# BIOPRODUCTION OF (R)-MANDELIC ACID FROM STYRENE, L-PHENYLALANINE, GLUCOSE, OR GLYCEROL VIA NOVEL ARTIFICIAL BIOSYNTHETIC PATHWAY

## Field of Invention

This invention relates to the bioproduction of useful and valuable enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof. More particularly, the present invention provides methods of bioproduction of enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof by one or more recombinant 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, glycerol, L-phenylalanine or substituted L-phenylalanine, styrene or substituted styrene.

## 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 or is common general knowledge.

(*R*)-mandelic acid (MA) is an important and useful chiral molecule for the production of antibiotics such as cephalosporins and semi-synthetic penicillins, and antitumor and anti-obesity agents. (*R*)-MA is also intensively applied in the resolution of both racemic amines and alcohols as the chiral resolving agent, and was used as a chiral template to synthesize chirally imprinted mesoporous materials (T. Yutthalekha *et al.*, *Electroanalysis* **2015**, *27*, 2209–2213).

Currently, the synthesis of (*R*)-MA is mainly achieved via chemical methods. The cyanide-based method involves two-step reactions, including cyanation of benzaldehyde using either NaCN or transition metal catalysts, such as titanium or vanadium complexes of chiral ligands, and the hydrolysis of mandelonitrile using hydrochloric acid, to give enantiopure (*R*)-MA (WO 2002066410 A1; and B. B. Corson *et al.*, *Org. Synth.* **1926**, *6*, 58). This method requires the use of highly toxic cyanide, expensive transition metal catalyst together with chiral ligands, but gives unsatisfactory *ee*, low overall yields, and generates a lot of by-products and large amount of waste. The dichloroacetophenone-based method involves the chlorination of acetophenone with chlorine ($Cl_2$), followed by alkaline hydrolysis by sodium hydroxide at 65 °C and acidolysis with hydrogen chloride. This method requires the use of toxic and dangerous

$Cl_2$, high temperature, and also suffers from problems associated with side products (mono, tri-choloroacetophenone) (J. G. Aston*et al.*, *Org. Synth.* **1943**, *23*, 48). Practically, chemical methods are mainly used to produce racemic MA, then followed by a resolution process to get enantiopure (*R*)- or (*S*)-MA. The separation of the racemic mixture to get enantiopure MA is still challenging and thus, the kinetic resolution is often required but the theoretical maximum yield is only 50%. Therefore, chemical methods generally involve multi-step reactions and the isolation of intermediates is required. As a result, chemical methods can be costly and will generate by-products and a lot of waste.

Biosynthesis of high-value chemicals from renewable feedstocks for sustainable manufacturing of chemicals has received an increasing attention in attribution to the change of the global climate and oil depletion. Compared with chemical methods, biological method is greener and more sustainable due to the non-toxicity, mild reaction conditions, high chemo-, regio- and stereo-selectivity of the biocatalysts. The replacement of the chemical synthesis with clean, green, and high-yielding biocatalytic preparation is considered as one of the most attractive strategies to realize the sustainable manufacturing of chemicals. However, the current biological methods for the production of (*R*)-MA also suffer from some drawbacks. The single step kinetic resolution catalyzed by enzymes such as nitrilases (NLases), lipases, esterases, or dehydrogenases usually have low efficiency and low yield, and the racemic MA feedstocks are still derived from chemical synthesis. Biosynthesis of (*R*)-MA from glucose was reported but this method gave low product concentrations (≤1 g/L) due to the use of inefficient nature pathways and enzymes (e.g. hydroxymandelate synthase (HmaS), Z. Sun *et al.*, *Microb. Cell Fact.* **2011**, *10*, 71). Currently, the fermentative production of (*R*)-MA is still challenging for practical industrial application due to the lack of efficient pathways.

A pathway to produce (*R*)-MA from *L*-phenylalanine (*L*-Phe) and glucose involving HmaS is known (see FIG. 1a). However, this is not particularly efficient or high yielding. In order to improve the production, it is expected that people working in this field would try to:

- shorten the existing pathway by reducing the number of enzymes, rather than adding more enzymes;
- replace the rate-limiting enzymes with other enzymes having better activity; or
- delete some enzymes in host cells to avoid the conversion of intermediates to other unrelated products; or
- combinations of the above.

The development of biosynthetic pathways with high activity and desired selectivity is one of the most promising ways for efficient direct synthesis of (*R*)-MA. However, corresponding technological development remains challenging due to the lack of novel pathways and suitable enzymes. Therefore, there exists a need to develop efficient, sustainable, and non-chemical synthesis methods which use mild conditions, non-toxic reagents and catalysts for the production of enantiopure MA.

**Summary of Invention**

The invention will now be described by reference to the following numbered clauses.

1. A method for producing an enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid 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 Clause 1, wherein the method produces enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof, comprising the steps of:

(a) generating enantiomerically pure or enantiomerically enriched 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; and

(b) generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof from enantiomerically pure or enantiomerically enriched phenylethane diol or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol to a carboxylic acid using an oxidation enzyme.

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 oxidation enzyme is an alcohol oxidase, optionally wherein the alcohol oxidase is from *Streptomyces coelicolor,* or its mutants or similar enzymes with more than 50% identity.

4. 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, pRSF-AldO.

5. 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.

6. The method according to Clause 5 wherein, the ammonia lyase is phenylalanine ammonia lysase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity.

7. The method according to Clause 5 or Clause 6 wherein, the decarboxylate is phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity.

8. The method according to any one of Clauses 5 to 7, 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-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO.

9. The method according to any one of Clauses 6 to 8, wherein the method further comprises cells that overexpress the natural *L*-phenylalanine biosynthetic pathway, which cells convert glucose of glycerol to *L*-phenylalanine.

10. The method according to Clause 9 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.

11. The method according to Clause 10, wherein the microbial cells producing *L*-phenylalanine from glucose or glycerol overexpress at least one enzyme is one in which the where the microbial cells are mutated for deletion or inactivation of crr and/or tyrA genes.

12. The method according to any one of Clauses 9 to 11, wherein the microbial cells that overexpress the natural *L*-phenylalanine biosynthetic pathway are *E. coli* NST74-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO, which is used to produce (*R*)-mandelic acid from glucose or glycerol.

13. The method according to any one of Clauses 9 to 12, wherein the microbial cells that overexpress the natural *L*-phenylalanine biosynthetic pathway are *E. coli* NST74-Phe, which is used in combination with *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO to provide (*R*)-mandelic acid from glucose or glycerol.

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

15. The method according to any one of Clauses 1 to 13, 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).

16. 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.

17. 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 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;

(c) an oxidation enzyme for generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol 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.

18. The isolated nucleic acid of clause 17, encoding a plurality of said catalytic enzymes.

19. The isolated nucleic acid molecule of clause 18, 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 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 (*R*)-mandelic acid or a derivative thereof; and

iii) 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.

20. An expression construct comprising at least one nucleic acid molecule as described in any one of Clauses 17 to 19.

21. 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 20.

22. The one or more recombinant prokaryotic or eukaryotic cells according to Clause 21, wherein said cells are recombinant bacterial cells.

23. The one or more recombinant prokaryotic or eukaryotic cells cells according to Clause 21 or Clause 22, 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 oxidase from *Streptomyces coelicolor*;

(d) a phenylalanine ammonia lysase from *Arabidopsis thaliana*;

(e) a phenylacrylic acid decarboxylase from *Aspergillus niger*.

24. The one or more recombinant cells of clause 23, wherein said cells are selected from:

(a) *E. coli* T7-pCDF-SMO-StEH, pRSF-AldO;

(b) *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO; and

(c) *E. coli* NST74-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO.

25. A kit comprising at least one isolated nucleic acid according to any one of Clauses 17 to 19.

**Drawings**

**FIG. 1** depicts (a) production of (*R*)-MA from *L*-Phe and glucose involving hydroxymandelate synthase (HmaS); and (b-c) the enzyme cascades for the bioproduction of (*R*)-MA from *L*-Phe, glucose or glycerol. (b) The five-enzyme artificial cascade containing phenylalanine ammonia lyase (PAL), phenylacrylic acid decarboxylase (PAD), styrene monooxygenase (SMO), selective epoxide hydrolase (StEH), and alcohol oxidase (AldO) for the conversion of *L*-Phe to (*R*)-MA; and (c) Biotransformation of glycerol or glucose to (*R*)-MA by combining *L*-Phe biosynthesis pathway with five-enzyme artificial cascade.

**FIG. 2** depicts the time course of producing (*R*)-MA from (*R*)-1,2-phenylethanediol (PED), styrene, and *L*-Phe. (a) Biotransformation of (*R*)-PED to (*R*)-MA with *E. coli* (AldO) cells (15 g cdw/L) in potassium phosphate (KP) buffer (200 mM, pH 8.0); (b) Biotransformation of styrene to (*R*)-MA with *E. coli* (S2A) cells (15 g cdw/L) in a mixture of KP buffer and *n*-hexadecane (1:1; v/v); and (c) Biotransformation of *L*-Phe to (*R*)-MA with *E. coli* (P2S2A) cells (15 g cdw/L) in a mixture of KP buffer and *n*-hexadecane (1:1; v/v). All reactions were performed at 30 °C and 250 rpm.

**FIG. 3** depicts the engineered *E. coli* cells coexpressing SMO, StEH and AldO for biotransformation of styrene to (*R*)-MA.

**FIG. 4** depicts the high pressure liquid chromatography (HPLC) chromatograms. (a) Standard (*R*)-MA; and (b) (*R*)-MA produced during the biotransformation of styrene (20 mM) with resting cells of *E. coli* (S2A) cells (15 g cdw/L) in a mixture of KP buffer (200 mM, pH 8.0) containing 0.5% glucose and *n*-hexadecane (1:1, v/v) at 30 °C for 24 h. Benzyl alcohol was used as the internal standard (I.S.).

**FIG. 5** depicts the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total intracellular protein of *E. coli* cells used for the bioproduction of (*R*)-MA. Lane A: *E. coli* (AldO); Lane B: *E. coli* (S2A); and Lane C: *E. coli* (P2S2A).

**FIG. 6** depicts the construction of *E. coli* cells co-expressing multiple enzymes for the bioproduction of (*R*)-MA. (a) *E. coli* (P2S2A) strain harbouring plasmid pET-PAL-PAD encoding PAL and PAD, plasmid pCDF-SMO-StEH encoding SMO and StEH, and plasmid pRSF-AldO encoding AldO for the conversion of *L*-Phe to (*R*)-MA; (b) *E. coli* NST74 (P2S2A) strain harbouring plasmid pET-PAL-PAD encoding PAL and PAD, plasmid pCDF-SMO-StEH encoding SMO and StEH, and plasmid pRSF-AldO encoding AldO for the conversion of glycerol or glucose to (*R*)-MA; (c) Coupled *E. coli* NST74-Phe strain over-expressing five limiting enzymes in Shikimate pathway with *E. coli* (P2S2A) expressing five-enzyme artificial cascade for the conversion of glycerol or glucose to (*R*)-MA; and (d) SDS-PAGE. Lane M: protein marker; Lane A: total intracellular protein of *E. coli* NST74; and Lane B: total intracellular protein of E. coli NST74 (P2S2A).

**FIG. 7** depicts the HPLC chromatograms. (a) Standard (*R*)-MA; and (b) (*R*)-MA produced during the biotransformation of *L*-Phe (15 mM) with resting cells of *E. coli* (P2S2A) cells (15 g cdw/L) in a mixture of KP buffer (200 mM, pH 8.0) containing 0.5% glucose and n-hexadecane (1:1, v/v) at 30 °C for 24 h. Benzyl alcohol was used as I.S.

**FIG. 8** depicts the construction of *E. coli* strain for the bioproduction of (*R*)-MA from glucose or glycerol. (a) Engineered *E. coli* cells coexpressing *L*-Phe biosynthetic pathway involved enzymes for the fermentative production of *L*-Phe from either glucose or glycerol; and (b) Engineered *E. coli* cells coexpressing *L*-Phe biosynthetic pathway involved enzymes, PAL, PAD, SMO, StEH and AldO for biotransformation of glucose or glycerol to (*R*)-MA.

**FIG. 9** depicts the time course of producing (*R*)-MA from glycerol or glucose with single engineered *E. coli* NST74 (P2S2A) at 25 °C. (a) Fermentation from glycerol in a single aqueous phase system; (b) Fermentation from glucose in a single aqueous phase system; (c)

Fermentation from glycerol in *n*-hexadecane-aqueous phase system (1:2; v/v); and (d) Fermentation from glucose in *n*-hexadecane-aqueous phase system (1:2; v/v).

**FIG. 10** depicts the time course of producing (*R*)-MA from glycerol via coupled *E. coli* (NST74-Phe) and *E. coli* (P2S2A). (a) Fermentative production of *L*-Phe from glycerol with *E. coli* (NST74-Phe); and (b) Biotransformation of *L*-Phe to (*R*)-MA by adding *E. coli* (P2S2A) cells into glycerol-fermented media of *E. coli* (NST74-Phe).

**FIG. 11** depicts the time course of biotransformation of glucose to (*R*)-MA via coupling *E. coli* NST74-Phe and *E. coli* (P2S2A) cells in aqueous-*n*-hexadecane two-phase system.

**FIG. 12** depicts the chiral HPLC chromatograms of (*R*)-MA. (a) Racemic MA standard; (b) (*R*)-MA standard; and (c) Isolated (*R*)-MA products from biotransformation of *L*-Phe to (*R*)-MA with *E. coli* (P2S2A) cells.

**Description**

For the production of (*R*)-mandelic acid from *L*-phenylalanine, *L*-phenylalanine can be converted to phenylpyruvate in *E. coli* cells by endogenous transaminases. Only 3 enzymes (hydroxymandelate synthase (HmaS), 4-hydroxymandelate oxidase (Hmo) and D-mandelate dehydrogenase (DMD)) are needed for this purpose as shown in the pathway of FIG. 1a. However, the new pathway disclosed herein contains 5 enzymes (see FIG. 1b), and actually perform 6 reactions (AldO catalyse double oxidation reaction). This is longer than the existing pathway. However, our new pathway is surprisingly more efficient than existing pathways for producing (*R*)-mandelic acid.

In a first aspect of the invention, there is provided a method for producing an enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid 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, the 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.

As disclosed herein, these multiple reactions 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*)-mandelic acid 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*)-mandelic acid 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.

Styrene and derivatives thereof can be manufactured on very large scales 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.

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.

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, $NH_2$, 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 $C_{1-10}$ alkyl and, more preferably, $C_{1-6}$ 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 $C_{3-12}$ cycloalkyl and, more preferably, $C_{5-10}$ (e.g. $C_{5-7}$) 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 $C_{6-14}$ (such as $C_{6-13}$ (e.g. $C_{6-10}$)) 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. $C_{6-14}$ 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, azetidinyl, 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, sulfolanyl, 3-sulfolenyl, tetrahydropyranyl, tetrahydrofuranyl, tetrahydropyridyl (such as 1,2,3,4-tetrahydropyridyl and 1,2,3,6-tetrahydropyridyl), thietanyl, thirranyl, thiolanyl, thiomorpholinyl, trithianyl (including 1,3,5-trithianyi), 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, isothiaziolyl, 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 $CF_3$ or an alkyl group bearing $Cl_3$), 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, $CH_3$ or $OCH_3$. 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*-$CH_3$, *p*-$CH_3$, or *p*-$OCH_3$.

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 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 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 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 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 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 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 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. 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., 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 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) 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 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).

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 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 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

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, 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 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: 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 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.

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. 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

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. 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 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 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 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**, l23: 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 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.

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. 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.

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.

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.

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.

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.

The method in the first aspect of the invention that produces enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof, may comprise the steps of:

(a) generating enantiomerically pure or enantiomerically enriched 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; and

(b) generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof from enantiomerically pure or enantiomerically enriched phenylethane diol or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol to a carboxylic acid using an oxidation enzyme.

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

(a) the epoxidase may be 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, may be from *Solanum tuberosum* or its mutants or similar enzymes with more than 50% identity; and/or

(c) the oxidation enzyme may be an alcohol oxidase, optionally wherein the alcohol oxidase is from *Streptomyces coelicolor,* or its 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 (i.e. the one or more recombinant microbial cells genetically engineered to overexpress multiple enzymes). For example, the recombinant microbial cells may be *E. coli* T7-pCDF-SMO-StEH, pRSF-AldO. This recombinant microbial cell may be particularly useful when seeking to generate (*R*)-mandelic acid or a derivative thereof from styrene or a derivative thereof. However, if may be of limited utility (unless used in conjunction with other recombinant cells) when seeking to generate (*R*)-mandelic acid or a derivative thereof from *L*-phenylalanine or a derivative thereof or from glucose or glycerol.

Thus, in a further embodiment 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*-cinnamic acid or a derivative thereof in a decarboxylation reaction catalyzed by a decarboxylase. Any suitable ammonia lyase may be used in these embodiments. For example, the ammonia lysase may be phenylalanine ammonia lysase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity. Any suitable decarboxylate may be used in these embodiments. For example, the decarboxylate may be phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity. In particular embodiments that may be mentioned herein, 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-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO.

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-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO, which may also be used to produce (*R*)-mandelic acid from glucose or glycerol. Alternatively, the microbial cells that overexpress the natural *L*-phenylalanine biosynthetic pathway may be *E. coli* NST74-Phe, which can be used in combination with *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO to provide (*R*)-mandelic acid from glucose or glycerol. Without wishing to be bound by theory, it is believed that the combination of one strain of microbial cells (e.g. *E. coli* NST74-Phe) to produce *L*-phenylalanine from glycose and/or glycerol in combination with a second strain of microbial cells to convert *L*-phenylalanine to (*R*)-mandelic acid may be an optional configuration for the method, as discussed herein.

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 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.

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 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;

(c) an oxidation enzyme for generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol 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.

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 (*R*)-mandelic acid or a derivative thereof; and

iii) 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 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 oxidase from *Streptomyces coelicolor*;

(d) a phenylalanine ammonia lysase from *Arabidopsis thaliana*;

(e) 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, pRSF-AldO;

(b) *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO; and

(c) *E. coli* NST74-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO.

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*)-mandelic acid or a derivative thereof from a suitable starting material.

It has been surprisingly found that the natural *L*-phenylalanine biosynthesis pathway and an artificial cascade can be made compatible with one another. Without wishing to be bound by theory, it is believed that the natural biosynthesis pathway and artificial cascade are incompatible due to the difference in optimal conditions. To overcome the issue, the inventors have devised a coupled-cells strategy that successfully overcomes this incompatibility.

Another common issue is the metabolic burden in *E. coli* due to overexpression of many enzymes. Given this, it is preferred not to combine multiple enzymes into one system. Using the coupled-cells strategy disclosed herein, it is believed that the metabolic burden due to overexpressing many enzymes is overcome. This is clearly demonstrated by the successful production of (*R*)-mandelic acid from glucose/glycerol.

The processes disclosed herein use stable and less toxic starting materials, such as styrene and biocompatible substrates such as *L*-phenylalanine, glucose and glycerol. It is noted that these processes avoid the use of highly toxic cyanide and expensive transition metal catalysts that are commonly required in conventional (*R*)-mandelic acid synthetic approaches. The process disclosed herein is also synergistic and may result in the higher production of the desired product than possible using conventional methods.

For the production of (*R*)-mandelic acid from glucose, people working in this field are more willing likely to use a single strain, rather than two strains. However, the coupled strain strategy disclosed herein produced much more (*R*)-mandelic acid than that using single strain. This is an unexpected result.

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

**Examples**

Materials

*D*-glucose (99%), glycerol (98%), *L*-phenylalanine (*L*-Phe, 99%), styrene (99%), (*R*)-PED (98%), (*R*)-MA (99%), (*S*)-styrene oxide (97%), *n*-hexadecane (99%), streptomycin sulfate salt (95%), kanamycin, chloramphenicol, ampicillin sodium salt (96%), trifluoroacetic acid (TFA, 99%), D-(+)-Glucose (99.5%), $NH_4OH$ (99%; 28% $NH_3$ in $H_2O$), *n*-hexane (99%, HPLC Grade), Glycerol (99.5%), $MgSO_4$ (99%), $FeCl_3$ (99%), NaCl (99.5%), $KH_2PO_4$ (99%), benzyl alcohol (99%), $Na_2HPO_4 \cdot 2H_2O$ (99%), $CaCl_2$ (99%), $NH_4Cl$ (99%), NaOH (99%), $Na_2SO_4$ (99%), $NaMoO_4$ (99%), $CoCl_2 \cdot 6H_2O$ (99%), HCl (99%), $H_3BO_4$ (99%), $CuCl_2$ (99%), $ZnCl_2 \cdot 4H_2O$ (98%), N,N,N',N'-tetramethylethylenediamine (TEMED, 99.5%), sodium dodecyl sulfate (SDS, 99%), and ammonium persulfate (98%) were purchased from Sigma Aldrich. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 99%) was bought from Gold Biotechnology. $K_2HPO_4 \cdot 3H_2O$ (99%) was bought from Merck. Acetonitrile (ACN, HPLC grade) and ethyl acetate (HPLC grade) were purchased from Tedia. Isopropyl alcohol (99.5%) was obtained from Fischer Chemicals. Yeast extract and Tris buffer (pH 7.5, ultra pure grade) were purchased from FirstBase. Bacto Tryptone was purchased from Gibco. Luria broth (LB) medium was purchased from Biomed Diagnostics. 30% Acrylamide/bis-acrylamide solution (37.5:1) was obtained from Bio-Rad. Hydrochloric acid (HCl, 37%) was purchased from VWR.

Tris-HCl solution was prepared by adding hydrochloric acid into the freshly-prepared Tris-buffer until the desired pH level was achieved.

M9 was used as a culture medium, containing (per Liter): 6 g yeast extract, 20 g glucose, 0.5 g NaCl, 6 g $Na_2HPO_4$, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mL trace metals, 1 g $NH_4Cl$, and 3 g $KH_2PO_4$. Terrific broth (TB) medium was used as an alternative culture medium, containing (per Liter): 24 g yeast extract, 20 g tryptone, and 4 mL glycerol.

Analytical techniques

*Reverse phase high performance liquid chromatography (HPLC)*

*L*-Phe, (*R*)-PED, and (*R*)-MA were analyzed using reverse phase HPLC (Shimadzu Prominence HPLC System). Column: Agilent Poroshell 120 SB-C18 (150 mm x 4.6 mm x 2.7 µm). Mobile phase: 30% ACN and 70% water containing 0.1% TFA. Flow rate: 0.4 mL/min, temperature: 25 °C. Detector: photodiode array detector. Wavelength: 210 nm. Retention time:

5.5 min for (*R*)-1,2-phenylethanediol, 5.8 min for (*R*)-MA, 4.1 min for *L*-phenylalanine, and 8.3 min for benzyl alcohol (internal standard).

*Normal phase HPLC*

The *ee* of (*R*)-MA was determined by normal phase HPLC with a chiral column. Column: Daicel Chiralpak IC-3 (250 × 4.6 mm, 3 µm). Mobile phase: 10% isopropyl alcohol and 90% *n*-hexane. Flow rate: 0.8 mL/min. Detection: 210 nm. Temperature: 25 °C.

*Gas chromatography (GC)*

Styrene and (*S*)-styrene oxide were analyzed using Agilent 7890A GC. Column: Agilent HP-5 (30 m x 0.32 mm x 0.25 mm). Temperature programme: initial temperature at 70 °C, increase 25 °C/min until it reached 200 °C; subsequently increase to 250 °C at 50 °C/min, then hold for 1 min; Lastly, increase to 270 °C at 20 °C/min. Retention time: 2.8 min for styrene, 4.0 min for (*S*)-styrene oxide, and 3.8 min for benzyl alcohol (internal standard).

*SDS-PAGE*

Gel Preparation:

10 mL of separating gel containing $H_2O$ (3.2 mL), 8% (w/v) acrylamide/bis-acrylamide (4 mL), 1.5 M tris (pH 8.8, 2.6 mL), 10% (w/v) SDS (0.1 mL), 10% (w/v), ammonium persulfate (0.1 mL), and TEMED (0.01 mL) was firstly prepared. Subsequently, 5 mL of stacking gel containing $H_2O$ (2.975 mL), 0.5 M Tris-HCl (pH 6.8, 1.25 mL), 10% (w/v) SDS (0.05 mL), 8% (w/v) acrylamide/bis-acrylamide (0.67 mL), 10% (w/v) SDS (0.05 mL), 10% (w/v) ammonium persulfate (0.05 mL), and TEMED (5 µL) was added on to the solidified separating gel.

Sample Preparation:

The designated strains were initially inoculated and cultured with respective antibiotics, followed by harvesting and centrifugation at 5000 g for 10 min. The cell pellet was resuspended by using $H_2O$ to a cell density of 1 g cdw/L. SDS-PAGE loading dye was subsequently added, and each sample was subsequently subjected into heating at 95 °C for 15 min. 10 µL of each sample was loaded into the well accordingly, and SDS-PAGE was done for 90 minutes.

*Nuclear magnetic resonance (NMR) spectroscopy*

A Bruker AVANCE™ III HD 400 MHz spectrometer was used for both $^{1}H$ and $^{13}C$ NMR analysis.

<u>General procedure of fed-batch growing cells fermentation for the production of *L*-Phe from glycerol or glucose</u>

*E. coli* NST74-Phe was inoculated in 100 mL LB medium containing 50 μg/mL streptomycin at 37 °C and 250 rpm for 12 h. The inoculated strain was subsequently transferred and cultured in 1.5-liter bioreactor with the modified $NH_4$-media containing 10 g/L $(NH_4)_2SO_4$, 5 g/L $KH_2PO_4$, 5 g/L $MgSO_4$, 5 g/L yeast extract, 50 μg/mL streptomycin, and 10 g/L carbon source (glycerol or glucose) for 28 h. 30% $NH_4OH$, and 80% carbon sources were used as the feed to control the carbon source at pH 6.8 and 5-10 g/L, respectively, without acetate accumulation. Aeration and stirrer speed were also modified between 0.5-3 litre per minute and 500-2000 rpm, respectively, to maintain the dissolved oxygen (DO) level at 20-30%. IPTG was added to a final concentration of 0.1 mM at 8 h to induce the protein expression. In the end of the fermentation, 30% $NH_4OH$ was added to adjust the reaction mixture to pH 8.0 before performing further biotransformation.

General procedure for culturing *E. coli* cells

*E. coli* strains were inoculated with LB medium containing appropriate antibiotics (50 μg/mL kanamycin, 50 μg/mL chloramphenicol, 50 μg/mL streptomycin, or 100 μg/mL ampicillin) at 37 °C and 250 rpm for 8-10 h. The inoculated strain was subsequently transferred and grown into a 250 mL-baffled culture flask with M9 medium containing appropriate antibiotics in a total volume of 50 mL. When cell density ($OD600_{nm}$) reached ~0.6, IPTG was added to a final concentration of 0.5 mM to induce the protein expression at 22 °C. The cells were harvested after 14 h of growth by centrifugation (4000 g, 10 min).

**Example 1. Development of artificial enzyme cascade for the conversion of styrene to (*R*)-MA**

*Genetic engineering of recombinant E. coli strains*

The gene of alditol oxidase (AldO) from *Streptomyces coelicolor* A3(2) was codon optimized and synthesized based on the reported gene sequence (GenBank accession No. SCO6147) by GenScript. The gene fragments of AldO was amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase with forward primer 5′-TATATCATGAGCGACATTACCGTTACCAAC-3′ and reverse primer 5′-CTCGAATTCTTAACCTGCCAGAACGCCACGAAC-3′ (restriction sites are underlined). The PCR products were digested with *Bsp*HI and *Eco*RI and then ligated with *Nco*I and *Eco*RI digested pRSFDuet-1 to give pRSF-AldO. *E. coli* (AldO) strain was made by transforming pRSF-AldO into chemical competent *E. coli* T7 cells.

E. coli (S2A) strain was made by co-transformation of pRSF-AldO and previously constructed pCDF-SMOStEH (Y. Zhou, S. Wu & Z. Li, *Angew. Chem. Int. Ed.* **2016**, *55*, 11647–11650) into chemical competent *E. coli* T7 cells. *E. coli* (P2S2A) strain was made by transforming previously constructed pET-PAL-PAD (Y. Zhou, S. Wu & Z. Li, *Angew. Chem. Int. Ed.* **2016**, *55*, 11647–11650) into chemical competent *E. coli* (S2A) cells. The engineering of *E. coli* NST74 (P2S2A) strain was similar to above mentioned procedures using our previously constructed *E. coli* NST74 (DE3) cells (Y. Zhou *et al.*, *ChemSusChem* **2018**, *11*, 2221-2228). All the strains and enzymes used in this study are listed in Tables 1-2.

**Table 1.** Strains used in this study.

| Strains | Genotype and plasmids contained | Source |
|---|---|---|
| *E. coli* T7 | *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10* | New England Biolabs |
| *E. coli* NST74 | *aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr) ,pheO352, aroG397(fbr)* | ATCC 31884 |
| *E. coli* NST74 (DE3) | *aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr) ,pheO352, aroG397(fbr) (DE3)* | Y. Zhou *et al.*, *ChemSusChem* **2018**, *11*, 2221-2228 |
| *E. coli* (AldO) | pRSF-AldO | This study |
| *E. coli* (S2A) | pCDF-SMO-StEH, pRSF-AldO | This study |
| *E. coli* (P2S2A) | pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO | This study |
| *E. coli* NST74 (P2S2A) | pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO | This study |
| *E. coli* NST74-Phe | pCDF-Phe | B. S. Sekar, B. R. Lukito & Z. Li *ACS Sustainable Chem. Eng.* **2019**, *7*, 14, 12231–12239 |

**Table 2.** Enzymes used in this study.

| Enzyme | Reaction | Source | Descriptions | References |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Phenylalanine ammonia lyase (PAL) | Convert *L*-Phe to *trans*-cinnamic acid | *Arabidopsis thaliana* | Encoded by *Atpal* 2 | F. C. Cochrane, L. B. Davin & N. G. Lewis, *Phytochemistry* **2004**, *65*, 1557-1564 |
| Phenylacrylic acid decarboxylase (PAD) | Convert *trans*-cinnamic acid to styrene | *Aspergillus niger* | PAD consists of ferulic acid decarboxylase (FDC1) and phenylacrylic acid decarboxylase (PAD1), which are encoded by *fdc1* and *pad1*, respectively | K. A. P. Payne *et al.*, *Nature* **2015**, *522*, 497-501 |
| Styrene monooxygenase (SMO) | Convert styrene to (*S*)-styrene oxide | *Pseudomonas sp. VLB120* | SMO consists of StyA and StyB, which are encoded by *styA* and *styB*, respectively | S. Panke *et al.*, *Appl. Environ. Microbiol.* **1998**, *64*, 2032-2043 |
| Epoxide hydrolase (StEH) | Convert (*S*)-styrene oxide to (*R*)-PED | *Solanum tuberosum* | Encoded by *Steh* 1 | D. Lindberg, A. Gogoll & M. Widersten, *The FEBS Journal* **2008**, *275*, 6309-6320 |
| Alditol oxidase (AldO) | Convert (*R*)-PED to (*R*)-MA | *Streptomyces coelicolor* A3(2) | The gene of AldO was codon optimized and synthesized based on the reported gene sequence (GenBank accession No. SCO6147) | D. P. H. M. Heuts *et al.*, *J. Biol. **Chem**.* 2007, *282*, 20283–20291; and E. W. van Hellemond *et al.*, *Adv. Synth. Catal.* **2009**, |

**Example 2. Biotransformation of (*R*)-PED to (*R*)-MA**

*Biotransformation of (R)-PED to (R)-MA with resting cells of E. coli (AldO) in aqueous phase*
*E. coli* (AldO) cells were cultured with Kanamycin antibiotics (50 mg/L), harvested, and resuspended in 10 mL of KP buffer (potassium phosphate buffer, 100–200 mM, pH 8.0) containing 2.76 g/L (20 mM) of (*R*)-PED to a cell density of 10–15 g cdw/L to perform the biotransformation at 30 °C and 250 rpm for 24 h. 50 µL of the aqueous phase was collected and diluted with 450 µL ultrapure water containing 0.5% TFA and 500 µL ACN containing 2 mM benzyl alcohol. The samples were analysed using HPLC to quantify the concentration of (*R*)-MA and (*R*)-PED.

Results and discussion

Alditol oxidase (AldO) from *Streptomyces coelicolor* A3(2) was chosen for the conversion of (*R*)-PED to (*R*)-MA (FIG. 1b, D. P. H. M. Heuts *et al.*, *J. Biol.* ***Chem.*** 2007, *282*, 20283–20291; and E. W. van Hellemond *et al.*, *Adv. Synth. Catal.* **2009**, *351*, 1523–1530). AldO gene was cloned in pRSFDuet-1 plasmid and transformed into *E. coli* T7 strain to give *E. coli* (AldO). Biotransformation of (*R*)-PED to (*R*)-MA was investigated with *E. coli* (AldO) cells (10–15 g cdw/L) in KP buffer system (200 mM, pH 8.0) at 30 °C for 24 h, and 15 g cdw/L of cells gave the best results. The time course is shown in FIG. 2a. (*R*)-MA was produced at a linear rate within the first 8 h with the fast consumption of (*R*)-PED. After 8 h of biotransformation, 1.9 g/L of (*R*)-MA was produced, giving a space–time yield of 237.5 mg/L/h. These results clearly demonstrate that AldO catalyzed the conversion of (*R*)-PED to (*R*)-MA. As the reaction continued, the reaction rate gradually decreased, and 2.43 g/L (16 mM) of (*R*)-MA was produced at 24 h in 80% yield, while 600 mg/L (4 mM) of (*R*)-PED remain unreacted. This is probably because of the high $K_m$ (101 mM) toward (*R*)-PED (E. W. van Hellemond *et al.*, *Adv. Synth. Catal.* **2009**, *351*, 1523–1530) and relatively low activity of AldO. Enzyme evolution might be needed for increasing its affinity with (*R*)-PED as well as the activity. Although $H_2O_2$ could be produced during the reaction with 20 mM substrate, the low level of the produced $H_2O_2$ in cells did not influence the enzymatic reactions. This is probably because the *E. coli* cells produced endogenous catalase which could decompose $H_2O_2$. The co-expression of the catalase from *E. coli* with AldO was also tried. However, the product yield was not increased.

**Example 3. One-pot bioproduction of (*R*)-MA from styrene via whole cell biotransformation in *n*-hexadecane-aqueous two-phase system**

Styrene is a good industrial feedstock because it can be produced from ethylbenzene by dehydrogenation at a very large scale and low cost. Direct production of (*R*)-MA from styrene provides an economic and cyanide-free synthetic approach. Therefore, a synthetic pathway including SMO, StEH and AldO was designed for the bioconversion of styrene to (*R*)-MA (FIG. 1b). The *E. coli* (S2A) cells coexpressing SMO, StEH and AldO were used as whole cell catalyst for the bioconversion of styrene to (*R*)-MA. (FIG. 3).

*Biotransformation of styrene to (R)-MA with resting cells of E. coli (S2A) in two-phase system*

*E. coli* (S2A) cells were inoculated and cultured with antibiotics (50 mg/L Kanamycin and 50 mg/L Streptomycin), then subsequently harvested and resuspended in 10 mL of KP buffer (200 mM, pH 8.0) containing 0.5% glucose to a cell density of 15 g cdw/L. 10 mL of *n*-hexadecane containing 2.08 g/L (20 mM) of styrene was added to perform the biotransformation at 30 °C and 250 rpm for 24 h. 50 µL of the aqueous phase was collected and diluted with 450 µL ultrapure water containing 0.5% TFA and 500 µL ACN containing 2 mM benzyl alcohol. The samples were analysed using HPLC to quantify the concentration of (*R*)-MA and (*R*)-PED. 50 µL of the organic phase was collected and diluted with 950 µL ethyl acetate containing 2 mM benzyl alcohol, and the samples were analysed using GC to quantify the concentration of styrene and (*S*)-styrene oxide.

Results and discussion

pRSF-AldO were co-transformed together with pCDF-SMO-StEH to give *E. coli* (S2A) co-expressing SMO, StEH, and AldO for the direct conversion of styrene to (*R*)-MA (FIG. 1b). Cascade biotransformation of styrene to (*R*)-MA was conducted using *E. coli* (S2A) cells (15 g cdw/L) in a mixture of KP buffer (200 mM, pH 8.0) containing 0.5% glucose and *n*-hexadecane (1:1; v/v) at 30 °C for 24 h. The organic phase functions as a reservoir for toxic substrate styrene and intermediate styrene oxide. As shown in FIG. 4, (*R*)-MA was produced during the biotransformation of styrene (20 mM) with resting cells of *E. coli* (S2A) cells in *n*-hexadecane-aqueous two-phase system. The time course on the production of (*R*)-MA from styrene is shown in FIG. 2b. As time went on, the styrene concentration rapidly decreased and the concentration of (*R*)-MA and (*R*)-PED increased gradually. 1.07 g/L (7 mM) of (*R*)-MA was successfully obtained within the first 10 h, which corresponds to a space–time yield of 107 mg/L/h. After 24 h reaction, 1.52 g/L (10 mM) of (*R*)-MA (>99% *ee*) was produced from 2.08 g/L (20 mM) of styrene, together with the remaining (*R*)-PED (800 mg/L; 6 mM) and styrene (300 mg/L; 3 mM). A small amount of mass imbalance (about 1 mM) was observed, which is probably due to styrene evaporation.

**Example 4. One-pot bioproduction of (*R*)-MA from *L*-Phe via whole cell biotransformation in *n*-hexadecane-aqueous two-phase system**

*L*-Phe is a renewable bio-based starting material which can be produced via microbial fermentation at a very large scale and low cost. Direct production of (*R*)-MA from *L*-Phe provides an economic and sustainable synthetic approach. To achieve this goal, PAL and PAD were chosen and employed for the conversion of *L*-Phe to styrene. Then, these two enzymes were added into the artificial pathway described in Example 3 which converts styrene to *(R)*-MA for building a new synthetic pathway.

*Development of artificial enzyme cascade for the conversion of L-Phe to (R)-MA*

To develop an enzyme cascade for producing (*R*)-MA from *L*-Phe, deamination reaction catalyzed by PAL from *Arabidopsis thaliana* and decarboxylation reaction catalyzed by PAD [consisting of ferulic acid decarboxylase (FDC1) and phenylacrylic acid decarboxylase (PAD1)] from *Aspergillus niger* were introduced into the above mentioned epoxidation-hydrolysis-double oxidation artificial enzyme cascade, yielding a new five enzyme cascade. The addition of PAL and PAD enables the biotransformation of *L*-Phe to (*R*)-MA (FIG. 1b).

To engineer *E. coli* strains expressing the five-enzyme artificial cascade, the previously constructed pET-PALPAD (Y. Zhou, S. Wu & Z. Li, *Angew. Chem. Int. Ed.* **2016**, *55*, 11647–11650) were transformed into the *E. coli* (S2A) cells to give *E. coli* (P2S2A). The enzyme expression in *E. coli* (P2S2A) was analysed by SDS-PAGE (FIG. 5). The corresponding protein bands of PAL, PAD, SMO, StEH, and AldO were clearly observed, indicating these enzymes were well-expressed. The *E. coli* (P2S2A) cells coexpressing PAL, PAD, SMO, StEH and AldO were used as whole cell catalyst for the bioconversion of *L-Phe* to (*R*)-MA (FIG. 6).

*Biotransformation of L-Phe to (R)-MA with resting cells of E. coli (P2S2A) in two-phase system*

*E. coli* (P2S2A) cells were inoculated with LB medium containing 100 μg/mL ampicillin, 50 μg/mL kanamycin and 50 μg/mL streptomycin. Then, *E. coli* (P2S2A) cells were cultured, harvested and resuspended in 10 mL of KP buffer (200 mM, pH 8.0) containing 0.5% glucose and 2.48 g/L (15 mM) of *L*-Phe to a cell density of 15 g cdw/L. 10 mL of *n*-hexadecane was added to perform the biotransformation at 30 °C and 250 rpm for 24 h. 50 μL of the aqueous phase was collected and diluted with 450 μL ultrapure water containing 0.5% TFA and 500 μL ACN containing 2 mM benzyl alcohol. The samples were analysed using HPLC to quantify the concentration of (*R*)-MA, (*R*)-PED, and *L*-Phe. 50 μL of the organic phase was collected and diluted with 950 μL ethyl acetate containing 2 mM benzyl alcohol, and the samples were analysed using GC to quantify the concentration of styrene and (*S*)-styrene oxide.

Results and discussion

Cascade biotransformation of *L*-Phe to (*R*)-MA was conducted at 30 °C and 250 rpm using *E. coli* (P2S2A) cells (15 g cdw/L) in a mixture of KP buffer (200 mM, pH 8.0) containing 0.5% glucose and 2.48 g/L (15 mM) *L*-Phe and *n*-hexadecane (1:1; v/v). The organic phase functions as a reservoir for toxic intermediates such as styrene and styrene oxide. As shown in FIG. 7, (*R*)-MA was produced during the biotransformation of 15 mM *L*-Phe with resting cells of *E. coli* (P2S2A) cells. The time course of reaction is shown in FIG. 2c. With the rapid reduction of *L*-Phe, styrene, (*R*)-PED and (*R*)-MA gradually increased. After six hours of the biotransformation, 650 mg/L of (*R*)-MA was produced, giving a space–time yield of 108.3 mg/L/h. 913 mg/L (6 mM) of (*R*)-MA (>99% *ee*) was obtained after 24 h biotransformation of 2.48 g/L (15 mM) *L*-Phe. These results indicate the successful production of (*R*)-MA from *L*-Phe by the newly developed five-enzyme cascades, and represent the highest (*R*)-MA concentration among bioproduction from natural resources. Considering the remaining unreacted *L*-Phe (3 mM), and intermediates, styrene (1 mM) and (*R*)-PED (6 mM), a small amount of mass imbalance (about 1 mM) was observed, which is also possibly due to styrene evaporation. Further, the (*R*)-MA titer is much higher than those previously reported (236 mg/L in yeast cells and 680 mg/L in E. coli cells, M. Reifenrath & E. Boles, *Metab. Eng.* **2018**, *45*, 246–254; and Z. Sun *et al.*, *Microb. Cell Fact.* **2011**, *10*, 71).

The biotransformation was also performed using 5 mM or 10 mM *L*-Phe under the same reaction condition. 3 mM and 6 mM of (*R*)-MA was produced with 60% conversion in both cases, which is higher than the conversion obtained from 15 mM *L*-Phe. Although the product titer is much higher than previously reported, it is insufficient for commercial application. The expression of AldO was in a relatively low level when many different enzymes were co-expressed in *E. coli* cells (FIG. 6d). In addition, AldO showed insufficient activity. These were probably the reasons for the accumulation of (*R*)-PED. Further optimizations, such as the expression of different enzymes in high level and at the good ratio, as well as engineering of much more efficient enzyme, are needed to increase the conversion, enhance the product titer, and reduce the accumulation of intermediates.

**Example 5. One-pot fermentative production of (*R*)-MA from glycerol or glucose using a *L*-Phe-overproducing *E. coli* strain expressing the five-enzyme artificial cascade**

Glucose or glycerol are commonly used feedstocks in many microbial fermentation processes for fine chemicals production at large scale and low cost. Direct production of (*R*)-MA from

glucose or glycerol provides a prosperous approach for the green, economic and sustainable biosynthesis of pharmaceuticals and high-value chemicals.

For single strain approach, *L*-Phe and (*R*)-MA were produced in the same cell. The combination of natural *L*-Phe biosynthetic pathway and the artificial pathway described in Example 4 which converts *L*-Phe to (*R*)-MA led to a new pathway, enabling the bioconversion of glucose or glycerol to (*R*)-MA in one cell (FIG. 1c).

*Engineering of E. coli strains for the production of (R)-MA from glycerol or glucose*

For synthesis of (*R*)-MA from glycerol or glucose, the *E. coli* T7 host was changed into *E. coli* NST74 strain. The latter is a *L*-Phe-overproducing strain, producing *L*-Phe from glycerol or glucose (Y. J. Choi & D. E. Tribe, *Biotechnol. Lett.* **1982**, *4*, 223–228). pRSF-AldO, pET-PAL-PAD, and pCDF-SMO-StEH were transformed into the previously engineered *E. coli* NST74(DE3) strain (Y. Zhou, S. Wu & Z. Li, *Angew. Chem. Int. Ed.* **2016**, *55*, 11647–11650) to give the *E. coli* NST74 (P2S2A) strain (FIG. 6c). The protein expression in *E. coli* NST74 (P2S2A) was analyzed by SDS-PAGE (FIG. 6d). Similar to the results from *E. coli* (P2S2A), all enzymes involved in the artificial enzyme cascade were well-expressed in *E. coli* NST74 (P2S2A) strain. The *E. coli* NST74 (PS2S2A) cells coexpressing *L*-Phe biosynthetic pathway involved enzymes, PAL, PAD, SMO, StEH and AldO, were used as whole cell catalyst for the bioconversion of glucose or glycerol to (*R*)-MA (FIG. 8).

*Fermentative production of (R)-MA from glycerol or glucose with single E. coli strain in aqueous system*

*E. coli* NST74 (P2S2A) strains were inoculated with LB medium containing appropriate antibiotics (50 μg/mL kanamycin, 50 μg/mL streptomycin, and 100 μg/mL ampicillin) at 37 °C and 250 rpm for 8–10 h. The inoculated strain was subsequently transferred and grown in a 250 mL-baffled culture flask with M9 medium or TB medium (containing 0.4% (v/v) glycerol as carbon source) and appropriate antibiotics in a total volume of 50 mL. IPTG was added at a final concentration of 0.1 mM to induce the protein expression when $OD600_{nm}$ reached ~0.6. The cells were grown at 25 °C for another 24 h. After 24 h of growth, the cells were harvested by centrifugation (4000 g, 10 min). The concentration of (*R*)-MA, *L*-Phe and (*R*)-PED were analyzed by HPLC. The concentration of styrene in the reaction sample was analyzed by GC.

*Fermentative production of (R)-MA from glycerol or glucose with single E. coli strain in n-hexadecane-aqueous two-phase system*

The fermentative production of (*R*)-MA from glycerol or glucose in *n*-hexadecane-aqueous two-phase system was performed by following the fermentative production protocol above

except 25 mL of *n*-hexadecane (*n*-hexadecane:aqueous = 1:2) was added after the addition of IPTG.

Results and discussion

Fermentative biotransformation of glycerol (FIG. 9a) and glucose (FIG. 9b) successfully gave a (*R*)-MA concentration of 75 mg/L (0.49 mM) and 48 mg/L (0.31 mM), respectively. The fermentation of glycerol gave higher (*R*)-MA titer in comparison with the one performed with glucose as carbon source, suggesting that the cells tend to consume the glucose more for their growth than for the (*R*)-MA production. Notably, high accumulation of styrene in the aqueous phase was observed during the fermentation, which caused toxicity to the cells and result in low product yield.

To tackle the styrene toxicity issue, *n*-hexadecane-aqueous two-phase system was used for the fermentation. Here, *n*-hexadecane serves as a reservoir of toxic substrates and intermediates for reducing their toxicity to cells. *E. coli* NST74 (P2S2A) cells were grown in M9 or TB medium under the above-mentioned conditions. Time courses of the fermentation are shown in FIG. 9c-d. The concentration of (*R*)-PED and (*S*)-styrene oxide were very low (FIG. 9), possibly caused by the low concentration of *L*-Phe produced from glycerol or glucose. Compared to the previous single aqueous system, the two-phase system still significantly increased the product concentration. While the *L*-Phe, (*R*)-PED, and (*R*)-MA were formed in the aqueous phase, the intermediates styrene and (*S*)-styrene oxide remained in the organic phase to avoid the toxicity issue. The best results, 228 mg/L (1.5 mM) and 152 mg/L (1 mM) of (*R*)-MA, were obtained from the fermentative biotransformation of glycerol and glucose in the two-phase system, respectively, with the significant decrement of styrene accumulation.

**Example 6. One-pot production of (*R*)-MA from glycerol or glucose by coupling of two *E. coli* strains expressing the artificial enzyme cascade and *L*-Phe biosynthesis pathway, respectively.**

For coupling strains approach, *L*-Phe was produced by one *E. coli* strain, and the production of (*R*)-MA was catalyzed by a second *E. coli* strain. *E. coli* is able to produce *L*-Phe from glycerol or glucose via Shikimate pathway.

*Bioproduction of (R)-MA from glycerol or glucose via coupling two E. coli strains in n-hexadecane-aqueous two-phase system*

For enhancing the *L*-Phe producing ability of *E. coli*, *L*-Phe biosynthetic pathway involved enzymes including OAHP synthase aroG* (feedback inhibition resistant mutant AroG-D146N),

shikimate dehydrogenase ydiB, shikimate kinase aroK, chorismate mutase/prephenate dehydratase pheA* (feedback inhibition resistant mutants, truncated version of PheA with 1-300 AA) and tyrosine aminotransferase tyrB, were chosen, and these enzymes are also overexpressed in *E. coli.* These enzymes were cloned into pCDF Duet-1 vector and resulting plasmid pCDF-Phe was transformed into *E. coli* NST74.

Firstly, *L*-Phe was produced from glycerol or glucose by microbial fermentation production with *E. coli* NST74-Phe strain (FIG. 8a, B. S. Sekar, B. R. Lukito & Z. Li *ACS Sustainable Chem. Eng.* **2019**, *7*, 14, 12231–12239) in a fed-batch bioreactor. *E. coli* (NST74-Phe) was inoculated from glycerol stock into 100 mL of LB containing streptomycin (50 mg/L) and grown in a 250 mL flask at 37 °C until the $OD600_{nm}$ reached to 3-4. The culture was transferred to a 3 L bioreactor containing 1 L of fermentation medium (10 g/L glucose or glycerol, 10 g/L $(NH_4)_2SO_4$, 5 g/L $KH_2PO_4$, 5 g/L $MgSO_4$, 5 g/L yeast extract, 15 mg/L $FeSO_4$, 15 mg/L $MnSO_4$, and 1 g/L betaine). During the fermentation, 600 g/L glucose or 800 g/L glycerol solution was fed to maintain the concentration of glucose or glycerol as 5-10 g/L. The pH was controlled at 6.8 by $NH_4OH$ solution. The DO was maintained at 20-30% by supplying air or pure oxygen at 0.5-3 L/min and varying the agitation speed between 500 and 1500 rpm. IPTG was added to a final concentration of 0.1 mM when the $OD600_{nm}$ reached to 15-20. The fermentation was stopped when the culture reached a stationary phase. The fermentation culture containing biosynthesized *L*-Phe was used directly for the following biotransformation experiments.

During the time period of fermentative production of *L*-Phe, *E. coli* (P2S2A) strains were inoculated with LB medium containing 100 µg/mL ampicillin, 50 µg/mL kanamycin and 50 µg/mL streptomycin. Then, *E. coli* (P2S2A) cells were cultured, harvested and resuspended in 3-4 mL KP buffer (4 M, pH 8.0). Concentrated *E. coli* (P2S2A) cell suspension, KP buffer (4 M, pH 8.0), and 40% glucose solution were added into the fermented media containing *L*-Phe (67 mM from glucose or 78 mM from glycerol) and *E. coli* (NST74-Phe) cells to give 5 mL reaction mixture containing 60 or 72 mM of *L*-Phe, KP buffer (200 mM, pH 8.0), 1% glucose, and 15 g cdw/L of *E. coli* (P2S2A) cell suspension. 5 mL *n*-hexadecane was added to perform the biotransformation of *L*-Phe to (*R*)-MA at 30 °C and 250 rpm for 24 h (FIG. 10a). The final reaction mixture contained 1.65 g/L (10 mM) of *L*-Phe, 200 mM KP buffer (pH 8.0), 1% glucose, 15 g cdw/L of *E. coli* (P2S2A) cells and *n*-hexadecane (1:1, v/v). The reaction samples were analyzed by HPLC to quantify the concentration of (*R*)-MA, *L*-Phe and (*R*)-PED. The concentration of styrene in the reaction sample was analyzed by GC.

Results and discussion

Production of (*R*)-MA from glycerol or glucose was examined by coupling *E. coli* NST74-Phe which overexpresses five limiting enzymes to convert glycerol or glucose to *L*-Phe via the Shikimate pathway (B. S. Sekar, B. R. Lukito & Z. Li *ACS Sustainable Chem. Eng.* **2019**, *7*, 14, 12231–12239), and *E. coli* (P2S2A) expressing the novel artificial cascade to convert *L*-Phe to (*R*)-MA (FIG. 2c). *E. coli* NST74-Phe was first grown in modified $NH_4$-media containing 10 g/L glycerol or glucose for 28 h to produce 12.88 g/L (78 mM, FIG. 10a) and 11.07 g/L (67 mM) of *L*-Phe from glycerol and glucose, respectively.

Subsequently, freshly made *E. coli* (P2S2A) cells, concentrated KP buffer (pH 8.0), and glucose solution were directly added into fermentation medium, resulting in a reaction mixture containing 1.65 g/L of L-Phe, 200 mM KP buffer (pH 8.0), 1% glucose, and 15 g cdw/L of *E. coli* (P2S2A) cells. *n*-Hexadecane was added to give a ratio of 1:1 (v/v) for aqueous and organic phases. The biotransformation was performed at 30 °C and 250 rpm for 24 h. 760 (5 mM) and 455 mg/L (3 mM) of (*R*)-MA was obtained from glycerol-fermented media and glucose-fermented media, respectively (FIG. 10b and FIG. 11), with some amount of (*R*)-PED and styrene remaining in the reaction system (Table 3). While the optimal temperature for expression and production of *L*-Phe from glycerol or glucose is 37 °C (B. S. Sekar, B. R. Lukito & Z. Li *ACS Sustainable Chem. Eng.* **2019**, *7*, 14, 12231–12239), the optimal temperature for the expression of artificial enzyme cascade is 22 °C and for cascade biotransformation of *L*-Phe to (*R*)-MA, the optimal temperature is 30 °C. Therefore, the single-cell fermentation at 25 °C gave lower product concentration. In contrary, the two-cells system could be operated at their respective optimum temperatures to give higher productivity. The use of glycerol as the feedstock showed higher (*R*)-MA titer than those previously reported (236 mg/L in yeast cells and 680 mg/L in *E. coli* cells, M. Reifenrath & E. Boles, *Metab. Eng.* **2018**, *45*, 246–254; and Z. Sun *et al.*, *Microb. Cell Fact.* **2011**, *10*, 71).

**Table 3.** Biotransformation of glucose or glycerol to (*R*)-MA via coupling *E. coli* (NST74-Phe) and *E. coli* (P2S2A) cells.

| Carbon Sources | Cell Density (g cdw/L) | (*R*)-MA Concentration (mM)[a)] | Remaining Substrate & Intermediates | | |
|---|---|---|---|---|---|
| | | | *L*-Phe (mM) | Styrene (mM) | (*R*)-PED (mM) |
| Glycerol | 15 | 5 | 1 | 2 | 2 |
| Glucose | 15 | 3 | 1 | 2 | 4 |

[a)] Reaction was performed at 30 °C and 250 rpm for 24 h.

Here, the development of novel artificial enzyme cascades for (*R*)-MA production from cheap and easily available styrene, bio-based *L*-Phe (FIG. 6a), as well as the combination of natural *L*-Phe biosynthesis pathway and the artificial enzyme cascade for the production of (*R*)-MA from glucose or glycerol in single strain (FIG. 6b) and coupled cells biotransformation (FIG. 6c), respectively, is reported. These results successfully demonstrate the enhancement and synthetic potential for producing (*R*)-MA from cheap and easily available renewable feedstocks.

**Example 7. Preparative Biotransformation of *L*-Phe to (*R*)-MA in aqueous-*n*-hexadecane two-phase system**

*Preparative biotransformation of L-Phe to (R)-MA in aqueous-n-hexadecane two-phase system*

50 mL KP buffer (100 mM, pH 8.0) containing *E. coli* (P2S2A) cells (15 g cdw/L), 0.5% glucose, and 10 mM *L*-Phe was mixed with 50 mL *n*-hexadecane to perform the biotransformation at 30 °C and 250 rpm for 24 h. The reaction mixture was subsequently centrifuged (5000 g, 15 min) to separate the aqueous phase from the organic phase and the cell pellet. The aqueous phase was collected, saturated with NaCl, and adjusted to pH 1 by adding 10 M HCl, followed by extraction with ethyl acetate (3 x 50 mL). The organic phase was then collected and dried using $Na_2SO_4$, followed by filtration. The organic phase was subsequently evaporated by using the rotary evaporator to remove the ethyl acetate. The crude product was subjected to crystallization in ethyl acetate by dissolving at 65 °C and slowly cooling down to -20 °C. The crystals were taken by filtration, and the mother liquor was evaporated and subjected to crystallization again. The collected crystals were dried under vacuum for 12 h for further chiral HPLC and NMR analysis.

(*R*)-MA

$^1$H NMR spectrum (400 MHz, $CD_3OD$), δ 7.47 (dd, *J* = 15.1, 7.2 Hz, 2H), 7.31 (ddd, *J* = 10.7, 9.0, 5.1 Hz, 3H), 5.19 (s, 1H). $^{13}$C NMR spectrum (101 MHz, $CD_3OD$), δ 176.27, 140.75, 129.53, 129.33, 127.96, 74.21.

Results and discussion

Preparative biotransformation of *L*-Phe to (*R*)-MA was performed in a two-phase system with *E. coli* (P2S2A) cells, which yielded 6 mM (*R*)-MA in 24 h with 60% conversion. After purification, the pure product was obtained with an isolated yield of 46%. $^1$H and $^{13}$C NMR analysis confirmed the chemical structure of (*R*)-MA products. Chiral HPLC analysis showed > 99% *ee* of the produced (*R*)-MA (FIG. 12).

## Claims

1. A method for producing an enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid 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 (*R*)-mandelic acid or a derivative thereof, comprising the steps of:

(a) generating enantiomerically pure or enantiomerically enriched 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; and

(b) generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof from enantiomerically pure or enantiomerically enriched phenylethane diol or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol to a carboxylic acid using an oxidation enzyme.

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 oxidation enzyme is an alcohol oxidase, optionally wherein the alcohol oxidase is from *Streptomyces coelicolor,* or its mutants or similar enzymes with more than 50% identity.

4. 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, pRSF-AldO.

5. 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.

6. The method according to Claim 5 wherein, the ammonia lyase is phenylalanine ammonia lysase from *Arabidopsis thaliana*, or its mutants or similar enzymes with more than 50% identity.

7. The method according to Claim 5 or Claim 6 wherein, the decarboxylate is phenylacrylic acid decarboxylase from *Aspergillus niger*, or its mutants or similar enzymes with more than 50% identity.

8. The method according to any one of Claims 5 to 7, 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-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO.

9. The method according to any one of Claims 6 to 8, wherein the method further comprises cells that overexpress the natural *L*-phenylalanine biosynthetic pathway, which cells convert glucose of glycerol to *L*-phenylalanine.

10. The method according to Claim 9 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.

11. The method according to Claim 10, wherein the microbial cells producing *L*-phenylalanine from glucose or glycerol overexpress at least one enzyme is one in which the where the microbial cells are mutated for deletion or inactivation of crr and/or tyrA genes.

12. The method according to any one of Claims 9 to 11, wherein the microbial cells that overexpress the natural *L*-phenylalanine biosynthetic pathway are *E. coli* NST74-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO, which is used to produce (*R*)-mandelic acid from glucose or glycerol.

13. The method according to any one of Claims 9 to 12, wherein the microbial cells that overexpress the natural *L*-phenylalanine biosynthetic pathway are *E. coli* NST74-Phe, which is used in combination with *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO to provide (*R*)-mandelic acid from glucose or glycerol.

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

15. The method according to any one of Claims 1 to 13, 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).

16. 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.

17. 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 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;

(c) an oxidation enzyme for generating enantiomerically pure or enantiomerically enriched (*R*)-mandelic acid or a derivative thereof by selective oxidation of a primary alcohol on the phenylethane diol 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.

18. The isolated nucleic acid of claim 17, encoding a plurality of said catalytic enzymes.

19. The isolated nucleic acid molecule of claim 18, 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 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 (*R*)-mandelic acid or a derivative thereof; and

iii) 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.

20. An expression construct comprising at least one nucleic acid molecule as described in any one of Claims 17 to 19.

21. 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 20.

22. The one or more recombinant prokaryotic or eukaryotic cells according to Claim 21, wherein said cells are recombinant bacterial cells.

23. The one or more recombinant prokaryotic or eukaryotic cells cells according to Claim 21 or Claim 22, 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 oxidase from *Streptomyces coelicolor*;
(d) a phenylalanine ammonia lysase from *Arabidopsis thaliana*;
(e) a phenylacrylic acid decarboxylase from *Aspergillus niger*.

24. The one or more recombinant cells of claim 23, wherein said cells are selected from:

(a) *E. coli* T7-pCDF-SMO-StEH, pRSF-AldO;
(b) *E. coli* T7-pET-PAL-PAD, pCDF-SMO-StEH, pRSF-AldO; and

(c) *E. coli* NST74-pCDF-Phe, pET-PAL-PAD, pACYC-SMO-StEH, pRSF-AldO.

25. A kit comprising at least one isolated nucleic acid according to any one of Claims 17 to 19.