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(71) Applicant: **PHYTOGENE, INC.** [US/US]; 30111 Tomas Street, Rancho Santa Margarita, California 92688 (US).

(72) Inventors: **BHUIYA, Mohammad Wadud**; 1126 Bonhomme Lake Drive, St. Louis, Missouri 63132 (US). **CHEN, Hui**; 1127D Olive Lake Drive, Olivette, Missouri 63132 (US). **CAI, Xianpeng**; 1126E Bonhomme Lake Drive, Olivette, Missouri 63132 (US). **HAN, Jixiang**; 15 Birchwood Lane Ct., Maryland Heights, Missouri 63043 (US). **YU, Xiaodan**; 231 Renaldo Dr., Chesterfield, Missouri 63017 (US).

(74) Agents: **BOETTLER, Jeannie M.** et al.; Armstrong Teasdale LLP, 7700 Forsyth Blvd., Suite 1800, St. Louis, Missouri 63105-1847 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) Title: ENZYMES AND METHODS FOR STYRENE SYNTHESIS

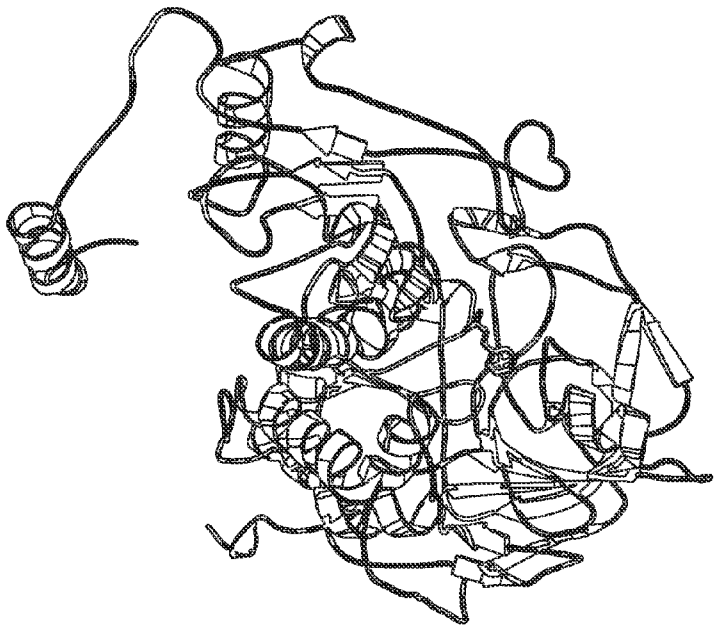

FIG. 1A

(57) Abstract: The subject technology generally relates to biosynthesis of styrene. Certain embodiments of the subject technology is based, in part, on the recognition that phenylalanine can be converted to styrene by a two-step pathway of deamination and decarboxylation, with trans-cinnamic acid (tCA) as the intermediate. Two types of enzymes are directly involved in this process, phenylalanine ammonia lyase (PAL), which converts phenylalanine to tCA, and cinnamic acid decarboxylase, which coverts tCA to styrene. Host cells expressing these two types of enzymes can be cultured in bioreactor to produce styrene from renewable substrates such as glucose.

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8 May 2014

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 7/40, 7/22, 5/00; C12N 1/26, 9/88, 9/00 (2014.01)
USPC - 435/71.1, 69.7, 156, 136, 166, 252.3, 252.11, 257.2, 419, 325
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12P 7/40, 7/22, 5/00, 21/04; C12N 15/00, 11/00, 1/26, 5/10, 9/88, 9/00 (2014.01)
USPC: 435/71.1, 69.7, 69.1, 41, 156, 136, 132, 166, 252.3, 254.11, 257.2, 419, 325

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; ProQuest; 'phenylalanine ammonia lyase (PAL),' cinnam*, 'phenyacrylic acid decarboxylase (PAD),' styrene, 'glycine-rich peptide linkers,' crystal*6, 'SrpABC'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	RANGARAJAN, ED. et al. Crystal Structure Of A Dodecameric FMN-Dependent UbiX-Like Decarboxylase (Pad1) From Escherichia Coli O157: H7. Protein Sci. 2004 November. Vol. 13, No. 11; pages 3006-3016; abstract; page 3007, column 1, paragraph 3; page 3010, column 2, paragraphs 4-5; page 3011, column 1, paragraphs 1-2; page 3012, column 2, paragraph 3; page 3013, column 1, paragraph 1; page 3014, column 2, paragraphs 1-3; page 3015, column 1, paragraph 1; figures 1a, 5.	30-33 ----- 34-36
X --- Y --- A	MCKENNA, R, et al. Styrene Biosynthesis From Glucose By Engineered E. Coli. Metab. Eng. September 2011. Vol. 13, No. 5: pages 544-554; abstract; page 545, column 1, paragraphs 2-3; page 545, column 2, paragraphs 1-2; page 546, column 2, paragraphs 1 and 3; page 547, column 1, paragraphs 1 and 3-5; page 547, column 2, paragraphs 2-7; page 548, column 1, paragraph 1-5; page 548, column 2, paragraphs 1 and 5-6; figures 1-4 and 6; Tables 1-3.	1, 9-12, 45, 46 ----- 2, 6-8, 20, 23, 24 ----- 4, 13, 16-19, 21
Y	COCHRANE, FC et al. The Arabidopsis Phenylalanine Ammonia Lyase Gene Family: Kinetic Characterization Of The Four PAL Isoforms. Phytochemistry. June 2004. Vol. 65, No. 11: pages 1557-1564; Table 1	2
Y	Registry of Standard Biological Parts: Protein domains/Linker. International Genetically Engineered Machine (iGEM) Foundation. 2008-2011. Retrieved from the internet 20 February 2014. < http://parts.igem.org/Protein_domains/Linker>; paragraph 1; Table.	6-8
Y	US 2007/0259409 A1 (WERY, G) November 8, 2007; abstract; paragraphs [0015], [0016]	20, 23, 24
Y	SMITS, SHJ. et al. A Structural Basis For Substrate Selectivity And Stereoselectivity In Octopine Dehydrogenase From Pecten Maximus. J. Mol. Biol. 01 August 2008, Vol. 381, No. 1: pages 200-211; abstract; figures 1-3, 4b and 5; page 209, column 2, paragraph 6.	34

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2014 (21.02.2014)	07 MAR 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/47098

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIGMA. Additive Screening Kit [datasheet]. Buchs, Switzerland. 2004; page 1, column 1, paragraph 2; page 3, Table.	35
Y	HAMPTON RESEARCH CORP. Temperature As A Crystallization Variable. California, U.S.A. 2001; page 1, column 1, paragraph 5; page 1, column 2, paragraph 9; page 1, column 2, paragraph 4.	36
A	MUKAI, N. et al. PAD1 And FDC1 Are Essential For The Decarboxylation Of Phenylacrylic Acids In Saccharomyces Cerevisiae. J. Biosci. Bioeng. June 2010. Vol. 109, No. 6: pages 564-569; page 564, column 2, paragraph 2.	4, 13, 16-19, 21
A	HUANG, HK et al. An Endogenous Factor Enhances Ferulic Acid Decarboxylation Catalyzed By Phenolic Acid Decarboxylase From Candida Guilliermondii. A.M.B. Express. 04 January 2012. Vol. 2, No. 1: 4; page 3, column 2, paragraphs 3, 5.	4, 13, 16-19, 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/47098

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☒ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/47098

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

-***-Please See Supplemental Page-***-

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

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

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

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

Groups I+: Claims 1, 2, 4, 6-13, 16-21, 23, 24, 30-36, 45, 46, SEQ ID NOs: 2, 8, 39-44

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

-***-Continued from Box No. III: Observations Where Unity Of Invention Is Lacking:

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

Groups I+, Claims 1-24, 30-37, 45 and 46 are directed toward a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase; a cinnamic acid decarboxylase comprising a mutation at an amino acid residue position corresponding to a position selected from the group consisting of: 155, 156, 159, 162, 163, 164, 172, 173, 174, 175, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 226, 227, 280, 285, 286, 287, 291, 326, 331, 360, 361, 395, 396, 398, 440, 441 of SEQ ID NO: 8, and combinations thereof; a host cell comprising: (a) a recombinantly expressed phenylalanine ammonia lyase; (b) a recombinantly expressed cinnamic acid decarboxylase; and (c) a recombinantly expressed membrane-bound transporter; a method of crystallizing a cinnamic acid decarboxylase, the method comprising: (a) providing a cinnamic acid decarboxylase solution at a concentration of from about 1 mg/ml to about 50 mg/ml; (b) mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of from about 1:10 to about 10:1; and (c) maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal; and a method for simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase; (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof; and (d) detecting the amount of the remaining substrate, or the amount of the product, or both.

A fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase, a cinnamic acid decarboxylase comprising a mutation at an amino acid residue position, a host cell comprising: (a) a recombinantly expressed phenylalanine ammonia lyase; (b) a recombinantly expressed cinnamic acid decarboxylase; and (c) a recombinantly expressed membrane-bound transporter; a method of crystallizing a cinnamic acid decarboxylase; and a method for simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities will be searched wherein the phenylalanine ammonia lyase consists of an amino acid sequence consisting of SEQ ID NO; 2; and the cinnamic acid decarboxylase consists of an amino acid sequence consisting of SEQ ID NO: 8, or mutations thereof. Applicant is invited to elect additional phenylalanine ammonia lyase sequences and/or additional cinnamic acid decarboxylase sequences to be searched, within the scope of the relevant claims, by specifying the relevant phenylalanine ammonia lyase sequence or cinnamic acid decarboxylase sequence and by paying an additional fee for each elected sequence. It is believed that claims 1, 2(in part), 3(in part), 4-14, 16-21, 22(in part), 23, 24, 30-37, 45 and 46 read on this first named invention and thus these claims will be searched without fee to the extent that they read on these sequences. Additional sequences will searched upon the payment of additional fees. Applicants must specify the claims that read on any additionally elected sequences. Applicants must further indicate, if applicable, the claims which read on the first named invention, if different than what was indicated above for this group. Exemplary elections would be phenylalanine ammonia lyase sequence SEQ ID NO: 4, or cinnamic acid decarboxylase sequence SEQ ID NO: 10. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. (It should be further noted that, as Group III, below, is directed toward SEQ ID NO: 16, should the applicants opt for said group to be searched, SEQ ID NO: 16 would also be included in the sequences searched under Group I without further additional payment).

Group II, Claims 25-29 and 41-44 are directed toward a method for screening candidate proteins for mutated cinnamic acid decarboxylase activity, the method comprising: (a) providing a protein sample comprising a candidate protein, and a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (b) combining the protein sample and the substrate sample to form a mixture, and incubating the mixture under a condition that allows a mutated cinnamic acid decarboxylase to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and combination thereof; and (c) exposing the mixture to a detection material that comprises a polymeric resin that absorbs the product vapor; and a method for producing styrene, the method comprising: (a) contacting a host cell with a fermentable carbon substrate, the host cell comprising (i) a phenylalanine ammonia lyase; and (ii) a cinnamic acid decarboxylase; and (b) culturing the host cell in a culture medium for a time sufficient to produce styrene, wherein the vapor of the styrene product is absorbed by an absorbing material.

Group III, Claims 38-40 are directed toward a crystal of cinnamic acid decarboxylase, wherein the cinnamic acid decarboxylase is in a complex with 3-hydroxyl cinnamic acid.

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Page:

The inventions listed as Groups I+, II and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase, a cinnamic acid decarboxylase comprising a mutation at an amino acid residue position, a host cell comprising a recombinantly expressed membrane-bound transporter; a method of crystallizing a cinnamic acid decarboxylase, the method comprising: (a) providing a cinnamic acid decarboxylase solution at a concentration of from about 1 mg/ml to about 50 mg/ml; (b) mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of from about 1:10 to about 10:1; and (c) maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal; and a method for simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase; (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof; and (d) detecting the amount of the remaining substrate, or the amount of the product, or both, not present in either of Groups II or III; the special technical features of Group II include a method for screening candidate proteins for mutated cinnamic acid decarboxylase activity, the method comprising: (a) providing a protein sample comprising a candidate protein, and a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (b) combining the protein sample and the substrate sample to form a mixture, and incubating the mixture under a condition that allows a mutated cinnamic acid decarboxylase to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and combination thereof; and (c) exposing the mixture to a detection material that comprises a polymeric resin that absorbs the product vapor, and a method of producing styrene, wherein the vapor of the styrene product is absorbed by an absorbing material, not present in either of Groups I+ or III; the special technical features of Group III include a crystal of cinnamic acid decarboxylase, wherein the cinnamic acid decarboxylase is in a complex with 3-hydroxyl cinnamic acid, not present in either of Groups I+ or II.

Groups I+, II and III share the technical features including cinnamic acid decarboxylase. Groups I+ share the technical features including a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase; a cinnamic acid decarboxylase comprising a mutation at an amino acid residue position; a host cell comprising: (a) a recombinantly expressed phenylalanine ammonia lyase; (b) a recombinantly expressed cinnamic acid decarboxylase; and (c) a recombinantly expressed membrane-bound transporter; a method of crystallizing a cinnamic acid decarboxylase, the method comprising: (a) providing a cinnamic acid decarboxylase solution at a concentration of from about 1 mg/ml to about 50 mg/ml; (b) mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of from about 1:10 to about 10:1; and (c) maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal; and a method for simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase; (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof; and (d) detecting the amount of the remaining substrate, or the amount of the product, or both. Groups I+ and II share the technical features including mutated cinnamic acid decarboxylase, a host cell comprising (i) a phenylalanine ammonia lyase; and (ii) a cinnamic acid decarboxylase, and a method of screening comprising a cinnamic acid decarboxylase comprising: providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; and incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof. Groups I and III share the technical features including a crystal of cinnamic acid decarboxylase.

However, these shared technical features are previously disclosed by WO 1994/008036 A1 to Clausen et al. (hereinafter 'Clausen') in view of the article 'Crystal structure of a dodecameric FMN-dependent UbiX-like decarboxylase (Pad1) from Escherichia coli 0157:H7' by Rangarajan et al. (hereinafter 'Rangarajan') and US 2009/0311760 A1 to Wery et al. (hereinafter 'Weryl'). Clausen discloses cinnamic acid decarboxylase (phenylalanine decarboxylase (cinnamic acid decarboxylase); page 3, lines 3-11, page 29, lines 9-24), a host cell (host cell; page 5, lines 30-31) comprising: (a) a recombinantly expressed (over-expressed (recombinantly expressed); page 10, lines 12-13) phenylalanine ammonia lyase (phenylalanine ammonia lyase (PAL); page 10, lines 12-13, Claim 1); and (b) a recombinantly expressed cinnamic acid decarboxylase (recombinantly expressed PAD mutants (recombinantly expressed cinnamic acid decarboxylase); page 29, line 26-page 30, line 3, Claims 1, 3); and screening phenylalanine ammonia lyase (screening phenylalanine ammonia lyase activity; page 16, lines 18-27, page 19, line 23-page 20, line 4) and cinnamic acid decarboxylase activities (PAD enzyme activity (cinnamic acid decarboxylase activity); page 28, line 18-page 29, line 7) comprising: (a) providing a phenylalanine ammonia lyase (phenylalanine ammonia lyase; page 16, lines 18-27), and (ii) a cinnamic acid decarboxylase (PAD (cinnamic acid decarboxylase); page 28, line 18-page 29, line 7); (b) providing a substrate (providing a substrate; page 16, lines 32-34, page 28, line 18-page 29, line 7) selected from the group consisting of phenylalanine, trans-cinnamic acid (cinnamic acid; page 16, lines 32-34), tyrosine, coumaric acid, and combinations thereof; (c) incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product (incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product; page 28, line 18-page 29, line 7) selected from the group consisting of styrene (styrene; page 28, line 32), 4-hydroxystyrene, and a combination thereof; and (d) detecting the amount of the remaining substrate, or the amount of the product, or both (detecting the amount of the remaining substrate or the amount of the product or both; page 17, lines 16-20), cinnamic acid decarboxylase mutated at an amino acid position (Pad1 mutants (mutated cinnamic acid decarboxylase); page 29, line 25-page 30, line 3) and screening (isolating (screening); page 29, lines 26-27) a mutated cinnamic acid decarboxylase (Pad1 mutants (mutated cinnamic acid decarboxylase); page 29, line 25-page 30, line 3) comprising: providing a substrate (providing a substrate; page 29, line 25-page 30, line 3) selected from the group consisting of phenylalanine, trans-cinnamic acid (t-cinnamic acid; page 29, lines 26-30), tyrosine, coumaric acid, and combinations thereof. Clausen further discloses incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product (incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product; page 28, line 18-page 29, line 7) selected from the group consisting of styrene (styrene; page 28, line 32), 4-hydroxystyrene, and a combination thereof. Clausen further discloses wherein the cinnamic acid decarboxylase may be attached to a surface (page 13, lines 9-18).

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Page:

Clausen does not disclose a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase; (c) a recombinantly expressed membrane-bound transporter; a method of crystallizing a cinnamic acid decarboxylase, the method comprising: (a) providing a cinnamic acid decarboxylase solution at a concentration of from about 1 mg/ml to about 50 mg/ml; (b) mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of from about 1:10 to about 10:1; and (c) maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal; simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, and and screening a mutated cinnamic acid decarboxylase comprising: providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; and incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, a crystal of cinnamic acid decarboxylase.

Wery discloses a cell comprising phenylalanine ammonia lyase (a cell comprising phenylalanine ammonia lyase; paragraph [0016]) and a recombinantly expressed membrane-bound transporter (recombinantly expressed membrane-bound transporter; paragraph [0022]). It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Clausen regarding recombinant cells comprising PAL and cinnamate dehydrogenase and that produce styrene with the membrane-bound transporter of Wery in order to enhance the elimination of the styrene from the cell, and produce a higher product yield (Wery, paragraph [0019]).

Rangarajan discloses a method of crystallizing (method of crystallizing; page 3014, column 2, paragraph 2) a cinnamic acid decarboxylase (Pad1; abstract, page 3013, column 1, paragraph 1), the method comprising: (a) providing a cinnamic acid decarboxylase solution at a concentration of from about 1 mg/ml to about 50 mg/ml (providing a cinnamic acid decarboxylase solution at a concentration of 7.5 mg/ml (from about 1 mg/ml to about 50 mg/ml); page 3014, column 2, paragraph 2); (b) mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of from about 1:10 to about 10:1 (mixing the cinnamic acid decarboxylase solution with a reservoir solution at a volume ratio of 1:1 (from about 1:10 to about 10:1); page 3014, column 2, paragraph 2); and (c) maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal (maintaining the mixture of step (b) at a temperature suitable for the formation of the cinnamic acid decarboxylase crystal; page 3015, column 1, paragraph 1). It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have combined the disclosure of Clausen regarding cinnamic acid decarboxylases, particularly mutant decarboxylases, with the disclosure of Rangarajan in order to enable the crystallization of mutant forms of the enzyme in order to characterize the structural changes of the polypeptides due to the mutations.

Further, although, as above, Clausen does not disclose a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase, as above, Clausen teaches wherein the cinnamate decarboxylase may be functionally attached to another entity, such as a surface. It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have further attached the PAL enzyme to either the surface or to the cinnamic acid decarboxylase using known methods, such as by crosslinking the polypeptides, or, more preferably, to producing a fusion protein comprising: (a) a first domain comprising a phenylalanine ammonia lyase, and (b) a second domain comprising a cinnamic acid decarboxylase, to avoid losses associated with deactivation of the enzymes using crosslinking agents and to increase the effective concentration of the product of the Pal enzyme, which was the substrate for the cinnamic acid dehydrogenase, thus increasing the efficiency of the overall reaction. Furthermore, although Clausen does not disclose simultaneously screening phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, and screening a mutated cinnamic acid decarboxylase comprising: providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; and incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, since Clausen, discloses the screening of the individual enzyme activities in host cells comprising the enzymes, including mutants of the cinnamic acid decarboxylase, it would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have simultaneously screened for both activities in cells comprising a fusion polypeptide having both activities, in particular to use the methods disclosed by Clausen to simultaneously screen phenylalanine ammonia lyase and cinnamic acid decarboxylase activities, the method comprising: (a) providing a fusion protein comprising: (i) a first domain comprising a phenylalanine ammonia lyase, and (ii) a second domain comprising a cinnamic acid decarboxylase (b) providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; (c) incubating the fusion protein and the substrate under a condition that allows the fusion protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, and screening a mutated cinnamic acid decarboxylase comprising: providing a substrate selected from the group consisting of phenylalanine, trans-cinnamic acid, tyrosine, coumaric acid, and combinations thereof; and incubating the protein and the substrate under a condition that allows the protein to convert the substrate to a product selected from the group consisting of styrene, 4-hydroxystyrene, and a combination thereof, in order to select the most active forms of the enzymes, and to screen for mutants of the cinnamic acid decarboxylase portion of the molecule having desirable properties.

Since none of the special technical features of the Groups I+, II and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Clausen, Rangarajan and Wery references, unity of invention is lacking.