(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
08 April 2021 (08.04.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/067974 A1

(51) International Patent Classification:
C12N 1/18 (2006.01) *C12N 15/81* (2006.01)
C12N 15/63 (2006.01) *C12N 9/10* (2006.01)

(21) International Application Number:
PCT/US2020/054323

(22) International Filing Date:
05 October 2020 (05.10.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/911,116 04 October 2019 (04.10.2019) US

(71) Applicant: **CONAGEN INC.** [US/US]; 15 DeAngelo Drive, Bedford, MA 01730 (US).

(72) Inventors: **WU, Yisheng**; 270 South Road, Bedford, MA 01730 (US). **LU, Wanli**; 12211 Bay Drive, Westford, MA 01886 (US). **COURTNEY, Jacob, T.**; 22B South Main Street, Milford, MA 01757 (US). **NUNN, David**; 141 John Street, Lowell, MA 01852 (US). **YU, Oliver**; 39 Drummer Boy Way, Lexington, MA 02420 (US).

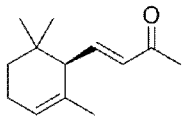
(74) Agent: **CHAN, Karen, K.**; Conagen Inc., 15 DeAngelo Drive, Bedford, MA 01730 (US).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, 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, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

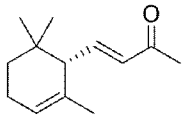
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: BIOSYNTHESIS OF ALPHA-IONONE AND BETA-IONONE

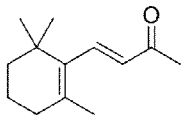
(57) Abstract: Provided herein are recombinant nucleic acid molecules, nucleic acid constructs, fusion enzymes, transformed host cells, and methods for making aroma compounds alpha-ionone or beta-ionone.



(*R*)-alpha-ionone



(*S*)-alpha-ionone



beta-ionone

FIG. 1

WO 2021/067974 A1

[Continued on next page]

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

BIOSYNTHESIS OF ALPHA-IONONE AND BETA-IONONE

RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Application No. 62/911,116, filed on October 4, 2019, the content of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[002] The field of the invention relates to compositions and processes for the biosynthetic production of carotenoid compounds including aroma compounds such as alpha-ionone and beta-ionone. More specifically, the present methods and processes make use of microbial host cells that have been transformed to include a heterologous nucleic acid that encodes a fusion enzyme having a lipid body compartmentalization signal tag (LBT) domain coupled to a carotenoid cleavage dioxygenase (CCD) domain.

BACKGROUND

[003] Consumers demand foods, fragrances, and cosmetics that have pleasant tastes and odors. In many cases, these traits of pleasant smells and tastes are provided through derivatives of carotenoids, such as alpha-ionone and beta-ionone, that have exceptional aroma characteristics as their odor threshold is at the sub-ppb range. Current supply for alpha-ionone and beta-ionone is mainly addressed through either extraction processes from plants or chemical synthesis. As more consumers prefer natural ingredients, the market demand for ionones from natural sources has increased dramatically. However, approximately one hundred tons of raspberries, or twenty hectares of agricultural area are required to yield approximately one gram of alpha-ionone. In addition, plant extraction-based production has significant disadvantages such as weather effects on the strength and abundance of the compounds of interest, risks of plant diseases and/or poor harvests, the stability of the compound, the environmental impact of increased production, and trade restrictions. Structurally alpha-ionone has a chiral center. Natural alpha-ionone from plants (such as raspberry) is (R)-(+)-(E)-alpha-ionone. In contrast, chemically synthesized α-ionone has two isomers (R and S) (FIG. 1). The R-enantiomer has a unique and strong floral flavor and aroma, described as a violet-like, fruit-like or raspberry-like flavor, while the S-enantiomer has a woody scent similar to beta-ionone. Therefore, chemically synthesized

alpha-ionone exists as a racemic mixture and contains enantiomers with two different scents. Its utility for the fragrance industry is therefore limited. Although new methods have been reported for the enantio-selective synthesis of (S)-alpha-ionone or (R)-alpha-ionone, the highest enantiomeric purity of the synthesized (R)-alpha-ionone report to date is about 97%, meaning substantial amounts of the (S)-enantiomer are still present. Moreover, chemical synthesis production can cause environmental issues, may use toxic precursors, and the production process itself may be subject to increased costs due to the costs of key starting materials.

[004] Accordingly, there is a need in the art for novel methods to produce "natural" alpha-ionone and beta-ionone economically and reliably without the limitations posed by plant extraction and chemical synthesis.

SUMMARY OF THE INVENTION

[005] According to the present teachings, alpha-ionone and beta-ionone can be reliably produced at high yields by enzyme engineering and fermentation technology using microbial cell cultures. These microbial cell cultures can synthesize "natural" alpha-ionone or beta-ionone de novo in commercially significant yields. Hence, new biosynthetic methods are provided herein to reduce costs of alpha-ionone and beta-ionone production and lessen the environmental impact of large-scale cultivation and processing of natural sources from which these aroma compounds are extracted conventionally.

[006] More specifically, the present teachings encompass methods and compositions for making ionone compounds (e.g., alpha-ionone, beta-ionone) by microbial fermentation, where such methods and compositions involve the transformation of a eukaryotic cell with a heterologous nucleic acid molecule that encodes a fusion enzyme having a lipid body compartmentalization signal tag (LBT) domain coupled to a carotenoid cleavage dioxygenase (CCD) domain.

[007] Fermentative production of alpha-ionone and beta-ionone has been hindered by the efficiency of the reaction step by which their respective precursor epsilon-carotene or beta-carotene is cleaved. The present disclosure is based, in part, on the finding that certain compartmentalization signal tags can target lipid bodies in which epsilon-carotene and beta-carotene accumulate, thereby increasing the binding affinity of a CCD enzyme to such lipid

bodies and thus dramatically increasing the catalytic efficiency of the CCD enzyme. By combining the overexpression of a nucleic acid molecule encoding a lipid body compartmentalization signal tag fused CCD, and the overexpression of various carotenoid precursor biosynthetic genes, the production of ionone compounds can be improved by providing much higher titers than alternative strategies such as the use of soluble tags, site-directed mutagenesis, or by creating an EC-CCD fusion enzyme alone. The present disclosure, therefore, provides economical and reliable methods for producing "natural" alpha-ionone and beta-ionone without the disadvantages associated with chemical synthesis or plant extraction.

[008] In one aspect, the present teachings relate to a recombinant microbial production host cell for producing an ionone compound, where the host cell includes at least one nucleic acid construct containing a coding sequence that encodes a fusion enzyme, where the fusion enzyme includes a first domain capable of functioning as a lipid body compartmentalization signal tag fused to a second domain having carotenoid cleavage activity. In various embodiments, the lipid body compartmentalization signal tag (LBT) can be a lipid body structural protein (e.g., oleosins and caleosins), an oleaginicity inducing lipid droplet protein (e.g., Oil1), an orange carotenoid-binding protein, and a membrane transporter protein (e.g. a sugar transporter such as STL1). The second domain can be a carotenoid cleavage dioxygenase (CCD) or a functional equivalent such as other monooxygenases, dioxygenases, and peroxidases that can cleave the appropriate bonds of epsilon-carotene and beta-carotene to provide alpha-ionone and beta-ionone.

[009] In various embodiments, the present teachings relate to a synthetic or recombinant nucleic acid molecule (i.e., an LBT-CCD fusion enzyme coding sequence) that includes a first nucleic acid sequence and a second nucleic acid sequence, where the first nucleic acid sequence and the second nucleic acid sequence together encode a fusion enzyme, where such fusion enzyme comprises a first domain capable of functioning as a lipid body compartmentalization signal tag and a second domain having carotenoid cleavage activity. In some embodiments, the lipid body compartmentalization signal tag can be selected from the group consisting of a lipid body structural protein, an oleaginicity-inducing lipid droplet protein, an orange carotenoid binding protein, and a membrane transporter protein. In certain embodiments, the lipid body compartmentalization signal tag can be a small lipid body structural protein, such as a lipid body structural protein that has a molecular weight of between 15 kDa

and 26kDa. For example, its molecular weight can be less than 25 kDa, less than 20kDa, or around 17 or 18 kDa. In preferred embodiments, the first domain of the fusion enzyme can be an oleosin or a caleosin. In specific embodiments, the first domain of the fusion enzyme can include an amino acid sequence having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30. In some embodiments, the lipid body compartmentalization signal tag can be a caleosin from *Arabidopsis thaliana*, *Ricinus communis*, *Glycine max*, *Sesamum indicum*, *Coix lacryma*, *Aspergillus niger*, or *Neurospora crassa*.

[0010] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Zea mays 16 kDa oleosin protein or a functional variant thereof that can bind to lipid bodies. For example, the Zea mays 16 kDa oleosin protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 1. In certain embodiments, the Zea mays 16 kDa oleosin protein can comprise the amino acid sequence of SEQ ID NO: 1. In other embodiments, the Zea mays 16 kDa oleosin protein can consist of the amino acid sequence of SEQ ID NO: 1. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 2.

[0011] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Sesamum indicum 17 kDa oleosin protein or a functional variant thereof that can bind to lipid bodies. For example, the Sesamum indicum 17 kDa oleosin protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 3. In certain embodiments, the Sesamum indicum 17 kDa oleosin protein can comprise the amino acid sequence of SEQ ID NO: 3. In other embodiments, the Sesamum indicum 17 kDa oleosin protein can consist of the amino acid sequence of SEQ ID NO: 3. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 4.

[0012] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Limnospira maxima orange carotenoid binding protein or a functional variant thereof that can bind to lipid bodies. For example, the orange carotenoid binding protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 5. In certain embodiments, the orange carotenoid binding protein

can comprise the amino acid sequence of SEQ ID NO: 5. In other embodiments, the orange carotenoid binding protein can consist of the amino acid sequence of SEQ ID NO: 5. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 6.

[0013] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Yarrowia lipolytica membrane transporter Stl1p protein or a functional variant thereof that can bind to lipid bodies. For example, the membrane transporter Stl1p protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 7. In certain embodiments, the membrane transporter Stl1p protein can comprise the amino acid sequence of SEQ ID NO: 7. In other embodiments, the membrane transporter Stl1p protein can consist of the amino acid sequence of SEQ ID NO: 7. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 8.

[0014] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Yarrowia lipolytica lipid droplet protein Oil1p or a functional variant thereof that can bind to lipid bodies. For example, the lipid droplet protein Oil1p can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 9. In certain embodiments, the lipid droplet protein Oil1p can comprise the amino acid sequence of SEQ ID NO: 9. In other embodiments, the lipid droplet protein Oil1p can consist of the amino acid sequence of SEQ ID NO: 9. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 10.

[0015] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be an Arabidopsis thaliana caleosin or a functional variant thereof that can bind to lipid bodies. For example, the Arabidopsis thaliana caleosin can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 31. In certain embodiments, the Arabidopsis thaliana caleosin can comprise the amino acid sequence of SEQ ID NO: 31. In other embodiments, the Arabidopsis thaliana caleosin can consist of the amino acid sequence of SEQ ID NO: 31. Accordingly, the first nucleic acid

sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 32.

[0016] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Sesamum indicum caleosin or a functional variant thereof that can bind to lipid bodies. For example, the Sesamum indicum can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 33. In certain embodiments, the Sesamum indicum can comprise the amino acid sequence of SEQ ID NO: 33. In other embodiments, the Sesamum indicum can consist of the amino acid sequence of SEQ ID NO: 33. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 34.

[0017] In various embodiments, the fusion enzyme according to the present teachings does not include a solubility-enhancement tag. Examples of such solubility-enhancement tags include glutathione-S-transferase (GST), small ubiquitin-like modifier (SUMO) proteins, maltose binding proteins (MBP), or thioredoxin (TrxA).

[0018] In various embodiments, the second domain having carotenoid cleavage activity can be a carotenoid-cleavage dioxygenase (CCD). For example, the CCD enzyme can be a CCD1 or CCD4 enzyme capable of cleaving the 5,6 (5',6'), 7,8 (7',8') and 9,10 (9',10') double bonds of a wide range of carotenoids. For example, the second domain can be a Petunia x hybrida CCD1 (PhCCD1) or a functional variant thereof. For example, the second or CCD domain can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 11. In certain embodiments, the second or CCD domain can comprise the amino acid sequence of SEQ ID NO: 11. In other embodiments, the second or CCD domain can consist of the amino acid sequence of SEQ ID NO: 11. Accordingly, the second nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 12 or SEQ ID NO: 13.

[0019] In another embodiment, the second domain can be an Osmanthus fragrans CCD1 enzyme or a functional variant thereof. For example, the second or CCD domain can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 14. In certain embodiments, the second or CCD domain can comprise the amino acid sequence of SEQ ID NO: 14. In other embodiments, the second or CCD domain can consist

of the amino acid sequence of SEQ ID NO: 14. Accordingly, the second nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 15.

[0020] In yet another embodiment, the second domain can be a Zea mays CCD1 or a functional variant thereof. For example, the second or CCD domain can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 16. In certain embodiments, the second or CCD domain can comprise the amino acid sequence of SEQ ID NO: 16. In other embodiments, the second or CCD domain can consist of the amino acid sequence of SEQ ID NO: 16. Accordingly, the second nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 17.

[0021] In other embodiments, the second domain can be a carotenoid oxygenase, a 9-cisepoxycarotenoid dioxygenase, or functional variants thereof, from various cyanobacteria such as, but not limited to, Nostocales cyanobacterium, Calothrix sp. Calothrix brevissima, and Scytonema millei. For example, the second or CCD domain can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. In certain embodiments, the second or CCD domain can comprise the amino acid sequence of SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. In other embodiments, the second or CCD domain can consist of the amino acid sequence of SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. Accordingly, the second nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, or SEQ ID NO: 25.

[0022] In some embodiments, the second or CCD domain can comprise a CCD1 enzyme from *Petunia x hybrid*, *Osmanthus fragrans*, *Zea mays*, *Arabidopsis thaliana*, *Vitis vinifera*, *Daucus carota*, *Lycopersicon esculentum*, and *Rubus idaeus*. In other embodiments, the second or CCD domain can comprise a CCD4 enzyme from *Arabidopsis thaliana*, *Bixa Orellana*, *Chrysanthemum morifolium*, *Crocus sativus*, *Musa acuminata*, *Malus domestica*, *Prunus persica*, *Rosa damascene*, *Vitis vinifera*, and *Osmanthus fragrans*. In embodiments where the desired ionone compound is alpha-ionone, the second or CCD domain can comprise a

CCD1 enzyme from *Petunia x hybrida* or *Osmanthus fragrans*, a CCD4 enzyme from *Chrysanthemum morifolium*, or a functional homolog thereof. In embodiments where the desired ionone compound is beta-ionone, the second or CCD domain can comprise a CCD1 enzyme from *Petunia x hybrida* or *Osmanthus fragrans*, a CCD4 enzyme from *Prunus persica*, or a functional homolog thereof.

[0023] In various embodiments, the nucleic acid molecule can include a third nucleic acid sequence operably linked to the first nucleic acid sequence at one end and the second nucleic acid sequence at another end, where the third nucleic acid sequence encodes a linker that couples the first domain to the second domain. For example, the linker can include 3 to 30 amino acids in length. The linker can include amino acids selected from the group of glycine, serine, threonine, and combinations thereof. In some embodiments, the linker can be a glysine-serine linker. In certain embodiments, the linker can include one or more units of GGGGS. In specific embodiments, the linker can be GGGGS. In alternative embodiments, the linker can be GGGGSGGGGSGGGGSGGGGS.

[0024] Another aspect of the present teachings relates to a nucleic acid construct. The nucleic acid construct can include the various embodiments of the nucleic acid molecule described above operably linked to a heterologous nucleic acid sequence. For example, the heterologous nucleic acid sequence can encode an expression control sequence, such as a promoter sequence. In some embodiments, the nucleic acid construct also can include a heterologous nucleic acid sequence encoding a lycopene epsilon-cyclase such as a *Lactuca sativa* lycopene epsilon-cyclase (EC) or a functional homolog thereof. In some embodiments, the nucleic acid construct also can include a heterologous nucleic acid sequence encoding a phyotoene synthase (CarRP).

[0025] Yet another aspect of the present teachings relates to a fusion enzyme that includes a lipid body compartmentalization signal tag (LBT) domain coupled to a carotenoid cleavage dioxygenase (CCD) domain as described herein. The LBT domain can be coupled to the CCD domain via a linker. For example, the linker can include 3 to 30 amino acids in length. The linker can include amino acids selected from the group of glycine, serine, threonine, and combinations thereof. In some embodiments, the linker can be a glysine-serine linker. In certain embodiments, the linker can include one or more units of GGGGS. In specific embodiments, the

linker can be GGGGS. In alternative embodiments, the linker can be GGGGSGGGGSGGGGSGGGGS. In various embodiments, the fusion enzyme can consist of an LBT domain fused to a CCD domain via a linker.

[0026] Yet another aspect of the present teachings can relate to a method of transforming a host cell. The method can include introducing into a host cell any of the present nucleic acid molecules or nucleic acid constructs described above, and selecting or screening for a transformed host cell that can be used for the production of carotenoid (e.g., ionone) compounds. In some embodiments, the host cell can be microbial cell such as a bacterial cell, a yeast cell, an algal cell, or a fungal cell. In other embodiments, the host cell can be a plant cell that do not naturally produce the carotenoids or ionones of interest. In certain embodiments, the host cell can be selected from Yarrowia, Escherichia; Salmonella; Bacillus; Acinetobacter; Streptomyces; Corynebacterium; Methylosinus; Methylomonas; Rhodococcus; Pseudomonas; Rhodobacter; Synechocystis; Saccharomyces; Zygosaccharomyces; Kluyveroumyces; Candida; Hansenula; Debaryomyces; Mucor; Pichia; Torulopsis; Aspergillus; Arthrobotlys; Brevibacteria; Microbacterium; Arthrobacter; Citrobacter; Klebsiella; Pantoea; and Clostridium. In particular embodiments, the host cell can be *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, or *Corynebacterium glutamicum*.

[0027] Yet another aspect of the present teachings can relate to a recombinant cell that includes any of the present nucleic acid molecules described above. The recombinant cell can be a prokaryotic cell or a eukaryotic cell. In some embodiments, the recombinant cell can be microbial cell such as a bacterial cell, a yeast cell, an algal cell, or a fungal cell. In other embodiments, the recombinant cell can be a plant cell that do not naturally produce the ionones of interest. In certain embodiments, the recombinant cell can be selected from Yarrowia, Escherichia; Salmonella; Bacillus; Acinetobacter; Streptomyces; Corynebacterium; Methylosinus; Methylomonas; Rhodococcus; Pseudomonas; Rhodobacter; Synechocystis; Saccharomyces; Zygosaccharomyces; Kluyveroumyces; Candida; Hansenula; Debaryomyces; Mucor; Pichia; Torulopsis; Aspergillus; Arthrobotlys; Brevibacteria; Microbacterium; Arthrobacter; Citrobacter; Klebsiella; Pantoea; and Clostridium. In particular embodiments, the recombinant cell can be *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, or *Corynebacterium glutamicum*.

[0028] Yet another aspect of the present teachings can relate to a method for producing an ionone compound such as alpha-ionone or beta-ionone. The method can include culturing a recombinant host cell according to the present teachings in a suitable medium (e.g., a medium including glucose or glycerol) under conditions whereby an ionone compound is produced. The recombinant host cell can include any of the present nucleic acid molecules or constructs described above, and where the culturing step leads to the expression of a fusion enzyme according to the present teachings, resulting in the increased production of alpha-ionone or beta-ionone by the recombinant host cell. In some embodiments, the recombinant host cell can be further transformed to overexpress one or more genes involved in the mevalonate pathway. For example, to increase production of lycopene, a precursor to the production of both alpha-ionone and beta-ionone, the transformed host cell can be transformed to overexpress one or more of hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), isopentenyl diphosphate isomerase (IPI), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS). In some embodiments, the transformed host cell can be transformed to overexpress a synthetic nucleic acid molecule that encodes a fusion enzyme including an FPPS fused in-frame to a GGPPS (FPPS::GGPPS). In some embodiments, the transformed host cell can be further transformed to overexpress a phytoene dehydrogenase (CarB), a bifunctional lycopene cyclase/phytoene synthase (CarRP) or a mutant thereof (e.g., the mutant CarRP-E78K (CarRP*), where the mutation knocks out the lycopene cyclase activity of the CarRP enzyme). For example, to increase the production of beta-carotene, the immediate precursor to beta-ionone, the transformed host cell can be transformed to overexpress CarRP and CarB, which in turn increase the production of beta-ionone. Alternatively, to increase the production of epsilon-carotene, the transformed host cell can be transformed to overexpress CarRP*, CarB, and an epsilon-cyclase (EC) gene. For example, the epsilon-cyclase gene can be a Lactuca sativa EC gene. In such embodiments, the transformed host cell can be transformed to overexpress a nucleic acid molecule having a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 26. In certain embodiments, the transformed host cell can be transformed to overexpress a nucleic acid molecule that encodes a fusion enzyme having a first EC domain and a second CCD domain, where the EC domain is coupled to the CCD domain via a linker according to the present teachings, to further improve the production of epsilon-carotene, and in turn, alpha-ionone.

[0029] The present teachings also encompass a recombinant host cell for producing a carotenoid compound. The host cell can include at least one nucleic acid construct that includes a coding sequence encoding a fusion enzyme having an LBT domain (e.g., an oleosin or caleosin polypeptide) fused to a beta-carotene ketolase (BKT or CrtW) or a beta-carotene hydroxylase (CrtR-B). In some embodiments, the host cell can include both a coding sequence encoding an LBT-BKT fusion enzyme and a coding sequence encoding an LBT-CrtR-B fusion enzyme. The beta-carotene ketolase and the beta-carotene hydroxylase can be from *Haematococcus pluvialis*, *Chlorella zofingiensis*, *Chlamydomonas reinhardtii*, *Paracoccus sp.*, and *Pantoea ananatis*. In one embodiment, the present invention relates to a method of producing astaxanthin which encompasses culturing a recombinant host cell that has been transformed with both a coding sequence encoding an LBT-BKT fusion enzyme and a coding sequence encoding an LBT-CrtR-B fusion enzyme under conditions whereby astaxanthin is produced. In another embodiment, the present invention relates to a method of producing canthaxanthin, where the method includes culturing a recombinant host cell that has been transformed with a coding sequence encoding an LBT-CrtW fusion enzyme under conditions whereby canthaxanthin is produced.

[0030] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawing and will herein be described in detail. It should be understood, however, that the drawings and detailed description presented herein are not intended to limit the disclosure to the particular embodiment disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present disclosure as defined by the appended claims.

[0031] Other features and advantages of this invention will become apparent in the following detailed description of preferred embodiments of this invention, taken with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0033] FIG. 1 shows the chemical structures of (R)-alpha-ionone (top), (S)-alpha-ionone (center), and beta-ionone (bottom).

[0034] FIG. 2 illustrates the biosynthesis pathway to alpha-ionone and beta-ionone according to the present teachings. The top portion illustrates the mevalonate pathway which can take place endogenously in a selected host cell. The bottom portion illustrates the heterologous biosynthetic pathway from geranylgeranyl diphosphate (GGPP) to phytoene to lycopene, which depending on whether an epsilon-cyclase (EC) gene or a bifunctional lycopene cyclase/phytoene synthase (CarRP) is overexpressed, leads to either epsilon-carotene or beta-carotene, respectively. In the presence of the LBT-CCD fusion enzyme according to the present teachings, epsilon-carotene is converted to alpha-ionone, and beta-carotene is converted to beta-ionone. Genes shaded in gray are those that are overexpressed in the transformed host cell.

[0035] FIG. 3 shows microscope images of the accumulation of carotenoids in lipid bodies or lipid droplets in Yarrowia lipolytica (A and B) and Corynebacterium glutamicum (C).

[0036] FIG. 4 shows the plasmids the inventors have generated to overexpress the various genes highlighted in FIG. 2. These gene cassettes were integrated into the chromosome of Yarrowia lipolytica ATCC 90811 strain as described in more detail in Examples 1, 3, 5, 7 and 8.

[0037] FIG. 5 shows HPLC profile and UV spectra confirming epsilon-carotene production in cell cultures of a transformed Yarrowia lipolytica strain.

[0038] FIG. 6 shows HPLC profile and UV spectra confirming beta-carotene production in cell cultures of a transformed Yarrowia lipolytica strain.

[0039] FIG. 7 shows GC/MS spectra confirming alpha-ionone production in cell cultures of a transformed Yarrowia lipolytica strain.

[0040] FIG. 8 compares the production titer of alpha-ionone by Yarrowia lipolytica strains that have been transformed to overexpress a heterologous CCD gene (PhCCD1) using different strategies as described in Example 7.

[0041] FIG. 9 shows GC/MS spectra confirming beta-ionone production in cell cultures of a transformed Yarrowia lipolytica strain.

[0042] FIG. 10 illustrates the astaxanthin biosynthesis pathways in *Haematococcus pluvialis* (left) and *Chlorella zofingiensis* (right). Enzymes are named according to the designation of their genes. BKT, beta-carotenoid ketolase; CrtR-B, beta-carotenoid hydroxylase. Canthaxanthin can be synthesized from beta-carotene via beta-carotenoid ketolase CrtW to echinenone, and from echinenone via CrtW to canthaxanthin.

[0043] FIG. 11 shows increased production of astaxanthin (AS) and zeaxanthin (ZX) when using a recombinant microbial production strain that has been transformed with a nucleic acid construct including a coding sequence encoding an LBT-CrtRB fusion enzyme and a coding sequence encoding an LBT-BKT fusion enzyme (Oleosin::crtRB-Oleosin::BKT), compared to a strain that has been transformed with a nucleic acid construct including a coding sequence encoding a CrtRB enzyme and a coding sequence encoding a BKT enzyme (crtRB-BKT). CX stands for canthaxanthin and BC stands for beta-carotene.

[0044] FIG. 12 shows increased production of canthaxanthin (CX) when using a recombinant microbial production strain that has been transformed with a nucleic acid construct including a coding sequence encoding an LBT-CrtW fusion enzyme (Oleosin::crtW), compared to a strain that has been transformed with a nucleic acid construct including a coding sequence encoding a CrtW enzyme (crtW). BC stands for beta-carotene.

DETAILED DESCRIPTION

[0045] The oleaginous yeast Yarrowia lipolytica is one of the most prolific heterologous hosts for carotenoid production due to its large intercellular pool size of acetyl-CoA (the starting materials of the carotenoid backbone, see FIG. 2), and well-established genetic toolboxes. The inventors have screened a large number of combinations of different carotenoid precursor biosynthetic genes. Through co-overexpression of HMGS, HMGR, IPI, FPPS and GGPPS genes, the inventors have generated various highly efficient host strains for carotenoid production. Overexpression of CarRP*/CarB, CarRP/CarB, or carRP*/CarB/EC genes in the Yarrowia lipolytica carotenoid host strain results in high-titer production of lycopene (2 g/L), beta-carotene (4 g/L) or epsilon-carotene (1.5 g/L).

[0046] Using lycopene-producing Yarrowia lipolytica strain as a host, the inventors screened 28 CCD enzymes for ionone production. Among the various CCD enzymes that were screened, PhCCD1 (SEQ ID NO: 11) originated from the plant Petunia hybrida exhibits the

highest activity. However, strains that were transformed with the wild-type PhCCD1 could only produce less than 3 mg/L of alpha-ionone from test-tube based cell cultures. It was reported that the catalytic efficiency of CCD enzyme is the key bottleneck of fermentation production of ionone compounds. The oleaginous nature of the Yarrowia lipolytica host cell is very helpful for the accumulation of carotenoids. As shown in FIGS. 3A and 3B, red-colored lycopene and orange-colored epsilon-carotene accumulate in lipid bodies in Yarrowia lipolytica cells. Similarly, lipid droplets containing carotenoids has been observed in Corynebacterium glutamicum carotene-producing strain (FIG. 3C). The inventors therefore hypothesized that the accumulation of carotene in lipid bodies (droplets) may impede the carotene substrate molecules' binding to the CCD enzyme. Therefore, besides protein engineering efforts on the PhCCD1 enzyme itself, the inventors decided to investigate a novel approach based upon lipid body based subcellular compartment engineering by screening a number of fusion enzymes that include PhCCD1 coupled to different putative compartmentalization signal tags. As the Examples show, this approach led to significant improvements in the efficiency of the carotenoid cleavage enzymes. The present invention therefore provides an economical and reliable approach for producing "natural" alpha-ionone and beta-ionone that is suitable for commercial scale-up production.

Methods of Making Alpha-Ionone and Beta-Ionone

[0047] Methods described herein, in some embodiments, provide for the production of "natural" alpha-ionone and beta-ionone using fusion enzymes and recombinant host cells that have been transformed to express such fusion enzymes. In various embodiments, the present methods involve cellular systems that include growing cells that have been transformed with a nucleic acid molecule, where such nucleic acid molecule includes a first nucleic acid sequence and a second nucleic acid sequence, where such first and second nucleic acid sequence together encodes a heterologous fusion enzyme that includes a first domain capable of functioning as a lipid body compartmentalization signal tag (sometimes referred herein as an LBT domain) and a second domain having carotenoid cleavage activity (sometimes referred herein as a carotenoid cleavage dioxygenase (CCD) domain). In some embodiments, the cellular systems can comprise bacterial cells, yeast cells, plant cells that do not naturally produce the ionones of interest, algal cells, bacterial cells and/or fungal cells that do not naturally encode the lipid body compartmentalization signal tag and/or the CCD described herein. In particular embodiments,

the cellular system can comprise growing bacteria and/or yeast cells selected from the group consisting of Yarrowia, Escherichia; Salmonella; Bacillus; Acinetobacter; Streptomyces; Corynebacterium; Methylosinus; Methylomonas; Rhodococcus; Pseudomonas; Rhodobacter; Synechocystis; Saccharomyces; Zygosaccharomyces; Kluyveroumyces; Candida; Hansenula; Debaryomyces; Mucor; Pichia; Torulopsis; Aspergillus; Arthrobotlys; Brevibacteria; Microbacterium; Arthrobacter; Citrobacter; Klebsiella; Pantoea; and Clostridium.

Pathways and Enzymes for Biosynthesis Alpha-Ionone or Beta-Ionone

[0048] Referring to FIG. 2, biosynthetic production of alpha-ionone and beta-ionone from carbon sources like glucose or glycerol can be performed via *in vivo* overexpression of a series of native and heterologous genes in microbial host cells. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the C5 building blocks for making geranylgeranyl diphosphate (GGPP), which is the direct precursor of carotenoids. In plant or fungal host cells, IPP and DMAPP can be generated from the mevalonate (MVA) pathway. Two molecules of acetyl-CoA are condensed into one molecule of acetoacetyl-CoA by acetyl-CoA acetyltransferase (AtoB). Acetoacetyl-CoA is converted into mevalonic acid via an intermediate hydroxymethylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR), respectively. Then IPP is produced from mevalonic acid by three enzymes, mevalonate kinase (MevK), phosphomevalonate kinase (PMK) and phosphomevalonate decarboxylase (PMD). MVA pathway requires isopentenyl diphosphate isomerase (IPI) to generate DMAPP from IPP. Through the coupling of multiple IPP and DMAPP molecules, the GGPP is formed. Through the condensation of two GGPP units catalyzed by a bifunctional lycopene cyclase/phytoene synthase CarRP, phytoene is produced, which is then converted to lycopene by phytoene dehydrogenase CarB. Lycopene can be cyclized to either epsilon-carotene by epsilon-cyclase (EC), or beta-carotene by CarRP. The cleavage of epsilon-carotene or beta-carotene by a CCD enzyme results in the production of alpha-ionone or beta-ionone, respectively.

[0049] With continued reference to FIG. 2, genes that are overexpressed in the transformed microbial host cells according to the present teachings are highlighted/shaded in gray. Specifically, these include: HMGS (NCBI RefSeq XP_506052.1); HMGR (GenBank accession No. RDW25091.1); IPI (NCBI RefSeq XP_504974.1); FPPS (NCBI RefSeq XP_503599.1); GGPPS (NCBI RefSeq XP_502923.1); CarRP (UniProtKB/Swiss-Prot:

Q9UUQ6.1); CarRP* (a mutant CarRP-E78K with glutamic acid mutated to lysine at position 78); CarB (GenBank accession No. OAD07725.1); EC (GenBank accession No. AAK07434.1); and PhCCD1 (GenBank accession No. AAT68189.1).

Lipid Body Compartmentalization Signal Tags

[0050] Referring to FIG. 3, the inventors have observed accumulation of carotene substrates in lipid bodies (droplets) in Yarrowia lipolytica (A and B) and Corynebacterium glutamicum (C). Lipid bodies (droplets) are ubiquitous organelles that store metabolic energy in the form of neutral lipids such as triacylglycerols and steryl esters. Putative lipid body compartmentalization signal tags can include lipid synthesis enzymes that promote lipid body formation, and structural or membrane proteins for lipid body assembly. In addition, the orange carotenoid binding protein can be useful because of its high affinity for carotenoids. After performing rigorous screening of a large number of candidate proteins, the inventors have identified certain putative lipid body compartmentalization signal tags that have led to improvements in the *in vivo* carotenoid cleavage efficiency of CCD enzymes in Yarrowia lipolytica cells when the lipid body compartmentalization signal tag and the CCD enzyme are expressed together as a fusion enzyme. These lipid body compartmentalization signal tags can include lipid body structural proteins (e.g., various oleosins and caleosins), oleaginicity inducing lipid droplet proteins (e.g., Oil1), orange carotenoid-binding proteins, and membrane transporter proteins (e.g. a sugar transporter such as STL1). In some embodiments, the lipid body compartmentalization signal tag can be an oleosin comprising an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 29 or SEQ ID NO: 30. In some embodiments, the lipid body compartmentalization signal tag can be a caleosin from *Arabidopsis thaliana*, *Ricinus communis*, *Glycine max*, *Sesamum indicum*, *Coix lacryma*, *Aspergillus niger*, or *Neurospora crassa*. In some embodiments, the lipid body compartmentalization signal tag can be selected from the group consisting of *Limnospira maxima* orange carotenoid binding protein, *Yarrowia lipolytica* membrane transporter Stl1p protein, *Yarrowia lipolytica* lipid droplet protein Oil1p, *Zea mays* 16 kDa oleosin, *Sesamum indicum* 17 kDa oleosin, *Arabidopsis thaliana* caleosin, and *Sesamum indicum* caleosin. Without wishing to be bound by any particular theory, the inventors believe that polypeptides including an amino acid sequence having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30, which corresponds to

the conserved domain of *Zea mays* oleosin and *Sesamum indicum* oleosin, respectively, can be useful as lipid body compartmentalization signal tags according to the present teachings.

[0051] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Zea mays 16 kDa oleosin protein or a functional variant thereof that can bind to lipid bodies. For example, the Zea mays 16 kDa oleosin protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 1. In certain embodiments, the Zea mays 16 kDa oleosin protein can comprise the amino acid sequence of SEQ ID NO: 1. In other embodiments, the Zea mays 16 kDa oleosin protein can consist of the amino acid sequence of SEQ ID NO: 1. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 2.

[0052] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Sesamum indicum 17 kDa oleosin protein or a functional variant thereof that can bind to lipid bodies. For example, the Sesamum indicum 17 kDa oleosin protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 3. In certain embodiments, the Sesamum indicum 17 kDa oleosin protein can comprise the amino acid sequence of SEQ ID NO: 3. In other embodiments, the Sesamum indicum 17 kDa oleosin protein can consist of the amino acid sequence of SEQ ID NO: 3. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 4.

[0053] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Limnospira maxima orange carotenoid binding protein or a functional variant thereof that can bind to lipid bodies. For example, the orange carotenoid binding protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 5. In certain embodiments, the orange carotenoid binding protein can comprise the amino acid sequence of SEQ ID NO: 5. In other embodiments, the orange carotenoid binding protein can consist of the amino acid sequence of SEQ ID NO: 5. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 6.

[0054] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Yarrowia lipolytica membrane transporter Stl1p protein or a functional variant thereof that can bind to lipid bodies. For example, the membrane transporter Stl1p protein can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 7. In certain embodiments, the membrane transporter Stl1p protein can comprise the amino acid sequence of SEQ ID NO: 7. In other embodiments, the membrane transporter Stl1p protein can consist of the amino acid sequence of SEQ ID NO: 7. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 8.

[0055] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Yarrowia lipolytica lipid droplet protein Oil1p or a functional variant thereof that can bind to lipid bodies. For example, the lipid droplet protein Oil1p can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 9. In certain embodiments, the lipid droplet protein Oil1p can comprise the amino acid sequence of SEQ ID NO: 9. In other embodiments, the lipid droplet protein Oil1p can consist of the amino acid sequence of SEQ ID NO: 9. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 10.

[0056] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be an Arabidopsis thaliana caleosin or a functional variant thereof that can bind to lipid bodies. For example, the Arabidopsis thaliana caleosin can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 31. In certain embodiments, the Arabidopsis thaliana caleosin can comprise the amino acid sequence of SEQ ID NO: 31. In other embodiments, the Arabidopsis thaliana caleosin can consist of the amino acid sequence of SEQ ID NO: 31. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 32.

[0057] In various embodiments, the lipid body compartmentalization signal tag fused to the CCD enzyme can be a Sesamum indicum caleosin or a functional variant thereof that can bind to lipid bodies. For example, the Sesamum indicum can comprise an amino acid sequence

having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 33. In certain embodiments, the Sesamum indicum can comprise the amino acid sequence of SEQ ID NO: 33. In other embodiments, the Sesamum indicum can consist of the amino acid sequence of SEQ ID NO: 33. Accordingly, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 34.

Carotenoid-Cleavage Domain

[0058] Enzymes that exhibit carotenoid cleavage activity can include various carotenoid cleavage dioxygenases (CCD). For example, the carotenoid-cleavage domain of the present fusion enzyme can be a CCD1 or CCD4 enzyme capable of cleaving the 5,6 (5',6'), 7,8 (7',8') and 9,10 (9',10') double bonds of a wide range of carotenoids. For example, the CCD can be a Petunia x hybrida CCD (PhCCD1) or a functional variant thereof. For example, the CCD can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 11. In certain embodiments, the CCD can comprise the amino acid sequence of SEQ ID NO: 11. In other embodiments, the CCD can consist of the amino acid sequence of SEQ ID NO: 11. In other embodiments, the CCD can be an Osmanthus fragrans CCD or a functional variant thereof. For example, the CCD can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 14. In certain embodiments, the CCD can comprise the amino acid sequence of SEQ ID NO: 14. In other embodiments, the CCD can consist of the amino acid sequence of SEQ ID NO: 14. In yet other embodiments, the CCD can be a Zea mays CCD or a functional variant thereof. For example, the CCD can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 16. In certain embodiments, the CCD can comprise the amino acid sequence of SEQ ID NO: 16. In other embodiments, the CCD can consist of the amino acid sequence of SEQ ID NO: 16.

[0059] In some embodiments, the CCD can be a homolog of PhCCD1. For example, the CCD can be a carotenoid oxygenase, a 9-cisepoxycarotenoid dioxygenase, or functional variants thereof. Such homologs can originate from various cyanobacteria such as, but not limited to, Nostocales cyanobacterium, Calothrix sp. Calothrix brevissima, and Scytonema millei. For example, the CCD can comprise an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. In certain embodiments, the CCD can comprise the amino acid sequence of SEQ ID

NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24. In other embodiments, the CCD can consist of the amino acid sequence of SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24.

[0060] In some embodiments, the CCD domain can comprise a CCD1 enzyme from *Arabidopsis thaliana*, *Vitis vinifera*, *Daucus carota*, *Lycopersicon esculentum*, and *Rubus idaeus*. In other embodiments, the CCD domain can comprise a CCD4 enzyme from *Arabidopsis thaliana*, *Bixa Orellana*, *Chrysanthemum morifolium*, *Crocus sativus*, *Musa acuminata*, *Malus domestica*, *Prunus persica*, *Rosa damascene*, *Vitis vinifera*, and *Osmanthus fragrans*. In embodiments where the desired ionone compound is alpha-ionone, the CCD domain can comprise a CCD1 enzyme from *Petunia x hybrida* or *Osmanthus fragrans*, a CCD4 enzyme from *Chrysanthemum morifolium*, or a functional homolog thereof. In embodiments where the desired ionone compound is beta-ionone, the CCD domain can comprise a CCD1 enzyme from *Petunia x hybrida* or *Osmanthus fragrans*, a CCD4 enzyme from *Prunus persica*, or a functional homolog thereof.

Fusion Enzymes

[0061] Fusion proteins including fusion enzymes have been developed as a class of novel biomolecules with multi-functional properties. By genetically fusing two or more protein domains together, the fusion protein product may obtain many distinct functions derived from each of their component moieties. The successful construction of a recombinant fusion protein requires two indispensable elements: the component proteins and the linkers. The choice of the component proteins is based on the desired functions of the fusion protein product and, according to the present teachings, includes an LBT domain and a CCD domain as described above. The selection of a suitable linker to join the protein domains together can be complicated, because an unsuitable linker can lead to misfolding of the fusion proteins, low yield in protein production, or impaired bioactivity.

[0062] In some embodiments, the linker used to couple the first LBT domain to the second CCD domain can include 3 to 30 amino acids in length. The linker can include amino acids selected from the group of glycine, serine, threonine, and combinations thereof. In some embodiments, the linker can be a glysine-serine linker. In certain embodiments, the linker can

include one or more units of GGGGS. In specific embodiments, the linker can be GGGGS. In alternative embodiments, the linker can be GGGGSGGGGSGGGGSGGGGS.

[0063] The present fusion enzyme can consist of an LBT domain fused to a CCD domain via a linker. The fusion enzyme can exclude any component that comprises glutathione-S-transferase (GST), small ubiquitin-like modifier (SUMO) protein, maltose binding protein (MBP), thioredoxin (TrxA), or any functional homologs thereof.

Nucleic Acids and Nucleic Acid Constructs

[0064] Nucleic acid molecules according to the present teachings include synthetic and recombinant nucleic acid molecules having a first nucleic acid sequence and a second acid sequence which together encode a fusion enzyme including an LBT domain and a CCD domain. In various embodiments, the first nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or SEQ ID NO: 10. In various embodiments, the second nucleic acid sequence can comprise a nucleic acid sequence having at least 80%, 85%, 90%, 95%, 97%, 98%, or 99% identity to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, or SEQ ID NO: 25. In various embodiments, the present nucleic acid molecule also includes a third nucleic acid sequence that encodes a linker as described herein.

[0065] A nucleic acid construct according to the present teachings can include a synthetic or recombinant nucleic acid molecule as provided herein which can be operably linked to other heterologous nucleic acid sequences. For example, a construct as provided herein can include a nucleic acid sequence that encodes one or more fusion enzymes as described herein, in which the nucleic acid sequence further comprises a promoter operably linked to other heterologous nucleic acid sequences. In some embodiments, the heterologous nucleic acid sequences can include a regulatory element. In some embodiments, a nucleic acid sequence that encodes one or more fusion enzymes as described herein can be operably linked to a terminator sequence. In some embodiments, the nucleic acid construct is functional in a Yarrowia cell. In some embodiments, the nucleic acid construct as provided herein is further defined as an expression cassette or a vector.

Cellular Systems

[0066] As referred herein, a cellular system according to the present methods can include any cell or cells that can be used to express the present fusion enzyme which includes an LBT domain coupled to a CCD domain. Such cellular system can include, but are not limited to, bacterial cells, yeast cells, plant cells, and animal cells. In some embodiments, the cellular system comprises bacterial cells, yeast cells, or a combination thereof. In some embodiments, the cellular system comprises prokaryotic cells, eukaryotic cells, and combinations thereof.

[0067] Bacterial cells of the present disclosure include, without limitation, Escherichia spp., Streptomyces spp., Zymomonas spp., Acetobacter spp., Citrobacter spp., Synechocystis spp., Rhizobium spp., Clostridium spp., Corynebacterium spp., Streptococcus spp., Xanthomonas spp., Lactobacillus spp., Lactococcus spp., Bacillus spp., Alcaligenes spp., Pseudomonas spp., Aeromonas spp., Azotobacter spp., Comamonas spp., Mycobacterium spp., Rhodococcus spp., Gluconobacter spp., Ralstonia spp., Acidithiobacillus spp., Microlunatus spp., Geobacter spp., Geobacillus spp., Arthrobacter spp., Flavobacterium spp., Serratia spp., Saccharopolyspora spp., Thermus spp., Stenotrophomonas spp., Chromobacterium spp., Sinorhizobium spp., Saccharopolyspora spp., Agrobacterium spp., Pantoea spp., and Vibrio natriegens.

[0068] Yeast cells of the present disclosure include, without limitation, Saccharomyces spp., Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Candida boidinii, and Pichia. According to the current disclosure, a yeast as claimed herein are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. Yeasts are unicellular organisms which evolved from multicellular ancestors but with some species useful for the current disclosure being those that can develop multicellular characteristics by forming strings of connected budding cells known as pseudo hyphae or false hyphae.

Cell Culture

[0069] A cell culture refers to any cell or cells that are in a culture. Culturing or incubating is the process in which cells are grown under controlled conditions, typically outside of their natural environment. For example, cells, such as yeast cells, may be grown as a cell suspension in liquid nutrient broth. A cell culture includes, but is not limited to, a bacterial cell

culture, a yeast cell culture, a plant cell culture, and an animal cell culture. In some embodiments, the cell culture comprises bacterial cells, yeast cells, or a combination thereof.

[0070] A bacterial cell culture of the present disclosure comprises bacterial cells including, but not limited to, Escherichia spp., Streptomyces spp., Zymomonas spp., Acetobacter spp., Citrobacter spp., Synechocystis spp., Rhizobium spp., Clostridium spp., Corynebacterium spp., Streptococcus spp., Xanthomonas spp., Lactobacillus spp., Lactococcus spp., Bacillus spp., Alcaligenes spp., Pseudomonas spp., Aeromonas spp., Azotobacter spp., Comamonas spp., Mycobacterium spp., Rhodococcus spp., Gluconobacter spp., Ralstonia spp., Acidithiobacillus spp., Microlunatus spp., Geobacter spp., Geobacillus spp., Arthrobacter spp., Flavobacterium spp., Serratia spp., Saccharopolyspora spp., Thermus spp., Stenotrophomonas spp., Chromobacterium spp., Sinorhizobium spp., Saccharopolyspora spp., Agrobacterium spp., Pantoea spp, and Vibrio natriegens.

[0071] A yeast cell culture of the present disclosure comprises yeast cells including, but not limited to Saccharomyces spp., Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia, Candida boidinii, and Pichia.

[0072] In some embodiments, a cell culture as described herein can be an aqueous medium including one or more nutrient substances as known in the art. Such liquid medium can include one or more carbon sources, nitrogen sources, inorganic salts, and/or growth factors. Suitable carbon sources can include glucose, fructose, xylose, sucrose, maltose, lactose, mannitol, sorbitol, glycerol, and corn syrup. Examples of suitable nitrogen sources can include organic and inorganic nitrogen-containing substances such as peptone, corn steep liquor, mean extract, yeast extract, casein, urea, amino acids, ammonium salts, nitrates and mixtures thereof. Examples of inorganic salts can include phosphates, sulfates, magnesium, sodium, calcium, and potassium salts. The liquid medium also can include one or more vitamins and/or minerals.

[0073] In some embodiments, cells are cultured at a temperature of 16°C to 40°C. For example, cells may be cultured at a temperature of 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C or 40°C.

[0074] In some embodiments, cells are cultured at a pH range from about 3 to about 9, preferably in the range of from about 4 to about 8. The pH can be regulated by the addition of an

inorganic or organic acid or base such as hydrochloric acid, acetic acid, sodium hydroxide, calcium carbonate, ammonia, or by the addition of a buffer such as phosphate, phthalate or Tris ®.

[0075] In some embodiments, cells are cultured for a period of 0.5 hours to 96 hours, or more. For example, cells may be cultured for a period of 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, or 72 hours. Typically, cells, such as bacterial cells, are cultured for a period of 12 to 24 hours. In some embodiments, cells are cultured for 12 to 24 hours at a temperature of 37°C. In some embodiments, cells are cultured for 12 to 24 hours at a temperature of 16°C.

[0076] In some embodiments, cells are cultured to a density of 1 x 108 (OD600< 1) to 2 x 1011 (OD ~ 200) viable cells/ml cell culture medium. In some embodiments, cells are cultured to a density of 1 x 108, 2 x 108, 3 x 108, 4 x 108, 5 x 108, 6 x 108, 7 x 108, 8 x 108, 9 x 108, 1 x 109, 2 x 109, 3 x 109, 4 x 109, 5 x 109, 6 x 109, 7 x 109, 8 x 109, 9 x 109, 1 x 1010, 2 x 1010, 3 x 1010, 4 x 1010, 5 x 1010, 6 x 1010, 7 x 1010, 8 x 1010, 9 x 1010, 1 x 1011, or 2 x 1011 viable cells/ml. (Conversion factor: OD 1 = 8 x 108 cells/ml).

Synthetic Biology

[0077] Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described, for example, by Sambrook, J., Fritsch, E. F. and Maniatis, T. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1989 (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. EXPERIMENTS WITH GENE FUSIONS; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1984; and Ausubel, F. M. et al., IN CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, published by GREENE PUBLISHING AND WILEY-INTERSCIENCE, 1987; the entirety of each of which is hereby incorporated herein by reference.

Microbial Production Systems

[0078] Expression of proteins in transformed host cells is most often carried out in a bacterial or yeast host cell 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: l) 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 vectors are within the scope of the present disclosure.

[0079] In an embodiment, the expression vector includes those genetic elements for expression of the recombinant polypeptide in microbial cells. The elements for transcription and translation in the microbial cell can include a promoter, a coding region for the protein complex, and a transcriptional terminator.

[0080] Persons of ordinary skill in the art will be aware of the molecular biology techniques available for the preparation of expression vectors. The polynucleotide used for incorporation into the expression vector of the subject technology, as described herein, can be prepared by routine techniques such as polymerase chain reaction (PCR).

[0081] A number of molecular biology techniques have been developed to operably link DNA to vectors via complementary cohesive termini. In one embodiment, complementary homopolymer tracts can be added to the nucleic acid molecule to be inserted into the vector DNA. The vector and nucleic acid molecule are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

[0082] In an alternative embodiment, synthetic linkers containing one or more restriction sites provide are used to operably link the polynucleotide of the subject technology to the expression vector. In an embodiment, the polynucleotide is generated by restriction endonuclease digestion. In an embodiment, the nucleic acid molecule is treated with bacteriophage T4 DNA polymerase or E. coli DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities and fill-in recessed 3'-ends with their polymerizing activities, thereby generating blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the product of the reaction is a polynucleotide carrying polymeric linker sequences at its ends. These polynucleotides are then cleaved with the

appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the polynucleotide.

[0083] Alternatively, a vector having ligation-independent cloning (LIC) sites can be employed. The required PCR amplified polynucleotide can then be cloned into the LIC vector without restriction digest or ligation (Aslanidis and de Jong, NUCL. ACID. RES. 18 6069-74, (1990), Haun, et al, BIOTECHNIQUES 13, 515-18 (1992), which is incorporated herein by reference to the extent it is consistent herewith).

[0084] In an embodiment, in order to isolate and/or modify the polynucleotide of interest for insertion into the chosen plasmid, it is suitable to use PCR. Appropriate primers for use in PCR preparation of the sequence can be designed to isolate the required coding region of the nucleic acid molecule, add restriction endonuclease or LIC sites, place the coding region in the desired reading frame.

[0085] In an embodiment, a polynucleotide for incorporation into an expression vector of the subject technology is prepared by the use of PCR using appropriate oligonucleotide primers. The coding region is amplified, whilst the primers themselves become incorporated into the amplified sequence product. In an embodiment, the amplification primers contain restriction endonuclease recognition sites, which allow the amplified sequence product to be cloned into an appropriate vector.

[0086] The expression vectors can be introduced into plant or microbial host cells by conventional transformation or transfection techniques. Transformation of appropriate cells with an expression vector of the subject technology is accomplished by methods known in the art and typically depends on both the type of vector and cell. Suitable techniques include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran mediated transfection, lipofection, chemoporation or electroporation.

[0087] Successfully transformed cells, that is, those cells containing the expression vector, can be identified by techniques well known in the art. For example, cells transfected with an expression vector of the subject technology can be cultured to produce polypeptides described herein. Cells can be examined for the presence of the expression vector DNA by techniques well known in the art.

[0088] The host cells can contain a single copy of the expression vector described previously, or alternatively, multiple copies of the expression vector,

[0089] In some embodiments, the transformed cell can be a bacterial cell, a yeast cell, an algal cell, a fungal cell, a plant cell, an insect cell or an animal cell. In some embodiments, the cell is a plant cell selected from the group consisting of: canola plant cell, a rapeseed plant cell, a palm plant cell, a sunflower plant cell, a cotton plant cell, a corn plant cell, a peanut plant cell, a flax plant cell, a sesame plant cell, a soybean plant cell, and a petunia plant cell.

[0090] Microbial host cell expression systems and expression vectors containing regulatory sequences that direct high-level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct vectors for expression of the recombinant polypeptide of the subjection technology in a microbial host cell. These vectors could then be introduced into appropriate microorganisms via transformation to allow for high level expression of the recombinant polypeptide of the subject technology.

[0091] Vectors or cassettes useful for the transformation of suitable microbial host cells are well known in the art. Typically the vector or cassette contains sequences directing transcription and translation of the relevant polynucleotide, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the polynucleotide which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is preferred for both control regions to be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a host.

[0092] Initiation control regions or promoters, which are useful to drive expression of the recombinant polypeptide in the desired microbial host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the subject technology including but not limited to CYCI, HIS3, GALI, GALIO, ADHI, PGK, PH05, GAPDH, ADCI, TRPI, URA3, LEU2, ENO, TPI (useful for expression in Saccharomyces); TEF (useful for expression in Yarrowia); AOXI (useful for expression in Pichia); and lac, trp, JPL, IPR, T7, tac, and trc (useful for expression in Escherichia coli).

[0093] Termination control regions may also be derived from various genes native to the microbial hosts. A termination site optionally may be included for the microbial hosts described herein.

[0094] In plant cells, the expression vectors of the subject technology can include a coding region operably linked to promoters capable of directing expression of the recombinant polypeptide of the subject technology in the desired tissues at the desired stage of development. For reasons of convenience, the polynucleotides to be expressed may comprise promoter sequences and translation leader sequences derived from the same polynucleotide. 3' non-coding sequences encoding transcription termination signals should also be present. The expression vectors may also comprise one or more introns in order to facilitate polynucleotide expression.

[0095] For plant host cells, any combination of any promoter and any terminator capable of inducing expression of a coding region may be used in the vector sequences of the subject technology. Some suitable examples of promoters and terminators include those from nopaline synthase (nos), octopine synthase (ocs) and cauliflower mosaic virus (CaMV) genes. One type of efficient plant promoter that may be used is a high-level plant promoter. Such promoters, in operable linkage with an expression vector of the subject technology should be capable of promoting the expression of the vector. High level plant promoters that may be used in the subject technology include the promoter of the small subunit (s) of the ribulose-l,5-bisphosphate carboxylase for example from soybean (Berry-Lowe et al., J. MOLECULAR AND APP. GEN., 1:483-98 (1982), the entirety of which is hereby incorporated herein to the extent it is consistent herewith), and the promoter of the chlorophyll binding protein. These two promoters are known to be light-induced in plant cells (see, for example, GENETIC ENGINEERING OF PLANTS, AN AGRICULTURAL PERSPECTIVE, A. Cashmore, Plenum, N.Y. (1983), pages 29-38; Coruzzi, G. et al., THE JOURNAL OF BIOLOGICAL CHEMISTRY, 258: 1399 (1983), and Dunsmuir, P. et al., JOURNAL OF MOLECULAR AND APPLIED GENETICS, 2:285 (1983), each of which is hereby incorporated herein by reference to the extent they are consistent herewith).

Analysis of Sequence Similarity Using Identity Scoring

[0096] As used herein, “sequence identity” refers to the extent to which two optimally aligned polynucleotide or peptide sequences are invariant throughout a window of alignment of

components, e.g., nucleotides or amino acids. An “identity fraction” for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence.

[0097] As used herein, the term “percent sequence identity” or “percent identity” refers to the percentage of identical nucleotides in a linear polynucleotide sequence of a reference (“query”) polynucleotide molecule (or its complementary strand) as compared to a test (“subject”) polynucleotide molecule (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and preferably by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG® Wisconsin Package® (Accelrys Inc., Burlington, MA). An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more polynucleotide sequences may be to a full-length polynucleotide sequence or a portion thereof, or to a longer polynucleotide sequence. For purposes of this disclosure "percent identity" may also be determined using BLASTX version 2.0 for translated nucleotide sequences and BLASTN version 2.0 for polynucleotide sequences.

[0098] The percent of sequence identity is preferably determined using the “Best Fit” or “Gap” program of the Sequence Analysis Software Package™ (Version 10; Genetics Computer Group, Inc., Madison, WI). “Gap” utilizes the algorithm of Needleman and Wunsch (Needleman and Wunsch, JOURNAL OF MOLECULAR BIOLOGY 48:443-53, 1970) to find the alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. “BestFit” performs an optimal alignment of the best segment of similarity between two sequences and inserts gaps to maximize the number of matches using the local homology

algorithm of Smith and Waterman (Smith and Waterman, ADVANCES IN APPLIED MATHEMATICS, 2:482-489, 1981, Smith et al., NUCLEIC ACIDS RESEARCH 11:2205-2220, 1983). The percent identity is most preferably determined using the "Best Fit" program.

[0099] Useful methods for determining sequence identity are also disclosed in the Basic Local Alignment Search Tool (BLAST) programs which are publicly available from National Center Biotechnology Information (NCBI) at the National Library of Medicine, National Institute of Health, Bethesda, Md. 20894; see BLAST Manual, Altschul et al., NCBI, NLM, NIH; Altschul et al., J. MOL. BIOL. 215:403-10 (1990); version 2.0 or higher of BLAST programs allows the introduction of gaps (deletions and insertions) into alignments; for peptide sequence BLASTX can be used to determine sequence identity; and, for polynucleotide sequence BLASTN can be used to determine sequence identity.

[00100] As used herein, the term "substantial percent sequence identity" refers to a percent sequence identity of at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity. Thus, one embodiment of the disclosure is a polynucleotide molecule that has at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity with a polynucleotide sequence described herein.

Identity and Similarity

[00101] Identity is the fraction of amino acids that are the same between a pair of sequences after an alignment of the sequences (which can be done using only sequence information or structural information or some other information, but usually it is based on sequence information alone), and similarity is the score assigned based on an alignment using some similarity matrix. The similarity index can be any one of the following BLOSUM62, PAM250, or GONNET, or any matrix used by one skilled in the art for the sequence alignment of proteins.

[00102] Identity is the degree of correspondence between two sub-sequences (no gaps between the sequences). An identity of 25% or higher implies similarity of function, while 18-25% implies similarity of structure or function. Keep in mind that two completely unrelated or

random sequences (that are greater than 100 residues) can have higher than 20% identity. Similarity is the degree of resemblance between two sequences when they are compared. This is dependent on their identity.

Explanation of Terms Used Herein:

[00103] As used herein, the singular forms “a, an” and “the” include plural references unless the content clearly dictates otherwise.

[00104] To the extent that the term “include,” “have,” or the like is used in the description or the claims, such term is intended to be inclusive in a manner similar to the term “comprise” as "comprise" is interpreted when employed as a transitional word in a claim.

[00105] The word “exemplary” is used herein to mean "serving as an example, instance, or illustration." Any embodiment described herein as "exemplary" is not necessarily to be construed as preferred or advantageous over other embodiments.

[00106] The term “complementary” is to be given its ordinary and customary meaning to a person of ordinary skill in the art and is used without limitation to describe the relationship between nucleotide bases that are capable to hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the subjection technology also includes isolated nucleic acid fragments that are complementary to the complete sequences as reported in the accompanying Sequence Listing as well as those substantially similar nucleic acid sequences.

[00107] The terms “nucleic acid” and “nucleotide” are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art and are used without limitation to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified or degenerate variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated.

[00108] “Coding sequence” is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence that encodes for a specific amino acid sequence.

[00109] The term “isolated” is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and when used in the context of an isolated nucleic acid or an isolated polypeptide, is used without limitation to refer to a nucleic acid or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as, for example, in a transgenic host cell.

[00110] The terms “incubating” and “incubation” as used herein means a process of mixing two or more chemical or biological entities (such as a chemical compound and an enzyme) and allowing them to interact under conditions favorable for producing the desired product.

[00111] The term “degenerate variant” refers to a nucleic acid sequence having a residue sequence that differs from a reference nucleic acid sequence by one or more degenerate codon substitutions. Degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed base and/or deoxyinosine residues. A nucleic acid sequence and all of its degenerate variants will express the same amino acid or polypeptide.

[00112] The terms “polypeptide,” “protein,” and “peptide” are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art; the three terms are sometimes used interchangeably, and are used without limitation to refer to a polymer of amino acids, or amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "polypeptide" as used herein refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms "protein," "polypeptide," and "peptide" are used interchangeably herein when referring to a polynucleotide product. Thus, exemplary polypeptides include polynucleotide products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing.

[00113] The terms "polypeptide fragment" and "fragment," when used in reference to a reference polypeptide, are to be given their ordinary and customary meanings to a person of ordinary skill in the art and are used without limitation to refer to a polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both.

[00114] The term "functional fragment" of a polypeptide or protein refers to a peptide fragment that is a portion of the full-length polypeptide or protein, and has substantially the same biological activity, or carries out substantially the same function as the full-length polypeptide or protein (e.g., carrying out the same enzymatic reaction).

[00115] The terms "variant polypeptide," "modified amino acid sequence" or "modified polypeptide," which are used interchangeably, refer to an amino acid sequence that is different from the reference polypeptide by one or more amino acids, e.g., by one or more amino acid substitutions, deletions, and/or additions. In an aspect, a variant is a "functional variant" which retains some or all of the ability of the reference polypeptide.

[00116] The term "functional variant" further includes conservatively substituted variants. The term "conservatively substituted variant" refers to a peptide having an amino acid sequence that differs from a reference peptide by one or more conservative amino acid substitutions and maintains some or all of the activity of the reference peptide. A "conservative amino acid substitution" is a substitution of an amino acid residue with a functionally similar residue. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one charged or polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between threonine and serine; the substitution of one basic residue such as lysine or arginine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another; or the substitution of one aromatic residue, such as phenylalanine, tyrosine, or tryptophan for another. Such substitutions are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. The phrase "conservatively substituted variant" also includes peptides wherein a

residue is replaced with a chemically-derivatized residue, provided that the resulting peptide maintains some or all of the activity of the reference peptide as described herein.

[00117] The term "variant," in connection with the polypeptides of the subject technology, further includes a functionally active polypeptide having an amino acid sequence at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical to the amino acid sequence of a reference polypeptide.

[00118] "Percent (%) amino acid sequence identity" with respect to the variant polypeptide sequences of the subject technology refers to the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues of a reference polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

[00119] Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For example, the % amino acid sequence identity may be determined using the sequence comparison program NCBI-BLAST2. The NCBI-BLAST2 sequence comparison program may be downloaded from ncbi.nlm.nih.gov. NCBI BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask yes, strand=all, expected occurrences 10, minimum low complexity length=15/5, multi-pass e-value=0.01, constant for multi-pass=25, dropoff for final gapped alignment=25 and scoring matrix=BLOSUM62. In situations where NCBI-BLAST2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain %

amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y where X is the number of amino acid residues scored as identical matches by the sequence alignment program NCBI-BLAST2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

[00120] In this sense, techniques for determining amino acid sequence "similarity" are well known in the art. In general, "similarity" refers to the exact amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" may then be determined between the compared polypeptide sequences. Techniques for determining nucleic acid and amino acid sequence identity also are well known in the art and include determining the nucleotide sequence of the mRNA for that gene (usually via a cDNA intermediate) and determining the amino acid sequence encoded therein, and comparing this to a second amino acid sequence. In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more polynucleotide sequences can be compared by determining their "percent identity", as can two or more amino acid sequences. The programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, Wis.), for example, the GAP program, are capable of calculating both the identity between two polynucleotides and the identity and similarity between two polypeptide sequences, respectively. Other programs for calculating identity or similarity between sequences are known by those skilled in the art.

[00121] An amino acid position "corresponding to" a reference position refers to a position that aligns with a reference sequence, as identified by aligning the amino acid sequences. Such alignments can be done by hand or by using well-known sequence alignment programs such as ClustalW2, Blast 2, etc.

[00122] Unless specified otherwise, the percent identity of two polypeptide or polynucleotide sequences refers to the percentage of identical amino acid residues or nucleotides across the entire length of the shorter of the two sequences.

[00123] The term "homologous" in all its grammatical forms and spelling variations refers to the relationship between polynucleotides or polypeptides that possess a "common evolutionary origin," including polynucleotides or polypeptides from super families and homologous polynucleotides or proteins from different species (Reeck et al., CELL 50:667, 1987). Such polynucleotides or polypeptides have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or the presence of specific amino acids or motifs at conserved positions. For example, two homologous polypeptides can have amino acid sequences that are at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 900 at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical.

[00124] "Suitable regulatory sequences" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

[00125] "Promoter" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters,

which cause a gene to be expressed in most cell types at most times, are commonly referred to as "constitutive promoters." It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[00126] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[00127] The term "expression" as used herein, is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the subject technology. "Over-expression" refers to the production of a gene product in transgenic or recombinant organisms that exceeds levels of production in normal or non-transformed organisms.

[00128] "Transformation" is to be given its ordinary and customary meaning to a person of reasonable skill in the field, and is used without limitation to refer to the transfer of a polynucleotide into a target cell for further expression by that cell. The transferred polynucleotide can be incorporated into the genome or chromosomal DNA of a target cell, resulting in genetically stable inheritance, or it can replicate independent of the host chromosomal. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[00129] The terms "transformed," "transgenic," and "recombinant," when used herein in connection with host cells, are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a cell of a host organism, such as a plant or microbial cell, into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host cell, or the nucleic acid molecule can be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or subjects are

understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

[00130] The terms "recombinant," "heterologous," and "exogenous," when used herein in connection with polynucleotides, are to be given their ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a polynucleotide (e.g., a DNA sequence or a gene) that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of site-directed mutagenesis or other recombinant techniques. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position or form within the host cell in which the element is not ordinarily found.

[00131] Similarly, the terms "recombinant," "heterologous," and "exogenous," when used herein in connection with a polypeptide or amino acid sequence, means a polypeptide or amino acid sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, recombinant DNA segments can be expressed in a host cell to produce a recombinant polypeptide.

[00132] The terms "plasmid," "vector," and "cassette" are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art and are used without limitation to refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene

and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[00133] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred materials and methods are described below.

[00134] The disclosure will be more fully understood upon consideration of the following non-limiting Examples. It should be understood that these Examples, while indicating preferred embodiments of the subject technology, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of the subject technology, and without departing from the spirit and scope thereof, can make various changes and modifications of the subject technology to adapt it to various uses and conditions.

EXAMPLES

Example 1: Generation of epsilon-carotene producing *Yarrowia lipolytica* strains

[00135] In general, the constitutive TEF promoter and the XPR2 terminator were used for the overexpression of individual genes in Yarrowia lipolytica ATCC 90811 host cells. A pUC57-Kan based vector carrying both the TEF promoter and the XPR2 terminator was cloned. Individual genes were cloned in between the TEF promoter and the XPR2 terminator using Gibson assembly. Cassettes containing the TEF promoter, one or more genes for overexpression, and the XPR2 terminator were amplified with PCR and then cloned into high copy number Yarrowia integration vectors such as pYlex-SDH, pYconVec-Leu, and pYconVec-Leu, which has auxotrophic lysine, leucine and uracil markers, respectively.

[00136] In order to boost the carotenoids precursors supply, three genes including genes encoding HMGS, HMGR and IPI, along with their own TEF promoter and XPR2 terminator, were cloned into pYlex-SDH vector to generate a pYlex-SDH-TEF-IPI-TEF-HMGR-TEF-HMGS construct (FIG. 4A). The pYlex-SDH-TEF-IPI-TEF-HMGR-TEF-HMGS construct was

then used to transform Yarrowia lipolytica ATCC 90811 host and select on minimal media plate without lysine.

[00137] Two genes for lycopene production, specifically genes encoding CarRP* (a mutant CarRP that has completely lost its lycopene cyclase activity) and CarB, and a gene encoding a fusion protein comprising FPPS fused in-frame to GGPPS, along with their own TEF promoter and XPR2 terminator, were cloned into pYconVec-Leu vector to generate a pYconVec-Leu-TEF-carB-TEF-carRP*-TEF-GGPPS::FPPS construct (FIG. 4B). The pYconVec-Leu-TEF-carB-TEF-carRP*-TEF-GGPPS::FPPS construct was then used to transform Yarrowia lipolytica ATCC 90811 strain hosting pYlex-SDH-TEF-IPI-TEF-HMGR-TEF-HMGS and select on minimal media plate without leucine. The red-colored clones were Yarrowia lipolytica lycopene producers. The colony with the highest lycopene content was named LYC-40 and chosen as the host for epsilon-carotene and alpha-ionone production.

[00138] The epsilon-cyclase (EC) gene, along with a TEF promoter and an XPR2 terminator, were cloned into a pYconVec-Ura vector to generate a pYconVec-Ura-TEF-EC construct. The pYconVec-Ura-TEF-EC was then used to transform lycopene-producing Yarrowia lipolytica ATCC 90811 host LYC-40 and select on minimal media plate without uracil. Thus, the orange-colored epsilon-carotene producing Yarrowia lipolytica strain was generated.

Example 2: Extraction of epsilon-carotene for HPLC analysis

[00139] The epsilon-carotene producing Yarrowia lipolytica strain was streaked onto YPD agar plate and grown at 30°C. After 2 days of incubation, the orange-colored colonies were grown in 5 ml of YPD liquid medium at 250 rpm and at 30°C for 4 days. 0.2 ml of cell culture was harvested by centrifugation and the cell pellet was resuspended with 0.8 ml of methanol and 0.2 ml of dichloromethane. 0.5 mm diameter glass beads were then added. The Yarrowia lipolytica cells were disrupted for 1 minute in a bead beater homogenizer. Then the mixture was centrifuged for 10 min at 15,000 rpm and the supernatant was collected for HPLC analysis.

[00140] HPLC analysis of epsilon-carotene was performed using an Ultimate 3000 HPLC System (Dionex, Sunnyvale, CA) that included a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Gemini C18 (150mm × 4.6mm 3 μm) with guard column was used for the characterization of lycopene, delta-carotene and epsilon-carotene. A flow rate of 1.0 ml/min was applied, and the mobile phase was

composed of isopropanol (A) and acetonitrile (B). The program is 0-5 min 85% B; 5-13 min 85%-60% B; 13-14 min 60%-85% B; 14-16 min 85% B. The detection wavelengths were 474 nm for lycopene, delta-carotene and epsilon-carotene.

[00141] As shown in FIG. 5, the Yarrowia lipolytica strain hosting genes of HMGS, HMGR, IPI, CarRP*, CarB, FPPS:: GGPPS and EC accumulated lycopene (10 min peak), delta-carotene (11.5 min peak) and epsilon-carotene (13 min peak) by comparing the retention time (top) and the UV spectrum (bottom) of the extracted products against those of known standards.

Example 3: Generation of beta-carotene producing *Yarrowia lipolytica* strains

[00142] Two genes for beta-carotene production, specifically genes encoding CarRP and CarB, and a gene encoding a fusion protein comprising FPPS fused in-frame to GGPPS, along with their own TEF promoter and XPR2 terminator, were cloned into pYconVec-Leu vector to generate a pYconVec-Leu-TEF-carB-TEF-carRP-TEF-GGPPS::FPPS construct. The pYconVec-Leu-TEF-carB-TEF-carRP-TEF-GGPPS::FPPS construct was then used to transform a Yarrowia lipolytica ATCC 90811 strain hosting pYlex-SDH-TEF-IPI-TEF-HMGR-TEF-HMGS and select on minimal media plate without leucine. The orange-colored clones were Yarrowia lipolytica beta-carotene producers. The colony with the highest beta-carotene content was named as BC-3 and chosen as the host for beta-ionone production.

Example 4: Extraction of beta-carotene for HPLC analysis

[00143] The beta-carotene producing Yarrowia lipolytica strain was streaked onto a YPD agar plate and grown at 30°C. After 2 days of incubation, the orange-colored colonies were grown in 5 ml of YPD liquid medium at 250 rpm and at 30°C for 4 days. 0.2 ml of cell culture was harvested by centrifugation and the cell pellet was resuspended with 0.8 ml of methanol and 0.2 ml of dichloromethane. 0.5 mm diameter glass beads were then added. The Yarrowia lipolytica cells were disrupted for 1 minute in a bead beater homogenizer. Then the mixture was centrifuged for 10 min at 15,000 rpm and the supernatant was collected for HPLC analysis.

[00144] HPLC analysis of beta-carotene was performed using an Ultimate 3000 HPLC System (Dionex, Sunnyvale, CA) that included a quaternary pump, a temperature-controlled column compartment, an auto sampler and a UV absorbance detector. Gemini C18 (150mm × 4.6mm 3 μm) with guard column was used for the characterization of beta-carotene. A flow rate of 1.0 ml/min was applied, and the mobile phase was composed of isopropanol (A) and

acetonitrile (B). The program is 0-5 min 85% B; 5-13 min 85%-60% B; 13-14 min 60%-85% B; 14-16 min 85% B. The detection wavelengths were 474 nm for beta-carotene.

[00145] As shown in FIG. 6, the Yarrowia lipolytica hosting genes of HMGS, HMGR, IPI, carRP, carB and FPPS:: GGPPS accumulates beta-carotene (13.5 min peak) by comparing the retention time (top) and the UV spectrum (bottom) of the extracted beta-carotene against those of the beta-carotene standard.

Example 5: Identifying candidate CCD enzymes with 9,10 (9', 10') carotenoid cleavage activity

[00146] Carotenoid cleavage dioxygenase (CCD) enzymes can catalyze the cleavage of a variety of carotene substrates (including epsilon-carotene shown in FIG. 5 and beta-carotene shown in FIG. 6) to generate various ionone compounds (including alpha-ionone and beta-ionone). Referring to FIG. 2, an efficient CCD is the determining factor and key bottleneck in the biosynthetic pathway to alpha-ionone or beta-ionone.

[00147] It has been reported that some plants or cyanobacteria can make ionone compounds. Based on published literature and sequence similarity network (SSN) analysis of the known CCD gene sequences, 28 candidate CCD genes were selected for in vivo screening of the most efficient CCD enzymes in Yarrowia lipolytica. Candidate CCD genes have been individually cloned into pUC57-Kan-TEF-XPR2 vector. Each candidate CCD gene, along with its own TEF promoter and XPR2 terminator, were cloned into pYconVec-Ura-TEF-EC vector to generate pYconVec-Ura-TEF-EC-TEF-CCD constructs. Each pYconVec-Ura-TEF-EC-TEF-CCD construct was then used to transform lycopene-producing Yarrowia lipolytica ATCC 90811 host LYC-40 and select on minimal media plate without uracil. Thus, the orange-colored or yellow-colored alpha-ionone producing Yarrowia lipolytica strains were generated. Among all of the CCD enzymes that were screened, the inventors identified PhCCD1 (originated from the plant Petunia hybrida) as the CCD enzyme with the highest activity, although its alpha-ionone titer from test-tube based cell cultures was still very low (< 3 mg/L). Three different versions of codon-optimized PhCCD1 gene (including SEQ ID NO: 12 and SEQ ID NO: 13) were then screened using the same strategy to identify the most efficient PhCCD1 gene in Yarrowia lipolytica ATCC 90811 (SEQ ID NO: 13).

Example 6: Extraction of alpha-ionone for GC-MS analysis

[00148] The putative orange-colored or yellow-colored alpha-ionone producing Yarrowia lipolytica clones were streaked onto minimal media plate without uracil and grown at 30°C. After 3 days of incubation, the colonies were grown in 5 ml of YPD liquid medium at 250 rpm and at 30°C for 5 days. To analyze alpha-ionone production, 1.0 ml of the Yarrowia lipolytica cell culture was taken and the yeast slurry was extracted with 1.0 ml of n-hexane with at least 1-minute vortex for thorough mixing. After centrifugation at 2,000 rpm for 10 min, the n-hexane phase was used for GC/MS analysis.

[00149] GC/MS analysis was conducted on Shimadzu GC-2010 system coupled with GCMS-QP2010S detector. The analytical column is SHRXI-5MS (thickness 0.25 μm; length 30 m; diameter 0.25 mm) and the injection temperature is 265 °C under split mode. The temperature gradient is 0-1 min 110 °C; 1-8.75 min 110 °C to 265 °C, a gradient of 20; 8.75-9.75 min, 265 °C.

[00150] As shown in FIG. 7, the Yarrowia lipolytica strain hosting genes of HMGS, HMGR, IPI, CarRP*, CarB, FPPS:: GGPPS, EC and CCD was shown to accumulate alpha-ionone (4.3 min peak) when comparing the retention time and the mass spectrometry data of the extracted product (top, center) against the authentic alpha-ionone standard (bottom).

Example 7: Identifying candidate lipid body compartmentalization signal tags to improve PhCCD1 9,10 (9', 10') carotenoid cleavage activity

[00151] Referring to FIG. 2, the last enzyme in the biosynthetic pathway to alpha-ionone and beta-ionone, i.e., CCD, is the key bottleneck for ionone production due to its poor activity. Efforts have been made to improve CCD carotenoid cleavage activity using E. coli as carotenoids platform. It was reported that multiple strategies including adding soluble tags to CCD, using site-directed mutagenesis, and creating carotene cyclase-CCD fusion enzymes could improve the catalytic properties of OfCCD1 in E. coli. It also has been reported that combination of site-directed mutagenesis and expressing peptide-CCD could slightly improve the PhCCD1 activity using a Saccharomyces cerevisiae platform. However, to the inventors' knowledge, no success has been achieved to improve CCD activity using the Yarrowia lipolytica platform. Besides protein engineering efforts on CCD enzymes, the inventors have chosen to investigate lipid body based subcellular compartment engineering approach to improve the cleavage activity of CCDs such as PhCCD1.

[00152] Compartmentalization of biosynthetic pathways or enzymes is an efficient strategy to increase chemical production. Pathway or enzyme compartmentalization aids chemical production by placing enzymes closer to substrates or essential co-factors, increasing the effective concentration of substrates, or providing a more suitable chemical environment for enzymatic reaction. Referring to FIG. 3, the inventors observed under microscope that red-colored lycopene and orange-colored carotene accumulated in lipid bodies in Yarrowia lipolytica cells and lipid droplets in Corynebacterium glutamicum cells. The inventors, therefore, decided to perform in vivo screening of a number of putative lipid body compartmentalization signal tags (including various lipid synthesis enzymes, membrane proteins for lipid body assembly, and lipid body structural proteins, as well as orange carotenoid binding proteins) using the Yarrowia lipolytica lycopene-producing strain LYC-40 as host.

[00153] A pYconVec-Ura-TEF-EC-TEF-PhCCD1 vector was created with a BsaI site in between the TEF promoter and the PhCCD1 gene that has been codon-optimized. Candidate genes encoding putative lipid body compartmentalization signal tags (LBT) have been individually cloned into the BsaI site to generate pYconVec-Ura-TEF-EC-TEF-LBT_CCD constructs (FIG. 4C). Each construct was then used to transform lycopene-producing Yarrowia lipolytica ATCC 90811 host LYC-40 and select on minimal media plate without uracil. Thus, the yellow-colored putative alpha-ionone producing Yarrowia lipolytica strains were generated for GC/MS screening of the most efficient lipid body compartmentalization signal tags.

[00154] As shown in FIG. 8, comparing with strategies of adding soluble tags (e.g., TrxA tagged PhCCD1, the amino acid sequence and codon-optimized nucleic acid sequence for TrxA are provided as SEQ ID NO. 27 and SEQ ID NO. 28), site-directed mutagenesis (PhCCD1-K164L, PhCCD1-T170A, PhCCD1-A327S, PhCCD1-S428N, PhCCD1-K164L_T170A_A327S, PhCCD1-K164L_T170A_A327S_S428N, which correspond to mutants of SEQ ID NO: 11), and creating epsilon cyclase (EC)-CCD fusion enzyme (EC-LGS-PhCCD1), a number of lipid body compartmentalization signal tags could dramatically improve the in vivo carotenoids cleavage efficiency of PhCCD1 in Yarrowia lipolytica cells. These lipid body compartmentalization signal tags include Limnospira maxima orange carotenoid binding protein, Yarrowia lipolytica membrane transporter Stl1p protein, Yarrowia lipolytica lipid droplet protein Oil1p, Zea mays 16 kDa oleosin and Sesamum indicum 17 kDa oleosin. With continued reference to FIG. 8, it was shown that instead of being limited to a ~5X increase in titer via the alternative strategies

described, the present methods which make use of a fusion enzyme including a lipid body compartmentalization signal tag coupled to the CCD enzyme, the increase in titer ranges from 11x to over 33x.

Example 8: Generation of beta-ionone producing *Yarrowia lipolytica* strain

[00155] Gene encoding lipid body compartmentalization tagged PhCCD1, along with TEF promoter and XPR2 terminator, was cloned into pYconVec-Ura vector to generate pYconVec-Ura-TEF-LBT_PhCCD1 constructs. The pYconVec-Ura-TEF-LBT_PhCCD1 construct was then used to transform beta-carotene producing Yarrowia lipolytica ATCC 90811 host BC-3 and select on minimal media plate without uracil. Thus, the yellow-colored beta-ionone producing Yarrowia lipolytica strains were generated. The yellow-colored clones were streaked onto minimal media plate without uracil and grown at 30°C. After 3 days incubation, the colonies were grown in 5 ml YPD liquid medium at 250 rpm and at 30°C for 5 days. To analyze beta-ionone production, 1.0 ml of the Yarrowia lipolytica cell culture was taken and the yeast slurry was extracted with 1.0 ml of n-hexane with at least 1-minute vortex for thorough mixing. After centrifugation at 2,000 rpm for 10 min, the n-hexane phase was used for GC/MS analysis.

[00156] GC/MS analysis was conducted on Shimadzu GC-2010 system coupled with GCMS-QP2010S detector. The analytical column is SHRXI-5MS (thickness 0.25 μm; length 30 m; diameter 0.25 mm) and the injection temperature is 265 °C under split mode. The temperature gradient is 0-1 min 110 °C; 1-8.75 min 110 °C to 265 °C, a gradient of 20; 8.75-9.75 min, 265 °C.

[00157] As shown in FIG. 9, the Yarrowia lipolytica strain hosting genes of HMGS, HMGR, IPI, CarRP, CarB, FPPS:: GGPPS and lipid body compartmentalization tagged PhCCD1 accumulated beta-ionone (4.7 min peak) by comparing the retention time and mass spectrometry data of the extracted product (top, center) against those of authentic beta-ionone standard (bottom).

Example 9: Increased production of astaxanthin by expressing fusion proteins Oleosin::crtRB and Oleosin::BKT

[00158] The LBT peptide described herein can be fused to other carotenoid-generating enzyme to increase production of other carotenoid compounds. FIG. 11 shows increased production of astaxanthin (AS) and zeaxanthin (ZX) when using a recombinant microbial production strain that has been transformed with a nucleic acid construct including a coding

sequence encoding an LBT-CrtRB fusion enzyme and a coding sequence encoding an LBT-BKT fusion enzyme (Oleosin::crtRB-Oleosin::BKT), compared to a strain that has been transformed with a nucleic acid construct including a coding sequence encoding an untagged CrtRB enzyme and a coding sequence encoding an untagged BKT enzyme (crtRB-BKT).

Example 10: Increased production of canthanxanthin by expressing fusion protein Oleosin::crt

[00159] FIG. 12 shows increased production of canthaxanthin (CX) when using a recombinant microbial production strain that has been transformed with a nucleic acid construct including a coding sequence encoding an LBT-CrtW fusion enzyme (Oleosin::crtW), compared to a strain that has been transformed with a nucleic acid construct including a coding sequence encoding an untagged CrtW enzyme (crtW).

SEQUENCES OF INTEREST

***Zea mays* oleosin 16 kDa**

Amino acid sequence of the protein NP_001336922.1 (SEQ ID NO: 1):

MATAHHADDHRAGRRSEAVGENYMRGLYGDDDYNATHYGHQQQQRPPPAPMAVAK
ALATATAAFSMLLLSGLAVTGTVLALIVATPLMVIFSPVLVPAAITVALLTVGIVSSGGFG
VAAVAVLAWVYRYLQTTTSSSGQQPHIVKDWAQQHRLEQTRAH

Codon-optimized nucleotide sequence of NP_001336922.1 for *Yarrowia lipolytica* (SEQ ID NO: 2):

ATGGCCACGGCACACCATGCTGATGATCACAGAGCTGGTCGTCGATCTGAGGCCGT
CGGTGAAAATTACATGAGAGGCCTCTACGGAGACGACGACTACAATGCAACCCATT
ACGGCCACCAACAACAACAAAGACCCCCTCCTGCCCCTATGGCTGTTGCAAAAGCT
CTTGCAACTGCAACCGCCGCTTTCTCGATGCTCCTCCTTAGCGGTCTGGCCGTTACGG
GAACCGTGCTCGCACTGATTGTTGCCACGCCTCTCATGGTCATCTTCTCCCCCGTCCT
TGTGCCCGCCGCAATCACCGTGGCCCTCCTGACAGTCGGCATTGTCTCGTCTGGCGG
TTTTGGTGTCGCTGCTGTTGCAGTTCTCGCTTGGGTGTATCGTTACCTCCAAACGACA
ACTAGCAGCTCGGGTCAGCAGCCTCACATTGTTAAAGATTGGGCACAACAGCACCG
TCTTGAGCAAACCCGTGCTCAC

***Sesamum indicum* oleosin 17 kDa**

Amino acid sequence of the protein (SEQ ID NO: 3):

MADRDRPHPHQIQVHPQHPHRYEGGVKSLLPQKGPSTTQILAIITLLPISGTLLCLAGITL
VGTLIGLAVATPVFVIFSPVLVPAAILIAGAVTAFLTSGAFGLTGLSSLSWVLNSFRRATG
QGPLEYAKRGVQEGTLYVGEKTKQAGEAIKSTAKEGGREGTART

Codon-optimized nucleotide sequence of AAG23840.1 for *Yarrowia lipolytica* (SEQ ID NO: 4):

ATGGCCGACCGTGATAGACCTCACCCTCACCAAATTCAAGTGCATCCTCAACACCCC
CATAGATACGAGGGTGGTGTTAAGTCCCTCCTCCCTCAGAAAGGCCCCTCGACCACC
CAGATTCTCGCTATCATTACCCTTCTCCCTATTAGCGGAACACTGCTCTGCCTGGCCG
GAATCACGCTGGTTGGAACCCTCATCGGCCTCGCCGTCGCAACACCTGTCTTCGTTA
TTTTCTCTCCTGTGCTCGTCCCCGCAGCCATCCTGATCGCCGGTGCCGTTACAGCATT
TCTCACTTCTGGCGCATTTGGTCTTACCGGCCTCAGCTCTCTCTCCTGGGTGCTCAAC
TCGTTCAGACGAGCTACGGGACAGGGACCCCTTGAATACGCTAAGCGAGGTGTTCA
GGAGGGTACGCTCTACGTTGGTGAGAAAACGAAGCAAGCAGGCGAGGCAATCAAGT
CCACGGCCAAAGAAGGAGGTCGAGAGGGCACTGCCCGAACA

***Limnospira maxima* orange carotenoid binding protein**

Amino acid sequence of the protein P83689.1 (SEQ ID NO: 5):

MPFTIDTARSIFPETLAADVVPATIARFKQLSAEDQLALIWFAYLEMGKTITIAAPGAAN
MQFAENTLQEIRQMTPLQQTQAMCDLANRTDTPICRTYASWSPNIKLGFWYELGRFMD
QGLVAPIPEGYKLSANANAILVTIQGIDPGQQITVLRNCVVDMGFDTSKLGSYQRVAEPV
VPPQEMSQRTKVQIEGVTNSTVLQYMDNLNANDFDNLISLFAEDGALQPPFQKPIVGKE
NTLRFFREECQNLKLIPERGVSEPTEDGYTQIKVTGKVQTPWFGGNVGMNIAWRFLLNP
ENKVFFVAIDLLASPKELLNL

Codon-optimized nucleotide sequence of P83689.1 for *Yarrowia lipolytica* (SEQ ID NO: 6):

ATGCCTTTCACTATCGATACGGCTAGAAGCATCTTCCCTGAGACACTGGCAGCCGAC
GTTGTCCCCGCCACAATCGCCCGATTTAAGCAGCTCAGCGCAGAAGACCAGCTTGCC
CTTATCTGGTTCGCTTACCTCGAGATGGGAAAGACTATCACTATCGCAGCACCCGGA
GCTGCCAATATGCAGTTCGCCGAGAACACACTTCAAGAGATCCGGCAGATGACTCC
TCTCCAGCAGACCCAGGCCATGTGTGATCTGGCTAATCGTACCGATACTCCTATTTG
TAGAACTTACGCTTCTTGGTCCCCCAATATTAAACTTGGTTTCTGGTATGAACTCGGC
CGGTTTATGGACCAGGGTCTTGTGGCCCCCATTCCCGAAGGTTACAAGCTCTCTGCT
AATGCAAATGCAATTCTTGTGACCATCCAAGGAATTGATCCTGGACAGCAAATCACG
GTCCTGCGGAATTGCGTTGTTGATATGGGTTTCGACACATCTAAGCTCGGTTCGTAC
CAGCGGGTTGCTGAGCCCGTCGTTCCCCCCCAGGAGATGTCCCAACGAACTAAGGTT
CAAATTGAGGGAGTTACTAACTCTACCGTCCTTCAGTACATGGATAATCTGAACGCA
AACGATTTTGATAATCTTATCTCGCTTTTCGCCGAGGATGGTGCTCTTCAGCCCCCTT
TTCAAAAGCCTATCGTTGGCAAAGAGAATACACTCAGATTTTTCCGAGAGGAATGCC
AGAACCTCAAACTCATCCCTGAGCGTGGAGTTTCGGAACCCACAGAGGATGGATAC
ACACAGATTAAGGTCACGGGCAAAGTTCAGACACCCTGGTTCGGCGGAAATGTGGG
AATGAACATCGCTTGGCGTTTCCTTCTTAACCCTGAGAACAAGGTTTTCTTTGTTGCA
ATCGATCTGCTCGCAAGCCCCAAAGAGCTCCTCAACCTT

***Yarrowia lipolytica* membrane transporter Stl1p**

Amino acid sequence of the protein XP_501907.1 (SEQ ID NO: 7):

MKLQVPAFARSSSDVKTSFMGARGQKLHNLVAAIAGLGFLLFGYDQGVMGGLLTLDTF
IQQFPKMDTSDYLPPKVKTFNTTIQGTAVGIYEIGCMIGALFTMWAGDKLGRRYMIFFGS
IIMTIGAILQCASYSLGQFIAGRVISGIGNGFITATVPMLQSECAKPERRGKLVMLEGALIT
AGIALSYWIDFGFYWVRTNDADWRFPIAFQIVFSLVLTFTIMSLPESPRWLVKKQRFEEA
AGVFAALEDVPLDDPYVINQITSVKESIMMEQLAQLGVDGVDARRKIQSGEFQMGQELS
FIGQMKLMFTFGKKKNFHRTMLAYWNQVMQQVTGINLITYYAAYIYQTSVGMNATDS
RILAACNGTEYFMASWVAFYTIERFGRRKLMLFGAVGQACTMAILTGCVYAASKPEDG
GLDMQGAGIAAAVFLFVFNTFFAIGWLGMTWLYPAEISSLEIRAPANGLSTSGNWAFNF
MVVMITPVAFNSIKWKTYIIFACINAFMVPMVYFFYPETAGRSLEEIDMIFAESNPRTPW
DVVGIANRLPKNSVATYDDYEAGEQEEKAIVETAESVSHDSAEFQ

Codon-optimized nucleotide sequence of XP_501907.1 for *Yarrowia lipolytica* (SEQ ID NO: 8):

ATGAAACTGCAAGTGCCCGCTTTCGCTCGGTCCTCCTCCGACGTGAAGACCTCTTTC
ATGGGTGCACGTGGACAAAAACTTCATAACCTTGTTGCCGCAATCGCCGGTCTTGGA
TTTCTTCTGTTCGGATACGACCAAGGCGTCATGGGTGGCCTGCTTACGCTCGATACG
TTCATCCAACAGTTTCCCAAAATGGATACCTCCGATTATCTGCCCCCTAAGGTTAAG
ACGTTTAACACGACAATTCAGGGAACAGCTGTGGGAATTTACGAAATTGGTTGTATG
ATTGGCGCTCTCTTCACAATGTGGGCTGGTGATAAGCTGGGCCGACGATACATGATC
TTCTTTGGCTCTATTATCATGACCATCGGTGCCATTCTTCAGTGTGCCAGCTATTCCC
TGGGCCAATTCATTGCTGGAAGAGTGATTTCTGGTATCGGAAACGGTTTCATCACGG
CTACTGTCCCTATGCTTCAGTCGGAATGCGCCAAGCCCGAAAGACGTGGCAAACTG
GTGATGCTGGAAGGAGCTCTGATCACGGCAGGAATTGCACTCAGCTACTGGATTGAT
TTCGGTTTTTATTGGGTCCGAACTAACGATGCAGATTGGCGGTTTCCTATCGCTTTTC
AGATCGTGTTTTCTCTTGTTCTGACTTTTACTATTATGTCCCTTCCCGAGAGCCCCAG
ATGGCTCGTCAAAAAGCAGCGATTCGAGGAGGCTGCAGGTGTCTTCGCTGCACTTGA
GGATGTTCCCCTTGATGATCCCTACGTTATCAACCAGATCACATCCGTCAAAGAATC
TATCATGATGGAACAGCTGGCCCAACTTGGTGTTGACGGTGTTGATGCTAGAAGAAA
AATTCAGAGCGGAGAGTTCCAAATGGGTCAGGAGCTTTCTTTTATCGGCCAGATGAA
GCTGATGTTCACCTTCGGTAAGAAAAAAAATTTTCACCGTACAATGCTGGCCTATTG
GAATCAAGTGATGCAACAGGTGACAGGTATTAACCTCATCACTTACTATGCCGCCTA
CATTTACCAGACCAGCGTTGGAATGAATGCCACCGACTCCCGTATTCTCGCTGCCTG
CAATGGTACGGAATATTTCATGGCTTCCTGGGTTGCCTTCTACACGATTGAACGGTT
CGGTCGTCGGAAACTTATGCTGTTCGGCGCTGTGGGCCAAGCCTGCACTATGGCTAT
CCTCACGGGCTGCGTTTATGCAGCCTCCAAGCCCGAAGATGGAGGCCTCGACATGC
AAGGCGCCGGTATTGCTGCAGCTGTTTTCCTTTTCGTTTTCAACACATTCTTCGCCAT
CGGATGGCTTGGTATGACCTGGCTCTATCCCGCTGAAATTTCCTCGCTCGAGATTCG
AGCACCTGCTAATGGACTCAGCACTAGCGGTAACTGGGCTTTCAATTTCATGGTCGT
TATGATTACGCCCGTTGCATTTAATTCTATTAAATGGAAGACCTACATTATTTTTGCA
TGCATCAACGCATTTATGGTGCCTATGGTGTATTTCTTCTACCCCGAAACTGCAGGTC
GGTCCCTGGAGGAAATTGACATGATTTTCGCCGAATCCAATCCCCGAACACCCTGGG
ATGTCGTCGGCATTGCTAACCGGCTGCCCAAGAATAGCGTGGCCACGTATGATGATT
ACGAGGCTGGCGAGCAGGAAGAAAAAGCCATCGTCGAAACTGCAGAGTCTGTCTCC
CACGATTCCGCCGAGTTTCAA

***Yarrowia lipolytica* lipid droplet protein Oil1p**

Amino acid sequence of the protein XP_505819.1 (SEQ ID NO: 9):

MPIKDFTNPAFSNPQETSSQHTHTKMPSVADNTSNGPIEGNVLPQSKFIQHLSEYPAVAA
VTGFAASFPVVKIFASNAVPLIQAIQNRGAPVAEPVVKRAAPYISQIDNAADEALNRLDK
AVPSLKNTKPDEVYSRIVTQPLENVRGTVDKYADETKNTVSRVVVQPIRDVASRVQSQV
VTYYDAHGKPIVHARLDPIFHPLNDRLEALINAYLPKGQEIVTDAENELARAWRLTVVA
FDRARPLIEQQTSQIQEINQHTREHIQKVYDGKRSEIDDKKTVSGPVYATVATVRDLSQE
GLQYAQSILNAKKPEEKADSNSGVAPVSQPHTTSAVDNSLAAPTAVHEVTASA

Codon-optimized nucleotide sequence of XP_505819.1 for *Yarrowia lipolytica* (SEQ ID NO: 10):

ATGCCTATTAAGGATTTTACCAATCCTGCATTTTCCAATCCCCAAGAAACATCCTCTC
AGCACACACATACAAAGATGCCTTCGGTCGCTGATAATACCTCCAACGGCCCCATTG
AGGGAAACGTGCTGCCTCAATCCAAGTTCATTCAGCATCTCTCTGAGTACCCTGCAG
TTGCAGCAGTCACAGGTTTCGCCGCATCCTTCCCTGTTGTCAAAATTTTTGCCAGCAA
TGCTGTTCCCCTTATCCAAGCCATTCAGAACCGTGGAGCTCCCGTGGCAGAACCTGT
GGTCAAACGTGCAGCCCCTTATATCTCCCAGATTGATAACGCTGCCGACGAGGCTCT
GAATCGGCTGGATAAGGCAGTCCCTTCTCTCAAAAATACCAAACCCGATGAAGTTTA
TTCCAGAATTGTGACCCAACCCCTGGAAAACGTGCGTGGCACTGTGGACAAATATGC
AGATGAGACGAAAAACACAGTCTCCCGGGTTGTTGTTCAGCCTATCAGAGATGTTGC
CTCTAGAGTTCAGTCTCAGGTTGTTACATATTATGATGCTCATGGAAAACCCATCGT
GCATGCACGGCTCGATCCCATTTTCCACCCCCTTAATGATAGACTTGAAGCTCTGATT
AATGCTTACCTGCCTAAGGGACAGGAGATCGTGACGGATGCCGAGAACGAGCTCGC
ACGAGCCTGGCGACTTACAGTTGTTGCATTCGATCGAGCCCGTCCTCTTATTGAGCA
ACAAACCTCGCAGATTCAAGAGATCAATCAACACACTCGGGAGCATATTCAAAAGG
TCTACGACGGCAAACGATCCGAGATCGACGACAAGAAGACTGTTTCTGGACCTGTG
TATGCAACCGTGGCCACGGTTCGGGATCTGTCCCAAGAAGGACTTCAATATGCTCAA
TCTATTCTGAACGCAAAGAAGCCCGAAGAGAAAGCCGACAGCAACAGCGGCGTCGC
ACCCGTCTCGCAACCCCACACCACGTCCGCTGTGGATAATAGCCTTGCTGCCCCTAC
TGCTGTGCATGAAGTCACTGCCAGCGCA

***Petunia x hybrida* carotenoid cleavage dioxygenase 1**

Amino acid sequence of the protein AAT68189.1 (SEQ ID NO: 11):

MGRKESDDGVERIEGGVVVVNPKPKKGITAKAIDLLEKVIIKLMHDSSKPLHYLSGNFA
PTDETPPLNDLPIKGHLPECLNGEFVRVGPNPKFAPVAGYHWFDGDGMIHGLRIKDGKA
TYVSRYVRTSRLKQEEFFEGAKFMKIGDLKGLFGLFTVYMQMLRAKLKILDTSYGNGT
ANTALVYHHGKLLALSEADKPYALKVLEDGDLQTLGMLDYDKRLLHSFTAHPKVDPV
TGEMFTFGYAHEPPYITYRVISKDGIMQDPVPITIPEAIMMHDFAITENYAIMMDLPLCFR
PKEMVKNNQLAFTFDTTKKARFGVLPRYAKSEALIRWFELPNCFIFHNANAWEEGDEV
VLITCRLPHPDLDMVNGEVKENLENFSNELYEMRFNMKSGAASQKKLSESSVDFPRINE
NYTGRKQRYVYGTTLNSIAKVTGIIKFDLHAEPETGKKQLEVGGNVQGIFDLGPGRFGSE

AVFVPSQPGTECEEDDGYLIFFVHDENTGKSAVNVIDAKTMSAEPVAVVELPKRVPYGF
HAFFVTEEQIQEQAKL

Codon-optimized nucleotide sequence of AAT68189.1 for *Yarrowia lipolytica* version 1(SEQ ID NO: 12):

ATGGGGCGAAAGGAGTCGGACGACGGCGTTGAGCGAATCGAGGGCGGCGTGGTGG
TTGTTAACCCCAAGCCCAAGAAGGGCATCACCGCCAAGGCAATTGATCTCCTGGAG
AAGGTTATAATTAAGCTTATGCACGACTCCTCCAAGCCCCTGCATTACCTGTCTGGG
AACTTCGCTCCCACCGACGAGACTCCACCCCTGAACGATCTGCCCATAAAGGGGCA
CCTGCCCGAGTGTCTGAACGGAGAGTTCGTGCGAGTGGGACCGAACCCCAAGTTCG
CCCCCGTGGCCGGCTATCATTGGTTCGACGGCGACGGGATGATCCATGGGCTGCGCA
TCAAGGACGGCAAGGCTACGTACGTATCCAGATACGTTCGAACCAGTAGACTTAAG
CAGGAGGAGTTTTTCGAGGGCGCCAAGTTCATGAAGATCGGCGACCTGAAGGGGCT
TTTCGGACTTTTTACCGTGTACATGCAGATGCTTCGAGCTAAGCTGAAGATCCTGGA
TACAAGCTACGGCAACGGGACCGCCAACACCGCCCTTGTTTACCATCACGGGAAGC
TGCTGGCTCTGTCTGAGGCCGACAAGCCCTACGCTCTGAAGGTGCTTGAGGACGGCG
ACCTCCAGACCCTGGGGATGCTTGACTACGATAAGCGACTGCTGCACTCTTTTACCG
CACACCCCAAGGTGGATCCCGTTACCGGAGAGATGTTCACATTCGGGTATGCCCATG
AGCCGCCCTATATAACCTACCGAGTGATTTCCAAGGACGGCATCATGCAGGACCCG
GTACCCATTACCATTCCCGAGGCCATAATGATGCACGACTTCGCCATCACCGAGAAC
TACGCTATCATGATGGACCTTCCCCTGTGCTTTCGCCCCAAGGAGATGGTTAAGAAC
AATCAGCTTGCCTTCACATTTGACACCACCAAGAAGGCCCGGTTCGGCGTACTGCCC
CGGTACGCTAAGTCGGAGGCACTTATACGATGGTTTGAGCTGCCCAACTGTTTTATT
TTCCATAATGCAAACGCCTGGGAGGAGGGCGACGAGGTAGTTCTGATCACATGTCG
CCTGCCGCACCCCGATCTTGATATGGTAAACGGCGAGGTGAAGGAGAACCTGGAGA
ACTTTAGCAACGAGCTTTACGAGATGCGATTCAATATGAAGTCGGGAGCTGCCTCCC
AGAAGAAGCTGTCCGAGTCCTCTGTGGACTTCCCCCGCATAAACGAGAATTATACCG
GCCGAAAGCAGAGATACGTTTACGGCACAACCCTGAATAGCATCGCAAAGGTTACC
GGCATAATAAAGTTCGACCTGCACGCCGAGCCCGAAACCGGGAAGAAGCAGCTGGA
GGTGGGCGGCAACGTGCAGGGGATATTCGATCTTGGCCCCGGGCGATTCGGAAGTG
AGGCCGTTTTCGTGCCATCTCAGCCCGGAACCGAGTGCGAGGAGGACGACGGCTAT
CTGATTTTTTTTGTTCACGACGAGAATACCGGCAAGTCCGCCGTTAACGTTATCGAC
GCTAAGACCATGTCCGCTGAGCCCGTTGCTGTGGTGGAGCTTCCAAAGCGAGTGCCC
TACGGCTTTCACGCATTTTTCGTAACCGAGGAGCAGATCCAGGAGCAGGCAAAGCTT
TAA

Codon-optimized nucleotide sequence of AAT68189.1 for *Yarrowia lipolytica* version 2 (SEQ ID NO: 13):

ATGGGCCGAAAGGAGTCTGACGACGGCGTGGAGCGAATCGAGGGCGGCGTGGTGGT
GGTGAACCCCAAGCCCAAGAAGGGCATCACCGCCAAGGCCATCGACCTGCTGGAGA
AGGTGATCATCAAGCTGATGCACGACTCTTCTAAGCCCCTGCACTACCTGTCTGGCA
ACTTCGCCCCCACCGACGAGACTCCCCCCCTGAACGACCTGCCCATCAAGGGCCACC
TGCCCGAGTGCCTGAACGGCGAGTTCGTGCGAGTGGGCCCCAACCCCAAGTTCGCC
CCCGTGGCCGGCTACCACTGGTTCGACGGCGACGGCATGATCCACGGCCTGCGAAT
CAAGGACGGCAAGGCCACCTACGTGTCTCGATACGTGCGAACCTCTCGACTGAAGC
AGGAGGAGTTCTTCGAGGGCGCCAAGTTCATGAAGATCGGCGACCTGAAGGGCCTG

TTCGGCCTGTTCACCGTGTACATGCAGATGCTGCGAGCCAAGCTGAAGATCCTGGAC
ACCTCTTACGGCAACGGCACCGCCAACACCGCCCTGGTGTACCACCACGGCAAGCT
GCTGGCCCTGTCTGAGGCCGACAAGCCCTACGCCCTGAAGGTGCTGGAGGACGGCG
ACCTCCAGACCCTGGGCATGCTGGACTACGACAAGCGACTGCTGCACTCTTTCACCG
CCCACCCCAAGGTGGACCCCGTGACCGGCGAGATGTTCACCTTCGGCTACGCCCACG
AGCCCCCCTACATCACCTACCGAGTGATCTCTAAGGACGGCATCATGCAGGACCCCG
TGCCCATCACCATCCCCGAGGCCATCATGATGCACGACTTCGCCATCACCGAGAACT
ACGCCATCATGATGGACCTGCCCCTGTGCTTCCGACCCAAGGAGATGGTGAAGAAC
AACCAGCTGGCCTTCACCTTCGACACCACCAAGAAGGCCCGATTCGGCGTGCTGCCC
CGATACGCCAAGTCTGAGGCCCTGATCCGATGGTTCGAGCTGCCCAACTGCTTCATC
TTCCACAACGCCAACGCCTGGGAGGAGGGCGACGAGGTGGTGCTGATCACCTGCCG
ACTGCCCCACCCCGACCTGGACATGGTGAACGGCGAGGTGAAGGAGAACCTGGAGA
ACTTCTCTAACGAGCTGTACGAGATGCGATTCAACATGAAGTCTGGCGCCGCCTCTC
AGAAGAAGCTGTCTGAGTCCTCTGTGGACTTCCCCCGAATCAACGAGAACTACACC
GGCCGAAAGCAGCGATACGTGTACGGCACCACCCTGAACTCTATCGCCAAGGTGAC
CGGCATCATCAAGTTCGACCTGCACGCCGAGCCCGAGACTGGCAAGAAGCAGCTGG
AGGTGGGCGGCAACGTGCAGGGCATCTTCGACCTGGGCCCCGGCCGATTCGGCTCT
GAGGCCGTGTTCGTGCCCTCTCAGCCCGGCACCGAGTGCGAGGAGGACGACGGCTA
CCTGATCTTCTTCGTGCACGACGAGAACACCGGCAAGTCTGCCGTGAACGTGATCGA
CGCCAAGACCATGTCTGCCGAGCCCGTGGCCGTGGTGGAGCTGCCCAAGCGAGTGC
CCTACGGCTTCCACGCCTTCTTCGTGACCGAGGAGCAGATCCAGGAGCAGGCCAAG
CTGTAA

***Osmanthus fragrans* carotenoid cleavage dioxygenase 1**

Amino acid sequence of the protein BAJ05401.1 (SEQ ID NO: 14):

MGMQGEDAQRTGNIVAVKPKPSQGLTSKAIDWLEWLFVKMMHDSKQPLHYLSGNFAP
VDETPPLKDLPVTGHLPECLNGEFVRVGPNPKFASIAGYHWFDGDGMIHGMRIKDGKA
TYVSRYVQTSRLKQEEFFGRAMFMKIGDLKGMFGLLMVNMQMLRAKLKVLDISYGIG
TANTALVYHHGKLLALSEADKPYAIKVLEDGDLQTIGLLDYDKRLAHSFTAHPKVDPFT
GEMFTFGYSHTPPYVTYRVISKDGAMNDPVPITVSGPIMMHDFAITENYAIFMDLPLYFK
PKEMVKDKKFIFSFDATQKARFGILPRYAKNELLIKWFELPNCFIFHNANAWEEGDEVV
LITCRLENPDLDMVNSTVKERLDNFKNELYEMRFNLQNGLASQKKLSVSAVDFPRVNES
YTTRKQRYVYGTTLDKIAKVTGIIKFDLHAEPETGKEKLELGGNVKGIFDLGPGRFGSEA
VFVPRHPGITSEEDDGYLIFFVHDENTGKSAVNVIDAKTMSPDPVAVVELPKRVPYGFH
AFFVTEDQLQEQAKV

Osmanthus fragrans CCD1 mRNA for carotenoid cleavage dioxygenase 1, nucleotide sequence of AB526197.1 (SEQ ID NO: 15):

ATGGGGATGCAAGGAGAGGATGCGCAGCGGACTGGGAATATTGTTGCTGTAAAGCC
GAAACCCAGTCAAGGGCTCACTTCCAAGGCCATAGACTGGTTGGAATGGCTGTTTGT
GAAAATGATGCATGACTCCAAACAGCCTCTCCATTATCTTTCTGGGAATTTTGCTCC
AGTTGATGAGACTCCTCCTCTTAAGGACCTTCCTGTTACAGGGCACCTCCCTGAGTG
TCTGAATGGTGAATTTGTGAGGGTTGGTCCTAACCCCAAGTTTGCTTCAATTGCTGGT
TATCATTGGTTTGATGGAGATGGAATGATTCATGGTATGCGGATAAAAGATGGGAA
AGCAACATATGTCTCGCGTTATGTGCAGACATCTCGCCTTAAACAAGAAGAGTTTTT

TGGCAGAGCTATGTTTATGAAGATTGGAGACCTGAAAGGGATGTTTGGATTGCTCAT
GGTTAACATGCAAATGCTCAGAGCAAAACTGAAAGTATTAGATATTTCCTATGGAAT
TGGTACAGCTAATACTGCTCTGGTATACCACCATGGAAAGCTTTTGGCACTTAGTGA
GGCAGATAAACCATATGCCATTAAAGTTTTGGAAGATGGAGATCTGCAAACTATTG
GCTTGCTGGACTATGATAAAAGACTGGCACATTCATTTACTGCTCATCCTAAGGTTG
ACCCATTTACCGGAGAGATGTTTACCTTTGGCTATTCACATACACCACCTTATGTCAC
ATACAGAGTTATATCGAAGGATGGAGCGATGAATGATCCCGTTCCAATAACAGTAT
CAGGCCCAATCATGATGCATGATTTTGCGATTACTGAAAATTATGCAATTTTTATGG
ACCTCCCTTTATACTTTAAGCCAAAGGAAATGGTGAAGGATAAAAAGTTTATTTTCA
GTTTTGATGCTACCCAGAAAGCTCGTTTTGGCATCCTTCCGCGGTATGCAAAGAATG
AGCTACTAATCAAATGGTTTGAGCTTCCAAATTGCTTCATATTCCATAATGCAAATG
CTTGGGAGGAAGGAGATGAAGTTGTTCTGATCACTTGTCGCCTTGAGAATCCAGATC
TTGACATGGTTAACAGCACCGTTAAAGAAAGGCTTGATAATTTCAAAAACGAACTGT
ACGAGATGAGGTTCAACCTCCAAAATGGTCTGGCTTCACAGAAAAAACTATCTGTAT
CCGCTGTAGATTTTCCAAGGGTGAACGAAAGTTACACTACTAGGAAACAGCGATAT
GTATATGGAACAACACTGGACAAGATTGCTAAGGTAACTGGGATTATCAAGTTTGAT
TTGCATGCGGAACCAGAGACTGGAAAAGAAAAGCTAGAACTTGGAGGAAATGTTAA
AGGTATCTTTGATCTAGGACCTGGAAGATTTGGTTCAGAGGCTGTCTTTGTTCCGCG
GCATCCTGGTATCACATCTGAAGAAGATGATGGCTACTTGATTTTCTTTGTACATGAT
GAGAACACTGGAAAGTCGGCAGTGAATGTAATTGATGCAAAAACAATGTCACCTGA
TCCTGTTGCTGTTGTCGAACTGCCCAAGAGAGTTCCGTACGGATTTCATGCCTTCTTC
GTGACAGAAGACCAACTTCAAGAACAGGCAAAGGTTTGA

***Zea mays* carotenoid cleavage dioxygenase 1**

Amino acid sequence of the protein ACG46084.1 (SEQ ID NO: 16):

MGTEAEQPDMDSHRNDGVVVVPAPRPRKGLASWALDLLESLAVRLGHDKTKPLHWLS
GNFAPVVEETPPAPNLTVRGHLPECLNGEFVRVGPNPKFAPVAGYHWFDGDGMIHAMR
IKDGKATYVSRYVKTARLKQEEYFGGAKFMKIGDLKGFFGLFMVQMQQLRKKFKVLD
FTYGFGTANTALIYHHGKLMALSEADKPYVVKVLEDGDLQTLGLLDYDKRLKHSFTAH
PKVDPFTDEMFTFGYSHEPPYCTYRVINKEGAMLDPVPITIPESVMMHDFAITENYSIFM
DLPLLFRPKEMVKNGEFIYKFDPTKKGRFGILPRYAKDDKLIRWFQLPNCFIFHNANAW
EEGDEVVLITCRLENPDLDKVNGYQSDKLENFGNELYEMRFNMKTGAASQKQLSVSAV
DFPRVNESYTGRKQRYVYCTILDSIAKVTGIIKFDLHAEPESGVKVLEVGGNVQGIYDLG
PGRFGSEAIFVPKHPGVSGEEDDGYLIFFVHDENTGKSEVNVIDAKTMSADPVAVVELP
NRVPYGFHAFFVTEDQLARQAEGQ

Zea mays carotenoid cleavage dioxygenase 1 (CCD1) mRNA, nucleotide sequence of DQ100346.1 (SEQ ID NO: 17):

ATGGGGACGGAGGCGGAGCAGCCGGACATGGACAGCCACCGAAACGACGGCGTCG
TGGTGGTGCCAGCGCCGCGCCCGCGTAAGGGGCTCGCTTCCTGGGCGCTCGACCTGC
TTGAGTCCCTCGCCGTGCGCCTCGGCCACGACAAGACCAAGCCGCTCCACTGGCTCT
CCGGCAACTTCGCCCCCGTCGTCGAGGAGACCCCGCCGGCCCCAAACCTTACCGTCC
GCGGACACCTCCCGGAGTGCTTGAATGGAGAGTTTGTCAGGGTTGGGCCTAATCCGA
AGTTTGCTCCTGTTGCGGGGTATCACTGGTTTGATGGAGACGGGATGATTCATGCCA
TGCGTATTAAGGATGGAAAAGCTACCTATGTATCAAGATATGTGAAGACTGCCCGCC

TCAAACAAGAGGAGTATTTTGGTGGAGCAAAGTTTATGAAGATTGGAGACCTTAAG
GGATTTTTTGGATTGTTTATGGTCCAAATGCAGCAACTTCGGAAAAAATTCAAAGTC
TTGGATTTTACCTATGGATTTGGGACAGCTAATACTGCACTTATATATCATCATGGTA
AACTCATGGCCTTGTCAGAAGCAGATAAGCCATATGTTGTTAAGGTCCTTGAAGATG
GAGACTTGCAGACTCTTGGCTTGTTGGATTATGACAAAAGGTTGAAACATTCTTTTA
CTGCCCATCCAAAGGTTGACCCTTTTACAGATGAAATGTTCACATTCGGATATTCAC
ATGAACCTCCATACTGTACATACCGTGTGATTAACAAAGAAGGAGCTATGCTTGATC
CTGTGCCAATAACAATACCGGAATCTGTAATGATGCATGATTTTGCCATCACAGAGA
ATTACTCTATTTTTATGGACCTCCCTTTATTGTTCCGACCAAAGGAAATGGTGAAGA
ACGGTGAGTTTATCTACAAGTTTGATCCTACAAAGAAAGGTCGTTTTGGTATTCTCC
CCCGCTATGCAAAGGATGACAAACTCATCAGATGGTTTCAACTCCCTAATTGTTTCA
TATTCCATAATGCTAATGCTTGGGAAGAGGGTGATGAAGTTGTTCTAATTACCTGCC
GCCTTGAGAATCCAGATTTGGACAAGGTGAATGGATATCAAAGTGACAAGCTCGAA
AACTTCGGGAATGAGCTGTACGAGATGAGATTCAACATGAAAACGGGTGCTGCTTC
ACAAAAGCAATTGTCTGTTTCTGCTGTGGATTTTCCTCGTGTTAATGAGAGCTATACT
GGCAGAAAGCAGCGGTATGTCTACTGCACTATACTTGACAGCATTGCGAAGGTGAC
TGGCATCATAAAGTTTGATCTGCATGCTGAACCGGAAAGTGGTGTGAAAGTACTTGA
AGTGGGAGGAAATGTACAAGGCATATATGACCTGGGACCTGGTAGATTTGGTTCAG
AGGCGATTTTTGTTCCCAAGCATCCAGGTGTGTCTGGAGAAGAAGATGACGGCTATT
TGATATTCTTTGTACACGACGAGAACACAGGGAAATCTGAAGTAAATGTTATCGATG
CAAAGACAATGTCTGCTGATCCAGTTGCGGTGGTTGAGCTTCCTAATAGGGTTCCTT
ATGGATTCCATGCCTTTTTTGTAACTGAGGACCAACTGGCTCGACAGGCGGAGGGGC
AGTGA

***Nostocales cyanobacterium* HT-58-2 9-cis-epoxycarotenoid dioxygenase**

Amino acid sequence of the protein WP_087544521.1 (SEQ ID NO: 18):

MAIKQVNPFLDGNFAPVEEEITTNTLQVIGELPPDLSGMFVRNGPNPQWTPIGQYHWFD
GDGMLHGVRISNGKATYRNRYVRTKGWKIENEAGKAVWTGLLEPRPKHTPHGQSKNT
ANTALVWHAGQLLALWEGGAPHAIKVPELKTIGEYTYNGKLVSAVTAHPKVDPVTGE
MMFFGYSFAPPYLQYSVVSPQGELLQTETIDIPMAVMMHDFAITEDYTIFMDLPLTFSQE
RIKRGEPLMMFERDKPSRFGIVPRYGNNSNIRWFESPACYIFHTVNAYEEGDEVVLVAC
RMSSTTVLGAPQDTHVDSEADIPRLHRWRFNLKTGKVSEEMLDDVPSEFPRVNENLLG
QKTRYGYTGRMAKSSLPLFDGLIKYDLNNGKSQTHEFGSGRYGGEAVFVPRPGATAED
DGWLVTFVHDTAKDTSELVVVSAQDVTGEPVARVLIPQRVPYGFHGAWVSEEQLKAS
M

Codon-optimized nucleotide sequence of WP_087544521.1 for *Yarrowia lipolytica* (SEQ ID NO: 19):

ATGGCAATTAAGCAGGTCAATCCCTTCCTTGACGGTAACTTCGCACCCGTTGAAGAG
GAGATCACAACCAACACGCTGCAGGTGATTGGAGAACTGCCCCCTGACCTCTCGGG
AATGTTTGTTCGAAATGGCCCTAATCCCCAGTGGACCCCTATCGGACAGTATCACTG
GTTCGACGGCGATGGCATGCTCCACGGCGTTCGTATTTCGAACGGCAAAGCTACTTA
CCGTAATCGGTACGTTCGAACAAAAGGATGGAAGATCGAAAACGAAGCTGGTAAAG
CCGTGTGGACCGGACTCCTTGAGCCCCGACCTAAACACACTCCTCATGGTCAATCGA
AGAACACAGCAAACACAGCCCTTGTGTGGCATGCAGGCCAACTGCTGGCACTCTGG

GAGGGCGGCGCCCCTCACGCAATCAAAGTTCCTGAGCTCAAGACGATCGGCGAATA
TACTTACAATGGAAAACTCGTGTCTGCTGTCACTGCCCATCCCAAAGTGGATCCCGT
CACGGGCGAGATGATGTTCTTTGGATACTCGTTCGCCCCTCCTTACCTGCAGTACTCT
GTGGTTTCCCCCCAGGGAGAACTTCTGCAAACGGAAACCATCGACATTCCTATGGCA
GTGATGATGCACGACTTTGCTATTACTGAAGATTATACTATTTTCATGGACCTTCCCC
TTACATTTTCTCAGGAGAGAATCAAGCGAGGAGAGCCCCTTATGATGTTTGAAAGAG
ATAAGCCTAGCCGATTCGGTATTGTGCCTCGTTATGGAAATAACTCTAACATTAGAT
GGTTCGAATCGCCCGCATGCTATATCTTCCACACGGTTAATGCATACGAAGAAGGAG
ATGAGGTTGTCCTGGTGGCCTGTCGAATGTCCTCTACGACTGTCCTGGGAGCACCTC
AAGACACACATGTCGATTCTGAAGCCGATATTCCCAGACTCCATCGATGGCGGTTCA
ATCTGAAGACTGGTAAGGTGTCGGAGGAAATGCTTGACGACGTCCCCTCGGAATTTC
CTCGAGTTAATGAAAATCTCCTGGGTCAAAAGACCCGATACGGTTACACTGGTCGGA
TGGCTAAGTCCTCCCTGCCTCTTTTTGACGGCCTCATCAAGTATGACCTGAACAATG
GCAAGAGCCAGACTCATGAATTCGGTTCTGGTCGATATGGAGGAGAAGCCGTGTTT
GTGCCTCGACCCGGTGCAACTGCTGAGGACGACGGCTGGCTGGTTACCTTCGTCCAC
GATACTGCTAAAGACACTTCCGAACTCGTGGTCGTGTCGGCACAGGACGTCACCGGT
GAGCCCGTCGCTAGAGTGCTTATCCCCCAGCGAGTTCCTTACGGTTTTCATGGCGCA
TGGGTGTCTGAGGAGCAACTGAAGGCATCGATGTGA

***Calothrix sp.* NIES-2098 carotenoid oxygenase**

Amino acid sequence of the protein BAY08575.1 (SEQ ID NO: 20):

MTTNTIPTTAINPYLDGNFAPVREEITTDTLQVVGQLPPDLAGMFVRNGPNPQWPPIGKY
HWFDGDGMLHGVRISNGQATYRNRYIQTRGWKIEREAGKAIWTGFMEPPRMDNPYGG
YKNTGNTALIWHAGQLLALNEGGAPHAMTLPELGTIGEYTYDGKLISAFTAHPKVDPVT
GEMMFFGYSFAPPYLQYSQVSAAGELLQTVPIDLPTPVMMHDFAITENYTIFMDLPLTLS
AERLQRGEPMLMFERDRSSRFGIVPRYGDNSNIRWFESSPCYVFHTLNAYEVGDEVVLI
GCRMSSTSVLVTEDNQTDADGNIPRLHQWRFNLKTGTVREQQLDDLAAEFPRVNEDFL
GRQTRYGYAGKLANSPLPLFDGVIKYDLNGETSQIHHFGQGRYGGEAVFAPRPGATHE
DDGWLITFVYDEGSDTSELVVINAQDITSEPVARVIIPQRVPYGFHGTWVSEEQLMTNS

Codon-optimized nucleotide sequence of BAY08575.1 for *Yarrowia lipolytica* (SEQ ID NO: 21):

ATGACCACAAATACGATCCCCACTACCGCCATCAACCCCTATCTTGACGGCAATTTC
GCACCTGTGCGGGAAGAAATCACTACGGACACCCTTCAAGTTGTTGGTCAACTCCCT
CCTGATCTGGCCGGCATGTTCGTGCGGAATGGTCCCAATCCCCAGTGGCCTCCTATC
GGTAAATATCACTGGTTCGATGGTGACGGCATGCTCCATGGAGTGCGTATCTCGAAT
GGTCAGGCAACCTATCGGAACCGATACATCCAAACCCGTGGATGGAAAATTGAGCG
TGAAGCCGGCAAAGCTATCTGGACTGGATTTATGGAGCCTCCTCGAATGGACAATCC
TTATGGTGGATATAAAAACACGGGCAATACAGCTCTGATTTGGCATGCAGGTCAGCT
TCTGGCACTTAACGAAGGTGGAGCCCCTCACGCTATGACTCTTCCTGAGCTCGGAAC
CATCGGAGAATACACCTATGACGGTAAACTCATCTCCGCATTCACTGCTCACCCCAA
GGTGGATCCTGTTACCGGCGAGATGATGTTCTTTGGATACTCCTTCGCACCTCCTTAT
CTTCAATACAGCCAGGTGTCTGCAGCAGGCGAGCTCCTTCAAACTGTGCCTATCGAT
CTCCCTACGCCTGTGATGATGCATGACTTTGCCATCACTGAGAACTACACAATCTTT
ATGGACCTTCCCCTGACGCTTTCGGCAGAGCGTCTTCAAAGAGGCGAACCTATGCTT

ATGTTCGAACGAGATCGATCGAGCCGGTTCGGTATCGTTCCCCGTTATGGTGATAAT
TCGAATATTCGTTGGTTTGAGTCCTCTCCCTGCTACGTGTTTCATACACTGAATGCCT
ATGAGGTGGGAGACGAGGTCGTGCTTATTGGATGCCGGATGAGCTCCACCTCCGTGC
TTGTGACGGAGGACAATCAGACCGACGCAGACGGTAATATTCCCAGACTTCACCAA
TGGAGATTTAATCTCAAGACTGGTACAGTTCGGGAGCAGCAGCTCGACGATCTCGCC
GCCGAATTTCCCCGAGTTAACGAGGACTTCCTCGGAAGACAAACTCGGTACGGCTAT
GCTGGAAAACTGGCTAACTCGCCCCTGCCTCTGTTTGACGGAGTTATCAAATATGAC
CTCAACGGCGAGACTTCCCAGATCCACCATTTTGGACAGGGAAGATACGGCGGCGA
AGCCGTTTTTGCCCCCAGACCTGGTGCCACCCATGAAGACGACGGATGGCTCATCAC
ATTTGTCTACGACGAGGGATCCGACACGAGCGAGCTCGTGGTCATCAACGCTCAAG
ATATTACGTCCGAACCCGTTGCTCGTGTGATTATTCCCCAACGTGTGCCCTACGGATT
TCACGGTACTTGGGTCAGCGAAGAACAGCTCATGACAAACAGCTAA

***Calothrix brevissima* NIES-22 carotenoid oxygenase**

Amino acid sequence of the protein BAY62420.1 (SEQ ID NO: 22):

MTSNIISTTADNPYLSGNFAPVGQEIATDSLPVLGELPPDLSGMFVRNGPNPQWPPIGNY
HWFDGDGMLHCVHVSEGKATYRNRYVQTQGWKIEREAGKAVWSGLLEPPQMDNPYG
AYKRTANTALIWHGGQMLALHEGSAPHAIKVPELETIGEYTYNDKLVSAFTAHPKVDP
VTGEMMFFGYSFTPPYLQYSVVSAVGELLQTVPIDLPVAVMMHDFAITKNYSIFLDLPLT
MRGERLQRGEPLFMFERDRPSRFGIIPRYGNNSNIRWFESEPCYIFHIYNAYEIGDEVVLL
GCRMSSTTVLGDDNSSDPDANVPRLHEWRFNLKTGTVSDQRLDDIPAEFPRINENYVGL
PTRYGYAGKAANTPVPLFDGLIKYDFSSGKSQTHEFGQGRFGGEAVFAPRPGATAEDDG
WLITFVHDETSDTSELLVVHAQDVSSEPVARVIIPQRVPYGFHGAWISEAQLKGA

Codon-optimized nucleotide sequence of BAY62420.1 for *Yarrowia lipolytica* (SEQ ID NO: 23):

ATGACTAGCAATATCATTTCCACTACAGCTGATAATCCCTACCTGTCCGGAAATTTC
GCTCCTGTCGGCCAGGAAATCGCCACAGATTCCCTCCCCGTCCTGGGTGAACTCCCT
CCCGATCTGAGCGGTATGTTCGTCCGGAATGGACCCAACCCCCAATGGCCTCCTATC
GGAAACTATCATTGGTTCGACGGAGATGGTATGCTGCACTGTGTTCACGTTTCGGAA
GGAAAAGCCACTTATAGAAATCGTTATGTGCAGACCCAGGGTTGGAAAATTGAGCG
GGAAGCTGGAAAGGCTGTTTGGTCGGGTCTGCTCGAACCTCCTCAGATGGACAATCC
TTACGGTGCATATAAAAGAACCGCTAATACAGCACTGATTTGGCACGGAGGTCAAA
TGCTCGCACTCCATGAGGGATCTGCACCCCACGCTATTAAGGTCCCCGAGCTGGAAA
CGATCGGCGAGTACACATATAATGATAAGCTGGTGTCGGCTTTTACGGCACATCCCA
AAGTGGATCCCGTGACGGGCGAGATGATGTTCTTTGGCTACTCCTTTACACCCCCCT
ATCTGCAATATTCGGTTGTGAGCGCTGTTGGTGAGCTGCTTCAGACTGTGCCCATCG
ATCTCCCCGTCGCCGTTATGATGCATGACTTTGCCATCACTAAAAACTACAGCATTTT
CCTGGATCTTCCTCTTACTATGCGTGGAGAGCGTCTTCAACGGGGAGAACCTCTTTTT
ATGTTTGAGCGGGACCGTCCTTCTCGATTTGGTATTATCCCTCGTTATGGTAATAATT
CTAATATTCGTTGGTTTGAAAGCGAACCTTGCTATATTTTCCACATCTACAACGCTTA
TGAAATCGGAGACGAGGTCGTGCTCCTGGGTTGTCGAATGAGCTCTACCACCGTTCT
GGGAGATGACAACTCGTCGGACCCCGACGCCAATGTTCCTCGACTTCATGAGTGGA
GATTTAATCTGAAGACGGGAACGGTGTCTGATCAACGACTTGACGACATTCCTGCTG
AATTCCCTCGAATCAACGAGAATTATGTCGGCCTCCCCACCAGATATGGCTACGCAG

GAAAGGCCGCAAACACGCCTGTCCCCCTCTTCGATGGACTTATTAAGTATGATTTTT
CGTCGGGTAAGAGCCAAACCCATGAATTTGGACAGGGCCGTTTTGGCGGCGAAGCT
GTCTTCGCACCCAGACCTGGTGCTACTGCTGAAGATGATGGATGGCTCATCACCTTT
GTCCACGACGAAACGTCTGACACCTCCGAACTTCTTGTCGTTCACGCTCAGGATGTG
TCTTCTGAGCCCGTTGCCCGAGTCATCATTCCTCAGCGGGTCCCCTACGGTTTCCACG
GAGCATGGATTTCTGAAGCTCAACTGAAAGGAGCCTAA

***Scytonema millei* 9-cis-epoxycarotenoid dioxygenase**

Amino acid sequence of the protein WP_069349917.1 (SEQ ID NO: 24):

MATTQVNPYLDGNFAPVREESTANTLQVIGELPPDLSGMFVRNGPNPQWTPIGKYHWF
DGDGMLHGVRISNGKATYRNRYVRTKKWKIENEAGKALLSGLLEPPQKKNPPGASKNT
ANTALVWHAGQLLALWEGGAPHAIRVPELKTKGEYTYNGKLASAFTAHPKVDPVTGE
MMFFGYGFAPPYLQYSVVSPEGELLQTEPIDIPMAVMMHDFAITQDYTIFMDLPLTFSQE
RRKRGEPMMKFERDKPSRFGIVPRYGNNSNIRWFESPACYIFHTLNAYEEGDEVVLIACR
MNSTTVLDVPQDTHTDSEADIPRLHRWRFNLKTGKVSEEMLDDTASEFPRINENLLGQK
TRYGYTGKMAKSSMPLFDGLIKYDFNTGKSQTHEFGRGRYGGEAVFVPRPGATAEDDG
WLVTFVHDTVEETSELVVVSAQDITGEPVARVLIPQRVPYGFHGAWVSEEQLKASV

Codon-optimized nucleotide sequence of WP_069349917.1 for *Yarrowia lipolytica* (SEQ ID NO: 25):

ATGGCAACTACCCAGGTTAACCCCTACCTGGACGGAAACTTCGCCCCCGTGAGAGA
AGAGAGCACCGCTAATACACTGCAGGTCATCGGAGAGCTTCCCCCTGATCTCTCGGG
CATGTTCGTTAGAAATGGTCCCAACCCTCAATGGACACCTATCGGTAAGTATCACTG
GTTTGACGGCGATGGCATGCTCCACGGCGTGCGTATCAGCAATGGCAAAGCTACCTA
TAGAAATCGGTACGTTCGGACCAAAAAGTGGAAGATTGAGAATGAAGCAGGAAAA
GCTCTCCTTTCTGGCCTTCTTGAACCCCCTCAAAAGAAAAACCCCCCTGGCGCTTCC
AAGAACACCGCCAATACCGCCCTTGTGTGGCATGCCGGACAACTTCTGGCTCTTTGG
GAAGGTGGTGCACCTCACGCAATTAGAGTCCCTGAGCTCAAGACGAAAGGTGAGTA
TACGTACAATGGAAAGCTGGCCAGCGCCTTTACAGCCCATCCCAAGGTTGATCCTGT
CACAGGAGAGATGATGTTTTTTGGTTACGGCTTTGCCCCTCCTTATCTGCAATATTCG
GTCGTTTCTCCCGAAGGCGAACTGCTGCAAACAGAGCCCATTGACATTCCTATGGCC
GTCATGATGCACGATTTTGCCATTACCCAGGATTACACCATTTTTATGGACCTGCCCC
TTACGTTTTCTCAGGAACGTCGGAAACGAGGTGAGCCCATGATGAAATTCGAGCGG
GATAAACCCTCGCGTTTCGGAATTGTCCCCAGATACGGAAATAATAGCAACATCCGT
TGGTTTGAAAGCCCTGCTTGTTACATTTTCCATACCCTCAACGCATACGAAGAAGGT
GACGAGGTGGTTCTTATCGCTTGCCGGATGAACTCGACAACAGTGCTTGATGTCCCT
CAAGACACCCATACCGATAGCGAGGCTGATATCCCCCGACTGCATCGATGGCGTTTT
AACCTTAAGACTGGAAAAGTCTCGGAAGAGATGCTTGATGACACGGCATCCGAATT
TCCTCGAATTAATGAAAACCTTCTGGGTCAGAAAACACGATATGGATACACCGGCA
AAATGGCTAAAAGCAGCATGCCTCTTTTCGACGGCCTCATTAAGTATGACTTTAACA
CGGGTAAATCCCAGACACACGAATTTGGACGTGGACGATATGGTGGTGAAGCCGTG
TTCGTCCCTCGACCTGGTGCCACCGCCGAGGATGATGGTTGGCTTGTCACGTTTGTCC
ACGACACTGTCGAGGAAACGTCGGAGCTCGTTGTCGTGTCTGCACAGGACATCACTG
GTGAACCCGTGGCCAGAGTCCTCATTCCTCAGCGGGTTCCCTATGGCTTCCATGGAG
CTTGGGTGAGCGAAGAACAGCTGAAGGCCTCGGTTTGA

***Lactuca sativa* lycopene epsilon-cyclase**

Codon-optimized nucleotide sequence of Lactuca sativa lycopene epsilon-cycalse for *Yarrowia lipolytica* (SEQ ID NO: 26):

ATGAAGTGCTCCGCGAAGTCCGATCGTTGCGTGGTGGATAAGCAGGGTATCTCCGTG
GCCGACGAAGAGGATTACGTCAAGGCCGGAGGCTCCGAACTTTTCTTCGTCCAGATG
CAGCGGACCAAGTCTATGGAGTCACAGAGCAAGTTGAGCGAAAAGCTCGCTCAGAT
TCCAATCGGAAACTGCATTTTGGATTTGGTTGTCATCGGCTGCGGCCCAGCTGGCCT
CGCTTTGGCGGCGGAATCGGCAAAGCTTGGCCTCAACGTGGGCCTGATCGGTCCAG
ATCTGCCATTCACCAACAACTACGGTGTGTGGCAGGACGAGTTCATTGGCCTGGGTC
TTGAAGGCTGCATCGAACACTCTTGGAAGGACACCCTCGTCTACCTGGATGACGCCG
ATCCAATCCGCATTGGACGTGCTTACGGACGCGTTCACCGTGACCTCCTGCACGAAG
AGCTGCTGCGTCGCTGCGTTGAGTCCGGCGTGTCTTACCTCAGCAGCAAGGTTGAAC
GCATCACCGAAGCACCAAACGGCTACTCACTCATCGAGTGCGAAGGTAACATCACC
ATCCCTTGCCGCCTCGCAACCGTGGCTTCGGGCGCCGCCTCCGGCAAGTTCCTGGAG
TACGAGTTGGGCGGACCTCGCGTGTGCGTGCAGACCGCGTACGGCATCGAGGTTGA
GGTCGAAAACAACCCATACGACCCTGATCTGATGGTTTTCATGGACTACCGTGACTT
CTCGAAGCACAAGCCTGAATCCCTTGAGGCAAAGTACCCAACTTTCCTTTACGTCAT
GGCTATGTCCCCAACCAAGATCTTCTTCGAGGAAACTTGCCTCGCTTCTCGCGAAGC
TATGCCTTTCAACTTGTTGAAGAGCAAGCTGATGTCCCGCCTGAAGGCTATGGGCAT
CCGCATCACCCGCACCTACGAGGAAGAATGGTCGTACATCCCGGTGGGCGGCAGCC
TGCCAAACACCGAGCAGAAGAACCTCGCCTTCGGTGCAGCAGCCTCGATGGTGCAC
CCAGCCACCGGTTACTCTGTGGTTCGCTCTCTGAGCGAAGCACCAAACTACGCCGCC
GTTATTGCGAAGATCCTGCGCCAGGACCAGTCAAAGGAGATGATTTCCCTCGGTAAG
TACACTAACATCTCCAAGCAGGCCTGGGAAACTCTCTGGCCACTTGAGCGCAAGCG
CCAGCGTGCCTTCTTCCTCTTCGGCTTGTCACACATCGTGCTCATGGACCTGGAAGG
CACTCGCACTTTCTTCCGTACCTTCTTCCGCCTGCCTAAGTGGATGTGGTGGGGCTTC
CTGGGCTCTTCGCTTTCCTCCACCGATTTGATTATCTTCGCTCTCTACATGTTCGTCAT
CGCTCCGCACTCCTTGCGTATGGAGCTCGTCCGTCACCTGTTGTCCGATCCGACCGG
TGCCACTATGGTGAAGGCATACCTGACCATCTAA

***E. coli* Thioredoxin (TrxA) tag**

Amino acid sequence of TrxA (SEQ ID NO. 27):

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKL
NIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA

Codon-optimized nucleotide sequence of trxA gene for *Yarrowia lipolytica* (SEQ ID NO: 28):

ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAA
GCGGACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAAT
GATCGCCCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGC
AAAACTGAACATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTA
TCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCA
CTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCG

Conserved domain in Zea mays oleosin

Amino acid sequence of conserved domain in Zea mays oleosin (SEQ ID NO. 29):

MAVAKALATATAAFSMLLLSGLAVTGTVLALIVATPLMVIFSPVLVPAAITVALLTVGIV
SSGGFGVAAVAVLAWVYRYL

Conserved domain in Sesamum indicum oleosin

Amino acid sequence of conserved domain in Sesamum indicum oleosin (SEQ ID NO. 30):

TQILAIITLLPISGTLLCLAGITLVGTLIGLAVATPVFVIFSPVLVPAAILIAGAVTAFLTSGA
FGLTGLSSLSWVLNSF

***Arabidopsis thaliana* caleosin**

Amino acid sequence of Arabidopsis thaliana caleosin (SEQ ID NO. 31):

MGSKTEMMERDAMATVAPYAPVTYHRRARVDLDDRLPKPYMPRALQAPDREHPYGT
PGHKNYGLSVLQQHVSFFDIDDNGIIYPWETYSGLRMLGFNIIGSLIIAAVINLTLSYATLP
GWLPSPFFPIYIHNIHKSKHGSDSKTYDNEGRFMPVNLELIFSKYAKTLPDKLSLGELWE
MTEGNRDAWDIFGWIAGKIEWGLLYLLARDEEGFLSKEAIRRCFDGSLFEYCAKIYAGIS
EDKTAYY

Nucleotide sequence of Arabidopsis thaliana caleosin (SEQ ID NO. 32):

ATGGGGTCAAAGACGGAGATGATGGAGAGAGACGCAATGGCTACGGTGGCTCCCTA
TGCGCCGGTCACTTACCATCGCCGTGCTCGTGTTGACTTGGATGATAGACTTCCTAA
ACCTTATATGCCAAGAGCATTGCAAGCACCAGACAGAGAACACCCGTACGGAACTC
CAGGCCATAAGAATTACGGACTTAGTGTTCTTCAACAGCATGTCTCCTTCTTCGATAT
CGATGATAATGGCATCATTTACCCTTGGGAGACCTACTCTGGACTGCGAATGCTTGG
TTTCAATATCATTGGGTCGCTTATAATAGCCGCTGTTATCAACCTGACCCTTAGCTAT
GCCACTCTTCCGGGGTGGTTACCTTCACCTTTCTTCCCTATATACATACACAACATAC
ACAAGTCAAAGCATGGAAGTGATTCAAAAACATATGACAATGAAGGAAGGTTTATG
CCGGTGAATCTTGAGTTGATATTTAGCAAATATGCGAAAACCTTGCCAGACAAGTTG
AGTCTTGGAGAACTATGGGAGATGACAGAAGGAAACCGTGACGCTTGGGACATTTT
TGGATGGATCGCAGGCAAAATAGAGTGGGGACTGTTGTACTTGCTAGCAAGGGATG
AAGAAGGGTTTTTGTCAAAAGAAGCTATTAGGCGGTGTTTCGATGGAAGCTTGTTCG
AGTACTGTGCCAAAATCTACGCTGGTATCAGTGAAGACAAGACAGCATACTACTAA

***Arabidopsis thaliana* caleosin**

Amino acid sequence of Sesamum indicum caleosin (SEQ ID NO. 33):

MATHVLAAAAERNAALAPDAPLAPVTMERPVRTDLETSIPKPYMARGLVAPDMDHPN
GTPGHVHDNLSVLQQHCAFFDQDDNGIIYPWETYSGLRQIGFNVIASLIMAIVINVALSY
PTLPGWIPSPFFPIYLYNIHKAKHGSDSGTYDTEGRYLPMNFENLFSKHARTMPDRLTLN

LFSKHARTMPDRLTLFGWIASKMEWTLLYILARDQDGFLSKEAIRRCYDGSLFEYCAKM
QRGAEDKMK

Nucleotide sequence of Sesamum indicum caleosin (SEQ ID NO. 34):

ATGGCAACTCATGTTTTGGCTGCTGCGGCGGAGAGAAATGCTGCGTTGGCGCCGGAC
GCCCCGCTTGCTCCGGTGACTATGGAGCGCCCAGTGCGCACTGACTTGGAGACTTCG
ATCCCGAAGCCCTATATGGCAAGAGGATTGGTTGCACCTGATATGGATCACCCCAAC
GGAACACCAGGCCATGTGCATGATAATTTGAGTGTGCTGCAACAGCATTGTGCTTTC
TTTGATCAGGATGATAACGGAATCATCTATCCATGGGAGACTTACTCTGGACTTCGC
CAAATTGGTTTCAATGTGATAGCTTCCCTTATAATGGCTATCGTCATTAATGTGGCGC
TGAGTTATCCTACTCTCCCGGGTTGGATTCCTTCTCCTTTTTTCCCCATATATTTGTAC
AACATACACAAGGCCAAACATGGAAGCGACTCCGGAACCTATGATACTGAAGGAAG
GTACCTACCTATGAATTTTGAGAACCTGTTCAGCAAGCATGCCCGGACAATGCCCGA
TAGGCTCACTCTAGGGGAGCTATGGAGCATGACTGAAGCTAACAGAGAAGCATTTG
ACATTTTCGGCTGGATCGCAAGCAAAATGGAGTGGACTCTCCTCTACATTCTTGCAA
GAGACCAGGACGGTTTCCTGTCGAAAGAAGCCATCAGGCGGTGTTACGATGGCAGT
TTGTTCGAGTACTGTGCAAAGATGCAAAGGGGAGCCGAGGACAAGATGAAATGA

CLAIMS

1. A recombinant microbial production host cell for producing an ionone compound, said host cell comprising at least one nucleic acid construct comprising a first coding sequence, wherein said first coding sequence encodes a fusion enzyme comprising a first domain capable of functioning as a lipid body compartmentalization signal tag (LBT) fused to a second domain having carotenoid cleavage activity, wherein said first domain is selected from the group consisting of a lipid body structural protein, an oleaginicity-inducing lipid droplet protein, an orange carotenoid binding protein, and a membrane transporter protein, and wherein said first domain does not comprise a glutathione-S-transferase (GST), a small ubiquitin-like modifier (SUMO) protein, a maltose binding protein (MBP), or thioredoxin (TrxA).
2. The host cell of claim 1, wherein the first domain comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9.
3. The host cell of claim 1, wherein the first domain comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 31, or SEQ ID NO: 33.
4. The host cell of claim 1, wherein the first domain is an oleosin polypeptide comprising an amino acid sequence having at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30.
5. The host cell of claim 1, wherein the first domain comprises a caleosin from *Arabidopsis thaliana*, *Ricinus communis*, *Glycine max*, *Sesamum indicum*, *Coix lacryma*, *Aspergillus niger*, or *Neurospora crassa*.
6. The host cell of claim 1, wherein the second domain is a carotenoid cleavage dioxygenase or a carotenoid oxygenase.
7. The host cell of claim 6, wherein the second domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24.
8. The host cell of claim 1, wherein the second domain comprises a CCD1 enzyme from *Petunia x hybrid*, *Osmanthus fragrans*, *Zea mays*, *Arabidopsis thaliana*, *Vitis vinifera*, *Daucus carota*, *Lycopersicon esculentum*, or *Rubus idaeus*, or a CCD4 enzyme from

Arabidopsis thaliana, *Bixa Orellana*, *Chrysanthemum morifolium*, *Crocus sativus*, *Musa acuminata*, *Malus domestica*, *Prunus persica*, *Rosa damascene*, *Vitis vinifera*, and *Osmanthus fragrans*.

9. The host cell of claim 1, wherein the first domain is fused to the second domain via a linker comprising 3 to 30 amino acids in length and comprising amino acids selected from the group of glycine, serine, threonine, and combinations thereof.
10. The host cell of claim 9, wherein the linker comprises a glysine-serine linker.
11. The host cell of claim 1, wherein said first coding sequence is operably linked to a TEF promoter.
12. The host cell of claim 1 for producing alpha-ionone, further comprising in the same nucleic acid construct or a different nucleic acid construct a second coding sequence encoding a lycopene epsilon-cyclase or a fusion enzyme comprising a lycopene epsilon-cyclase domain fused to a carotenoid cleavage dioxygenase or a carotenoid oxygenase domain.
13. The host cell of claim 1 for producing beta-ionone, further comprising in the same nucleic acid construct or a different nucleic acid construct a second coding sequence encoding a phytoene synthase.
14. The host cell of claim 13, further comprising in the same nucleic acid construct or a different nucleic acid construct a third coding sequence encoding a phytoene dehydrogenase.
15. The host cell of any one of claims 1-14, further comprising one or more nucleic acid constructs comprising one or more coding sequences, wherein said one or more coding sequences are selected from mevalonate pathway enzymes native to the host cell.
16. The host cell of claim 15, wherein said native mevalonate pathway enzymes are selected from the group consisting of hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), and isopentenyl-diphosphate-isomerase (IPI).
17. The host cell of claim 16, further comprising one or more nucleic acid constructs comprising one or more coding sequences, wherein said one or more coding sequences encode one or more enzymes selected from the group consisting of farnesyl diphosphate synthase (FPPS), geranylgeranyl diphosphate synthase (GGPPS), a GGPPS::FPPS fusion

enzyme, phytoene dehydrogenase (CarB), a bifunctional lycopene cyclase/phytoene synthase (CarRP), and a mutant CarRP having no lycopene cyclase activity (CarRP*).

18. The host cell of any one of claims 1-17, wherein the host cell is a yeast.

19. The host cell of any one of claims 1-17, wherein the host cell is selected from the group consisting of *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum*.

20. The host cell of claim 19, wherein the host cell is *Yarrowia lipolytica*.

21. A recombinant microbial production host cell for producing a carotenoid compound, said host cell comprising at least one nucleic acid construct comprising a coding sequence encoding a fusion enzyme comprising an oleosin polypeptide or a caleosin polypeptide fused to a beta-carotene ketolase or a beta-carotene hydroxylase.

22. The host cell of claim 21 comprising a first coding sequence encoding a first fusion enzyme comprising an oleosin polypeptide fused to a beta-carotene ketolase and a second coding sequence encoding a second fusion enzyme comprising an oleosin polypeptide or a caleosin polypeptide fused to a beta-carotene hydroxylase.

23. The host cell of claim 21 or 22, wherein the beta-carotene ketolase is an algal or bacterial BKT or CrtW.

24. The host cell of claim 23, wherein the beta-carotene ketolase is from *Haematococcus pluvialis*, *Chlorella zofingiensis*, *Chlamydomonas reinhardtii*, *Paracoccus sp.*, and *Pantoea ananatis*.

25. The host cell of claim 21 or 22, wherein the beta-carotene hydroxylase is an algal CrtR-B.

26. The host cell of claim 21 or 22, wherein the algal CrtR-b is from *Haematococcus pluvialis* or *Chlamydomonas reinhardtii.*

27. The host cell of any one of claims 21-26, wherein the oleosin polypeptide comprises the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30, and the caleosin polypeptide comprises the amino acid sequence of SEQ ID NO: 31 or SEQ ID NO: 33.

28. The host cell of claim 21, wherein the carotenoid compound is astaxanthin.

29. The host cell of claim 21, wherein the carotenoid compound is canthaxanthin.

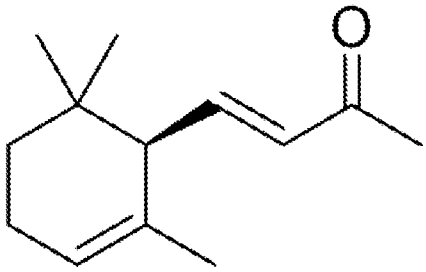
30. The host cell of any one of claims 21-29, wherein the host cell is *Yarrowia lipolytica*.

31. A synthetic or recombinant nucleic acid molecule comprising a first nucleic acid sequence and a second nucleic acid sequence, wherein the first nucleic acid sequence and the second nucleic acid sequence together encode a fusion enzyme, said fusion enzyme comprising a first domain capable of functioning as a lipid body compartmentalization signal tag and a second domain having carotenoid cleavage activity, wherein said first domain is selected from the group consisting of a lipid body structural protein, an oleaginicity-inducing lipid droplet protein, an orange carotenoid binding protein, and a membrane transporter protein, and wherein said first domain does not comprise a glutathione-S-transferase (GST), a small ubiquitin-like modifier (SUMO) protein, a maltose binding protein (MBP), or thioredoxin (TrxA).
32. The nucleic acid molecule of claim 31, wherein the first domain is a lipid body structural body having a molecule weight of less than 25 kDa.
33. The nucleic acid molecule of claim 32, wherein the first domain comprises the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30.
34. The nucleic acid molecule of claim 33, wherein the first domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 3.
35. The nucleic acid molecule of claim 33 or 34, wherein the first nucleic acid sequence comprises a nucleic acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 2 or SEQ ID NO: 4.
36. The nucleic acid molecule of claim 31, wherein the first domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 31, or SEQ ID NO: 33.
37. The nucleic acid molecule of claim 36, wherein the first nucleic acid sequence comprises a nucleic acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 32, or SEQ ID NO: 34.
38. The nucleic acid molecule of any one of claims 31-37, wherein the second domain is a carotenoid cleavage dioxygenase.
39. The nucleic acid molecule of claim 38, wherein the second domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 11, SEQ ID NO: 14, or SEQ ID NO: 16.

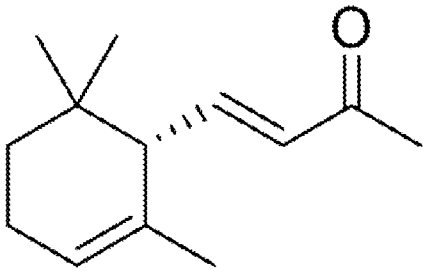
40. The nucleic acid molecule of claim 39, wherein the second nucleic acid sequence comprises a nucleic acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, or SEQ ID NO: 17.
41. The nucleic acid molecule of any one of claims 31-37, wherein the second domain is a carotenoid oxygenase or a 9-cis-epoxycarotenoid dioxygenase.
42. The nucleic acid molecule of claim 41, wherein the second domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24.
43. The nucleic acid molecule of claim 42, wherein the second nucleic acid sequence comprises a nucleic acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, or SEQ ID NO: 25.
44. The nucleic acid molecule of claim 31, wherein the second domain comprises a CCD1 enzyme from *Petunia x hybrid*, *Osmanthus fragrans*, *Zea mays*, *Arabidopsis thaliana*, *Vitis vinifera*, *Daucus carota*, *Lycopersicon esculentum*, or *Rubus idaeus*, or a CCD4 enzyme from *Arabidopsis thaliana*, *Bixa Orellana*, *Chrysanthemum morifolium*, *Crocus sativus*, *Musa acuminata*, *Malus domestica*, *Prunus persica*, *Rosa damascene*, *Vitis vinifera*, and *Osmanthus fragrans*.
45. The nucleic acid molecule of any one of claims 31-44 further comprising a third nucleic acid sequence operably linked to the first nucleic acid sequence at one end and the second nucleic acid sequence at another end, wherein the third nucleic acid sequence encodes a linker that couples the first domain to the second domain.
46. The nucleic acid molecule of claim 45, wherein the linker comprises 3 to 30 amino acids in length.
47. The nucleic acid molecule of claim 45 or 46, wherein the linker comprises amino acids selected from the group of glycine, serine, threonine, and combinations thereof.
48. The nucleic acid molecule of any one of claims 45-47, wherein the linker comprises a glysine-serine linker, optionally comprising one or more units of GGGGS.
49. The nucleic acid molecule of any one of claims 45-48, wherein the linker is GGGGS.
50. The nucleic acid molecule of any one of claims 45-49, wherein the linker is GGGGSGGGGSGGGGSGGGGS.

51. A nucleic acid construct comprising the nucleic acid molecule of any one of claims 31-50 operably linked to a heterologous nucleic acid sequence, optionally wherein said heterologous nucleic acid sequence is a promoter sequence.
52. The nucleic acid construct of claim 51, further comprising a heterologous nucleic acid sequence encoding an epsilon-cyclase or a phytoene synthase.
53. A fusion enzyme comprising a lipid body compartmentalization signal tag (LBT) domain coupled to a carotenoid cleavage dioxygenase (CCD) domain.
54. The fusion enzyme of claim 53, wherein the LBT domain comprises the amino acid sequence of SEQ ID NO: 29 or SEQ ID NO: 30.
55. The fusion enzyme of claim 54, wherein the LBT domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 3.
56. The fusion enzyme of claim 53, wherein the LBT domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 31, or SEQ ID NO: 33.
57. The fusion enzyme of any one of claims 53-56, wherein the CCD domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 11, SEQ ID NO: 14, or SEQ ID NO: 16.
58. The fusion enzyme of any one of claims 53-56, wherein the CCD domain comprises an amino acid sequence exhibiting at least 80% sequence identity to SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, or SEQ ID NO: 24.
59. The fusion enzyme of any one of claims 53-58, wherein the LBT domain is coupled to the CCD domain via a linker.
60. The fusion enzyme of claim 59, wherein the linker comprises 3 to 30 amino acids in length.
61. The fusion enzyme of claim 59 or 60, wherein the linker comprises amino acids selected from the group of glycine, serine, threonine, and combinations thereof.
62. The fusion enzyme of claim 61, wherein the linker comprises a glysine-serine linker.
63. The fusion enzyme of claim 62, wherein the linker optionally comprising one or more units of GGGGS.
64. A recombinant *Yarrowia lipolytica* cell comprising a nucleic acid molecule according to any one of claims 31 to 50 or a nucleic acid construct according to claim 51 or 52.

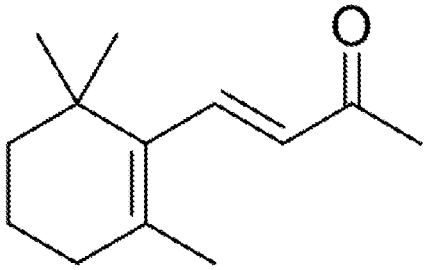
65. A method for producing an ionone compound, wherein the method comprises culturing a recombinant host cell of any one of claims 1-20 in a medium comprising glucose or glycerol under conditions whereby an ionone compound is produced.
66. The method of claim 65, wherein the ionone compound is alpha-ionone.
67. The method of claim 65, wherein the ionone compound is beta-ionone.
68. A method for producing a carotenoid compound, wherein the method comprises culturing a recombinant host cell of any one of claims 21-30 in a medium comprising glucose or glycerol under conditions whereby a carotenoid compound is produced.
69. The method of claim 68, wherein the carotenoid compound is astaxanthin.
70. The method of claim 68, wherein the carotenoid compound is canthaxanthin.



(*R*)-alpha-ionone



(*S*)-alpha-ionone



beta-ionone

FIG. 1

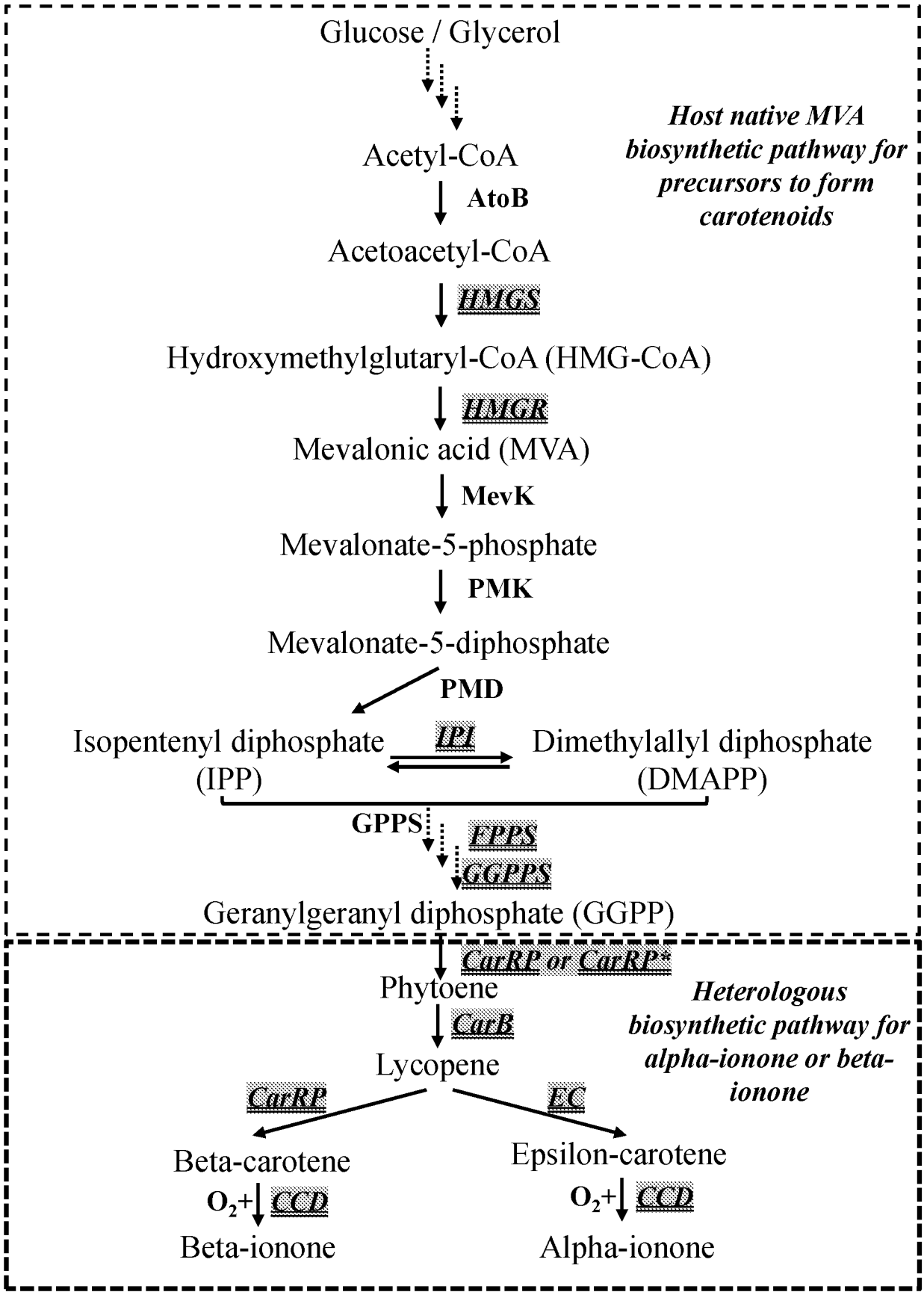

Glucose / Glycerol
Acetyl-CoA
AtoB
Acetoacetyl-CoA
HMGS
Hydroxymethylglutaryl-CoA (HMG-CoA)
HMGR
Mevalonic acid (MVA)
MevK
Mevalonate-5-phosphate
PMK
Mevalonate-5-diphosphate
PMD
Isopentenyl diphosphate (IPP)
IPI
Dimethylallyl diphosphate (DMAPP)
GPPS
FPPS
GGPPS
Geranylgeranyl diphosphate (GGPP)
Host native MVA biosynthetic pathway for precursors to form carotenoids
CarRP or CarRP*
Phytoene
CarB
Lycopene
CarRP
EC
Beta-carotene
Epsilon-carotene
O2+ CCD
O2+ CCD
Beta-ionone
Alpha-ionone
Heterologous biosynthetic pathway for alpha-ionone or beta-ionone

FIG. 2

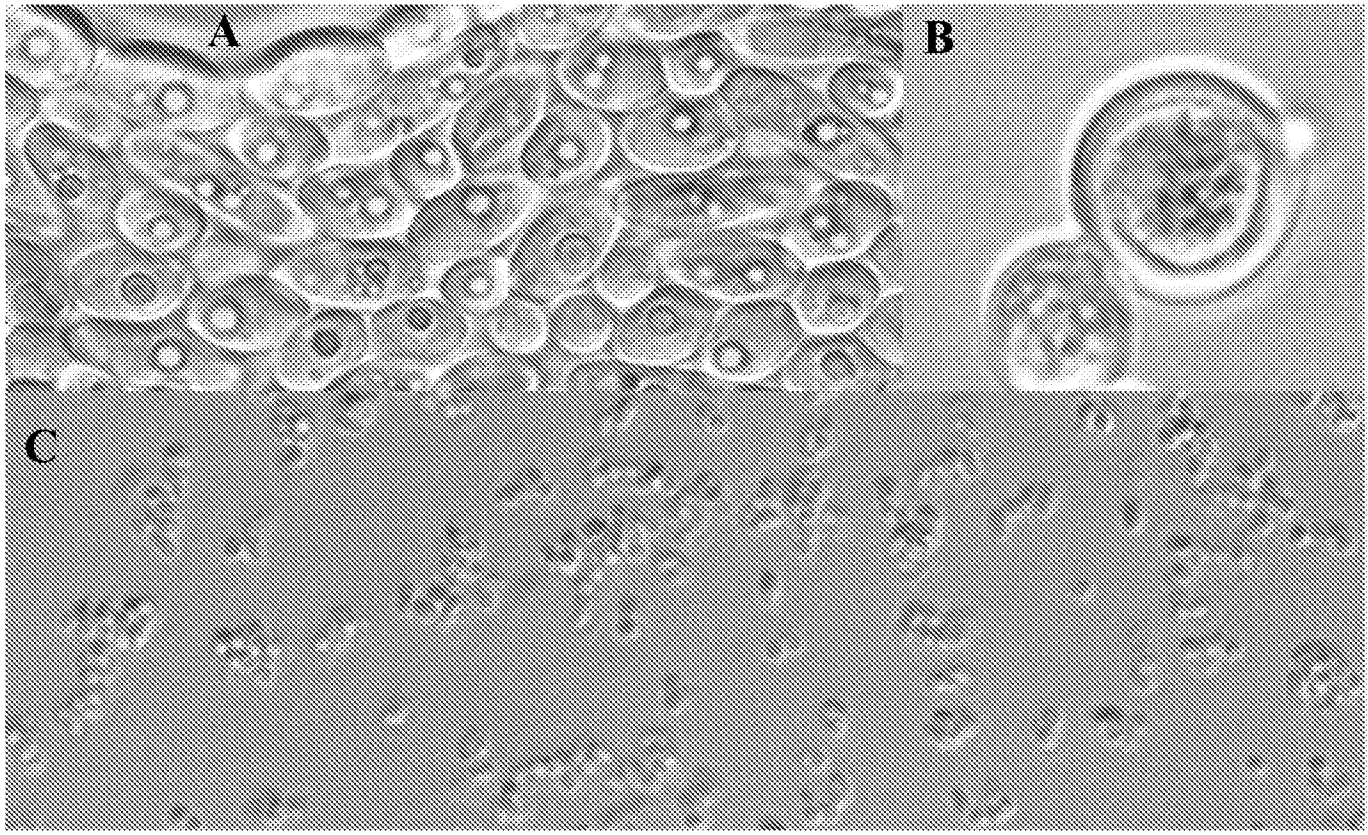
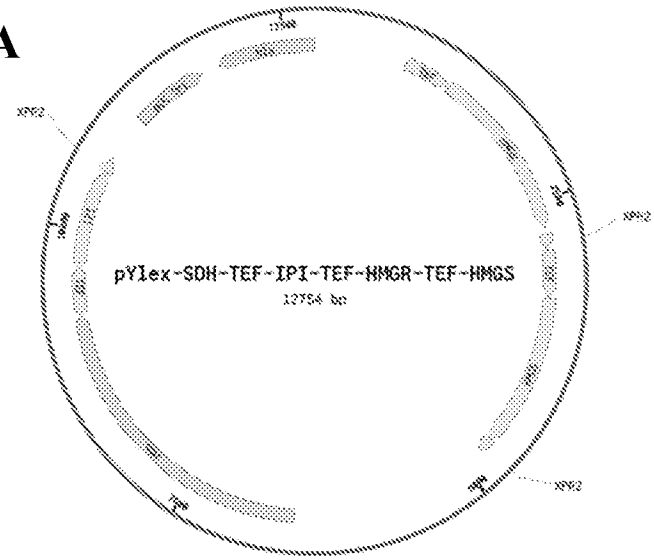


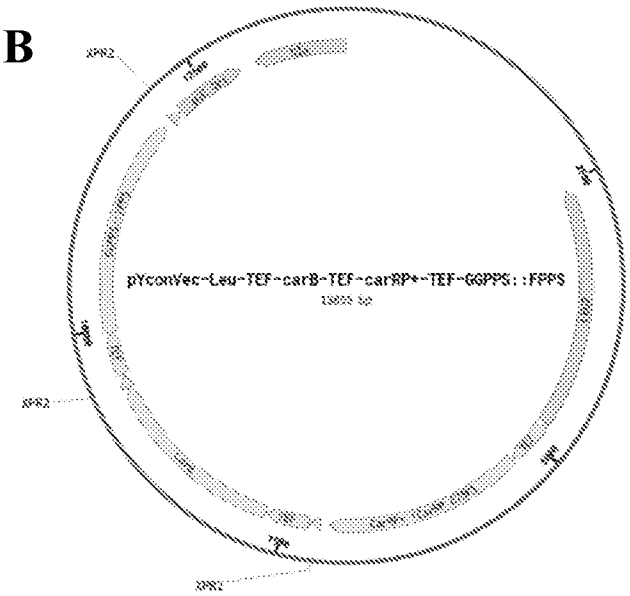
FIG. 3

A



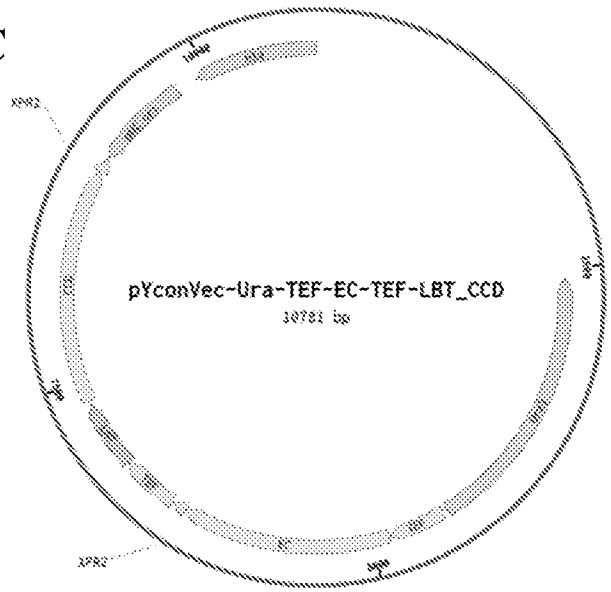
Sequence Name	Function
TEF	Constitutive promoter
XPR2	Terminator
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
IPI	Isopentenyl-diphosphate-isomerase
pUC Ori	Origin of replication
bla	Ampicillin resistance gene
SDH	Auxotrophic lysine marker

B



Sequence Name	Function
TEF	Constitutive promoter
XPR2	Terminator
CarRP*	Phytoene synthase (mutant carRP)
CarB	Phytoene dehydrogenase
GGPPS::FPPS	A fusion protein comprising FPPS fused to GGPPS
pUC Ori	Origin of replication
bla	Ampicillin resistance gene
LEU2	Auxotrophic leucine marker

C



Sequence Name	Function
TEF	Constitutive promoter
XPR2	Terminator
EC	Lycopene epsilon-cyclase
LBT	Putative lipid body compartmentalization signal tag
CCD	Carotenoid cleavage dioxygenase
pUC Ori	Origin of replication
bla	Ampicillin resistance gene
URA3	Auxotrophic uracil marker

FIG. 4

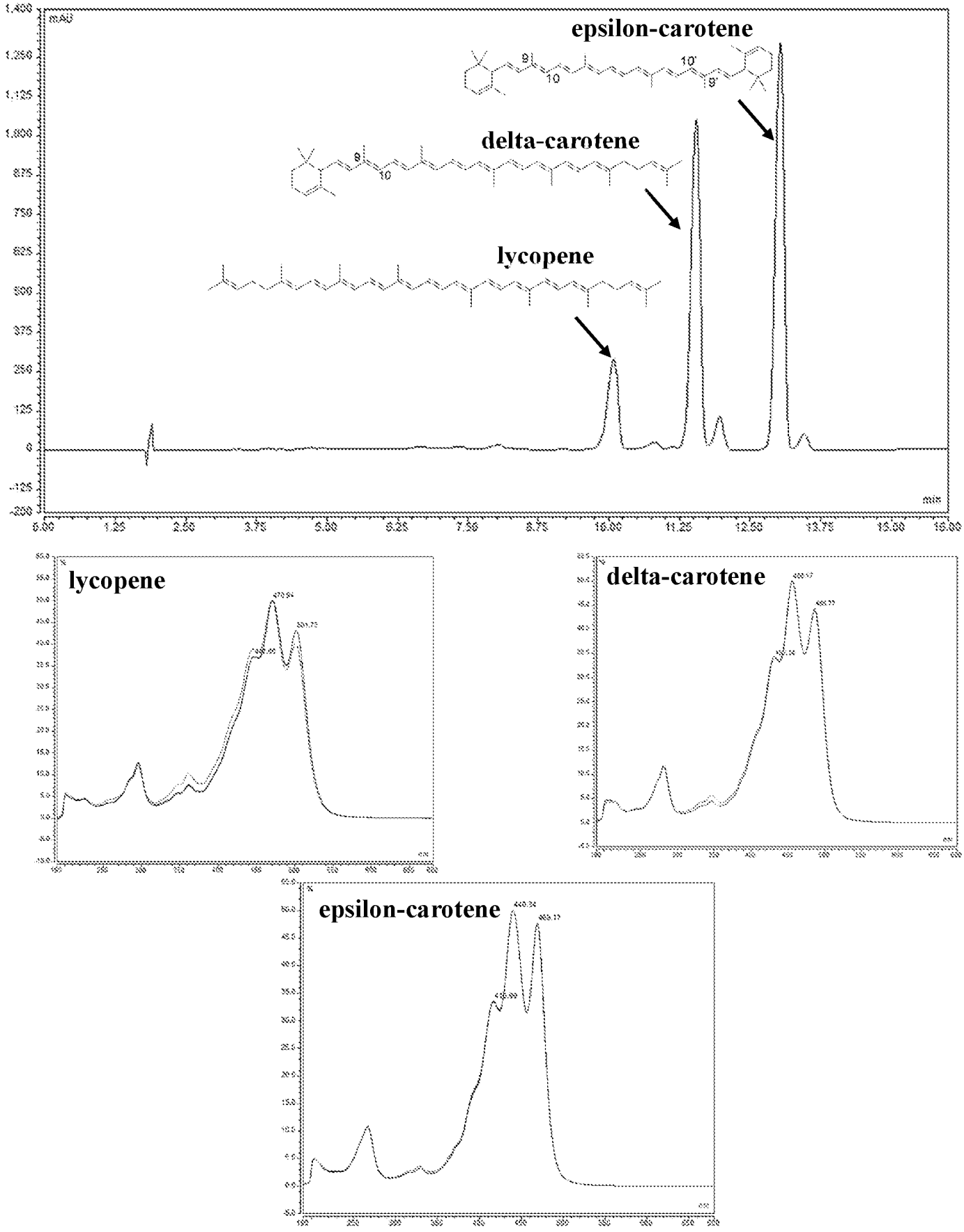
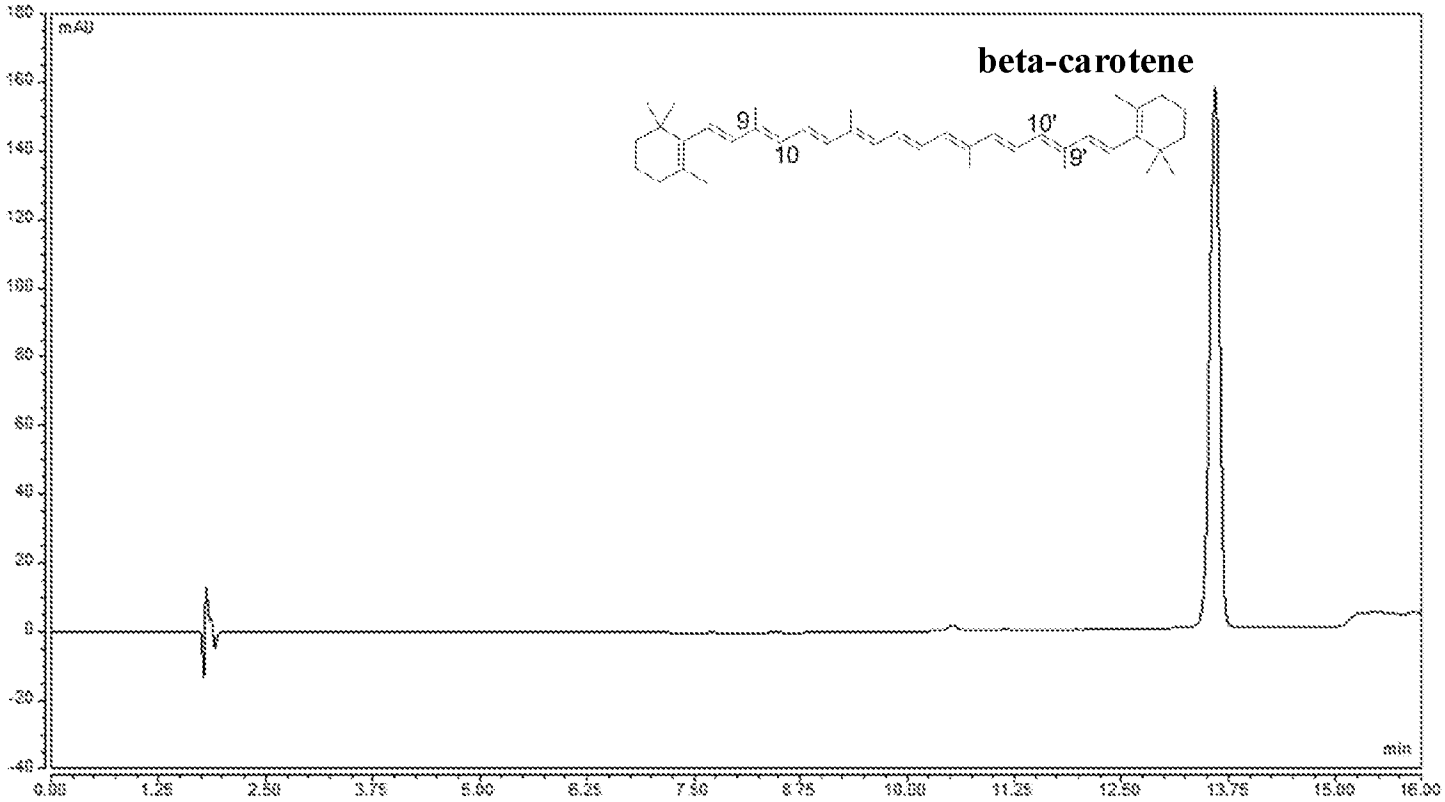

epsilon-carotene
delta-carotene
lycopene
lycopene
delta-carotene
epsilon-carotene

FIG. 5


beta-carotene
9
10
10'
9'
mAU
min

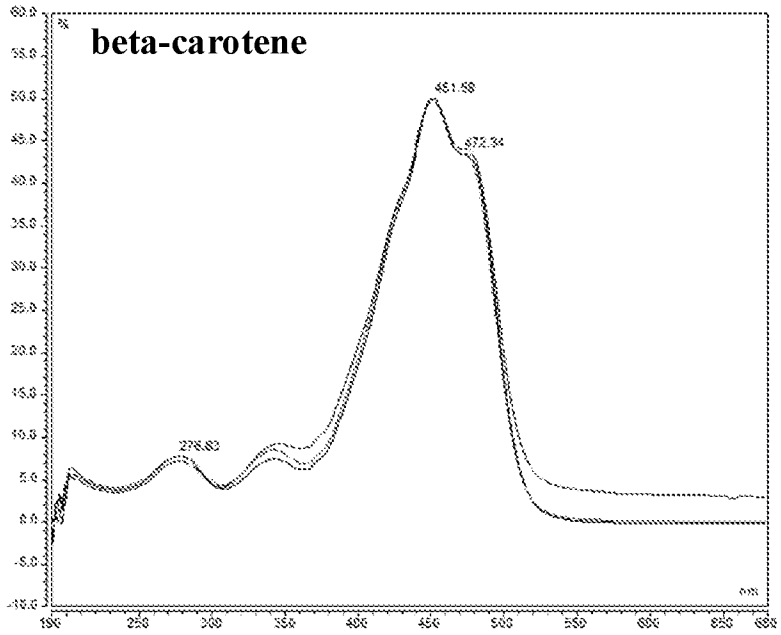

beta-carotene
451.58
482.34
276.80
nm

FIG. 6

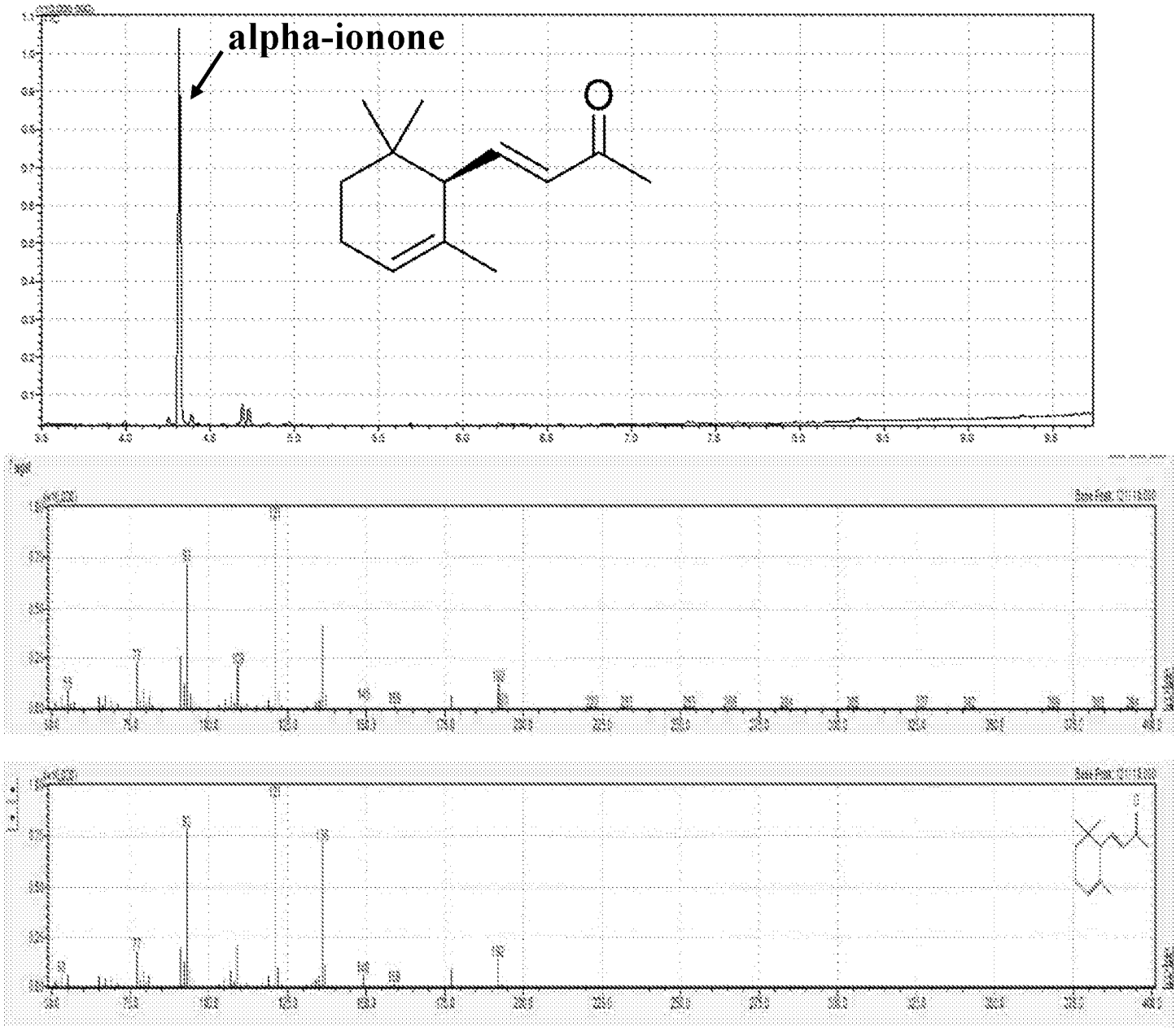
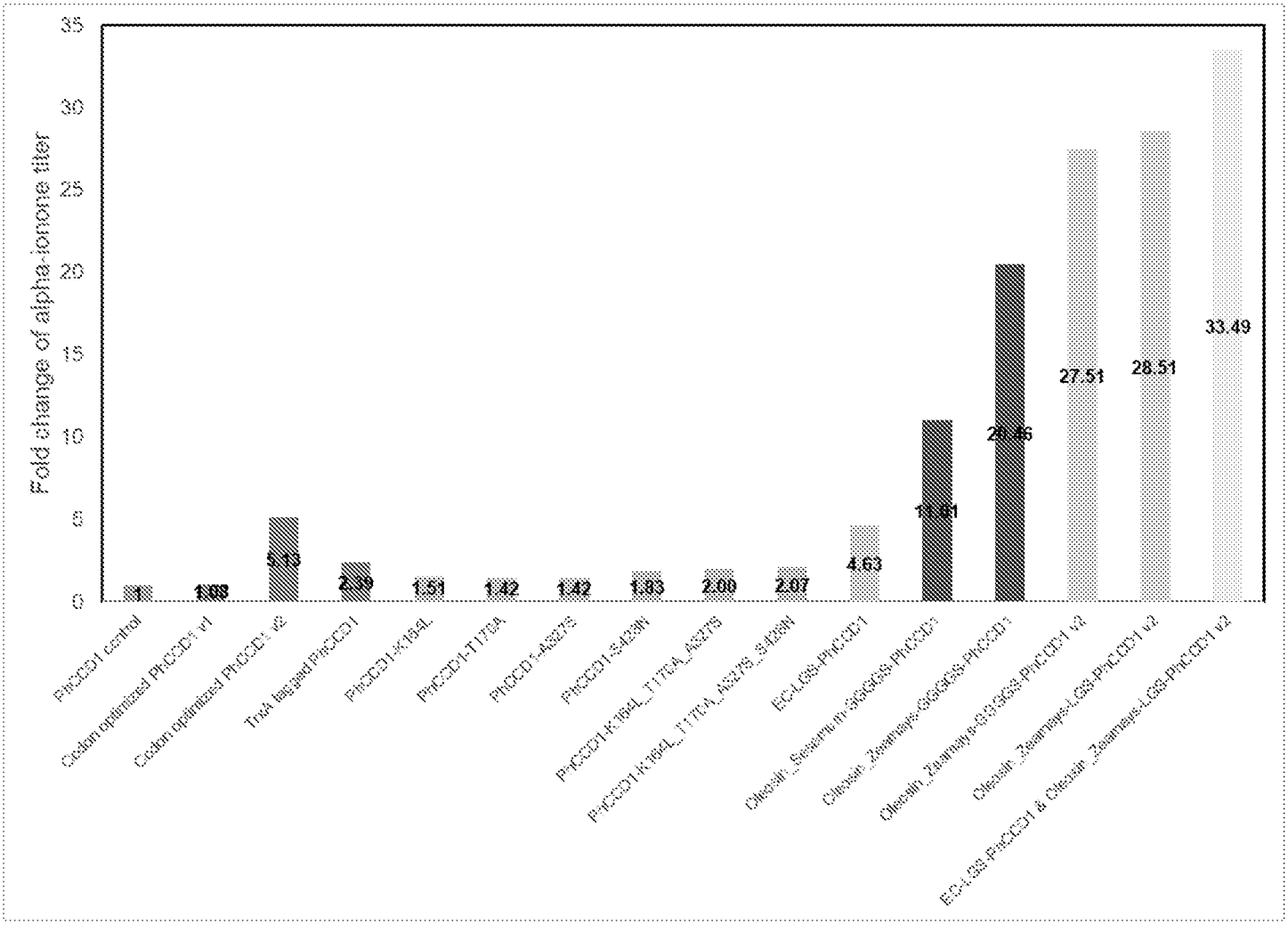


FIG. 7



Engineering strategy	Enzyme variants	Fold change of alpha-ionone titer
Control strain	PhCCD1 control	1
Codon optimization	Codon optimized PhCCD1 v1	1.08
	Codon optimized PhCCD1 v2	5.13
Solubility enhancement	TrxA tagged PhCCD1	2.39
Site-directed mutagenesis	PhCCD1-K164L	1.51
	PhCCD1-T170A	1.42
	PhCCD1-A327S	1.42
	PhCCD1-S428N	1.83
	PhCCD1-K164L_T170A_A327S	2.00
	PhCCD1-K164L_T170A_A327S_S428N	2.07
Fusing CCD1 and EC	EC-LGS-PhCCD1*	4.63
Lipid body compartmentalization signal tags	Oleosin_Sesamum-GGGGS-PhCCD1	11.01
	Oleosin_Zeamays-GGGGS-PhCCD1	20.46
Combination of beneficial strategies	Oleosin_Zeamays-GGGGS-PhCCD1 v2	27.51
	Oleosin_Zeamays-LGS-PhCCD1 v2	28.51
	EC-LGS-PhCCD1 & Oleosin_Zeamays-LGS-PhCCD1 v2	33.49

*LGS=GGGGSGGGGSGGGGSGGGGS

FIG. 8

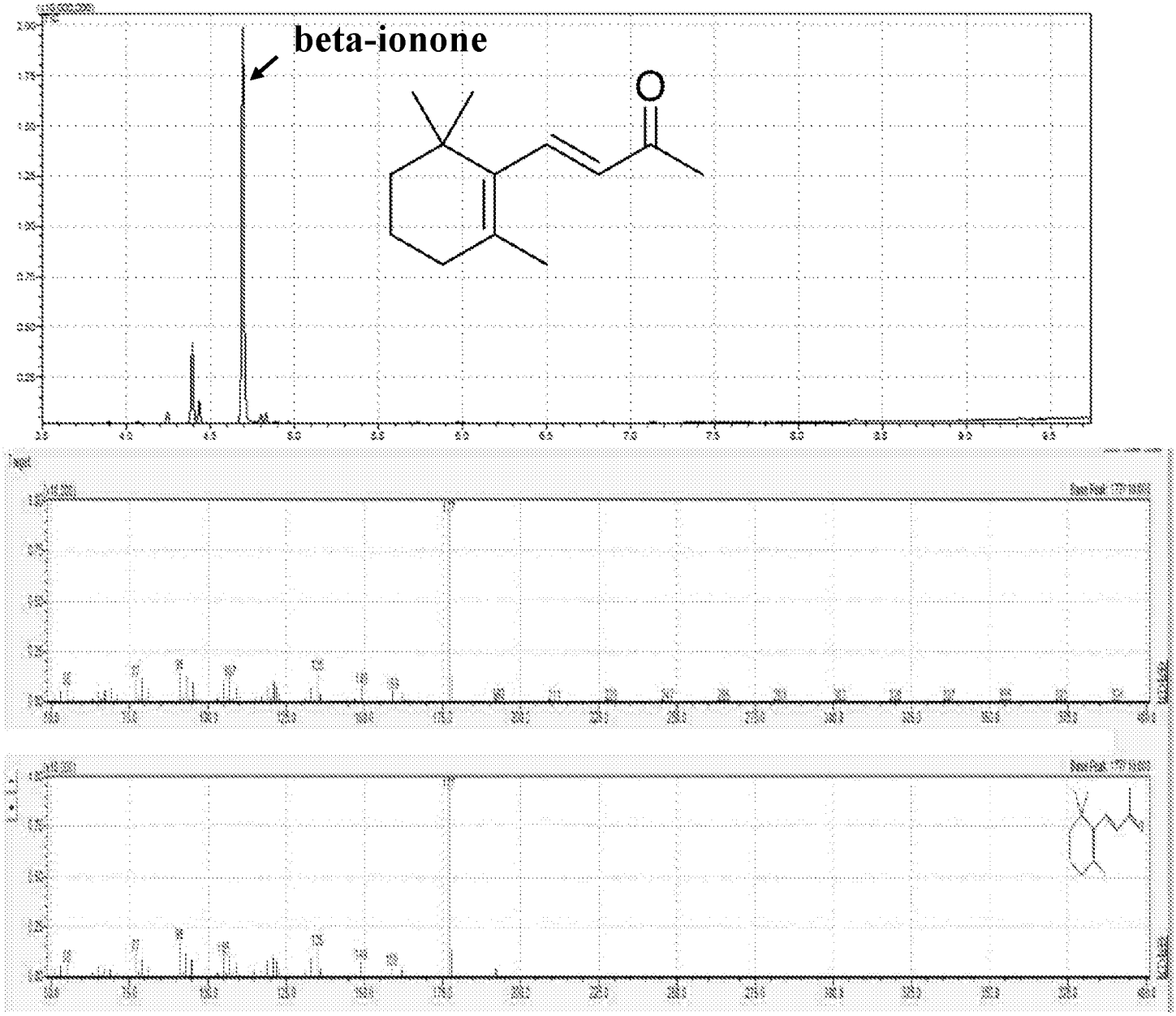


FIG. 9

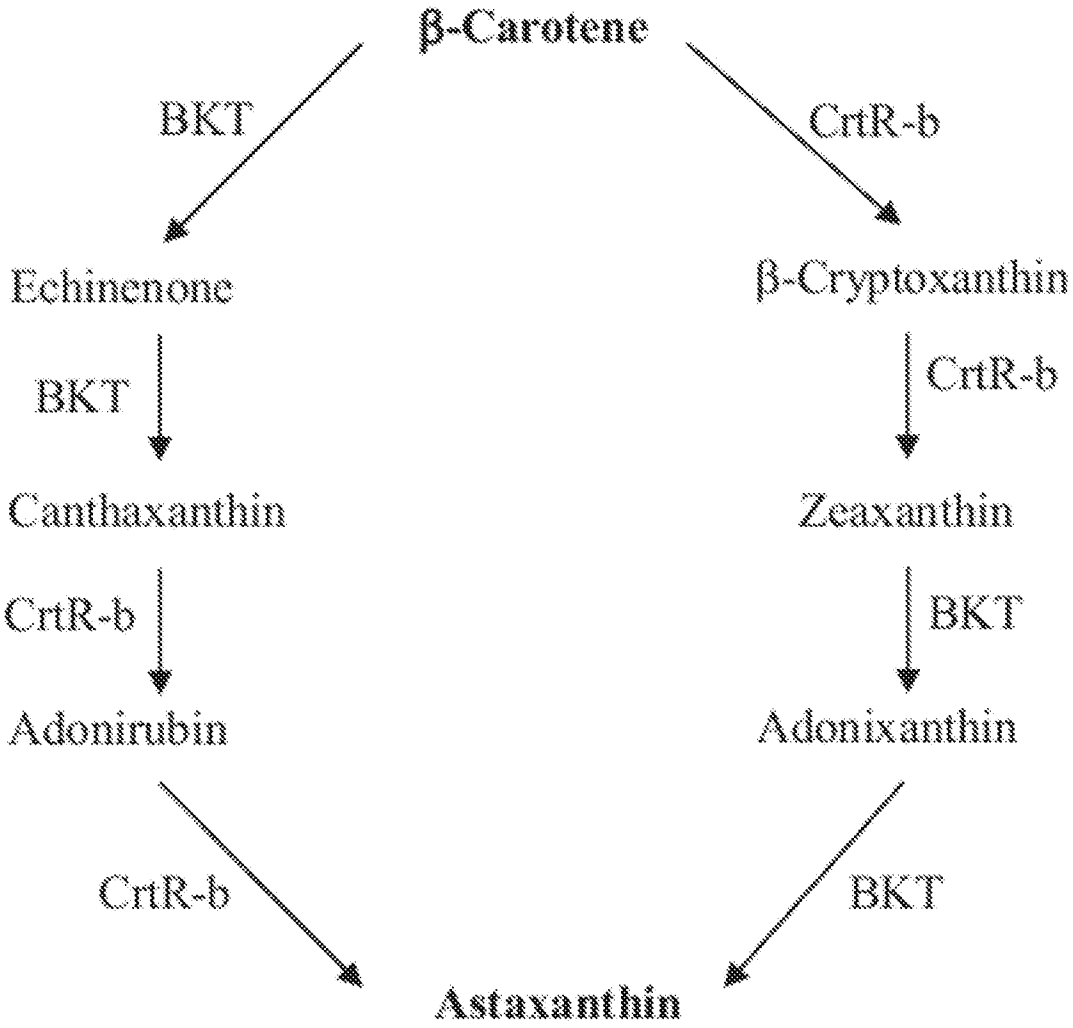

β-Carotene
BKT
CrtR-b
Echinenone
β-Cryptoxanthin
BKT
CrtR-b
Canthaxanthin
Zeaxanthin
CrtR-b
BKT
Adonirubin
Adonixanthin
CrtR-b
BKT
Astaxanthin

FIG. 10

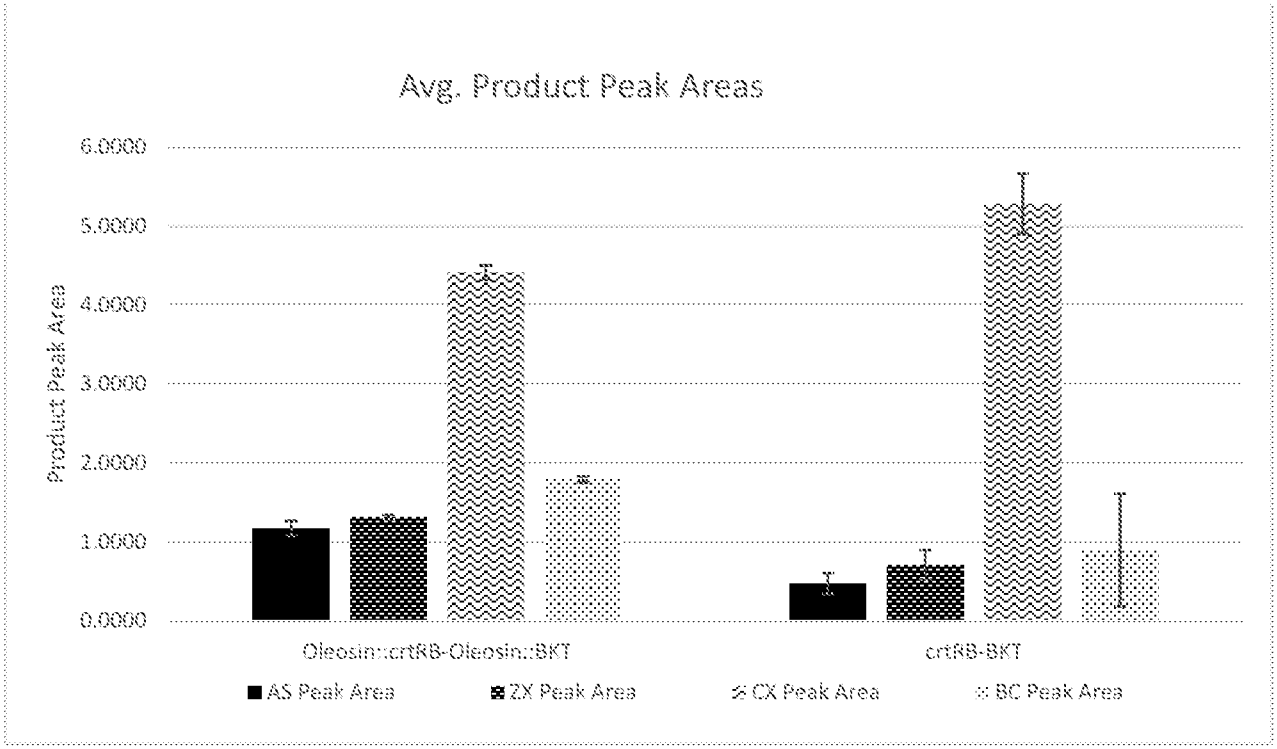

Avg. Product Peak Areas
Product Peak Area
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
0.0000
Oleosin::crtRB-Oleosin::BKT
crtRB-BKT
AS Peak Area
ZX Peak Area
CX Peak Area
BC Peak Area

FIG. 11

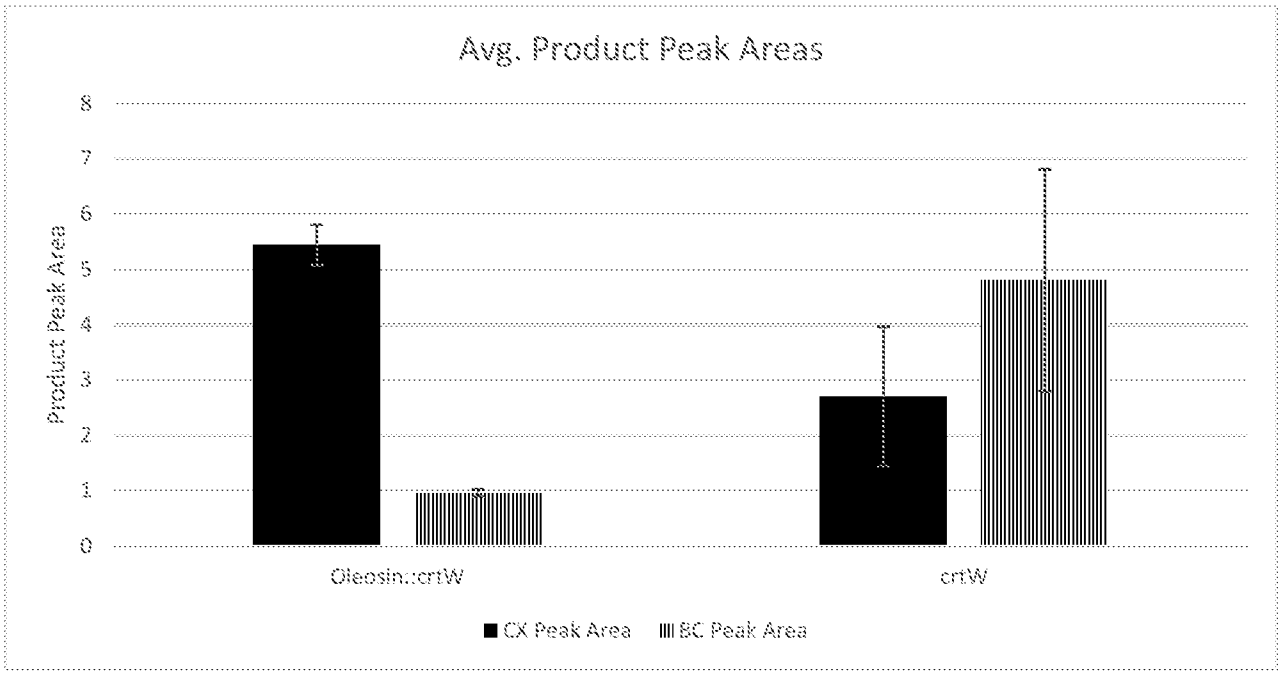

Avg. Product Peak Areas
Product Peak Area
8
7
6
5
4
3
2
1
0
Oleosin::crtW
crtW
CX Peak Area
BC Peak Area

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/54323

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:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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 forming part of 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/US 20/54323

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.: 18-20, 27, 30, 38-43, 45-52, 59-70
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

--continued on extra sheet--

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 6, 7, 9-12, 15-17(in part), 31, 36, limited to SEQ ID NOs: 5, 11, and alpha-ionone

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/54323

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 1/18, C12N 15/63, C12N 15/81, C12N 9/10 (2021.01)

CPC - C12N 15/52, C12N 15/81, C12N 9/0069, C12N 9/1085

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2019/0194699 A1 (CENTROME, INC. DBA ADVANCED BIOTECH INC.) 27 June 2019 (27.06.2019) abstract; para [0011]-[0016]; [0045]; claims 1-13; SEQ ID NO: 10	1-3, 6, 7, 9-12, 15-17, 31, 36
Y	CATALDO et al. Chemical vs. biotechnological synthesis of C 13-apocarotenoids: current methods, applications and perspectives. Applied microbiology and biotechnology. 1 July 2016, Vol 100, No 13, pp 5703-18; entire document, especially p. 11, col 2, para 3; p. 12, col 1, para 1	1-3, 6, 7, 9-12, 15-17, 31, 36
Y	UniProtKB Accession No P83689, Red carotenoid-binding protein, 03 April 2007 [online]. [Retrieved on 03 February 2021]. Retrieved from the internet: <URL: https://www.uniprot.org/uniprot/P83689> Entire document; sequence	2, 3, 15/(2,3), 36
Y	WO 2017/029129 A1 (GLYCOTOPE GMBH) 23 February 2017 (23.02.2017) p 2, para 5-6; p 3, para 2-3; p 3, para 6; p 10, para 7	9, 10, 15/(9,10)
Y	US 6,376,222 B1 (BABYAK et al.) 23 April 2002 (23.04.2002) claims 1-5	11, 15/11
Y	US 2014/0234928 A1 (DSM IP ASSETS B.V.) 21 August 2014 (21.08.2014) claims 19-20; para [0026]; [0072]; [0163]	16, 17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"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
04 February 2021	01 MAR 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

--continued from: Box No. III Observations where unity of invention is lacking--

Group I+, claims 1-17, 21-26, 28, 29, 31-37, 44, directed to a recombinant microbial production host cell for producing a carotenoid compound, said host cell comprising at least one nucleic acid construct comprising a coding sequence encoding a fusion enzyme comprising a first domain capable of functioning as a lipid body compartmentalization signal tag (LBT) fused to a second domain having enzymatic activity, or a synthetic or recombinant nucleic acid molecule encoding said fusion enzyme. The host cell and nucleic acid molecule will be searched to the extent that the first domain encompasses SEQ ID NO: 5 (an orange carotenoid binding protein), (note, this is the first claimed first domain sequence, claim 2), the second domain encompasses SEQ ID NO: 11 (a carotenoid cleavage dioxygenase), and the carotenoid compound produced encompasses alpha-ionone. It is believed that claims 1-3, 6, 7, 9-12, 15-17(in part), 31, 36 encompass this first named invention, and thus these claims will be searched without fee to the extent the first and second domains encompasses SEQ ID NOs: 5 and 11, and the product encompasses alpha-ionone. Additional first and/or second domain(s) and/or carotenoid compound(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected first and/or second domain(s) and/or carotenoid compound(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. 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. An exemplary election would be a first domain comprising SEQ ID NO: 7 (a membrane transporter protein), a second domain comprising SEQ ID NO: 11, and the product alpha-ionone (claims 1-3, 6, 7, 9-12, 15-17(in part), 31, 36).

Group II, claims 53-58, directed to a fusion enzyme.

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

Special technical features

Group I+ has the special technical feature of a composition comprising or consisting of a synthetic or recombinant nucleic acid molecule, that is not required by Group II.

Group II has the special technical feature of a composition consisting of a fusion enzyme, that is not required by Group I+.

The inventions of Group I+ each include the special technical feature of a unique fusion enzyme encoding nucleic acid sequence, and is considered a distinct technical feature.

Common technical features

The inventions of Group I+ and Group II share the common technical feature of a (nucleic acid sequence encoding) a fusion enzyme comprising a lipid body compartmentalization signal tag (LBT) domain coupled to a carotenoid cleavage dioxygenase (CCD) domain.

No technical features are shared between the fusion enzyme encoding nucleic acid sequences of Group I+ and accordingly, these groups lack unity a priori.

Additionally, even if the inventions listed as Group I+ were considered to share the technical features of including: a recombinant microbial production host cell for producing an ionone compound, said host cell comprising at least one nucleic acid construct comprising a first coding sequence, wherein said first coding sequence encodes a fusion enzyme comprising a first domain capable of functioning as a lipid body compartmentalization signal tag (LBT) fused to a second domain having carotenoid cleavage activity, wherein said first domain is selected from the group consisting of a lipid body structural protein, an oleaginicity inducing lipid droplet protein, an orange carotenoid binding protein, and a membrane transporter protein, and wherein said first domain does not comprise a glutathione-Stransferase (GST), a small ubiquitin-like modifier (SUMO) protein, a maltose binding protein (MBP), or thioredoxin (TrxA), these shared technical features are previously disclosed by the prior art, as further discussed below.

The feature shared by Groups I+ and II and the feature shared by the inventions listed as Group I+ are made obvious by US 2019/0194699 A1 to Centrome, Inc. dba Advanced Biotech Inc. (hereinafter 'Centrome'), in view of the article by Cataldo et al., entitled "Chemical vs. biotechnological synthesis of C 13-apocarotenoids: current methods, applications and perspectives", (Applied microbiology and biotechnology. 1 July 2016, Vol 100, No 13, pp 5703-18), (hereinafter 'Cataldo').

Centrome teaches a recombinant microbial production host cell for producing an ionone compound (claim 1 "method of producing enantiomerically pure (R)-(E)-(+)-alpha-ionone in Saccharomyces cervisiae"), said host cell comprising at least one nucleic acid construct comprising a first coding sequence that encodes an enzyme domain having carotenoid cleavage activity (CCD) (claim 1 "constructing recombinant yeast cells that overexpress native nucleic acids, or modified versions thereof, encoding at least one enzyme of the mevalonate pathway for synthesizing farnesyl pyrophosphate...modifying the recombinant yeast cells from step (a) to further comprise heterologous nucleic acids that encode enzymes of an apocarotenoid pathway for synthesizing alpha-ionone"; claim 13 "wherein the recombinant yeast cells are transformed to include nucleic acids having the sequences of... SEQ ID NO: 10", note that SEQ ID NO: 10, amino acids 1-546, is 100% identical to Applicant carotenoid cleavage sequence SEQ ID NO: 11 (see Applicant claim 7). Centrome does not specifically teach said first coding sequence encodes a fusion enzyme that further comprises a (first) domain capable of functioning as a lipid body compartmentalization signal tag (LBT) fused to the (second) domain having carotenoid cleavage activity, wherein said first domain is selected from the group consisting of a lipid body structural protein, an oleaginicity inducing lipid droplet protein, an orange carotenoid binding protein, and a membrane transporter protein, and wherein said first domain does not comprise a glutathione-Stransferase (GST), a small ubiquitin-like modifier (SUMO) protein, a maltose binding protein (MBP), or thioredoxin (TrxA). However, Centrome does teach the host cell is engineered to express multiple enzymes (claim 1).

--continued on next sheet--

--continued from previous sheet--

Cataldo discloses engineered microbial cells with increased synthesis of cartenoids including B-ionone due to expression of an exogenous enzyme having carotenoid cleavage activity (CCD) (p. 11, col 2, para 3 "optimization of the biosynthetic pathway of B-carotene...the oxidative cleavage of B-carotene could be increased by using CCDs with higher activity than those currently employed"). Cataldo further teaches high-level production of B-carotene in S.cerevisiae leads to B-carotene accumulation that causes membrane stress and a perturbation of membrane structure and dynamics that restricts the final yield (p. 12, col 1, para 1 "high-level production of B-carotene in S.cerevisiae leads to membrane stress and induces a pleiotropicdrug resistance response (Verwaal et al. 2010). This suggests that B-carotene accumulation may cause a perturbation of membrane structure and dynamics and restrict the final yield"), and suggests protein engineering to modify CCDs to achieve better accessibility, including addition of coding sequences with membrane binding activity (p. 12, col 1, para 1 "strategies to overcome this limitation. One alternative could be carotenoid-binding proteins (Bhosale and Bernstein 2007) that can buffer beta-carotene localization. However, the substrate accessibility of CCDs could decrease, and the high-energy cost associated with extra protein synthesis could be detrimental. An alternative approach benefits from the fact that the structural model of maize CCD1 shows a putative hydrophobic patch for membrane binding (Messing et al. 2010) that might be necessary to access membrane-embedded substrates. Accordingly, CCDs can be modified by protein engineering to achieve better accessibility"). In view of Cataldo, it would have been obvious to one of ordinary skill in the art to have modified the CCD coding sequence taught by Centrome to include a sequence that encodes a domain with carotenoid-binding activity, including those suggested by Cataldo, such that the host cell produces a fusion of the CCD enzyme comprising the domain with carotenoid-binding activity, in order to reduce B-carotene accumulation that causes membrane stress and a perturbation of membrane structure and dynamics that restricts the final yield.

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

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

NOTE, continuation of item 4 above: claims 18-20, 27, 30, 38-43, 45-52, 59-70 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).