3.2.1]octanyl, aziridinyl, azetidiny, dihydropyranyl, dihydropyridyl, dihydropyrrolyl (including 2,5-dihydropyrrolyl), dioxolanyl (including 1,3-dioxolanyl), dioxanyl (including 1,3-dioxanyl and 1,4-dioxanyl), dithianyl (including 1,4-dithianyl), dithiolanyl (including 1,3-dithiolanyl), imidazolidinyl, imidazolinyl, morpholinyl, 7-oxabicyclo[2.2.1]heptanyl, 6-oxabicyclo[3.2.1]octanyl, oxetanyl, oxiranyl, piperazinyl, piperidinyl, pyranyl, pyrazolidinyl, pyrrolidinonyl, pyrrolidinyl, pyrrolinyl, quinuclidinyl, sulfolanly, 3-sulfolenyl, tetrahydropyranyl, tetrahydrofuranyl, tetrahydropyridyl (such as 1,2,3,4-tetrahydropyridyl and 1,2,3,6-tetrahydropyridyl), thietanyl, thirranly, thiolanyl, thiomorpholinyl, trithianyl (including 1,3,5-trithianyl), tropanyl, benzothiadiazolyl (including 2,1,3-benzothiadiazolyl), isothiochromanyl and, more preferably, acridinyl, benzimidazolyl, benzodioxanyl, benzodioxepinyl, benzodioxolyl (including 1,3-benzodioxolyl), benzofuranyl, benzofurazanyl, benzothiazolyl, benzoxadiazolyl (including 2,1,3-benzoxadiazolyl), benzoxazinyl (including 3,4-dihydro- 2H-1,4-benzoxazinyl), benzoxazolyl, benzomorpholinyl, benzoselenadiazolyl (including 2,1,3-benzoselenadiazolyl), benzothienyl, carbazolyl, chromanyl, cinnolinyl, furanyl, imidazolyl, imidazo[1,2-a]pyridyl, indazolyl, indolinyl, indolyl, isobenzofuranyl, isochromanyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolyl, isoxazolyl, naphthyridinyl (including 1,6-naphthyridinyl or, preferably, 1,5-naphthyridinyl and 1,8-naphthyridinyl), oxadiazolyl (including 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl and 1,3,4-oxadiazolyl), oxazolyl, phenazinyl, phenothiazinyl, phthalazinyl, pteridinyl, purinyl, pyranyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolyl, quinazolinyl, quinolinyl, quinolizinyl, quinoxalinyl, tetrahydroisoquinolinyl (including 1,2,3,4-tetrahydroisoquinolinyl and 5,6,7,8-tetrahydroisoquinolinyl), tetrahydroquinolinyl (including 1,2,3,4-tetrahydroquinolinyl and 5,6,7,8-tetrahydroquinolinyl), tetrazolyl, thiadiazolyl (including 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl and 1,3,4-thiadiazolyl), thiazolyl, thiochromanyl, thiophenetyl, thienyl, triazolyl (including 1,2,3-triazolyl, 1,2,4-triazolyl and 1,3,4-triazolyl) and the like. Substituents on heterocyclic groups may, where appropriate, be located on any atom in the ring system including a heteroatom. The point of attachment of heterocyclic groups may be *via* any atom in the ring system including (where appropriate) a heteroatom (such as a nitrogen atom), or an atom on any fused carbocyclic ring that may be present as part of the ring system. Heterocyclic groups may also be in the N- or S- oxidised form. Heterocyclic

groups that may be mentioned herein include cyclic amino groups such as pyrrolidinyl, piperidyl, piperazinyl, morpholinyl or a cyclic ether such as tetrahydrofuranyl.

When used herein, the term "heterocyclic alkyl" is to be interpreted in line with the definitions provided hereinbefore for "alkyl" and "heterocyclic", where the point of attachment of the group to the rest of the compound of formula (I) or (II) is through the alkyl portion of the heterocyclic alkyl group.

The substituents mentioned herein may be substituted or unsubstituted. When the substituents are substituted, they may be substituted with one or more of the groups selected from the group of halogen (e.g., a single halogen atom or multiple halogen atoms forming, in the latter case, groups such as CF₃ or an alkyl group bearing Cl₃), cyano, nitro, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, heterocycle, aryl, OR_a, SR_a, S(=O)R_e, S(=O)₂R_e, P(=O)₂R_e, S(=O)₂OR_e, P(=O)₂OR_e, NR_bR_c, NR_bS(=O)₂R_e, NR_bP(=O)₂R_e, S(=O)₂NR_bR_c, P(=O)₂NR_bR_c, C(=O)OR_e, C(=O)R_a, C(=O)NR_bR_c, OC(=O)R_a, OC(=O)NR_bR_c, NR_bC(=O)OR_e, NR_dC(=O)NR_bR_c, NR_dS(=O)₂NR_bR_c, NR_dP(=O)₂NR_bR_c, NR_bC(=O)R_a, or NR_bP(=O)₂R_e, wherein R_a is hydrogen, alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, heterocycle, or aryl; R_b, R_c and R_d are independently hydrogen, alkyl, cycloalkyl, heterocycle, aryl, or said R_b and R_c together with the N to which they are bonded optionally form a heterocycle; and R_e is alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, heterocycle, or aryl. It will be appreciated that these substituted groups may be unsubstituted or are themselves substituted with one or more halogen atoms.

For the avoidance of doubt, in cases in which the identity of two or more may be the same, the actual identities of the respective substituents are not in any way interdependent unless otherwise specified.

Specific styrene and *L*-phenylalanine derivatives that may be mentioned herein include those in which the phenyl ring of styrene and/or phenylalanine is mono- or disubstituted by (a) substituent(s) selected from F, Cl, Br, CH₃ or OCH₃. For example, the phenyl ring of styrene and/or phenylalanine may be monosubstituted by *o*-F, *m*-F, *p*-F, *m*-Cl, *p*-Cl, *m*-Br, *p*-Br, *m*-CH₃, *p*-CH₃, or *p*-OCH₃.

In particular embodiments of the invention that may also be mentioned herein, the styrene derivative may be indene which is either unsubstituted or substituted as described for styrene above.

The terms "amino acid" or "amino acid sequence," as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and
5 like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

The term "isolated" is herein defined as a biological component (such as a nucleic acid, peptide or protein) that has been substantially separated, produced apart from, or purified
10 away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been isolated thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically
15 synthesized nucleic acids.

The phrases "nucleic acid" or "nucleic acid sequence," as used herein, refer to an oligonucleotide, nucleotide, polynucleotide, or any fragment thereof, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may
20 represent the sense or the antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material. In the context of the invention, "fragments" refers to those nucleic acid sequences which are greater than about 60 nucleotides in length, and most preferably are at least about 100 nucleotides, at least about 1000 nucleotides, or at least about 10,000 nucleotides in length which are not full-length native sequence but retain catalytic enzyme
25 activity.

The term "oligonucleotide," as used herein, refers to a nucleic acid sequence of at least about 6 nucleotides to 60 nucleotides, preferably about 15 to 30 nucleotides, and most preferably about 20 to 25 nucleotides, which can be used in PCR amplification or in a hybridization assay
30 or microarray. As used herein, the term "oligonucleotide" is substantially equivalent to the terms "amplimers," "primers," "oligomers," and "probes," as these terms are commonly defined in the art.

The terms 'variant' and 'mutant' are used interchangeably herein. The at least one nucleic acids encoding at least one catalytic enzyme may encode a variant or mutant of the exemplified catalytic enzyme which retains activity. A "variant" of a catalytic enzyme, as used
35 herein, refers to an amino acid sequence that is altered by one or more amino acids. The

variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both.

- 5 Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing catalytic activity may be found using computer programs well known in the art, for example, DNASTAR software. In some embodiments, variant enzymes are at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more, homologous or identical at the amino acid level to an exemplary amino acid sequence described herein (e.g.,
10 catalase, alcohol dehydrogenase, α -transaminase) or a functional fragment thereof— e.g., over a length of about: 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% of the length of the mature reference sequence.

- The terms 'phenylacetaldehyde reductase' (PAR) and 'alcohol dehydrogenase' (ADH), as
15 referred to herein, are used interchangeably.

- A vector can include one or more catalytic enzyme nucleic acid(s) in a form suitable for expression of the nucleic acid(s) in a host cell. Preferably the recombinant expression vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence(s)
20 to be expressed. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences such as the T7 IPTG-inducible promoters disclosed in the Examples herein. The design of the expression vector can depend on such factors as the
25 choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or polypeptides, including fusion proteins or polypeptides, encoded by nucleic acids as described herein (e.g., catalytic enzyme proteins, fusion proteins, and the like).

- 30 The recombinant expression vectors of the invention can be designed for expression of catalytic enzyme proteins in prokaryotic or eukaryotic cells. For example, polypeptides of the invention can be expressed in bacteria (e.g., *E. coli*), insect cells (e.g., using baculovirus expression vectors), yeast cells or mammalian cells. Suitable host cells are discussed further
35 in Goeddel, (1990) Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. Alternatively, the recombinant expression vector(s) can be

transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing
5 constitutive or inducible promoters directing the expression of either fusion or non-fusion
proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to
the amino terminus of the recombinant protein. Such fusion vectors typically serve three
purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the
10 recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a
ligand in affinity purification. Often, a proteolytic cleavage site is introduced at the junction of
the fusion moiety and the recombinant protein to enable separation of the recombinant protein
from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and
their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical
fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D. B. and Johnson,
15 K. S. *Gene* **1988**, 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5
(Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding
protein, or protein A, respectively, to the target recombinant protein.

To maximize recombinant protein expression in *E. coli* is to express the protein in a host
20 bacterium with an impaired capacity to proteolytically cleave the recombinant protein
(Gottesman, S. *Gene Expression Technology: Methods in Enzymology* **1990** 185, Academic
Press, San Diego, Calif. 119-128). Another strategy is to alter the nucleic acid sequence of
the nucleic acid to be inserted into an expression vector so that the individual codons for each
amino acid are those preferentially utilized in *E. coli* (Wada et al., *Nucleic Acids Res.* **1992** 20:
25 2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by
standard DNA synthesis techniques and is described in the Examples.

The catalytic enzyme expression vector can be a yeast expression vector, a vector for
expression in insect cells, e.g., a baculovirus expression vector, a vector for expression in
30 bacterial cells, e.g. a plasmid vector, or a vector suitable for expression in mammalian cells.

When used in mammalian cells, the expression vector's control functions can be provided by
viral regulatory elements. For example, commonly used promoters are derived from polyoma,
Adenovirus 2, cytomegalovirus and Simian Virus 40.
35

In a preferred embodiment, the promoter is an inducible promoter, e.g., a promoter regulated
by a steroid hormone, by a polypeptide hormone (e.g., by means of a signal transduction

pathway), by a chemical (e.g., Isopropyl β -D-1-thiogalactopyranoside (IPTG)) or by a heterologous polypeptide.

The methods described herein make use of enzymes to catalyse a sequence of reactions.

- 5 While these reactions may be performed individually or, more particularly, two or more of them in combination, all of the reactions may be combined into a cascade reaction sequence that provides the product from the initial starting material in one pot, thereby eliminating the need for isolation of the intermediates and, potentially, increasing the overall yield of the reaction sequence. These cascade reactions may involve the use of one or more reactive components
10 selected from the group consisting of cells, immobilized cells, cell extracts, isolated enzymes and immobilized enzymes in said reaction vessel.

Another aspect the invention provides at least one expression construct comprising at least one nucleic acid sequence that is heterologous according to any aspect of the invention.

- 15 Preferably the at least one construct comprises a plasmid suitable for expression of at least one catalytic enzyme in a bacterium.

Another aspect the invention provides a host cell which includes at least one nucleic acid molecule described herein, e.g., at least one catalytic enzyme nucleic acid molecule within a
20 recombinant expression vector or a catalytic enzyme nucleic acid molecule containing sequences which allow homologous recombination into a specific site of the host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to
25 either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

- A host cell can be any prokaryotic or eukaryotic cell. For example, at least one catalytic enzyme protein can be expressed in bacterial cells (such as *E. coli*), insect cells (such as
30 *Spodoptera frugiperda* Sf9 cells), yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells (African green monkey kidney cells CV-1 origin SV40 cells; Gluzman *Cell* **1981**, 123: 175-182)). Other suitable host cells are known to those skilled in the art.

- One or more vector DNAs can be introduced into host cells via conventional transformation or
35 transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation,

DEAE-dextran-mediated transfection, lipofection, or electroporation. For example, according to the invention a host cell may comprise one, two, three, four or more plasmids, each of which may express at least one catalytic enzyme directed to a chemical transformation in the pathway from an alkene starting material to an enantiomerically pure or enantiomerically enriched 1,2-amino alcohol or α -amino acid. The host cell may also include a vector to express catalytic enzymes for providing the alkene by, for example, generating a vinyl carboxylic acid from an α -amino acid by a deamination reaction catalyzed by an ammonia lyase and generating the alkene from the vinyl carboxylic acid in a decarboxylation reaction catalyzed by a decarboxylase.

10

In a preferred embodiment, the catalytic enzymes required to transform an alkene starting material to an enantiomerically pure or enantiomerically enriched 1,2-amino alcohol or α -amino acid are arranged on expression vectors as modules, wherein each module comprises a combination of catalytic enzymes to perform specific reactions within the overall system.

15

For example, a first module may comprise enzymes for the dihydroxylation of a terminal alkene to a 1,2-diol co-expressed on a plasmid; a second module may comprise enzymes for the oxidation-amination of a 1,2-diol to a 1,2-amino alcohol co-expressed on a plasmid; a third module may comprise enzymes for the double oxidation of a 1,2-diol to an α -hydroxy acid co-expressed on a plasmid; a fourth module may comprise enzymes for the oxidation-amination of α -hydroxy acid to α -amino acid co-expressed on a plasmid; and a fifth module may comprise enzymes for the deamination-decarboxylation of an α -amino acid to an alkene. Arrangements of such enzymes as modules allow flexibility in constructing a serial cascade of reactions in one pot. One or more modules may be engineered onto the same plasmid. For example, a host cell comprising the said first and third modules, on the same or separate plasmids, is capable of catalyzing the conversion of a terminal alkene to a 1,2-amino alcohol. Likewise a host cell comprising said first, a second and fourth module is capable of catalyzing the conversion of a terminal alkene to an α -amino acid. If module 5 is co-expressed in the host cell, an α -amino acid feed stock can be provided for the cell to generate its own terminal alkene for the cascade reactions.

25

30

In one aspect of the invention there is provided one or more recombinant prokaryotic or eukaryotic cells selected from the group comprising bacterial cells, yeast cells, mammalian cells and insect cells, wherein said cells comprise at least one expression construct and/or heterologous nucleic acid molecule that encodes at least one catalytic enzyme required in the pathway from alkene to enantiomerically pure or enantiomerically enriched 1,2-amino alcohol or from an α -amino acid to enantiomerically pure or enantiomerically enriched 1,2-amino alcohol.

35

The cells may contain a single expression vector or construct, such as a plasmid, which expresses a single catalytic enzyme or co-expresses a plurality of catalytic enzymes as described herein under the control of at least one regulatory element. The catalytic enzymes may be arranged in the plasmid as an individual artificial operon under the control of a promoter with one ribosome-binding site before every gene, or arranged with individual promoters. Accordingly, a one-pot synthesis cascade may be achieved with cells expressing all required catalytic enzymes on one or several plasmids, or with different recombinant cells which each express a specific repertoire of catalytic enzymes, providing the necessary cells are included for a particular chemical transformation.

10

In another preferred embodiment, said catalytic enzymes for use in the invention have at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more, homology or amino acid identity with at least one enzyme mentioned herein.

15 A host cell of the invention can be used to produce (i.e., express) one or more catalytic enzyme proteins. Accordingly, the invention further provides methods for producing one or more catalytic enzyme proteins, e.g., one or more catalytic enzyme proteins described herein, using the host cells of the invention. In one embodiment, the method includes culturing the host cell of the invention (into which one or more recombinant expression vector(s) encoding one or more catalytic enzyme proteins has/have been introduced) in a suitable medium such that one or more catalytic enzyme proteins is/are produced. In another embodiment, the method further includes isolating one or more catalytic enzyme proteins from the medium or the host cell.

25 According to another aspect of the invention there is provided a kit comprising at least one recombinant cell, expression construct or isolated nucleic acid according to any aspect of the invention. Preferably, the kit can be used to provide one or more components for a one-pot system to enzyme-catalyze the production of an enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof from a suitable starting material.

30 The method in the first aspect of the invention that produces enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof may comprise the steps of:

(a) generating phenylethane diol or a derivative thereof from styrene or a derivative thereof by conducting an epoxidation reaction catalyzed by an epoxidase to form styrene oxide or a derivative thereof and conducting a hydrolysis reaction catalyzed by an epoxide hydrolase on the epoxide, or by a dihydroxylation reaction catalyzed by a dioxygenase;

35

(b) generating 2-hydroxyacetophenone or a derivative thereof from the phenylethane diol or a derivative thereof by an oxidation reaction catalyzed by an alcohol dehydrogenase or a secondary alcohol oxidase; and

- (c) generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol
 5 or a derivative thereof from 2-hydroxyacetophenone or a derivative thereof by a transamination reaction catalyzed by a transaminase or a reductive amination reaction catalyzed by an amine dehydrogenase.

In the above enzymatic cascade, one or more of the following may apply:

- 10 (a) the epoxidase may be a styrene monooxygenase. For example, the styrene monooxygenase may be from *Pseudomonas sp. VLB120*, or its mutants or similar enzymes with more than 50% identity;
- (b) the epoxide hydrolase, when used, may be from *Solanum tuberosum* or its mutants or similar enzymes with more than 50% identity;
- 15 (c) the alcohol dehydrogenase, when used, may be from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* or their mutants or similar enzymes with more than 50% identity; and
- (d) the transaminase may be from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Marteella mediterranea*, *Neosartora fischeri*, *Arthrobacter sp.*, or *Aspergillus*
 20 *terreus* or their mutants or similar enzymes with more than 50% identity.

Yet more particularly, the epoxide hydrolase, when used, may be selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA) from *Bacillus subtilis* or its mutants or similar enzymes with more than
 25 50% identity and/or the transaminase may be selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri* or their mutants or similar enzymes with more than 50% identity.

As noted above, the currently disclosed invention may make use of one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes. Thus, in
 30 embodiments of the invention that may be mentioned herein, the one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) may be selected from one or more of the group consisting of:

- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
- 35 (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
- (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
- (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;

- (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
- (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.

In particular embodiments of the invention, the one of the one or more recombinant microbial
 5 cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) may be *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA.

In further embodiments of the invention that may be mentioned herein, the one of the one or
 10 more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) may be selected from one or more of the group consisting of:

- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA;
- (b) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
- 15 (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA;
- (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH;
- (e) *E. coli* T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
- (f) *E. coli* T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.

20 In particular embodiments of the invention, the one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) may be *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH.

25 As noted hereinbefore, the method may make use of other starting materials other than styrene or its derivatives. Thus, in further embodiments of the invention, the method may further comprise providing styrene or a derivative thereof by generating *trans*-cinnamic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof by a deamination reaction catalyzed by an ammonia lyase and generating styrene or a derivative thereof from the *trans*-
 30 cinnamic acid or a derivative thereof in a decarboxylation reaction catalyzed by a decarboxylase.

In the conversion of *L*-phenylalanine or a derivative thereof to styrene or a derivative thereof, one or more of the following enzymes may be used:

- 35 (a) the ammonia lyase may be phenylalanine ammonia lyase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity; and

(b) the decarboxylase may be phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity.

In particular embodiments of the invention, the conversion of *L*-phenylalanine or a derivative thereof to enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof may make use of one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes), said one or more recombinant microbial cells may be *E. coli* T7-*pACYC-PAL-PAD*, *pCDF-SMO-StEH*, *pET-BDHA-AlaDH*, *pRSF-BmTA*.

In additional or alternative embodiments of the invention, the conversion of *L*-phenylalanine or a derivative thereof to enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof may make use of one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes), said one or more recombinant microbial cells may be *E. coli* T7-*pACYC-PAL-PAD*, *pCDF-SMO-StEH*, *pET-BDHA-AlaDH*, *pRSF-NfTA*.

As noted above, the method disclosed herein may make use of the cellular apparatus to generate the feedstock for the enzymatic cascade from glucose or glycerol. Thus, in embodiments of the invention, the method may further comprise cells that overexpress the natural *L*-phenylalanine biosynthetic pathway, which cells convert glucose or glycerol to *L*-phenylalanine. For example, the microbial cells producing *L*-phenylalanine from glucose or glycerol may overexpress at least one enzyme that is selected from one or more of the group consisting of DAHP synthase (*AroG*), shikimate kinase (*AroK*), shikimate dehydrogenase (*YdiB*), chorismate mutase/prephenate dehydratase (*PheA*) and tyrosine aminotransferase (*TyrB*), or their mutants or similar enzymes with more than 50% identity. More particularly, the microbial cells producing *L*-phenylalanine from glucose or glycerol may overexpress at least one enzyme that is:

- (a) a feedback inhibition resistant mutant of *AroG*;
- (b) a feedback inhibition resistant mutant of *PheA*.

As will be appreciated, one or both of these enzymes may be overexpressed.

In particular embodiments of the invention, the microbial cells producing *L*-phenylalanine from glucose or glycerol overexpress at least one enzyme may be one in which the microbial cells are mutated for deletion or inactivation of *crr* and/or *tyrA* genes.

In particular embodiments of the invention, the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme may be *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA or *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA. Additionally or alternatively, the microbial
5 cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme may be a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA or may be a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.

10 The method may make use of any suitable medium to conduct the reaction. For example, the one-pot reaction system may comprise an aqueous medium. Alternatively or additionally, the one-pot reaction system may comprise use of a bi-phasic medium, optionally wherein the bi-phasic medium consists of an aqueous and solid resin medium, or an aqueous and organic solvent medium (e.g. the organic solvent medium is selected from one or more alkanes
15 solvents and/or one or more ester solvents). Alkane solvents that may be mentioned herein include, but are not limited to n-hexadecane.

In embodiments of the invention disclosed herein, the one-pot reaction system may comprise use of absorbent resins or nanomaterials for *in-situ* removal of product.

20

In a further aspect of the invention, there is provided an isolated nucleic acid molecule encoding at least one heterologous catalytic enzyme selected from the group comprising:

(a) an epoxidase for conducting an epoxidation reaction to form styrene oxide or a derivative thereof from styrene or a derivative thereof and an epoxide hydrolase for conducting
25 a hydrolysis reaction on the epoxide to provide phenylethane diol or a derivative thereof, or an oxygenase for generating phenylethane diol or a derivative thereof from styrene or a derivative thereof by a dihydroxylation reaction;

(b) an alcohol dehydrogenase or a secondary alcohol oxidase for generating 2-hydroxyacetophenone or a derivative thereof from phenylethane diol by an oxidation reaction;

30 and

(c) a transaminase or an amine dehydrogenase for generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof by a transamination reaction or a reductive amination from 2-hydroxyacetophenone or a derivative thereof; or

(d) an ammonia lyase for generating *trans*-cinnamic acid or a derivative thereof
35 from L-phenylalanine or a derivative thereof by a deamination reaction; and

(e) a decarboxylase for generating styrene or a derivative thereof from *trans*-cinnamic acid or a derivative thereof in a decarboxylation reaction.

As will be appreciated, the isolated nucleic acid molecule above may encode for two or more, such as three or more, such as four or more, or all of said catalytic enzymes. That is, the isolated nucleic acid may encode a plurality of said catalytic enzymes. In such embodiments, said plurality of catalytic enzymes may be arranged as at least one module selected from the group comprising:

- i) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms styrene or a derivative thereof to phenylethane diol or a derivative thereof;
- ii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms phenylethane diol or a derivative thereof to a 2-hydroxyacetophenone or a derivative thereof;
- iii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms 2-hydroxyacetophenone or a derivative thereof to enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof; and
- v) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms an L-phenylalanine or a derivative thereof to styrene or a derivative thereof, or any combination thereof.

A further aspect of the invention may relate to an expression construct comprising at least one nucleic acid molecule as described hereinbefore. The expression construct may comprise one or more recombinant prokaryotic or eukaryotic cells selected from the group comprising bacterial cells, yeast cells, mammalian cells and insect cells. For example, the one or more recombinant prokaryotic or eukaryotic cells may be one in which said enzymes (encoded by the at least one nucleic acid molecule) have at least 50% amino acid identity with at least one enzyme selected from the group comprising:

- (a) a styrene monooxygenase from *Pseudomonas sp. VLB120*;
- (b) an epoxide hydrolase from *Solanum tuberosum*;
- (c) an alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* (e.g. the epoxide hydrolase is selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA));
- (d) a transaminase from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Marteella mediterranea*, *Neosartora fischeri*, *Arthrobacter sp.*, or *Aspergillus terreus* (e.g. the transaminase is selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri*);
- (e) a phenylalanine ammonia lyase from *Arabidopsis thaliana*;
- (f) a phenylacrylic acid decarboxylase from *Aspergillus niger*.

In particular embodiments of the invention, the one or more recombinant cells may be selected from:

- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
- (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
- 5 (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
- (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;
- (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH;
- (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
- (g) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NfTA;
- 10 (g) *E. coli* T7-pCDF-SMO-StEH, pET-NfTA, pRSF-BDHA- AlaDH;
- (i) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NfTA;
- (j) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NfTA, pRSF-SMO-StEH;
- (k) *E. coli* T7-pCDF-NfTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
- (l) *E. coli* T7-pCDF-NfTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
- 15 (m) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA;
- (n) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA;
- (o) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA; and
- (p) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NfTA.

20

In more particular embodiments that may be mentioned herein, the one or more recombinant cells may be selected from:

- (i) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
- (ii) *E. coli* T7-pCDF-SMO-StEH, pET-NfTA, pRSF-BDHA- AlaDH;
- 25 (iii) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA; and
- (iv) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.

30

In a further aspect of the invention, there is provided a kit comprising at least one isolated nucleic acid as described hereinbefore.

Further aspects and embodiments of the invention will now be discussed by reference to the following non-limiting examples.

Examples

35

Materials

The following chemicals were purchased from Sigma-Aldrich: *L*-Phenylalanine **6** (99%), *D*-glucose **8** (99%), glycerol **9** (99%), (*R*)-phenylglycinol **1a** (99%), (*S*)-phenylglycinol **1a** (99%), (*R*)-phenylethanediol **4a** (99%), 2-hydroxyacetophenone **5a** (99%), styrene **2a** (99%), 2-fluorostyrene **2b** (98%), indene **2l** (98%), kanamycin sulfate (95%), ampicillin sodium salt
 5 (96%), streptomycin sulfate (95%), ammonium persulfate ($\geq 98\%$), sodium citrate ($\geq 98\%$), thiamine HCl (98%), ferrous sulfate (99%), ammonium hydroxide (28%, NH_3 in H_2O), NaCl (99.5%), betaine (99%), *n*-hexadecane (99%), ethyl oleate (99%), KH_2PO_4 (99%), trifluoroacetic acid (TFA, 99%), sulphuric acid ($\geq 99\%$), acetophenone (99%), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (99%), NH_4Cl (99%), NaOH (99%), Na_2SO_4 (99%), benzyl alcohol (99%), MgSO_4 (99%),
 10 MnSO_4 (99%), CaCl_2 (99%), FeCl_3 (99%), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (99%), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (99%), $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ (98%), NaMoO_4 (99%), H_3BO_4 (99%), HCl (99%), NaBH_4 ($>99\%$), 2-amino-2-(3-bromophenyl)acetic acid ($>99\%$), iodine (I_2 , $>99\%$), KOH ($>99\%$), methanol (MeOH , $>99\%$), tetrahydrofuran (THF, $>99\%$), ethyl acetate ($>99\%$), glucose ($>99\%$), glycerol ($>99\%$), dichloromethane (CH_2Cl_2 , $>99\%$), *D*-Alanine ($>98\%$), *L*-Glutamate ($>99\%$), *L*-Alanine ($>99\%$)
 15 and isopropylamine ($>99\%$).

The following chemicals were obtained from other suppliers: 3-fluorostyrene **2c** ($>97\%$), 4-fluorostyrene **2d** ($>98\%$), 3-chlorostyrene **2e** ($>98\%$), 4-chlorostyrene **2f** ($>98\%$), 3-bromostyrene **2g** ($>97\%$), 4-bromostyrene **2h** ($>95\%$), 3-methylstyrene **2i** ($>97\%$), 4-methylstyrene **2j** ($>96\%$), 4-methoxystyrene **2k** ($>98\%$), (1*R*,2*R*)-1-amino-2-indanol **1l** were
 20 purchased from TCI chemicals. (*R*)-2-amino-2-(2-fluorophenyl)ethanol **1b** (97%), 2-amino-2-(3-fluorophenyl)ethanol **1c** (97%), 2-amino-2-(4-fluorophenyl)ethanol **1d** (97%), (*R*)-2-amino-2-(4-fluorophenyl)ethanol **1d** (97%), 2-amino-2-(3-chlorophenyl)ethanol **1e** (97%), (*S*)-2-amino-2-(3-chlorophenyl)ethanol **1e** (97%), 2-amino-2-(4-chlorophenyl)ethanol **1f** (97%), (*S*)-
 25 2-amino-2-(3-bromophenyl)ethanol **1g** (97%), 2-amino-2-(4-bromophenyl)ethanol **1h** (97%), 2-amino-2-(3-methylphenyl)ethanol **1i** (97%), 2-amino-2-(4-methylphenyl)ethanol **1j** (97%), (*S*)-2-amino-2-(4-methylphenyl)ethanol **1j** (97%) and 2-amino-2-(4-methoxyphenyl)ethanol **1k** (97%) were purchased from Acrotein ChemBio Inc. Isopropyl β -D-1-thiogalactopyranoside (IPTG, 99%) was purchased from 1st base Singapore. Ethyl acetate (HPLC grade) and
 30 acetonitrile (HPLC grade) were obtained from Tedia. Luria broth (LB, Miller) powder and BactoTM yeast extract were purchased from Becton Dickinson Germany.

The plasmid isolation kit and gel extraction kit were purchased from Omega Bio-tek, Inc. USA. All DNA-modifying enzymes including DNA polymerases and restriction digestion enzymes
 35 required in gene cloning were purchased from Thermo Fisher Scientific USA. SDS sample loading dye and SDS-PAGE gels were purchased from Genscript Singapore.

Analytical techniques

High-performance liquid chromatography (HPLC) analysis

The samples were centrifuged at $30,000 \times g$ for 10 min to separate the cells, aqueous and organic phase. The products were present only in the aqueous phase and analyzed by reverse phase HPLC using a Shimadzu prominence HPLC system. The HPLC was equipped with Agilent Poroshell 120 SB-C18 column (150×4.6 mm, $2.7 \mu\text{m}$) and maintained at 25°C . A mobile phase of 80% water with 0.1% TFA, and 20% acetonitrile was used at a flow rate of 0.5 mL/min. The products were detected using photodiode array detector measuring UV absorbance at 210 nm.

10

Retention time: **4a**, 4.33 min; **5a**, 6.02 min; **6**, 3.55 min; **1a**, 4.22 min; **1b**, 4.25 min; **1c**, 4.62 min; **1d**, 4.71 min; **1e**, 6.12 min; **1f**, 6.71 min; **1g**, 6.93 min; **1h**, 7.74 min; **1i**, 5.62 min; **1j**, 6.20 min; **1k**, 4.78 min; and **1l**, 4.32 min.

15 *Analysis of ee values with HPLC*

The ee values of the products were analyzed using reverse phase HPLC system equipped with Daicel Crownpak CR (+) (150×4.0 mm, $5 \mu\text{m}$) column and maintained at 25°C . A mobile phase of water with 0.1% TFA was used at a flow rate of 1.0 mL/min. The ee values of products **1h** and **1k**, were analyzed as aforementioned but with modified mobile phase: 90% water with 0.1% TFA and 10% methanol (MeOH) was used at a flow rate of 1.0 mL/min. The products were detected using photodiode array detector measuring UV absorbance at 210 nm.

20

Retention time: (*R*)-**1a**, 4.62 min; (*S*)-**1a**, 4.19 min; (*R*)-**1b**, 4.19 min; (*R*)-**1c**, 5.81 min; (*R*)-**1d**, 6.03 min; (*R*)-**1e**, 19.78 min; (*R*)-**1f**, 18.74 min; (*R*)-**1g**, 4.66 min; (*R*)-**1h**, 13.61 min; (*R*)-**1i**, 13.48 min; (*R*)-**1j**, 12.81 min; (*R*)-**1k**, 4.53 min; and (*R*)-**1l**, 5.84 min.

25

SDS-PAGE analysis

The harvested cell pellets were resuspended in 20 mM Tris-HCl buffer (pH 8.0) to give a cell concentration of 10 OD_{600} . 1 mL of the cells was added with 1 g of 0.1 mm glass beads in a 2 mL lysis tube, followed by homogenization using FastPrep-24. The lysate was centrifuged at $2000 \times g$ for 5 min to remove whole cells and glass beads. 50 μL of the clear lysate was mixed with 10 μL of 5x SDS sample loading dye, heated at 95°C for 15 min, and resolved in 12% SDS-PAGE gel. The molecular weights of the expressed proteins were predicted from the DNA sequence using ExPASy Compute pI/Mw tool. Predicted molecular weights: AlaDH, 48.6 kDa; BDHA, 37.3 kDa; BmTA, 53 kDa; NfTA, 36 kDa; PAD, 55.2 kDa; PAL, 77.9 kDa; SMO, 46.4 kDa; StEH, 36.2 kDa.

35

Polymerase chain reaction (PCR)

PCR was performed using the Thermal cycler purchased from Biorad USA. PCR experiments were performed using Phusion polymerase from Thermo Scientific USA. The protocols from the manufacturers were followed for PCR amplifications.

5

NMR spectroscopy

The NMR was performed using the NMR spectrometer purchased from Bruker USA. The samples were prepared in CD₃OD. ¹H NMR and ¹³C NMR were performed at 400 MHz and 100 MHz, respectively.

10

Synthesis of racemic 2-amino-2-(3-bromophenyl)ethanol **1g**

Racemic 2-amino-2-(3-bromophenyl)ethanol **1g** was synthesized in our lab according to a reported method (WO/2014/106606).

15 A solution of racemic 2-amino-2-(3-bromophenyl)acetic acid (23 mg), and NaBH₄ (9 mg) in anhydrous THF (2 mL) were added dropwise into a solution of iodine (26 mg) in anhydrous THF (2 mL). The reaction mixture was stirred at 70 °C for 20 h, followed by quenching with MeOH at room temperature. The reaction mixture was treated with 20% aqueous KOH and stirred overnight. The crude **1g** was obtained by extraction with ethyl acetate, followed by
20 evaporation. The extracted compound was further purified by preparative thin layer chromatography (TLC) with CH₂Cl₂:MeOH:NH₃ (28% aqueous solution) of 100:10:1 as the mobile phase (*R_f* ≈ 0.3). The purified compound was checked by HPLC (>95% purity) and ¹H NMR.

25 ¹H NMR (400 MHz, CD₃OD): δ 7.57 (s, 1H), 7.41 (d, *J* = 7.82 Hz, 1H), 7.33 (d, *J* = 7.74 Hz, 1H), 7.25 (t, *J* = 7.78 Hz, 1H), 4.84 (s, 3H), 3.92 (dd, *J* = 7.82 5.08 Hz, 1H), 3.66 (dd, *J* = 4.97 10.81 Hz, 1H), 3.54 (dd, *J* = 7.44 10.81 Hz, 1H).

General procedure for the preparation of medium

30 *Medium for genetic engineering, seed culturing and strain maintenance*
LB medium – Tryptone (10 g/L), Yeast extract (5 g/L) and NaCl (10 g/L).

Medium for culturing E. coli strains for whole-cell biotransformation

35 Modified M9 medium - Na₂HPO₄ (6 g/L), KH₂PO₄ (3 g/L), NH₄Cl (1 g/L), NaCl (0.5 g/L), MgSO₄ (1 mM), CaCl₂ (0.1 mM), glucose (20 g/L), yeast extract (6 g/L), and trace metal solution (1 mL). Trace metal solution: CoCl₂·2H₂O (0.1 g/L), H₃BO₃ (0.1 g/L), Na₂MoO₄ (0.1 g/L), FeCl₃ (8.3 g/L), ZnCl₂ (0.84 g/L), CuCl₂·2H₂O (0.13 g/L) and MnCl₂ (0.016 g/L).

Medium for the production of L-phenylalanine by E. coli NST-Phe

Fermentation medium - Glucose or glycerol (10 g/L), KH₂PO₄ (5 g/L), (NH₄)₂SO₄ (10 g/L), MgSO₄ (5 g/L), yeast extract (5 g/L), betaine (1 g/L), FeSO₄ (15 mg/L), and MnSO₄ (15 mg/L). Glucose or glycerol was maintained at 10 g/L by feeding 500 g/L of solution, and the pH was maintained at ~6.8 with NH₄OH solution (30%).

General procedure for recombinant strain construction

The duet vectors such as pACYCDuet, pETDuet, pCDFDuet and pRSFDuet were used as expression plasmids. The insert DNA was either PCR amplified or synthesized as double stranded DNA, and ligated with appropriate expression plasmid using restriction-digestion cloning method. The ligated DNA fragments were transformed in *E. coli* DH5α competent cells using heat-shock method. The colonies were verified using colony PCR and the positive clones were sequenced. The positive clones were cultured in 5 mL of LB medium for 12 h at 37 °C and the expression plasmids were extracted. The isolated plasmids were transformed into *E. coli* T7 express to obtain recombinant *E. coli* strains for enzyme expression.

General procedure for the cultivation of *E. coli* strains

The glycerol stock of recombinant *E. coli* was inoculated in 2 mL of LB medium with appropriate antibiotics (50 µg/mL kanamycin, 25 µg/mL chloramphenicol, 50 µg/mL streptomycin, and 100 µg/mL ampicillin), and grown at 37 °C and 220 rpm for 6-8 h. 1 mL of the culture was added to 50 mL of modified M9 medium with appropriate antibiotics (50 µg/mL kanamycin, 25 µg/mL chloramphenicol, 50 µg/mL streptomycin, and 100 µg/mL ampicillin) in a 250 mL flask, and grown at 37°C and 220 rpm. Antibiotics were reduced to 50% for strains containing more than two plasmids. At 2 h, 0.5 mM of IPTG was added and the growth temperature was set at 22 °C. After 16 h of growth at 22 °C, the cells were harvested by centrifugation at 2680 × g for 10 min. The cell pellets were resuspended in appropriate volume of phosphate buffer and used for biotransformation.

Example 1. Recombinant strain construction

30

DNA sequences of recombinant genes

>BDHA (SEQ ID. NO: 1)
>CpSADH (SEQ ID. NO: 2)
>HnTA (SEQ ID. NO: 3)
35 >DpgTA (SEQ ID. NO: 4)
>EeaTA (SEQ ID. NO: 5)
>VFTA (SEQ ID. NO: 6)

- >CvTA (SEQ ID. NO: 7)
 >mVfTA (SEQ ID. NO: 8). Mutation of VfTA: Y150F and V153A.
 >BmTA (SEQ ID. NO: 9)
 >MmTA (SEQ ID. NO: 10)
 5 >NfTA (SEQ ID. NO: 11)
 >ArRTA (SEQ ID. NO: 12)
 >AtTA (SEQ ID. NO: 13)

10 *E. coli* strain expressing enzymes for the cascade conversion of styrene **2a** to (R)- and (S)-2-phenylglycinol **1a**

The genes coding for BDHA from *Bacillus subtilis* BGSC1A1, CpSADH from *Candida parapsilosis* and 11 transaminases were synthesized as gBlock fragments and amplified by PCR. BdhA and CpSADH genes were cloned in pETDuet and the transaminases genes were cloned in pRSFDuet. The genes coding for enzymes in the cascade (SMO, StEH, BdhA, 15 AlaDH and BmTA or NfTA) were cloned into three expression plasmids (pETDuet, pCDFDuet and pRSFDuet) with different copy numbers and antibiotic resistance. The expression plasmids were transformed into *E. coli* T7 to obtain 12 different recombinant strains (*E. coli*-SSBB-1-6 and *E. coli*-SSBN-1-6) with varying expression levels of the enzymes in the artificial cascade (Table 1).

20

Table 1. List of *E. coli* strains used in this study for the production of (R)- and (S)-2-phenylglycinol **1a**.

Strain	Recombinant plasmids	Reaction
<i>E. coli</i> -BDHA	<i>E. coli</i> T7-pETDuet-BDHA	4 to 5
<i>E. coli</i> -CpSADH	<i>E. coli</i> T7-pETDuet-CpSADH	4 to 5
<i>E. coli</i> -HnTA	<i>E. coli</i> T7-pRSFDuet-HnTA	5 to 1
<i>E. coli</i> -DpgTA	<i>E. coli</i> T7-pRSFDuet-DpgTA	5 to 1
<i>E. coli</i> -EeaTA	<i>E. coli</i> T7-pRSFDuet-EeaTA	5 to 1
<i>E. coli</i> -VfTA	<i>E. coli</i> T7-pRSFDuet-VfTA	5 to 1
<i>E. coli</i> -CvTA	<i>E. coli</i> T7-pRSFDuet-CvTA	5 to 1
<i>E. coli</i> -mVfTA	<i>E. coli</i> T7-pRSFDuet-mVfTA	5 to 1
<i>E. coli</i> -BmTA	<i>E. coli</i> T7-pRSFDuet-BmTA	5 to 1
<i>E. coli</i> -MmTA	<i>E. coli</i> T7-pRSFDuet-MmTA	5 to 1
<i>E. coli</i> -NfTA	<i>E. coli</i> T7-pRSFDuet-NfTA	5 to 1
<i>E. coli</i> -ArRTA	<i>E. coli</i> T7-pRSFDuet-ArRTA	5 to 1
<i>E. coli</i> -AtTA	<i>E. coli</i> T7-pRSFDuet-AtTA	5 to 1
<i>E. coli</i> -SSBB-1	<i>E. coli</i> T7-pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA	2 to (R)-1
<i>E. coli</i> -SSBB-2	<i>E. coli</i> T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA-AlaDH	2 to (R)-1

<i>E. coli</i> -SSBB-3	<i>E. coli</i> T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA	2 to (R)-1
<i>E. coli</i> -SSBB-4	<i>E. coli</i> T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH	2 to (R)-1
<i>E. coli</i> -SSBB-5	<i>E. coli</i> T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH	2 to (R)-1
<i>E. coli</i> -SSBB-6	<i>E. coli</i> T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH	2 to (R)-1
<i>E. coli</i> -SSBN-7	<i>E. coli</i> T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA	2 to (S)-1
<i>E. coli</i> -SSBN-8	<i>E. coli</i> T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH	2 to (S)-1
<i>E. coli</i> -SSBN-9	<i>E. coli</i> T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA	2 to (S)-1
<i>E. coli</i> -SSBN-10	<i>E. coli</i> T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH	2 to (S)-1
<i>E. coli</i> -SSBN-11	<i>E. coli</i> T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH	2 to (S)-1
<i>E. coli</i> -SSBN-12	<i>E. coli</i> T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH	2 to (S)-1
<i>E. coli</i> -PPSSBB	<i>E. coli</i> T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA	6 to (R)-1
<i>E. coli</i> -PPSSBN	<i>E. coli</i> T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA	6 to (S)-1
<i>E. coli</i> NST-PPSSBB	<i>E. coli</i> NST74(DE3)-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA	8 or 9 to (R)-1
<i>E. coli</i> NST-PPSSBN	<i>E. coli</i> NST74(DE3)-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA	8 or 9 to (S)-1
<i>E. coli</i> NST-Phe	<i>E. coli</i> NST74(DE3)-pCDF-AroG*-YdiB-AroK-PheA*-TyrB	8 or 9 to 6

E. coli strain expressing enzymes for the cascade conversion of L-phenylalanine **6** to (R)- and (S)-2-phenylglycinol **1a**

- 5 *E. coli*-SSBB-1 and *E. coli*-SSBN-2 were further engineered for the cascade conversion of L-phenylalanine **6** to phenylglycinol **1a**. pACYCDuet expressing PAL and PAD from our previous study (Y. Zhou, S. Wu & Z. Li, *Adv. Synth. Catal.* **2017**, 359, 4305-4316) was transformed into *E. coli*-SSBB-1 (pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA) and *E. coli*-SSBN-2 (pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH) to obtain *E. coli*-PPSSBB (produce (R)-**1a**) and *E. coli*-PPSSBN (produce (S)-**1a**), respectively.

10

E. coli strain expressing enzymes for the production of (R)- and (S)-2-phenylglycinol **1a** from glucose **8** and glycerol **9**

For the synthesis of phenylglycinol **1a** from glucose **8** and glycerol **9**, *E. coli* NST74 (DE3) was used as the expression strain, which was previously engineered to produce L-phenylalanine

(Y. Zhou *et al.*, *ChemSusChem* **2018**, *11*, 2221-2228). pCDF-SMO-StEH, pET-BdhA-AlaDH, pRSF-BmTA/NfTA and pACYC-PAL-PAD were transformed into *E. coli* NST74 (DE3) to obtain *E. coli* NST-PPSSBB (produce (R)-**1a**) and *E. coli* NST-PPSSBN (produce (S)-**1a**), respectively.

5

Example 2. Design of cascade biotransformation for the production of (R)- and (S)-2-phenylglycinol **1a** from styrene **2a**

To convert styrene **2a** to (R)- and (S)-2-phenylglycinol **1a**, we designed artificial cascade with
10 four enzymes for the epoxidation-hydrolysis-oxidation-transamination reactions (FIG. 1a).

Conversion of (R)-phenylethanediol **4a** to hydroxyacetophenone **5a**

Biotransformation was performed at 30 °C with 5 mL suspension of *E. coli*-BDHA or *E. coli*-CpSADH (10 g cdw/L) in phosphate buffer (200 mM, pH 8) containing 2 or 10 mM of **4a**. At 12
15 h, 50 µL of the aqueous sample was collected and analyzed in reverse-phase HPLC for the production of **5a**.

Whole cell biotransformation for hydroxyacetophenone **5a** to (R)- and (S)-Phenylglycinol **1a**

Whole cell biotransformation was performed at 30 °C for 24 h with 5 mL suspension of *E. coli*
20 strains expressing transaminases (20 g cdw/L) in potassium phosphate buffer (KP, 200 mM, pH 8) containing 10 mM of **5a**. Glucose (0.5%) and 200 mM of an amino donor selected from D-Alanine, Glutamate, L-Alanine and isopropylamine, were added to the reaction mixture at 0 h. Then, additional glucose (0.5%) and amino donor (100 mM) were added at 20 h. After 24 h reaction, the samples were analysed by reverse phase HPLC to determine the concentration and ee of **1a**. Biotransformations were performed in triplicates and the mean values were
25 presented.

Results and discussion

The conversion of styrene **2a** to (R)-phenylethanediol **4a** by epoxidation-hydrolysis reaction
30 were achieved by SMO from *Pseudomonas sp.* and StEH from *Solanum tuberosum* (S. Wu, J. Liu & Z. Li, *ACS Catal.* **2017**, *7*, 5225-5233; Y. Zhou, S. Wu & Z. Li, *Angew. Chem.* **2016**, *128*, 11819-11822; and S. Wu *et al.*, *ACS Catal.* **2014**, *4*, 409-420). Conversion of **4a** to 2-hydroxyacetophenone **5a** by oxidation reaction could be achieved by the use of alcohol dehydrogenase. Amination of **5a** to (R)- and (S)-2-phenylglycinol **1a**, respectively, could be
35 achieved by using (R) and (S)-enantioselective transaminases.

For the oxidation of (R)-phenylethanediol **4a** to 2-hydroxyacetophenone **5a**, BDHA from *Bacillus subtilis* BGSC1A1 and CpSADH from *Candida parapsilosis* were examined. These

enzymes were reported for the efficient production of hydroxy ketones (S. Wu, Z. Li, *ChemCatChem* **2018**, *10*, 2164-2178; J. Zhang *et al.*, *ACS Catal.* **2015**, *5*, 51-58; and J. Liu & Z. Li, *Biotechnol. Bioeng.* **2019**, *116*, 536-542). The genes coding for BDHA and CpSADH were cloned in individual pET28a plasmid under the control of T7 promoter, and the two recombinant plasmids were transformed into separate *E. coli* T7 strains to obtain *E. coli*-BDHA expressing BDHA and *E. coli*-CpSADH expressing CpSADH, respectively. The resting cells of *E. coli*-BDHA and *E. coli*-CpSADH (10 g cdw/L) were evaluated for oxidation of 2 mM of **4a**, giving 95-100% conversion to **5a** in both cases (FIG. 2a). The concentration of **4a** was increased to 10 mM, and the strains were evaluated. *E. coli*-BDHA efficiently produced 9.4 mM of **5a** with 94% conversion within 12 h, whereas *E. coli*-CpSADH produced only 6.24 mM of **5a** with 62% conversion at the same time. Therefore, BDHA was chosen as the enzyme for the enzyme cascade for the oxidation of **4a** in the cascade biotransformations.

Transaminase is the key enzyme in this cascade catalytic reaction for the production of enantiopure 2-phenylglycinol **1a**. For the conversion of **5a** to (*R*)-**1a**, databases were searched for (*S*)-enantioselective transaminases with substrate preference similar to **5a** and simple amino donors such as glutamate, L-alanine and isopropylamine. Eight (*S*)-enantioselective transaminases such as HnTA, DpgTA, EeaTA, VfTA, CvTA, mVfTA (A. Nobili *et al.*, *ChemCatChem* **2015**, *7*, 757-760), BmTA and MmTA, were identified with substrate preference similar to **5a**. The genes coding for the eight (*S*)-enantioselective transaminases were cloned in individual pRSFDuet-1 plasmid and transformed into *E. coli* T7 strains, each expressing one of the eight different transaminases (Tables 1-2). 10 g cdw/L of resting cells of *E. coli* strains expressing eight transaminases were used for the biotransformation of 10 mM of **5a** in KP buffer containing glucose (0.5%) and 200 mM of amino donor (*D*-, *L*-alanine or glutamate) for 24 h.

Table 2. Screening of transaminases for the conversion of hydroxyacetophenone **5a** to (*R*)- and (*S*)-Phenylglycinol **1a**.

Strain	Source of transaminase	Amino donor	Product	Conv. (%)	e.e. (%)
<i>E. coli</i> -HnTA	<i>Hyphomonas neptunium</i>	<i>D</i> -Alanine	-	-	-
<i>E. coli</i> -DpgTA	<i>Pseudomonas stutzeri</i>	Glutamate	-	-	-
<i>E. coli</i> -EeaTA	<i>Escherichia coli</i>	Glutamate	-	-	-

<i>E. coli</i> -VfTA	<i>Vibrio fluvialis</i>	L-Alanine	(R)-1	20	>99
<i>E. coli</i> -CvTA	<i>Chromobacterium violaceum</i>	L-Alanine	(R)-1	39	>99
<i>E. coli</i> -mVfTA	<i>Vibrio fluvialis</i>	L-Alanine	(R)-1	34	>99
<i>E. coli</i> -BmTA	<i>Bacillus megaterium</i>	L-Alanine	(R)-1	82	>99
<i>E. coli</i> -MmTA	<i>Martellella mediterranea</i>	L-Alanine	(R)-1	73	98
<i>E. coli</i> -NfTA	<i>Neosartora fischeri</i>	L-Alanine	(S)-1	24	91
<i>E. coli</i> -ArRTA	<i>Arthrobacter sp.</i>	L-Alanine	(S)-1	12	78
<i>E. coli</i> -AtTA	<i>Aspergillus terreus</i>	Isopropylamine	(S)-1	8	54

As shown in Table 2 and FIG. 2b, several transaminases were able to convert **5a** to (R)-**1a**, and the best result was obtained from *E. coli*-BmTA expressing the transaminase from *Bacillus megaterium*. BmTA was able to convert 10 mM of **5a** to (R)-**1a** with 82% efficiency and >99% ee with L-alanine as the amino donor. BmTA was thus chosen for the artificial cascade for the production of (R)-**1a**.

Similarly, (R)-enantioselective transaminases were searched in database and three transaminase candidates such as NfTA, ArRTA and AtTA, were identified for the examination on the conversion of **5a** to (S)-**1a**. The selected transaminases were cloned in individual pRSFDuet-1 plasmid, and transformed into three different *E. coli* T7 strains. 10 g cdw/L of resting cells of *E. coli* strains expressing three (R)-enantioselective transaminases were used for the biotransformation of 10 mM of **5a** in KP buffer containing glucose (0.5%) and 200 mM of amino donor (L-alanine or isopropylamine) for 24 h. As shown in Table 2 and in FIG. 2b, only *E. coli*-NfTA expressing transaminase from *Neosartora fischeri* was able to convert **5a** to (S)-**1a** with relatively high efficiency. NfTA was able to convert 10 mM of **5a** to (S)-**1a** in 24% and 91% ee with L-alanine as the amino donor. Therefore, NfTA was chosen for the artificial cascade for the production of (S)-**1a**.

Example 3. Engineering of *E. coli* co-expressing 5 enzymes of the artificial cascade for the conversion of styrene **2a to (R)- and (S)-2-phenylglycinol **1a****

For the efficient whole cell biotransformation of **2a** to (*R*)- and (*S*)-**1a** with a single strain, a combinatorial approach was attempted for the expression of enzymes in the artificial cascade. Combinatorial approach facilitates to obtain maximum conversion efficiency by optimizing the expression level of multiple enzymes in the cascade. This could be achieved by expressing the enzymes of the artificial cascade from three plasmids (pCDFDuet-1, pETDuet-1 and pRSFDuet-1) with varying copy numbers of 10 to >100 (N. H. Tolia & L. Joshua-Tor, *Nat. Methods* **2006**, 3, 55-64). Different combinations of these plasmids could result in different expression levels of the enzymes (Y. Zhou, S. Wu & Z. Li, *Angew. Chem.* **2016**, 128, 11819-11822; and S. Wu *et al.*, *Adv. Synth. Catal.* **2017**, 359, 2132-2141). Thus, SMO and StEH for the conversion of **2a** to **4a** were cloned together in pCDFDuet-1, pETDuet-1 and pRSFDuet-1. BDHA for the conversion of **4a** to **5a** and alanine dehydrogenase (S. Wu, J. Liu & Z. Li, *ACS Catal.* **2017**, 7, 5225-5233) for recycling amino donor (*L*-alanine) were cloned together in pCDFDuet-1, pETDuet-1 and pRSFDuet-1. BmTA for (*R*)-**1a** or NfTA for (*S*)-**1a** production from **5a** was cloned in pCDFDuet-1, pETDuet-1 and pRSFDuet-1. The recombinant pCDFDuet-1, pETDuet-1 and pRSFDuet-1 plasmids (FIG. 3) were transformed into twelve different *E. coli* strains so that all five enzymes were present in compatible plasmids in each *E. coli* strain (FIG. 4a,d and Table 1). The *E. coli* strains containing the five-enzyme cascade for the conversion of **2a** to (*R*)-**1a** and (*S*)-**1a**, were named *E. coli*-SSBB-1-6 and *E. coli*-SSBN-1-6, respectively. SDS-PAGE analysis of *E. coli*-SSBB-1-6 and *E. coli*-SSBN-1-6 were performed, which confirmed the expression of all five enzymes in the artificial cascade (FIG. 4b,e). The expression of BmTA was relatively higher in *E. coli*-SSBB-1 and *E. coli*-SSBB-2 compared to other strains

Example 4. One-pot biotransformation of styrene **2a to (*R*)- and (*S*)-2-phenylglycinol **1a** with *E. coli* co-expressing 5 enzymes of artificial cascade**

*Cascade conversion of styrene **2a** to (*R*)- and (*S*)-2-phenylglycinol **1a***

Biotransformation was performed at 30 °C in a two-phase system with 2 mL suspension of *E. coli*-SSBB-1-6 (20 g cdw/L) and *E. coli*-SSBN-1-6 (20 g cdw/L) in phosphate buffer (200 mM, pH 8) with *n*-hexadecane containing **2a** (final conc., 10 mM for (*R*)-**1a** and 5 mM for (*S*)-**1a**). Glucose (2%) and 100 mM of *L*-alanine were added to the reaction mixture at 0 h. Additional glucose (1%) and *L*-alanine (100 mM) were added together at 12 h and 20 h. The reaction was continued for 24 h and 48 h for the synthesis of (*R*)- and (*S*)-**1a**, respectively. (*R*)- and (*S*)-**1a** was present only in the aqueous phase and 50 µL of sample was taken to analyse its concentration using reverse phase HPLC.

Results and discussion

One-pot biotransformation of 10 mM of **2a** to (*R*)-**1a** was performed with resting cells of *E. coli*-SSBB-1-6 (20 g cdw/L) at 30 °C for 24 h. As shown in FIG. 4c, the difference in copy numbers of expression vectors showed huge variation in the synthesis of (*R*)-**1a** by *E. coli*-SSBB-1-6. *E. coli*-SSBB-1 (pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA) was the best strain to produce 6.83 mM of (*R*)-**1a** (>99% ee and 68% conversion) from **2a** (FIG. 4c). One-pot biotransformation of 5 mM of **2a** to (*S*)-**1a** was performed with resting cells of *E. coli*-SSBN-1-6 (20 g cdw/L) at 30 °C for 48 h. The engineered *E. coli* strains expressing the developed enzyme cascade converted styrene **2a** to (*S*)-**1a** in 91% ee at 315 mg/L. As shown in FIG. 4f, *E. coli*-SSBN-2 (pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH) showed the highest conversion of 23%. As NFTA was not efficient like BmTA, a relatively low production of (*S*)-**1a** was observed in *E. coli*-SSBN-1-6. Evolution of NFTA and search for new *R*-selective transaminase for further improvement of (*S*)-**1a** production is under progress.

Example 5. Optimization of reaction conditions for the conversion of styrene **2a** to enantiopure 2-phenylglycinol **1a** using *E. coli*-SSBB-1

To further improve the conversion of **2a** to (*R*)-**1a** by *E. coli*-SSBB-1, the reaction conditions for one-pot biotransformation described in Example 4, were optimized (Table 3).

Table 3. Optimization of reaction conditions for the production of (*R*)-2-phenylglycinol **1a** from styrene **2a** using *E. coli*-SSBB-1.

Strain	Cell conc. (g cdw L ⁻¹)	Organic phase	Organic phase : KP buffer ratio	Amino donor	Conv. (%)
<i>E. coli</i> -SSBB-1	20	<i>n</i> -Hexadecane	1:1	<i>L</i> -alanine and 100mM NH ₃ /NH ₄ Cl	68%
<i>E. coli</i> -SSBB-1	20	<i>n</i> -Hexadecane	1:1	100mM NH ₃ /NH ₄ Cl	69%
<i>E. coli</i> -SSBB-1	20	Ethyl oleate	1:1	100mM NH ₃ /NH ₄ Cl	68%
<i>E. coli</i> -SSBB-1	20	<i>n</i> -Hexadecane	0.2:1	<i>L</i> -alanine and 100mM NH ₃ /NH ₄ Cl	72%
<i>E. coli</i> -SSBB-1	20	<i>n</i> -Hexadecane	0.2:1	100mM NH ₃ /NH ₄ Cl	74%
<i>E. coli</i> -SSBB-1	20	Ethyl oleate	0.2:1	100mM NH ₃ /NH ₄ Cl	72%

Conversion of styrene **2a** to (*R*)- 2- phenylglycinol **1a** using *E. coli*-SSBB-1

Whole cell biotransformation was performed at 30 °C for 24 h in a two-phase system with 2 mL suspension of *E. coli*-SSBB-1 (20 g cdw/L) in phosphate buffer (200 mM, pH 8) with

different volumes of *n*-hexadecane or ethyl oleate containing **2a** (10 mM, final conc.). Glucose (2%) and 100 mM of an amino donor selected from *L*-alanine and 100mM NH₃/NH₄Cl, and 100 mM NH₃/NH₄Cl, were added to the reaction mixture at 0 h. Additional glucose (1%) and amino donor (100 mM) were added together at 12 h and 20 h.

5

Results and discussion

Firstly, the biotransformation of **2a** to (*R*)-**1a** was performed with *E. coli*-SSBB-1 (20 g cdw/L) with 0 mM or 100 mM supplementation of *L*-alanine. *E. coli* naturally produces *L*-alanine and *E. coli*-SSBB-1 expresses alanine dehydrogenase for recycling *L*-alanine using NH₃/NH₄Cl, therefore the supplementation of *L*-alanine might not be necessary. In fact, same conversion efficiency (68-69%) was obtained with or without *L*-alanine supplementation. Secondly, the organic phase *n*-hexadecane was replaced by ethyl oleate, and the synthesis of (*R*)-**1a** from **2a** was evaluated. The engineered *E. coli* strains expressing the developed enzyme cascade converted styrene **2a** to (*R*)-**1a** in >99% ee at 1015 mg/L. As *n*-hexadecane is obtained from petroleum, a greener and biocompatible organic phase such as ethyl oleate which can be obtained from biodiesel was tested. No significant difference was observed in the conversion (68-69%), suggesting that ethyl oleate could be used as a greener alternative. In addition, as the organic phase could damage the cell membrane of whole cells after long exposure during biotransformation, the volume of organic phase (*n*-hexadecane or ethyl oleate) was reduced by changing the ratio of organic phase:phosphate buffer ratio from 1:1 to 0.2:1. Surprisingly, improved conversion of **2a** to (*R*)-**1a** was observed with both *n*-hexadecane:phosphate buffer and ethyl oleate:phosphate buffer in 0.2:1 ratio, respectively. 74% conversion of **2a** to (*R*)-**1a** was achieved in biotransformation with *n*-hexadecane:phosphate buffer in 0.2:1 ratio. Reduced volume of organic phase usage increased the conversion efficiency and could also contribute to reducing operation cost in a large-scale production.

30

Example 6. One-pot biotransformation of styrenes 2a-2k and indene 2l to (*R*)-phenylglycinols 1a-1k and (1*R*,2*R*)-1-amino-2-indanol 1l with *E. coli* co-expressing 5 enzymes of artificial cascade

*Cascade conversion of styrenes 2b-l to (*R*)-2-phenylglycinols 1b-l*

Biotransformation was performed at 30 °C in a two-phase system with 2 mL suspension of *E. coli*-SSBB-1 in phosphate buffer (200 mM, pH 8) with 0.4 mL of *n*-hexadecane containing **2b-l** (final conc., 5-10 mM). Glucose (2%) and 100 mM of NH₃/NH₄Cl were added to the reaction mixture at 0 h. Additional glucose (1%) and NH₃/NH₄Cl (100 mM) were added together at 12 h and 20 h. After 48 h of biotransformation, (*R*)-**1b-l** were present only in the aqueous phase and their concentration were analysed using reverse phase HPLC.

35

Results and discussion

Substituted (*R*)-phenylglycinols **1a-1k** and (1*R*,2*R*)-1-amino-2-indanol **1l** are also important building blocks in the synthesis of pharmaceuticals. Therefore, *E. coli*-SSBB-1 cells were examined for the conversion of substituted styrenes **2a-k** and indene **2l** to (*R*)-**1a-1k** and (1*R*,2*R*)-1-amino-2-indanol **1l**. One-pot biotransformation was performed in the two-phase system with *n*-hexadecane and phosphate buffer (0.2:1) with 5-10 mM of **2a-2l** and *E. coli*-SSBB-1 (20 g cdw/L). The conversion to **1a-1l** are listed in Table 4. All the tested substrates (**2a-2k**) were converted to their respective (*R*)-phenylglycinols (**1a-1k**) and (1*R*,2*R*)-1-amino-2-indanol **1l** in 95->99% ee. Among the eleven substituted substrates, eight substrates were converted to their respective (*R*)-phenylglycinols with >50% conversion. Specifically, **2c**, **2d**, **2i**, **2k** showed >60% conversion. Thus, the developed artificial cascade is useful for the production of various (*R*)-2-phenylglycinols **1a-1k** and (1*R*,2*R*)-1-amino-2-indanol **1l**.

Table 4. Cascade biotransformation of styrenes **2a-2k** and indene **2l** to (*R*)-2-phenylglycinols-**1a-1k** and (1*R*,2*R*)-1-amino-2-indanol **1l** with resting cells of *E. coli*-SSBB-1.

Substrate a)	R	Substrate conc. b) (mM)	Cell conc. (g cdw L ⁻¹)	Product	Conv. c) (%)	ee c) (%)
2a	H	10	20	1a	74	>99
2b	<i>o</i> -F	10	20	1b	57	>99
2c	<i>m</i> -F	10	20	1c	69	>99
2d	<i>p</i> -F	10	20	1d	73	99
2e	<i>m</i> -Cl	10	20	1e	56	>99
2f	<i>p</i> -Cl	5	25	1f	53	>99
2g	<i>m</i> -Br	5	25	1g	34	95
2h	<i>p</i> -Br	5	25	1h	22	>99
2i	<i>m</i> -Me	10	20	1i	67	>99
2j	<i>p</i> -Me	5	25	1j	57	>99
2k	<i>p</i> -OMe	5	25	1k	61	>99
2l	-	5	25	1l	24	>99

a) Substrates **2a-2l** were converted to **1a-1l** by *E. coli* SSBB-1 (20 g cdw/L) at 30 °C for 48 h in a two-phase system of 2 mL phosphate buffer (200 mM, pH 8) and 0.4 mL *n*-hexadecane containing **2a-2l**. b) normalized to aqueous phase volume. c) Concentration and enantiomeric excess of **1a-1l** were analysed using reverse phase HPLC with SB-C18 and Crownpak CR (+) chiral column, respectively. Biotransformations were performed in duplicates and the mean values were presented.

Example 7. Scaled-up one-pot biotransformation of styrenes **2a, **2d**, **2e**, **2j**, **2k** to (*R*)-2-phenylglycinols **1a**, **1d**, **1e**, **1j**, **1k****

One-pot biotransformation of styrenes **2a**, **2d**, **2e**, **2j**, **2k** to (*R*)-2-phenylglycinols **1a**, **1d**, **1e**, **1j**, **1k** as described in Example 6, were scaled up.

Preparative biotransformation of styrenes (2a, 2d, 2e, 2j, 2k) to produce (R)-2-phenylglycinols (1a, 1d, 1e, 1j, 1k)

Preparative biotransformation was performed at 30 °C in a two-phase system with 100 mL suspension of *E. coli*-SSBB-1 (25 g cdw/L) in phosphate buffer (200 mM, pH 8) and 20 mL of *n*-hexadecane containing 50 mM of **2a**, **2d**, **2e**, **2j**, **2k**. Glucose (2%) and NH₃/NH₄Cl (100 mM) were added to the reaction at 0 h, 16 h and 24 h. After 48 h of biotransformation, the aqueous phase was separated by centrifugation at 4200 × g for 10 min. The aqueous phase was saturated with NaCl, adjusted to pH < 2 with HCl (10 M), and washed with ethyl acetate (2 × 30 mL) to remove trace *n*-hexadecane and other organic impurities. The aqueous phase was adjusted to pH > 12 with NaOH (10 M), followed by extraction with ethyl acetate (3 × 100 mL). The organic phase was separated and dried over Na₂SO₄. After filtration, the organic phase was subjected to evaporation. The crude phenylglycinols were purified by flash chromatography on a silica gel column with CH₂Cl₂:MeOH:NH₃ (28% aqueous solution) of 100:10:1 as eluent (*R*_f ≈ 0.3–0.6 for (*R*)-**1a**, **1d**, **1e**, **1j** and **1k**). The collected fraction containing the product was dried over Na₂SO₄. After filtration, the organic solvent was removed by evaporation, and the product was dried under vacuum overnight to obtain the pure product.

Results and discussion

Preparative biotransformation was performed with resting cells of *E. coli*-SSBB-1 (25 g cdw/L) in 100 mL of phosphate buffer with 20 mL of *n*-hexadecane containing **2a**, **2d**, **2e**, **2j**, **2k** (50 mM, normalized to aqueous phase volume). After reaction for 48 h at 30 °C, (*R*)-**1a**, **1d**, **1e**, **1j** and **1k** were isolated from the aqueous phase in high purity with 59%, 50%, 37%, 32%, and 44% yield, respectively.

1a

81 mg, 59% yield, HPLC purity: >98%. ¹H NMR (400 MHz, CDCl₃) δ 7.44 – 7.21 (m, 5H), 4.04 (dd, *J* = 8.4, 4.3 Hz, 1H), 3.73 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.56 (dd, *J* = 10.9, 8.4 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 142.57, 128.84, 127.75, 126.70, 68.02, 57.55.

1d

78 mg, 50% yield, HPLC purity: >98%. ¹H NMR (400 MHz, CDCl₃) δ 7.36 – 7.23 (m, 2H), 7.08 – 6.96 (m, 2H), 4.05 (dd, *J* = 8.2, 4.3 Hz, 1H), 3.70 (dd, *J* = 10.8, 4.3 Hz, 1H), 3.53 (dd, *J* = 10.8, 8.3 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 162.36 (d, *J* = 245.8 Hz), 128.33 (d, *J* = 8.0 Hz), 139.69, 115.66 (d, *J* = 21.3 Hz), 68.00, 56.85.

1e

64 mg, 37% yield, HPLC purity: >98%. ¹H NMR (400 MHz, CDCl₃) δ 7.40 – 7.14 (m, 4H), 4.03 (dd, *J* = 7.2, 4.0 Hz, 1H), 3.73 (dd, *J* = 10.6, 3.6 Hz, 1H), 3.53 (dd, *J* = 10.4, 8.1 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 145.03, 134.72, 130.10, 127.86, 126.96, 124.94, 68.03, 57.14.

5

1j

49 mg, 32% yield, HPLC purity: >98%. ¹H NMR (400 MHz, CDCl₃) δ 7.18 (dd, *J* = 23.7, 8.1 Hz, 4H), 4.01 (dd, *J* = 8.4, 4.3 Hz, 1H), 3.71 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.54 (dd, *J* = 10.8, 8.5 Hz, 1H), 2.33 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 139.53, 137.43, 129.51, 126.59, 68.02, 57.25, 21.24.

10

1k

73 mg, 44% yield, HPLC purity: >98%. ¹H NMR (400 MHz, CDCl₃) δ 7.30 – 7.20 (m, 2H), 6.92 – 6.84 (m, 2H), 4.00 (dd, *J* = 8.0, 4.1 Hz, 1H), 3.79 (s, 3H), 3.69 (dd, *J* = 10.8, 4.3 Hz, 1H), 3.59 – 3.48 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 159.03, 127.63, 114.05, 100.00, 67.86, 56.72, 55.30.

15

Example 8. Design of cascade biotransformations and development of resting-cells bioprocess to convert bio-based *L*-phenylalanine **6 to (*R*)- and (*S*)-2-phenylglycinol **1a****

20

For the conversion of bio-based *L*-phenylalanine **6** to enantiopure (*R*)- and (*S*)-**1a** (FIG. 1b), artificial enzyme cascade consisting of decarboxylation-deamination-epoxidation-hydrolysis-oxidation-transamination, PAL of *Arabidopsis thaliana* and PAD of *Saccharomyces cerevisiae* were expressed together in the cascade for **2a** to (*R*)- and (*S*)-**1a**.

25

Cascade conversion of L-phenylalanine 6 to (R)- and (S)-phenylglycinol 1a

Biotransformation of *L*-phenylalanine **6** using *E. coli*-PPSSBB and *E. coli*-PPSSBN was performed at 30 °C for 24 h for the synthesis of (*R*)- and (*S*)-**1a**, respectively. Reaction was performed in a two-phase system with 5 mL suspension of *E. coli*-PPSSBB or *E. coli*-PPSSBN (20 g cdw/L) in phosphate buffer (200 mM, pH 8) with 1 mL of *n*-hexadecane. **6** (10 mM), glucose (2%) and NH₃/NH₄Cl (100 mM) were added to the reaction at 0 h. Additional glucose (1%) and NH₃/NH₄Cl (100 mM) were added together at 12 h. At 24 h, 50 μL of the aqueous phase was collected to quantify the concentration of (*R*)- or (*S*)-**1a** using reverse phase HPLC.

30

Results and discussion

35

We previously proved that PAL and PAD efficiently produces **2a** from **6** (139 mM of **2a**, 92% conversion, B. R. Lukito *et al.*, *Adv. Synth. Catal.* **2019**, 361, 3560-3568; B. R. Lukito *et al.*,

ChemCatChem **2019**, *11*, 831-840; Y. Zhou, S. Wu & Z. Li, *Angew. Chem.* **2016**, *128*, 11819-11822; and Y. Zhou *et al.*, *ChemSusChem* **2018**, *11*, 2221-2228). Thus, PAL and PAD enzymes cloned in pACYCDuet-1 (pACYC-PAL-PAD, B. R. Lukito *et al.*, *ChemCatChem* **2019**, *11*, 831-840) were co-expressed in *E. coli*-SSBB-1 (pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA) and *E. coli*-SSBN-7 (pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA), and the resulted recombinant strains were named as *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively (FIG. 5a). SDS-PAGE analysis of the crude cell lysate of *E. coli*-PPSSBB and *E. coli*-PPSSBN was performed (FIG. 5b), showing the expression of all seven enzymes of the artificial cascade in *E. coli*-PPSSBB and *E. coli*-PPSSBN.

Cascade biotransformation of 10 mM of **6** to (*R*)- and (*S*)-**1a** was performed with the resting cells (20 g cdw/L) of *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively, in the two-phase system with *n*-hexadecane and phosphate buffer (0.2:1). After 24 h of reaction at 30 °C, 576 mg/L of (*R*)-**1a** (42% conversion efficiency) and 356 mg/L of (*S*)-**1a** (26% conversion efficiency) was produced by *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively (FIG. 5c). This is the first report on the synthesis of (*R*)- and (*S*)-**1a** from bio-based substrate **6**. Since the accumulation of intermediates such as **4a** and **5a** was observed, further enhancement of the productivity might be achieved by improving the activity of the alcohol dehydrogenase and transaminase.

Example 9. Design of cascade biotransformations and development of growing-cells bioprocess to convert renewable feedstocks glucose **8 and glycerol **9** to (*R*)- and (*S*)-2-phenylglycinol **1a****

E. coli naturally produces *L*-phenylalanine **6** by fermentation of low-cost renewable feedstocks such as glucose **8** and glycerol **9** through the Shikimate pathway (B. S. Sekar, B. R. Lukito & Z. Li, *ACS Sustain. Chem. Eng.* **2019**, *7*, 12231-12239; M. Weiner *et al.*, *Biochem. Eng. J.* **2014**, *83*, 62-69; and Y. Liu *et al.*, *BMC Biotechnol.* **2018**, *18*, 5). The natural *L*-phenylalanine biosynthesis pathway was combined with the artificial enzyme cascade of converting **6** to (*R*)- or (*S*)-**1a** (developed in Example 8), to transform **8** and **9** to (*R*)- and (*S*)-**1a**, respectively (FIG. 1c). As the biosynthesis of **6** is strictly regulated in *E. coli* by feedback regulation (S.-P. Liu *et al.*, *Process Biochem.* **2013**, *48*, 413-419), we engineered *E. coli* NST74 (DE3) containing natural *L*-phenylalanine biosynthesis pathway for the overproduction of **6** from **8** or **9** with relaxed feedback regulation (B. S. Sekar, B. R. Lukito & Z. Li, *ACS Sustain. Chem. Eng.* **2019**, *7*, 12231-12239; and Y. Zhou *et al.*, *ChemSusChem* **2018**, *11*, 2221-2228). This strain was used as the host.

The plasmids pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA and pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA were transformed into *E. coli* NST74 (DE3) to produce *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN, respectively (FIG. 6a). SDS-PAGE analysis was performed with the crude cell lysate of *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN (FIG. 6b). The expression of all seven enzymes in the artificial cascade were confirmed in *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN.

Fermentation of glucose 8 and glycerol 9 to produce (R)- or (S)-2-phenylglycinol 1a

E. coli NST-PPSSBB and *E. coli* NST-PPSSBN were inoculated, respectively, in 2 mL of LB medium containing appropriate antibiotics (50 µg/mL kanamycin, 25 µg/mL chloramphenicol, 50 µg/mL streptomycin, and 100 µg/mL ampicillin) and grown at 37 °C, 220 rpm for 6-8 h. 1 mL of the culture of *E. coli* NST-PPSSBB or *E. coli* NST-PPSSBN was added to modified M9 medium (50 mL) with 20 g/L of **8** or **9**, respectively, and appropriate antibiotics (50 µg/mL kanamycin, 25 µg/mL chloramphenicol, 50 µg/mL streptomycin, and 100 µg/mL ampicillin) in a 250 mL flask. After 2 h of growth at 37 °C, IPTG (0.1 mM) and *n*-hexadecane (10 mL) were added and the growth temperature was set at 25 °C. At 24 h, 50 µL of the aqueous phase was collected to quantify the concentration of (R)- or (S)-**1a** using reverse phase HPLC.

Results and discussion

The fermentation of **8** or **9** to (R)- and (S)-**1a** was performed with *E. coli* NST-PPSSBB and *E. coli* NST-PPSSBN, respectively, in 50 mL of modified M9 media containing 10 g/L of **8** or **9**, and 10 mL of *n*-hexadecane at 25 °C for 24 h (FIG. 7a). *E. coli* NST-PPSSBB produced 123 and 178 mg/L of (R)-**1a** from glucose **8** and glycerol **9**, respectively, while *E. coli* NST-PPSSBN produced 110 and 151 mg/L of (S)-**1a** from **8** and **9**, respectively (FIG. 6c). This is the first report on the bioproduction of (R)- and (S)-**1a** from low-cost renewable feedstocks such as **8** and **9**. However, further optimization of the reaction conditions and engineering of strains is necessary to improve the fermentative production and explore the possibilities of large-scale production, and is addressed in the following example.

Example 10. Production of (R)- and (S)-2-phenylglycinol 1a from renewable feedstocks glucose 8 and glycerol 9 by coupling two strains expressing L-phenylalanine biosynthesis pathway and the artificial cascade

The fermentative synthesis of (R)- and (S)-**1a** from sugars **8** and **9** could burden the cells due to overexpression of several enzymes in a single strain. In addition, the supplementation of organic phase to growing cells and the accumulation of reaction intermediates could inhibit the cell growth, thus reducing the fermentative synthesis of (R)- and (S)-**1a**. To overcome

these issues, we coupled fermentation-biotransformation (B. R. Lukito *et al.*, *Adv. Synth. Catal.* **2019**, 361, 3560-3568; B. S. Sekar, B. R. Lukito & Z. Li, *ACS Sustain. Chem. Eng.* **2019**, 7, 12231-12239; and Y. Zhou *et al.*, *Biotechnol. Bioeng.* **2020**, 117, 2340-2350) to fermentatively produce high concentrations of **6** from **8** or **9** and sequentially biotransform **6** to (R)- and (S)-**1a**, respectively.

Coupled fermentation-biotransformation for the production of (R)- or (S)-2-phenylglycinol 1a from glucose 8 or glycerol 9

Fermentative production of L-phenylalanine **6** from **8** or **9** by *E. coli* NST-Phe was performed as mentioned previously (B. S. Sekar, B. R. Lukito & Z. Li, *ACS Sustain. Chem. Eng.* **2019**, 7, 12231-12239), to achieve 67-80 mM of **6**, and the fermentation culture containing biosynthesized **6** and *E. coli* NST-Phe cells was stored at 4 °C or used directly in biotransformation. 5 mL of reaction mixture was prepared with 5 mL of fermentation broth containing biosynthesized **6** (final conc. 10 mM), phosphate buffer (200 mM, pH 8), glucose (2%), and NH₃/NH₄Cl (100 mM). Resting cells of *E. coli* NST-PPSSBB or *E. coli* NST-PPSSBN (20 g cdw/L) and 1 mL of *n*-hexadecane were added, and biotransformation was performed at 30 °C and 220 rpm. Additional glucose (1%) and NH₃/NH₄Cl (100 mM) were added together at 12 h. At 24 h, 50 µL of the aqueous phase was collected to quantify the concentration of (R)- or (S)-**1a** using reverse phase HPLC. Experiment was performed in duplicates with error bars showing ± SD.

Results and discussion

Fermentative production of L-phenylalanine **6** from sugars **8** and **9** was performed by *E. coli* NST-Phe to obtain high concentrations of **6**, followed by the one-pot biotransformation of **6** to (R)- and (S)-**1a** by the resting cells of *E. coli*-PPSSBB and *E. coli*-PPSSBN, respectively (FIG. 7b and 8a).

E. coli NST-Phe overexpressing five key enzymes of Shikimate pathway (AroG*, AroK, YdiB, PheA*, and TyrB) was employed to produce 67-80 mM of **6** (B. R. Lukito *et al.*, *Adv. Synth. Catal.* **2019**, 361, 3560-3568; and B. S. Sekar, B. R. Lukito & Z. Li, *ACS Sustain. Chem. Eng.* **2019**, 7, 12231-12239). Resting cells (20 g cdw/L) of *E. coli*-PPSSBB and *E. coli*-PPSSBN were added to the fermentation broth with biosynthesized **6**, to perform biotransformation of **6** to (R)- and (S)-**1a**, respectively. By coupled fermentation-biotransformation approach, 274 and 384 mg/L of (R)-**1a** were obtained from glucose **8** and glycerol **9**, respectively. For the synthesis of (S)-**1a**, the product titer of 274 and 301 mg/L were achieved from glucose **8** and glycerol **9**, respectively (FIG. 8b). Table 5 summarizes the production of (R)- and (S)-**1a** from glucose **8** and glycerol **9**.

Table 5. Bioproduction of (*R*)- and (*S*)-2-Phenylglycinol **1a** from renewable resources such as *L*-phenylalanine (*L*-Phe) **6**, glucose **8**, and glycerol **9**.

Experiment	Renewable Substrate	Strain	Product	Conc. (mM)
Resting cells biotransformation ^a	<i>L</i> -Phe 6	<i>E. coli</i> -PPSSBB	(<i>R</i>)- 1	4.2
Resting cells biotransformation ^a	<i>L</i> -Phe 6	<i>E. coli</i> -PPSSBN	(<i>S</i>)- 1	2.6
Growing cells fermentation ^b	Glucose 8	<i>E. coli</i> NST-PPSSBB	(<i>R</i>)- 1	1
Growing cells fermentation ^b	Glycerol 9	<i>E. coli</i> NST-PPSSBB	(<i>R</i>)- 1	1.3
Growing cells fermentation ^b	Glucose 8	<i>E. coli</i> NST-PPSSBN	(<i>S</i>)- 1	1
Growing cells fermentation ^b	Glycerol 9	<i>E. coli</i> NST-PPSSBN	(<i>S</i>)- 1	1.1
Coupled fermentation-biotransformation ^c	Glucose 8	<i>E. coli</i> NST-Phe + <i>E. coli</i> -PPSSBB	(<i>R</i>)- 1	2
Coupled fermentation-biotransformation ^c	Glycerol 9	<i>E. coli</i> NST-Phe + <i>E. coli</i> -PPSSBB	(<i>R</i>)- 1	2.8
Coupled fermentation-biotransformation ^c	Glucose 8	<i>E. coli</i> NST-Phe + <i>E. coli</i> -PPSSBN	(<i>S</i>)- 1	2
Coupled fermentation-biotransformation ^c	Glycerol 9	<i>E. coli</i> NST-Phe + <i>E. coli</i> -PPSSBN	(<i>S</i>)- 1	2.2

^{a)} see Example 8 for experimental protocol. ^{b)} see Example 9 for experimental protocol. ^{c)} see Example 10 for experimental protocol.

5

The developed strategies are potentially useful for producing high-value chemicals from cheap and renewable feedstocks.

Claims

1. A method for producing an enantiomerically pure or enantiomerically enriched (*R*)- or (*S*)-2-phenylglycinol or a derivative thereof using one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes, which method comprises subjecting styrene or a derivative thereof to multiple enzyme-catalyzed chemical transformations in a one-pot reaction system, optionally wherein at least one overexpressed enzyme is located on one or more plasmids or integrated in the chromosome of each of the one or more recombinant microbial cells.
2. The method of Claim 1, wherein the method produces enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof, comprising the steps of:
 - (a) generating phenylethane diol or a derivative thereof from styrene or a derivative thereof by conducting an epoxidation reaction catalyzed by an epoxidase to form styrene oxide or a derivative thereof and conducting a hydrolysis reaction catalyzed by an epoxide hydrolase on the epoxide, or by a dihydroxylation reaction catalyzed by a dioxygenase;
 - (b) generating 2-hydroxyacetophenone or a derivative thereof from the phenylethane diol or a derivative thereof by an oxidation reaction catalyzed by an alcohol dehydrogenase or a secondary alcohol oxidase; and
 - (c) generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof from 2-hydroxyacetophenone or a derivative thereof by a transamination reaction catalyzed by a transaminase or a reductive amination reaction catalyzed by an amine dehydrogenase.
3. The method according to Claim 2, wherein:
 - (a) the epoxidase is a styrene monooxygenase, optionally wherein the styrene monooxygenase is from *Pseudomonas* sp. VLB120, or its mutants or similar enzymes with more than 50% identity; and/or
 - (b) the epoxide hydrolase, when used, is from *Solanum tuberosum* or its mutants or similar enzymes with more than 50% identity; and/or
 - (c) the alcohol dehydrogenase, when used, is from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* or their mutants or similar enzymes with more than 50% identity; and/or
 - (d) the transaminase is from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Marteella mediterranea*, *Neosartora fischeri*, *Arthrobacter* sp., or *Aspergillus terreus* or their mutants or similar enzymes with more than 50% identity.

4. The method according to and one of Claim 3, wherein
- (i) the epoxide hydrolase, when used, is selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA) from *Bacillus subtilis* or its mutants or similar enzymes with more than 50% identity; and/or
 - (ii) the transaminase is selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri* or their mutants or similar enzymes with more than 50% identity.
5. The method according to any one of the preceding claims, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is selected from one or more of the group consisting of:
- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
 - (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
 - (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
6. The method according to Claim 5, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA.
7. The method according to any one of the preceding claims, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is selected from one or more of the group consisting of:
- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA;
 - (b) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
 - (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (f) *E. coli* T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
8. The method according to any one of the preceding claims, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e.

the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH.

9. The method according to any one of the preceding claims, wherein the method further comprises providing styrene or a derivative thereof by generating *trans*-cinnamic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof by a deamination reaction catalyzed by an ammonia lyase and generating styrene or a derivative thereof from the *trans*-cinnamic acid or a derivative thereof in a decarboxylation reaction catalyzed by a decarboxylase.

10. The method according to 9, wherein:

- (a) the ammonia lyase is phenylalanine ammonia lyase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity; and/or
- (b) the decarboxylase is phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity.

11. The method according to any one of Claims 1 to 6 and 9 to 10, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA.

12. The method according to any one of Claims 1 to 4 and 7 to 10, wherein one of the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes) is *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NFTA.

13. The method according to any one of Claims 9 to 12, wherein the method further comprises cells that overexpress the natural *L*-phenylalanine biosynthetic pathway, which cells convert glucose or glycerol to *L*-phenylalanine.

14. The method according to Claim 13 wherein the microbial cells producing *L*-phenylalanine from glucose or glycerol overexpress at least one enzyme is selected from one or more of the group consisting of DAHP synthase (AroG), shikimate kinase (AroK), shikimate dehydrogenase (YdiB), chorismate mutase/prephenate dehydratase (PheA) and tyrosine aminotransferase (TyrB), or their mutants or similar enzymes with more than 50% identity,

optionally wherein the microbial cells producing L-phenylalanine from glucose or glycerol overexpress at least one enzyme is:

- (a) a feedback inhibition resistant mutant of AroG;
- (b) a feedback inhibition resistant mutant of PheA.

15. The method according to Claim 14, wherein the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme is one in which the microbial cells are mutated for deletion or inactivation of *crr* and/or *tyrA* genes.

16. The method according to any one of Claims 13 to 15, wherein the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme is *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA or *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NfTA.

17. The method according to any one of Claims 13 to 15, wherein the microbial cells producing L-phenylalanine from glucose or glycerol that overexpress at least one enzyme is a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA or is a combination of *E. coli* NST74-Phe with *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.

18. The method according to any one of the preceding claims, wherein the one-pot reaction system comprises an aqueous medium.

19. The method according to any one of Claims 1 to 17, wherein the one-pot reaction system comprises use of a bi-phasic medium, optionally wherein the bi-phasic medium consists of an aqueous and solid resin medium, or an aqueous and organic solvent medium (e.g. the organic solvent medium is selected from one or more alkanes solvents and/or one or more ester solvents).

20. The method according to any one of the preceding claims, wherein the one-pot reaction system comprises use of absorbent resins or nanomaterials for in-situ removal of product.

21. An isolated nucleic acid molecule encoding at least one heterologous catalytic enzyme selected from the group comprising:

(a) an epoxidase for conducting an epoxidation reaction to form styrene oxide or a derivative thereof from styrene or a derivative thereof and an epoxide hydrolase for conducting a hydrolysis reaction on the epoxide to provide phenylethanediol or a derivative thereof, or an oxygenase for generating phenylethanediol or a derivative thereof from styrene or a derivative thereof by a dihydroxylation reaction;

(b) an alcohol dehydrogenase or a secondary alcohol oxidase for generating 2-hydroxyacetophenone or a derivative thereof from phenylethanediol by an oxidation reaction; and

(c) a transaminase or an amine dehydrogenase for generating enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof by a transamination reaction or a reductive amination from 2-hydroxyacetophenone or a derivative thereof; or

(d) an ammonia lyase for generating *trans*-cinnamic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof by a deamination reaction; and

(e) a decarboxylase for generating styrene or a derivative thereof from *trans*-cinnamic acid or a derivative thereof in a decarboxylation reaction.

22. The isolated nucleic acid of claim 21, encoding a plurality of said catalytic enzymes.

23. The isolated nucleic acid molecule of claim 22, wherein said plurality of catalytic enzymes is arranged as at least one module selected from the group comprising:

i) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms styrene or a derivative thereof to phenylethanediol or a derivative thereof;

ii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms phenylethanediol or a derivative thereof to a 2-hydroxyacetophenone or a derivative thereof;

iii) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms 2-hydroxyacetophenone or a derivative thereof to enantiomerically pure or enantiomerically enriched 2-phenylglycinol or a derivative thereof; and

v) a module comprising heterologous nucleic acid sequences that, when expressed, enzymatically transforms an *L*-phenylalanine or a derivative thereof to styrene or a derivative thereof;

or any combination thereof.

24. An expression construct comprising at least one nucleic acid molecule as described in any one of Claims 21 to 23.

25. One or more recombinant prokaryotic or eukaryotic cells selected from the group comprising bacterial cells, yeast cells, mammalian cells and insect cells, wherein said cells comprise at least one expression construct as described in Claim 24.
26. The one or more recombinant prokaryotic or eukaryotic cells according to Claim 25, wherein said cells are recombinant bacterial cells.
27. The one or more recombinant prokaryotic or eukaryotic cells according to Claim 25 or Claim 26, wherein said enzymes have at least 50% amino acid identity with at least one enzyme selected from the group comprising:
- (a) a styrene monooxygenase from *Pseudomonas sp. VLB120*;
 - (b) an epoxide hydrolase from *Solanum tuberosum*;
 - (c) an alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, *Bacillus subtilis* (e.g. the epoxide hydrolase is selected from secondary alcohol dehydrogenase from *Candida parapsilosis* or, more preferably, (*R,R*)-butanediol dehydrogenase (BDHA));
 - (d) a transaminase from *Bacillus megaterium*, *Chromobacterium violaceum*, *Vibrio fluvialis*, *Marteella mediterranea*, *Neosartora fischeri*, *Arthrobacter sp.*, or *Aspergillus terreus* (e.g. the transaminase is selected from *Bacillus megaterium*, *Vibrio fluvialis*, or *Neosartora fischeri*);
 - (e) a phenylalanine ammonia lyase from *Arabidopsis thaliana*;
 - (f) a phenylacrylic acid decarboxylase from *Aspergillus niger*.
28. The one or more recombinant cells of claim 27, wherein said cells are selected from:
- (a) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
 - (b) *E. coli* T7-pCDF-SMO-StEH, pET-BmTA, pRSF-BDHA- AlaDH;
 - (c) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-BmTA;
 - (d) *E. coli* T7-pCDF-BDHA- AlaDH, pET-BmTA, pRSF-SMO-StEH;
 - (e) *E. coli* T7-pCDF-BmTA, pET-SMO-StEH, pRSF-BDHA- AlaDH;
 - (f) *E. coli* T7-pCDF-BmTA, pET-BDHA- AlaDH, pRSF-SMO-StEH;
 - (g) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NFTA;
 - (g) *E. coli* T7-pCDF-SMO-StEH, pET-NFTA, pRSF-BDHA- AlaDH;
 - (i) *E. coli* T7-pCDF-BDHA- AlaDH, pET-SMO-StEH, pRSF-NFTA;
 - (j) *E. coli* T7-pCDF-BDHA- AlaDH, pET-NFTA, pRSF-SMO-StEH;
 - (k) *E. coli* T7-pCDF-NFTA, pET-SMO-StEH, pRSF-BDHA- AlaDH; and
 - (l) *E. coli* T7-pCDF-NFTA, pET-BDHA- AlaDH, pRSF-SMO-StEH.
 - (m) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA;
 - (n) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NFTA;

- (o) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA; and
- (p) *E. coli* NST74-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-NfTA.
29. The one or more recombinant cells of claim 28, wherein said cells are selected from:
- (i) *E. coli* T7-pCDF-SMO-StEH, pET-BDHA- AlaDH, pRSF-BmTA;
- (ii) *E. coli* T7-pCDF-SMO-StEH, pET-NfTA, pRSF-BDHA- AlaDH;
- (iii) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-BmTA; and
- (iv) *E. coli* T7-pACYC-PAL-PAD, pCDF-SMO-StEH, pET-BDHA-AlaDH, pRSF-NfTA.
30. A kit comprising at least one isolated nucleic acid according to any one of Claims 21 to 24.