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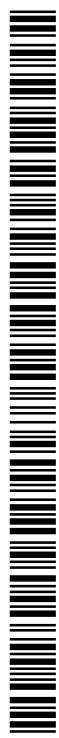
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(54) Title: NOVEL ENZYME

(57) Abstract: The present invention is related to novel oxygenase involved in biosynthetic production of retro-carotenoids, in particular rhodoxanthin. The invention also features polynucleotides and the corresponding polypeptides comprising the full-length sequences of the novel gene/polypeptide and fragments thereof, in particular functional equivalents of said gene/polypeptide. The invention further relates to genetically engineered microorganisms and their use, in particular for biosynthesis of retro-carotenoids, such as e.g. rhodoxanthin.



NOVEL ENZYME

The present invention is related to novel oxygenase involved in biosynthetic production of retro-carotenoids, in particular rhodoxanthin. The invention also features polynucleotides and the corresponding polypeptides comprising the full-length sequences of the novel gene/polypeptide and fragments thereof, in particular functional equivalents of said gene/polypeptide. The invention further relates to genetically engineered microorganisms and their use, in particular for biosynthesis of retro-carotenoids, such as e.g. rhodoxanthin.

10 Retro-carotenoids are carotenoids with a shift of one position of the single and double bonds of the respective conjugated polyene. There are indications that some of them have stronger antioxidative activity for lipid peroxidation induced by free radical and singlet oxygen than that of β -carotene, i.e. the non-retro type carotenoids.

15 Rhodoxanthin, an example of a retro-carotenoid, and which is found in nature in e.g. arils, berries, leaves or flowers of the poisonous yew (*Taxus*), Aloe or honeysuckle (*Lonicera* sp.), is widely used as a coloring material for foodstuffs and beverages as well as pharmaceutical and cosmetic preparations, imparting to them a yellow to red coloration. As a food additive, it is used under the E number E161f as a food coloring.

20 Chemical synthesis of rhodoxanthin is known since the 1970s (see e.g. US3941841A). Currently, there exists rising demand for so-called "natural" colorants, especially in the food, beverage or cosmetic sector, i.e. colorants which are produced biotechnologically, i.e. avoiding consumption of energy, water, organic and/or inorganic solvents, synthesis of undesired side products and the like as often the disadvantage of chemical processes.

The isolation of rhodoxanthin from its natural source has proven extremely disadvantageous. One main reason is that rhodoxanthin occurs only in small amounts in these plants or part of plants (berries, leaves, arils). Therefore, a great quantity of e.g. berries must be utilized in order to isolate a small amount of rhodoxanthin. Additionally, the process whereby rhodoxanthin is isolated from e.g. berries of green plants has proven extremely cumbersome and uneconomical. 30 Until now, there is no procedure known for biosynthesis of rhodoxanthin.

Thus, there is a strong need for a biotechnological production of rhodoxanthin and other retro-carotenoids, which can be used as e.g. coloring material in the food &

beverage, pharmaceutical and cosmetic industry in order to replace the chemical produced rhodoxanthin.

Surprisingly, we have now identified a novel gene encoding a polypeptide with enzymatic activity towards biosynthesis of retro-carotenoids, in particular

5 rhodoxanthin, from carotenoids, such as e.g. beta-carotene, which can be used in a novel biotechnological process towards production of retro-carotenoids, in particular rhodoxanthin, in a suitable host cell as defined herein.

In particular, the invention is directed to a hydroxylating enzyme, e.g. an oxygenase, such as e.g. a polypeptide having hydroxylase activity, in particular
10 beta-carotene hydroxylase activity, which is selected from a polypeptide with at least 63%, such as e.g. at least 65, 70, 75 80, 85, 90, 95, 97, 98, 99% or up to 100% identity to SEQ ID NO:2 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:1, in particular a recombinant nucleic acid molecule. In another embodiment, the invention is directed to a hydroxylating
15 enzyme, e.g. an oxygenase, such as e.g. a polypeptide having hydroxylase activity, in particular beta-carotene hydroxylase activity, which is selected from a polypeptide with at least 78 %, such as e.g. at least 80, 85, 90, 95, 97, 98, 99% or up to 100% identity to SEQ ID NO:4 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:3. In another embodiment, the invention is
20 directed to a hydroxylating enzyme, e.g. an oxygenase, such as e.g. a polypeptide having hydroxylase activity, in particular beta-carotene hydroxylase activity, which is selected from a polypeptide with at least 78 %, such as e.g. at least 80, 85, 90, 95, 97, 98, 99% or up to 100% identity to SEQ ID NO:6 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:5. Surprisingly, the
25 novel enzyme as defined herein is capable of extending the oxidation reaction beyond the formation of hydroxyl groups and to catalyzing the bond shift associated with retro-carotenoids and the two ketolation reactions leading to rhodoxanthin. This is a unique feature for beta-carotene hydroxylases.

The novel enzyme having beta carotene hydroxylase activity might be obtainable
30 from any carotenoid producing organism, including plant, algae, fungi or bacteria. Preferably, the polypeptide having hydroxylase activity, preferably beta-carotene hydroxylase activity, is obtainable from plants such as Taxus, in particular flowering plants, including but not limited to the genus Aloe, preferably Lonicera, such as e.g. from rhodoxanthin-producing tissues including berries, in particular
35 said polypeptide and/or the gene encoding said enzyme having hydroxylase

activity, as defined herein is originated from *Lonicera* sp., including but not limited to *L. morrowii* or *L. tatarica*.

Thus, in a particular embodiment, the polypeptide and/or the gene encoding said polypeptide acting as oxygenase, such as e.g. having hydroxylase activity, in particular such as e.g. beta-carotene hydroxylase activity, more particularly a
5 polypeptide with at least 63% identity to a polypeptide according to SEQ ID NO:2 is isolated from *Lonicera* sp., such as e.g. from *L. morrowii*, in particular from berries of *L. morrowii*.

The novel gene can be identified by isolation and sequencing of the cDNA from
10 rhodoxanthin-producing red *Lonicera* berries. Homologs can be identified via sequence identity to the novel gene according to SEQ ID NO:1. The highest identity with 62.8 % on amino acids level was found with the beta-carotene hydroxylase of *Vitis vinifera* (gi 526117836, ref:NP_001268126). None of the so-far identified homologous beta-carotene hydroxylating enzymes is known for
15 conversion of beta-carotene to rhodoxanthin.

The enzyme according to the present invention is capable of oxidation of carotenoids into their retro-carotenoids. Non-limited examples of such retro-carotenoids which can be generated by the enzymatic activity of the enzymes as defined herein include retrodehydro- γ -carotene (CAS 36210-84-1), tangeraxanthin
20 (CAS 1463442-20-7), anhydroeschtscholtzanthin (CAS 3484-56-8), isocarotene (CAS 3297-24-3), 7,7'-dihydro- β -carotene (CAS 4521-90-8), monoanhydroeschtscholtzanthin, eschtscholtzanthin (CAS 472-73-1), 5-hydroxyeschtscholtzanthin (CAS 292146-02-2), rhodoxanthin (CAS 116-30-3), eschtscholtzanthone (CAS 3484-59-1), loniceraxanthin (CAS 52073-89-9), (3R)-3-hydroxy-4',12'-retro-beta,beta-carotene-3',12'-dione (CAS 72755-64-7) and retro-C18-dione (CAS 336105-84-1), preferably rhodoxanthin.

Thus, the present invention is directed to a novel biosynthetic process for production of retro-carotenoids, in particular rhodoxanthin, which is generated via enzymatic conversion of a carotenoid, such as e.g. beta-carotene, said conversion
30 being catalyzed via the action of an oxygenase, preferably having hydroxylase activity, more preferably beta-carotene hydroxylase activity, even more preferred a polypeptide with an amino acids sequence with at least 78%, such as e.g. 80, 85, 90, 95, 97, 98, 99% or up to 100% identity to SEQ ID NO:2 or 4, 6 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:1, 3, or 5.

According to the present invention, the novel enzyme has activity of an oxygenase, in particular activity of a hydroxylase. The terms "oxygenase", "hydroxylase", "beta-carotene hydroxylase" or "BHYR" are used interchangeably herein. They refer to an enzyme which is involved in bio-conversion of a carotenoid, such as e.g. beta-carotene, which can catalyze the oxidation of such carotenoid into a particular retro-form, in particular which is involved in the bio-conversion of a carotenoid, such as e.g. beta-carotene, into rhodoxanthin. In particular, such term also includes functional equivalents or derivatives or fragments of such enzyme as defined above, as long as such functional equivalents, derivatives or fragments still have the same hydroxylase activity as defined herein.

As used herein, the term "% identity" and "identity" refers to the comparison of two amino acid sequences using a sequence analysis program as for instance Blast or Clustal Omega. Secondary structure prediction can be done by at least use of the Prime software from Schrödinger or by on-line software tools such as JPred. The term % identical refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence. If both amino acid sequences which are compared do not differ in any of their amino acids, they are identical or have 100% identity. With regards to plant oxidoreductases as e.g. with the BHYR-type enzyme as disclosed herein, the skilled person is aware of the fact that plant-derived enzymes might contain a chloroplast targeting signal which is to be cleaved via specific enzymes, such as e.g. chloroplast processing enzymes (CPEs).

Biosynthesis of retro-carotenoids, in particular rhodoxanthin, can be carried out with either wild-type enzymes derivable from nature, as e.g. isolated from *Lonicera* sp. or other suitable sources as defined herein, or modified, i.e. optimized, BHYR. Optimization includes modification which enables higher activity in a suitable host system as compared to activity of the wild-type BHYR. The skilled person is aware of techniques in order to optimize the enzymatic activity, including e.g. overexpression of a gene, removing of targeting signals, down-regulation of BHYR-specific repressors, codon-optimization or introduction of restriction sites.

Depending on the host cell, the polynucleotides as defined herein, such as e.g. the polynucleotide according to SEQ ID NO:1, might be optimized for expression in the respective host cell. The skilled person knows how to generate such modified polynucleotides. It is understood that the polynucleotides as defined herein also

encompass such host-optimized nucleic acid molecules as long as they still express the polypeptide with the respective activities as defined herein.

The novel enzyme according to the present invention also encompasses enzymes carrying amino acid substitution(s) which do not alter enzyme activity, i.e. which
5 show the same properties with respect to the wild-type enzyme and catalyze at least one of the abovementioned hydroxylation reactions. Such mutations are also called "silent mutations", which do not alter the (enzymatic) activity of the enzyme as described herein.

A nucleic acid molecule according to the invention may comprise only a portion or
10 a fragment of the nucleic acid sequence provided by the present invention, such as for instance the sequence shown in SEQ ID NO:1, 3, 5 for example a fragment which may be used as a probe or primer or a fragment encoding a portion of a protein according to the invention. The nucleotide sequence determined from the cloning of the BHyR gene allows for the generation of probes and primers
15 designed for use in identifying and/or cloning other BHyR family members, as well as BHyR homologues from other species. The probe/primer typically comprises substantially purified oligonucleotides which typically comprises a region of nucleotide sequence that hybridizes preferably under highly stringent conditions to at least about 12 or 15, preferably about 18 or 20, more preferably about 22 or 25,
20 even more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 or more consecutive nucleotides of a nucleotide sequence shown in SEQ ID NO:1 or a fragment or derivative thereof.

A preferred, non-limiting example of such hybridization conditions are hybridization in 6x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more
25 washes in 1x SSC, 0.1% SDS at 50°C, preferably at 55°C, more preferably at 60°C and even more preferably at 65°C.

Highly stringent conditions include, for example, 2 h to 4 days incubation at 42°C using a digoxigenin (DIG)-labeled DNA probe (prepared by using a DIG labeling system; Roche Diagnostics GmbH, 68298 Mannheim, Germany) in a solution such
30 as DigEasyHyb solution (Roche Diagnostics GmbH) with or without 100 µg/ml salmon sperm DNA, or a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 0.02% sodium dodecyl sulfate, 0.1% N-lauroylsarcosine, and 2% blocking reagent (Roche Diagnostics GmbH), followed by washing the filters twice for 5 to 15 minutes in 2x SSC and 0.1% SDS at room

temperature and then washing twice for 15-30 minutes in 0.5x SSC and 0.1% SDS or 0.1x SSC and 0.1% SDS at 65-68°C.

The biosynthetic process for production of retro-carotenoids, in particular rhodoxanthin, as described herein might be performed with isolated/purified

- 5 enzymes – either wild-type or modified – or as a whole cell biocatalyst in a biotransformation reaction, wherein the BHYR – either wild-type or modified – is expressed in a suitable host system as described herein.

- Expression of the enzymes/polynucleotides according to the present invention can be achieved in any host system, including (micro)organisms, which is suitable for
- 10 carotenoid production and which allows expression of the nucleic acids according to the invention, including functional equivalents or derivatives as described herein. Examples of suitable carotenoid-producing host (micro)organisms are bacteria, fungi, including yeasts, plant or animal cells. Preferred organisms are bacteria such as those of the genera *Escherichia*, such as, for example,
- 15 *Escherichia coli*, *Streptomyces*, *Pantoea* (*Erwinia*), *Bacillus*, *Flavobacterium*, *Synechococcus*, *Lactobacillus*, *Corynebacterium*, *Micrococcus*, *Mixococcus*, *Brevibacterium*, *Bradyrhizobium*, *Gordonia*, *Dietzia*, *Muricauda*, *Sphingomonas*, *Synochocystis*, *Paracoccus*, such as, for example, *Paracoccus xeaxanthinifaciens*, or eukaryotic microorganisms, in particular selected from *Saccharomyces*, such as
- 20 *Saccharomyces cerevisiae*, *Aspergillus*, such as *Aspergillus niger*, *Pichia*, such as *Pichia pastoris*, *Hansenula*, such as *Hansenula polymorpha*, *Phycomyces*, such as *Phycomyces blakesleanus*, *Mucor*, *Rhodotorula*, *Sporobolomyces*, *Xanthophyllomyces*, *Phaffia*, *Blakeslea*, such as e.g. *Blakeslea trispora*, or *Yarrowia*, such as *Yarrowia lipolytica*, or higher eukaryotic cells selected from
- 25 animals or plants, such as e.g. tobacco, potato, tomato, *Arabidopsis*, soybean, maize, cotton, wheat, rapeseed, Marigold, kale, spinach, California Poppy (*Eschscholzia California*), *Arabidopsis*, *Taxus*, as well as microalgae such as *Haematococcus*, *Chlorella*, *Dunaliella*, such as e.g. *Dunaliella salina*, *Neosporangium*, *Chlamydomonas*, *Murielopsus*, and *Scenedesmus*, in particular
- 30 plants selected from *Taxus*, California poppy, *Arabidopsis*, *Nicotiana*, such as e.g. *Nicotiana benthamiana*. In particularly preferred is expression in *Yarrowia* or *Escherichia*, more preferably expression in *Yarrowia lipolytica*.

As used herein, a carotenoid-producing host cell is a host cell wherein the respective polypeptides are expressed and active in vivo leading to production of

carotenoids. The genes and methods to generate carotenoid-producing host cells are known in the art. Depending on the carotenoid to be produced, different genes might be involved. As defined herein, a rhodoxanthin-producing host cell is capable of expressing a polypeptide having beta-carotene hydroxylase activity
5 with at least 78%, such as e.g. 80, 85, 90, 95, 97, 98, 99% or up to 100% identity to SEQ ID NO:2.

It is understood that the above-mentioned microorganisms also include synonyms or basonyms of such species having the same physiological properties, as defined by the International Code of Nomenclature of Prokaryotes or the International
10 Code of Nomenclature for algae, fungi, and plants (Melbourne Code).

Any of the BHYR as defined herein can be used for biotechnological production of retro-carotenoids, in particular rhodoxanthin, wherein the substrate, i.e. a carotenoid, such as beta-carotene, might be converted into the retro-carotenoid, in particular rhodoxanthin, by the action of said enzyme as described herein. The
15 produced retro-carotenoid, in particular rhodoxanthin, might be isolated and optionally further purified from the medium and/or host cell.

The host cell, i.e. microorganism, fungal or plant cell, which is able to express the BHYR according to the present invention may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic or anaerobic conditions
20 and as known by the skilled person for the different host cells. Optionally, such cultivation is in the presence of proteins and/or co-factors involved in transfer of electrons, as defined herein. The cultivation/growth of the host cell may be conducted in batch, fed-batch, semi-continuous or continuous mode. Depending on the host cell, preferably, production of retro-carotenoids such as e.g.
25 rhodoxanthin is performed in a fed-batch process using corn oil as carbon source.

When using a plant host cell, including but not limited to *Taxus*, California poppy, *Arabidopsis*, *Nicotiana*, the BHYR as defined herein might be used in a transient or stable expression system without any further modifications of the host for production of a retro-carotenoid, in particular rhodoxanthin. Stable transformation
30 of plants can be achieved using several methods, including electroporation and polyethylene glycol mediated transformation of protoplasts, particle bombardment, silicon carbide whiskers, *Agrobacterium*-mediated transformation, and *in planta* transformation.

When using a bacterial host cell, including but not limited to *E. coli*, the host cell expressing BHYR as defined herein might be further modified for ability to produce a retro-carotenoid, in particular rhodoxanthin. These further modifications include but are not limited to the introduction of bacterial, fungal or plant genes involved in lycopene biosynthesis, as they are known from e.g. *Pantoea* or *Lonicera*. In one embodiment, a bacterial host cell including *E. coli*, is further modified via introduction of lycopene biosynthetic genes and a gene encoding a polypeptide having a beta-carotene cyclase activity, such as e.g. known from *Lonicera*, including but not limited to a cyclase according to SEQ ID NO:14 or 16 which might be encoded by a polynucleotide according to SEQ ID NO:13 or 15.

When using a fungal host cell, including yeast, such as e.g. *Yarrowia lipolytica*, the host cell expressing BHYR as defined herein might be further modified/optimized in order to produce a retro-carotenoid, in particular rhodoxanthin. These further modifications include but are not limited to codon-optimization of the BHYR gene, such as e.g. shown in SEQ ID NO:5, and/or the introduction of oxidoreductases, in particular wherein the genes encoding such proteins are codon-optimized for expression in the respective host cell, such as e.g. *Yarrowia*, preferably the introduction of a protein-activity which is selected from the group consisting of ferredoxin, ferredoxin reductase, flavodoxin, flavodoxin reductase, putaredoxin, putaredoxin reductase, thioredoxin, thioredoxin reductase, cytochrome P450, cytochrome b5 reductase, monodehydroascorbate reductase, glutathione reductase, adrenodoxin, AOX (mitochondrial alternative oxidase), PTOX (plastid terminal oxidase) and combinations thereof. These proteins might be selected from different sources, such as e.g. *Aloe*, *E. coli*, *Lonicera* or might be originated from the endogenous gene(s) which are modified to increased their specific activities.

In one embodiment, the rhodoxanthin-producing host cell expressing the BHYR as defined herein, in particular a fungal host cell including yeast, preferably *Yarrowia*, furthermore comprises a protein having ferredoxin activity, in particular wherein the gene encoding said polypeptide being originating from *Aloe*, preferably with at least 59% identity to SEQ ID NO:8 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:7 or SEQ ID 17. Thus, a preferred fungal carotenoid producing host strain, including yeast, preferably *Yarrowia*, useful for conversion of beta-carotene into rhodoxanthin comprises (a) a polynucleotide expressing a polypeptide having BHYR activity with at least 78%, such as e.g. 80,

85, 90, 95, 97, 98, 99 or even 100% identity to SEQ ID NO:6 ? and (b) a polynucleotide expressing a polypeptide having ferredoxin activity with at least 59 %, such as e.g. at least 60, 65, 70, 75, 80, 90, 92, 95, 98, 99% or up to 100% identity to SEQ ID NO:8.

- 5 In a further embodiment, the rhodoxanthin-producing host cell expressing the BHYR as defined herein, in particular a carotenoid-producing fungal host cell including yeast, preferably *Yarrowia*, furthermore comprises a protein having ferredoxin reductase activity, in particular wherein the gene encoding said polypeptide being originating from *Aloe*, preferably with at least 82%, such as e.g.
- 10 85, 90, 92, 95, 97, 99 or event up to 100% identity to SEQ ID NO:10 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:9 or SEQ NO:18. Thus, a preferred fungal host strain, including yeast, preferably *Yarrowia*, useful for conversion of beta-carotene into rhodoxanthin comprises (a) a polynucleotide expressing a polypeptide having BHYR activity with at least 78%,
- 15 such as e.g. 80, 85, 90, 95, 97, 98, 99 or even 100% identity to SEQ ID NO:6, (b) a polynucleotide expressing a polypeptide having ferredoxin activity with at least 59 %, such as e.g. at least 60, 65, 70, 75, 80, 90, 92, 95, 98, 99% or up to 100% identity to SEQ ID NO:8, and (c) a polynucleotide expressing a polypeptide having ferredoxin reductase activity with at least 82%, such as e.g. 85, 90, 92, 95, 97, 99
- 20 or event up to 100% identity to SEQ ID NO:10.

- In even a further embodiment the rhodoxanthin-producing host cell expressing the BHYR as defined herein, in particular a carotenoid-producing fungal host cell including yeast, preferably *Yarrowia*, furthermore comprises a protein having chloroplast processing enzyme (CPE) activity, such as, e.g. a gene encoding
- 25 stromal processing peptidase (UniProtKB Q40983) from *Pisum sativum*, preferably with at least 80%, such as 85, 90, 92, 95, 97, 99 or even 100% identity to SEQ ID NO:12 which might be encoded by a polynucleotide including, but not limited to SEQ ID NO:11. Thus, a preferred fungal host strain, including yeast, preferably *Yarrowia*, useful for conversion of beta-carotene into rhodoxanthin comprises (a) a
- 30 polynucleotide expressing a polypeptide having BHYR activity with at least 78%, such as e.g. 80, 85, 90, 95, 97, 98, 99 or even 100% identity to SEQ ID NO:2, (b) a polynucleotide expressing a polypeptide having ferredoxin activity with at least 59 %, such as e.g. at least 60, 65, 70, 75, 80, 90, 92, 95, 98, 99% or up to 100% identity to SEQ ID NO:8, (c) a polynucleotide expressing a polypeptide having
- 35 ferredoxin reductase activity with at least 82%, such as e.g. 85, 90, 92, 95, 97, 99

or event up to 100% identity to SEQ ID NO:10, and (d) a polynucleotide expressing a polypeptide having CPE activity, such as e.g. stromal processing peptidase activity, including a polynucleotide expressing a polypeptide having CPE activity with at least 80% identity to SEQ ID NO:12.

- 5 As used herein, the term “ferredoxin activity” encompasses activity of all three ferredoxin isoforms, i.e. as ferredoxin 1, 2 and/or 3, including both leaf and heterotrophic forms

With regards to rhodoxanthin production using a carotenoid producing host cell as described herein, a yield of at least 20 mg/l, such as e.g. 50, 75, 100, 250, 500
10 mg/l, or even up to at least 1, 5, 10, 15, 20 or 50 g/l with a suitable substrate such as corn oil or glucose might be achieved compared to a beta-carotene producing host cell which is not comprising a polynucleotide expressing a BHYR as described herein.

According to a specific aspect, the present invention is directed to the use of
15 BHYR as defined herein in a process for production of retro-carotenoids via oxidation of their respective carotenoids, in particular in a process for production of rhodoxanthin, comprising the steps of:

- (a) isolation of a BHYr gene from a suitable source, such as e.g. *Lonicera* sp.;
(b) cloning of the BHYr gene into a suitable expression vector, wherein optionally
20 the BHYr gene is optimized for expression in said vector and/or a suitable host cell;
(c) introduction of said vector comprising the BHYr gene into a suitable carotenoid-producing host cell, such as a fungal cell including yeast, e.g. *Yarrowia*, preferably *Yarrowia lipolytica*;
25 (d) cultivation of said genetically modified host cell in the presence of a suitable carotenoid as substrate and under conditions such that the BHYr gene is active, i.e. BHYR is expressed and active in vivo, resulting in production of the retro-carotenoid, in particular rhodoxanthin produced from beta-carotene; and
(f) optionally, isolation and purification of the retro-carotenoid, in particular
30 rhodoxanthin.

Optionally, and as described herein, depending on the carotenoid-producing host cell, said process might include the introduction of further modifications into the host cell, such as e.g. the introduction of lycopene biosynthetic genes and a gene encoding a polypeptide having a cyclase activity, ferredoxin and/or ferredoxin

reductase activity and/or chloroplast processing enzyme activity as described above.

As defined herein, the BHYR according to the present invention is capable to catalyze the conversion/oxidation of a substrate selected from carotenoids into the respective retro-carotenoid, in particular conversion of beta-carotene into rhodoxanthin. A suitable substrate might be selected from isoprenoids and carotenoids, such as e.g. geranylgeranyl pyrophosphate (GGPP), phytoene, beta-carotene, lycopene, beta-cryptoxanthin, phytoene, lycopene, eschscholtzxanthin, zeaxanthin, preferably beta-carotene. The substrate is contacted with either the isolated form or with BHYR expressed/being active in vivo in a suitable host cell as defined herein. Depending on the host cell, the process according to the present invention might be carried out in the presence of proteins and/or co-factors, e.g. compounds used for transfer of electrons, i.e. acting as donor and/or acceptor of electrons, including but not limited to proteins and/or co-factors selected from the group consisting of NAD(H), NADP(H), ferredoxin, ferredoxin oxidoreductase, flavodoxin, flavodoxin oxidoreductase, putaredoxin, putaredoxin reductase, monodehydroascorbate reductase, glutathione reductase, PTOX, AOX and adrenodoxin. The use of such proteins and/or co-factors is known in the art. Specific useful proteins and/or co-factors which could be used preferably for production in a carotenoid-producing fungal host strain, including a yeast strain, such as e.g. *Yarrowia*, are selected from *Aloe*, such as in particular proteins having *Aloe* ferredoxin and/or ferredoxin reductase activity as described herein together with a polypeptide having chloroplast processing enzyme activity, such as e.g. a polypeptide with at least 80% identity with the CPE originating from *Pisum sativum*.

This is only one way of performing such process, the skilled person is able to adapt these conditions to other suitable host organisms and cultivation conditions as defined herein, including the selection of a suitable electron acceptor/donor.

As used herein, the term "specific activity" or "activity" with regards to enzymes means its catalytic activity, i.e. its ability to catalyze formation of a product from a given substrate. The specific activity defines the amount of substrate consumed and/or product produced in a given time period and per defined amount of protein at a defined temperature. Typically, specific activity is expressed in μmol substrate consumed or product formed per min per mg of protein. Typically, $\mu\text{mol}/\text{min}$ is abbreviated by U (= unit). Therefore, the unit definitions for specific activity of

$\mu\text{mol}/\text{min}/(\text{mg of protein})$ or $\text{U}/(\text{mg of protein})$ are used interchangeably throughout this document. An enzyme is active, if it performs its catalytic activity in vivo, i.e. within the host cell as defined herein or within a system in the presence of a suitable substrate. The skilled person knows how to measure enzyme activity, in particular activity of an oxygenase as defined herein. With regards to the present invention, "BHYR activity" is defined as the capability to catalyze the oxidation of beta-carotene into rhodoxanthin, which can be measured by HPLC as known by the skilled person (see Figures 1 to 4).

Rhodoxanthin as used herein includes any chemical form of rhodoxanthin found in aqueous solutions, including all isoforms. In particular, it includes a mixture of cis EZ-, EE and ZZ-rhodoxanthin.

The retro-carotenoid, in particular rhodoxanthin, produced via the action of the BHYR as described herein can be used as colorant, including as supplement to food, feed, beverages, cosmetics, pharmaceutical compositions including capsules or tablets. Depending on the final product, it might be further formulated as known in the art.

Retro-carotenoids, in particular rhodoxanthin, might be used in milled form to be supplemented into soft drinks, sports drinks, mineral drinks, carbonated drinks, fruit juices, sugar-containing beverages, diet beverages, gums, yellies, or edible coatings used in sugar syrup, sugar-free syrups and panned confection. It might furthermore be used in liquid formulations mixed with/embedded into modified food starch, saccharide and water. The skilled person is aware of such products and methods how to generate such forms.

Figures

Figure 1. HPLC chromatogram (left side) and UV spectra for the individual peaks (right side) of orange (Fig.1A) and red (Fig.1B) *Lonicera* sp. berries. Peak 1: beta-cryptoxanthin; peak 2: rhodoxanthin cis zz isomer; peak 3: rhodoxanthin cis ez isomer; peak 4: rhodoxanthin cis ee isomer; peak 5: zeaxanthin. Absorbance at 494 nm is shown on the y-axis, time in minutes is shown on the x-axis.

Figure 2. HPLC chromatogram and UV spectra for the individual peaks (right side) of *Nicotiana benthamiana* leaf extracts. Untransfected control (Fig.2A) is compared to leaf transfected with BHYr (Fig.2B). Peak 1: rhodoxanthin cis zz isomer; peak 2: rhodoxanthin cis ez isomer; peak 3: rhodoxanthin cis ee isomer;

peak 4: lutein; peak 5: zeaxanthin. Absorbance at 494 nm is shown on the y-axis, time in minutes is shown on the x-axis.

Figure 3. HPLC chromatogram (left side) and UV spectra for the individual peaks (right side) of *E. coli* extracts. *E. coli* transformed with crtE, crtB, crtI, and idi from *Pantoea*, and beta carotene cyclase from *Lonicera* (Fig.3A) is compared to *E. coli* transformed with crtE, crtB, crtI, and idi from *Pantoea*, beta carotene cyclase from *Lonicera* sp, and BHYr of *Lonicera* sp. (Fig.3B). Peak 1: carotenes; peak 2: beta-cryptoxanthin; peak 3: rhodoxanthin cis zz isomer; peak 4: rhodoxanthin cis ez isomer; peak 5: rhodoxanthin cis ee isomer; peak 6: zeaxanthin. Absorbance at 494 nm is shown on the y-axis, time in minutes is shown on the x-axis.

Figure 4. HPLC chromatogram (left side) and UV spectra for the individual peaks (right side) of *Yarrowia lipolytica* extracts. *Y. lipolytica* transformed with CarB and CarRP from *Mucor circinellioides*, and BHYr from *Lonicera* sp. (Fig.4A) is compared to *Y. lipolytica* transformed with CarB and CarRP from *Mucor circinellioides*, BHYr from *Lonicera* sp, CPE from *Pisum sativum*, ferredoxin (FD) and ferredoxin reductase (FNR) from *Aloe* sp. (Fig.4B). Peak 1: beta-cryptoxanthin; peak 2: rhodoxanthin cis zz isomer; peak 3: rhodoxanthin cis ez isomer; peak 4: rhodoxanthin cis ee isomer; peak 5: zeaxanthin. Absorbance at 494 nm is shown on the y-axis, time in minutes is shown on the x-axis.

Figure 5. Sequences 1 to 18 as used herein (for more explanation see text).

The following examples are illustrative only and are not intended to limit the scope of the invention in any way. The contents of all references, patent applications, patents and published patent applications, cited throughout this application are hereby incorporated by reference, in particular EP 3072400 or WO2016151084.

Examples

Example 1: Identification of BHYr gene from *Lonicera*

Lonicera sp. plants producing red and orange berries, respectively, were identified growing wild in Massachusetts. Berries were extracted with tetrahydrofuran, dried down, and resuspended in heptane:ethyl acetate: methylene chloride (2:2:1). A Phenomenex Luna Silica (2), 3 micro 150x 4.6 mm column with a security silica guard column kit was used to resolve carotenoids with a Waters 2998 PDA detector. Synthetic carotenoid samples, purchased from CaroteNature (GmbH, Im Budler 8, CH-4419 Lupsingen, Switzerland) or received from DSM Nutritional

Products Ltd, were used as reference. The mobile phase consisted of 1000 mL heptane, 60 mL isopropanol, and 0.1 mL acetic acid. The flow rate for each run was 0.6 mL per minute. Column temperature was ambient. The injection volume was 20 µL. The detector was a Waters 2998 photo-diode array detector from 210
 5 to 600 nm. HPLC analysis of the berries confirmed that the red berries produced rhodoxanthin, while orange berries lacked rhodoxanthin and contained high levels of zeaxanthin (Figure 1).

A transcriptomics approach was used to identify genes that were highly expressed in rhodoxanthin producing berries. A gene (BHYr; SEQ ID NO:1) with homology to
 10 beta carotene hydroxylases was found to be highly expressed in red berries compared to unripe green berries or ripe orange berries. Proteomic analysis was performed on red and orange berries to confirm the high levels of the BHYR protein in rhodoxanthin producing berries. Comparison of the Lonicera BHYR protein sequence with other plant BHY proteins revealed an overall sequence
 15 identify of 62.8% with regards to the full-length protein and a sequence identity of 77% when aligning the mature presumed protein, i.e. after cleavage of the presumed chloroplast targeting signal. The closest hit was found compared to beta-carotene hydroxylase of *Vitis vinifera* (gi 526117836, ref:NP_001268126).

20 **Example 2: Expression of BHYr and production of rhodoxanthin in a plant system**

A beta carotene hydroxylase-like gene (BHYr) was found to be highly expressed in red but not orange *Lonicera* berries (Fig.1B).

Lonicera red berry cDNA (350 pg) was used to amplify BHYr using primers SEQ ID NO:23/24. An 858 bp fragment was obtained and cleaved using NcoI and
 25 BstEII. The plant expression vector pCambia1304 was digested with NcoI and BstEII and ligated to the BHYr NcoI–BstEII fragment to produce pMB8082.

Table 1: Oligonucleotides used for PCR reactions.

Primer	Primer sequence 5' – 3'	SEQ ID NO:
MO11209	CACACCCATGGCGCAAGAGAGAACAG	19
MO11212	CACACcttaagTTAATCCTCTTTTTTACCTGC	20
MO11207	CACACCATATGCCACAAAAAAGGTCTCTC	21
MO11206	cacaccctaggTCAAATGGATTCAAGTGCAA	22

Primer	Primer sequence 5' – 3'	SEQ ID NO:
MO11378	CACACCCATGGCAACCGGAGTTCC	23
MO11379	CACACGGTGACCTTAATCCTCTTTTTCACCTGCACC	24

In order to test the cloned BHYr, *Nicotiana benthamiana* seeds were germinated for 1 week in a Lawn and Garden Pellet Greenhouse. The greenhouse was covered with a dome to maintain high humidity and a 16/8 h day/night cycle was used. After two weeks, pellets with germinated plants were transferred to pots (10.16 cm x 10.16 cm) with potting soil. The plants were grown for an additional 6 weeks.

Agrobacterium tumefaciens strain GV3101 (resistant to rifampicin and gentamicin) was grown on 5 ml YEB (5g/L bactopectone, 5g/L beef extract, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (supplemented with 40 µg/ml Gen and 25 µg/ml Rif) overnight at 30°C. The following day 50 ml YEB (no antibiotic) was inoculated with 2 ml of the overnight culture and grown at 30°C to and OD600 of 0.6. Cells were spun down for 15 min at 3000 g at 4°C. Cells were washed once with cold TE and spun down. The pellet was resuspended in 5 ml of cold TE. Aliquots of 0.2 ml were frozen in liquid nitrogen and stored at a temperature of -80°C. An aliquot of cells was thawed and 1 µg of pMB8085 was added, incubated on ice for 5 min and frozen in liquid nitrogen for 5 min. Cells were thawed by incubating for 5 min at 37°C. One ml of YEB medium was added and incubated 2 h at 30°C with shaking. After incubation, the tube was centrifuged for 30 sec at 3000g and the pellet was resuspended in 0.1 ml YEP medium. The resuspended cells were spread on YEB agar supplemented with 40 µg/ml Gen, 25 µg/ml Rif and 25 µg/ml Kan. Transformation plates were incubated 2 – 3 days. Two transformants were selected and frozen in 25% glycerol.

A single *A. tumefaciens* transformant harboring the BHYr gene (pMB8098) was spread on a fresh plate. An *A. tumefaciens* strain containing the P19 protein (in C58 genotype) was also spread on a fresh plate. Infiltration was done with BHYr plus P19 or with P19 alone as a control according to the protocol described by Schütze et al. (Plant Signal Transduction 479:189-202, 2009). Briefly, the plants were well hydrated by spraying the morning before infiltration and cell suspensions (OD600 0.7 – 0.8) of a 1:1 ratio of both strains or the P19-containing strain alone

were combined and infiltrated into leaves using a 10ml syringe. Five days after infiltration leaves were harvested. Approximately 0.2 – 0.5 g of leaves were extracted in a 7 ml Precellys tube using 3 ml Heptane:Ethyl Acetate 1:1 plus 0.01% BHT. Extracts were analyzed by normal phase HPLC. Leaves containing

5 BHYr plus P19, but not P19 alone, produced the distinctive rhodoxanthin peaks as shown in Figure 2.

Example 3: Expression of BHYr and production of rhodoxanthin in a bacterial system

Rhodoxanthin production was enabled in *E. coli* by the introduction of the genes

10 for lycopene biosynthesis from *Pantoea agglomerans*, along with the β -carotene cyclase and BHYr genes from *Lonicera* (see Example 1). To enable lycopene biosynthesis of pAC-LYCipi (Cunningham et al., *Eukaryot Cell*. 2007 Mar;6(3):533-45. Epub 2006 Nov 3. 10.1128/EC.00265-06) containing CrtE (geranylgeranyl diphosphate synthase), CrtI (phytoene dehydrogenase), CrtB (phytoene synthase)

15 and IDI (isopentenyl-diphosphate delta-isomerase) genes was synthesized with NotI ends and cloned into the NotI site of pET28a to create pMB8501 (Genescript).

A truncated version of the β -carotene cyclase gene (CCS18) was amplified from *Lonicera* sp. red berry cDNA (SEQ ID NO:13) with primers SEQ ID NO:21/22

20 which generated an NdeI site at the 5' end and an AvrII site on the 3' end. The resulting PCR product (SEQ ID NO:15) was digested with NdeI and AvrII, and cloned into the identical sites of pCDFDuet (Novagen) to generate plasmid pMB8103. Expression of the cyclase gene in the resulting plasmid is under control of the T7 promoter.

25 *E. coli* DB3 (BL21) was transformed simultaneously with pMB8501 and pMB8103 with selection on kanamycin (25 μ g/ml) and spectinomycin (25 μ g/ml) to generate strain MB8167. Outgrowth and plate growth was at 30°C or room temperature. Strain MB8167 was grown for 40 h on Overnight Express Autoinduction medium (Novagen) in LB with selection, i.e. in the presence of kanamycin and

30 spectinomycin (25°C, 48 h). Samples were extracted and analyzed by HPLC as described for *Nicotiana*. HPLC analysis revealed that strains containing both pMB8501 and pMB8103 produced a mixture of beta-carotene and lycopene (Fig.3A).

BHYr was amplified from *Lonicera* sp. cDNA using primers SEQ ID NO:19/20. The PCR product was digested with NcoI and AflIII and subcloned into pACYCDuet digested with the same enzymes, resulting in MB8088. Strain MB8167 (producing beta-carotene) was made competent, and transformed with pMB8088, with
5 selection on kanamycin, spectinomycin, and chloramphenicol (17 µg/ml). Resulting transformants were grown on inducing medium containing all three antibiotics for 48 h at 25°C. HPLC analysis of strain MB8168, which contains pMB8501, pMB8103 and pMB8088 is shown in Figure 3B. The strain produced a mixture including beta-cryptoxanthin, zeaxanthin, and the three rhodoxanthin isomers that
10 are present in natural rhodoxanthin from *Lonicera*.

Example 4: Expression of BHYr and production of rhodoxanthin in a yeast system

Rhodoxanthin production in *Yarrowia lipolytica* was enabled by introducing BHYr (truncated and optimized) into a beta-carotene producing *Yarrowia* strain.
15 Rhodoxanthin production was enabled by additionally introducing the full-length DNA encoding Aloe ferredoxin (SEQ ID NO:8) and Aloe ferredoxin reductase (SEQ ID NO:10), along with DNA expressing the truncated, *Yarrowia* codon optimized chloroplast processing enzyme from *Pisum sativum* (UniProtKB Q40983; SEQ ID NO:12) designed to cleave the chloroplast targeting signal from
20 the ferredoxin and ferredoxin reductase.

The BHYr gene of *Lonicera* was truncated to remove the presumed chloroplast targeting signal, and modified by codon optimization for expression in *Yarrowia* to result in SEQ ID NO:5. An NheI site was incorporated into the 5'-region, and an MluI site was incorporated into the 3'-region to allow subcloning into the *Yarrowia*
25 expression vector pMB6157 to generate plasmid pMB7918, where expression of BHYr is under the control of the *Yarrowia* Tef promoter. pMB7918 was transformed into the beta-carotene producing strain ML2461 which contains *Mucor circinellioides* CarB (phytoene dehydrogenase; CAB40843.1) and *M. circinellioides* CarRP (lycopene cyclase/phytoene synthase; CAB60272.1) and produces no
30 detectable xanthophylls (data not shown) to generate strain ML17461. ML17461 was grown on YPoil (yeast extract (10 g/l), peptone (20 g/l), tryptophan (0.15 g/l) and corn oil 2%) medium and samples were extracted and analyzed by HPLC as described for *Nicotiana*. Small amounts of beta-cryptoxanthin, zeaxanthin, and tentative rhodoxanthin EZ were produced (Fig.4A).

DNA sequences for Aloe ferredoxin, Aloe ferredoxin reductase and *P. sativum* CPE genes were *Yarrowia* codon-optimized (see SEQ ID NOs:7, 9, 11) and synthesized with an *NheI* site at the 5'-end and an *MluI* site at the 3'-end to allow cloning into *Yarrowia* expression vectors to generate pMB8059, pMB8056, and pMB7896, respectively. Expression of FD and FNR was with the TEF promoter, while expression of CPE was with the *Alk1* promoter of *Yarrowia*.

Strain ML 17769, which contains the *Mucor* *CarB* and *CarRP* genes for beta carotene production, the *P. sativum* chloroplast targeting enzyme (CPE), the Aloe ferredoxin and ferredoxin reductase, and the truncated *BHYr* gene produced significantly higher levels of all three rhodoxanthin isomers than the strain containing *CarB*, *CarRP* and *BHYR*, alone (Fig.4B).

Example 5: Rhodoxanthin-containing beverages

Rhodoxanthin produced in a method as described in Examples 1 to 4 is milled and added to a beverage as a dispersion (see e.g., Example 1 in EP 3072400). Optionally, the rhodoxanthin might be encapsulated in a matrix of modified food starch.

Example 6: Rhodoxanthin-containing gums and jellies

Rhodoxanthin produced in a method as described in Examples 1 to 4 is milled and added to gums or jellies, such as e.g. gummi bears as a dispersion. Preferably, the amount of the milled rhodoxanthin is in the range of from 1 to 50 ppm (based on total weight). Optionally, the rhodoxanthin might be encapsulated in a matrix of modified food starch (see e.g., Example 1 in WO2016151084).

Claims

1. A nucleic acid molecule encoding a polypeptide having beta-carotene hydroxylase activity, wherein said polypeptide is selected from a polypeptide with at least 78% identity to a polypeptide according to SEQ ID NO:2, 4 or 6.
- 5 2. The nucleic acid molecule according to claim 1, wherein the chloroplast targeting signal has been cleaved off.
3. The nucleic acid molecule according to claim 1 or 2 encoding a polypeptide catalyzing the conversion of carotenoids into their retro-carotenoids, preferably wherein the retro-carotenoid is selected from the group consisting of retrodehydro-
10 γ -carotene (CAS 36210-84-1), tangeraxanthin (CAS 1463442-20-7), anhydroeschscholtzxanthin (CAS 3484-56-8), isocarotene (CAS 3297-24-3), 7,7'-dihydro- β -carotene (CAS 4521-90-8), monoanhydroeschscholtzxanthin, eschscholtzxanthin (CAS 472-73-1), 5-hydroxyeschscholtzxanthin (CAS 292146-02-2), rhodoxanthin (CAS 116-30-3), eschscholtzxanthone (CAS 3484-59-1),
15 loniceraxanthin (CAS 52073-89-9), (3R)-3-hydroxy-4',12'-retro-beta,beta-carotene-3',12'-dione (CAS 72755-64-7) and retro-C18-dione (CAS 336105-84-1), more preferably rhodoxanthin.
4. The nucleic acid molecule according any one of claims 1 to 3 encoding a polypeptide catalyzing the conversion of beta-carotene into rhodoxanthin.
- 20 5. The nucleic acid molecule according to any one of claims 1 to 4 which is operatively linked to expression control element.
6. The nucleic acid molecule according to claim 5 which is transformed into and expressed in a plant, fungal, algae or bacterial host cell.
7. A carotenoid-producing host cell, preferably a microorganism, more
25 preferably selected from fungal, algae, bacterial or plant cell, said host comprising a polypeptide having beta-carotene hydrolase activity being encoded by a nucleic acid molecule according to any one of claims 1 to 6.
8. The host cell according to claim 7 for production of a retro-carotenoid, preferably wherein the retro-carotenoid is selected from the group consisting of
30 retrodehydro- γ -carotene (CAS 36210-84-1), tangeraxanthin (CAS 1463442-20-7), anhydroeschscholtzxanthin (CAS 3484-56-8), isocarotene (CAS 3297-24-3), 7,7'-dihydro- β -carotene (CAS 4521-90-8), monoanhydroeschscholtzxanthin, eschscholtzxanthin (CAS 472-73-1), 5-hydroxyeschscholtzxanthin (CAS 292146-

02-2), rhodoxanthin (CAS 116-30-3), eschscholtzxanthone (CAS 3484-59-1), loniceraxanthin (CAS 52073-89-9), (3R)-3-hydroxy-4',12'-retro-beta,beta-carotene-3',12'-dione (CAS 72755-64-7) and retro-C18-dione (CAS 336105-84-1), more preferably rhodoxanthin.

- 5 9. The host cell according to claim 7 or 8 which is a fungal cell, further comprising a nucleic acid molecule encoding a polypeptide having ferredoxin activity, wherein said polypeptide is selected from a polypeptide with at least 59 identity to a polypeptide according to SEQ ID NO:8 and/or further comprising a nucleic acid molecule encoding a polypeptide having ferredoxin reductase activity,
10 wherein said polypeptide is selected from a polypeptide with at least 82 identity to a polypeptide according to SEQ ID NO:10.

10. A process for conversion of a carotenoid into its retroform, preferably a carotenoid selected from the group consisting of lutein, beta-carotene, cantaxanthin, cryptoxanthin, phytoene, lycopene, eschscholtzxanthin and
15 zeaxanthin, wherein the carotenoid is contacted with the polypeptide encoded by the nucleic acid molecule according to any one of claims 1 to 6.

11. A process for biosynthesis of retro-carotenoids, preferably rhodoxanthin, comprising the steps:
(a) introduction of a nucleic acid molecule according to any one of claims 1 to
20 6 into a suitable carotene-producing host cell,
(b) conversion of a carotenoid present in said host cell into the retro-carotenoid in the presence of the polypeptide expressed by the nucleic acid introduced in step (a), and optionally
(c) isolation and/or purification of the retro-carotenoid from the host cell;
25 wherein optionally said process is conducted in the presence of a protein and/or co-factor involved in transfer of electrons, i.e. acting as donor and/or acceptor of electrons, including but not limited to proteins and/or co-factors selected from the group consisting of NAD(H), NADP(H), ferredoxin, ferredoxin oxidoreductase, flavodoxin, flavodoxin oxidoreductase, putaredoxin, putaredoxin reductase,
30 monodehydroascorbate reductase, glutathione reductase, PTOX, AOX and adrenodoxin.

12. The process according to claim 11, wherein the carotenoid is beta-carotene which is converted into rhodoxanthin.

13. Use of a polypeptide encoded by a nucleic acid molecule according to any one of claims 1 to 6 for conversion of a carotenoid, preferably beta-carotene, into a particular retro-carotenoid, preferably rhodoxanthin.
14. A process for production of a food, feed, beverage, cosmetic or
5 pharmaceutical composition comprising one or more process-steps according to any one of claims 10 to 12.

Figure 1

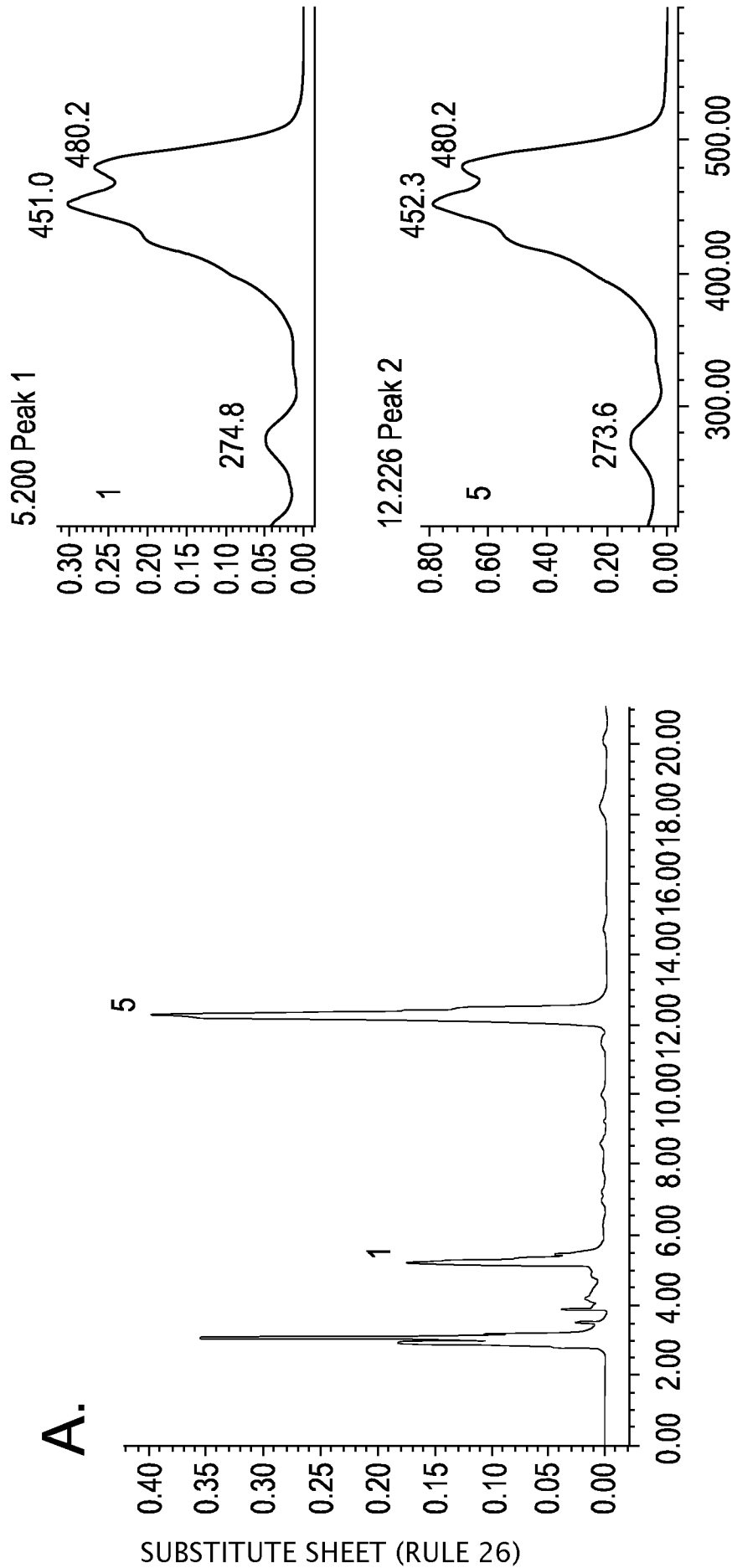


Figure 1

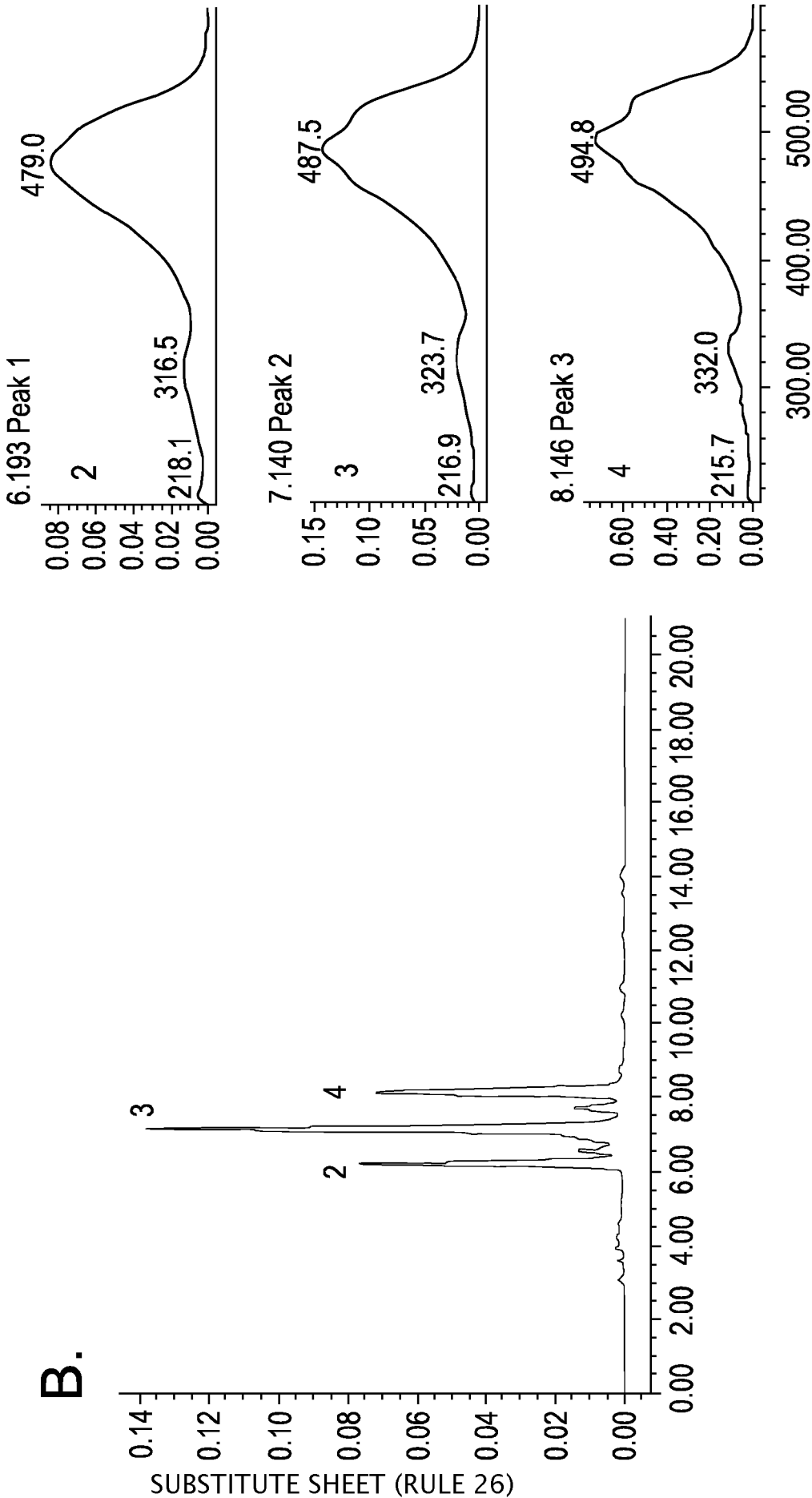


Figure 2

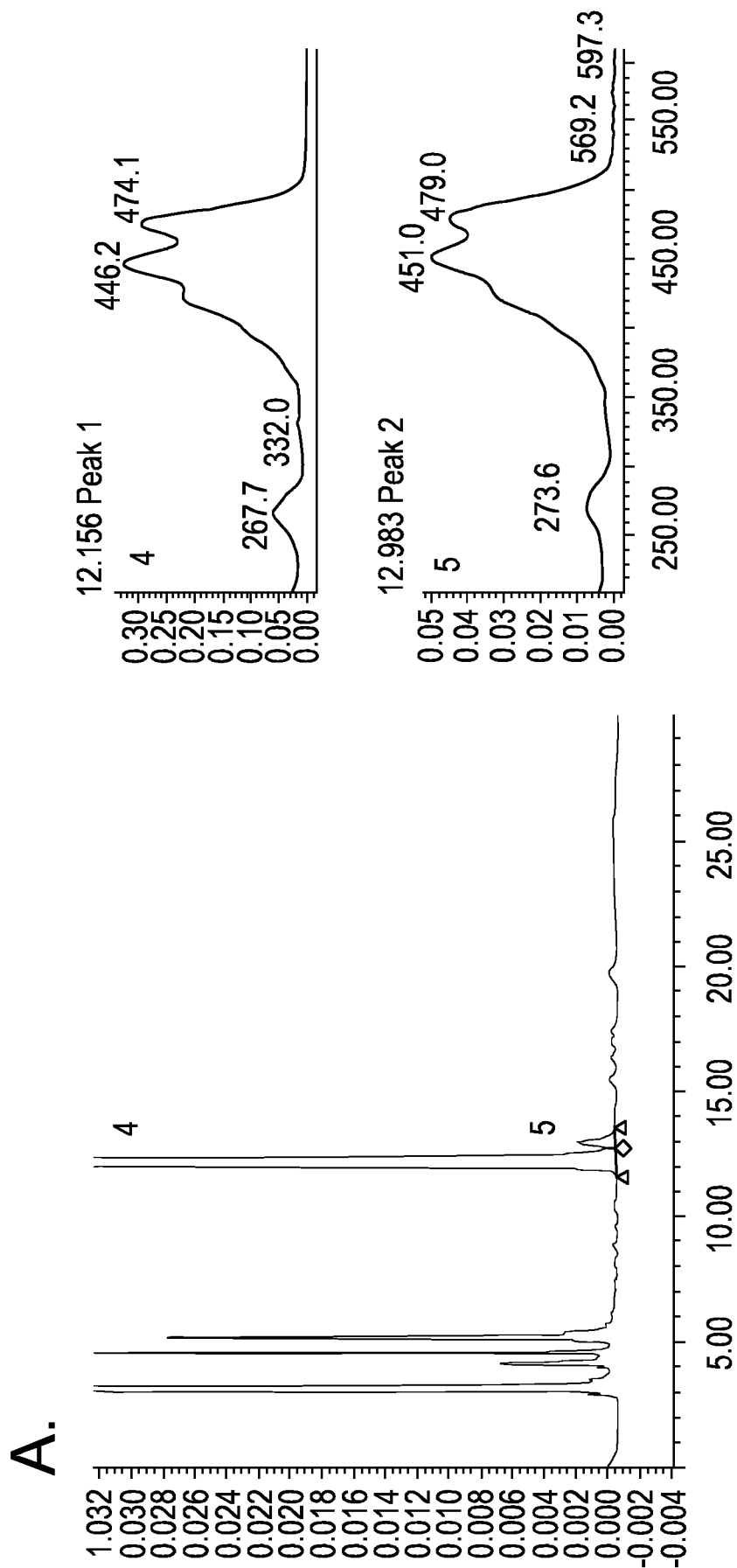


Figure 2

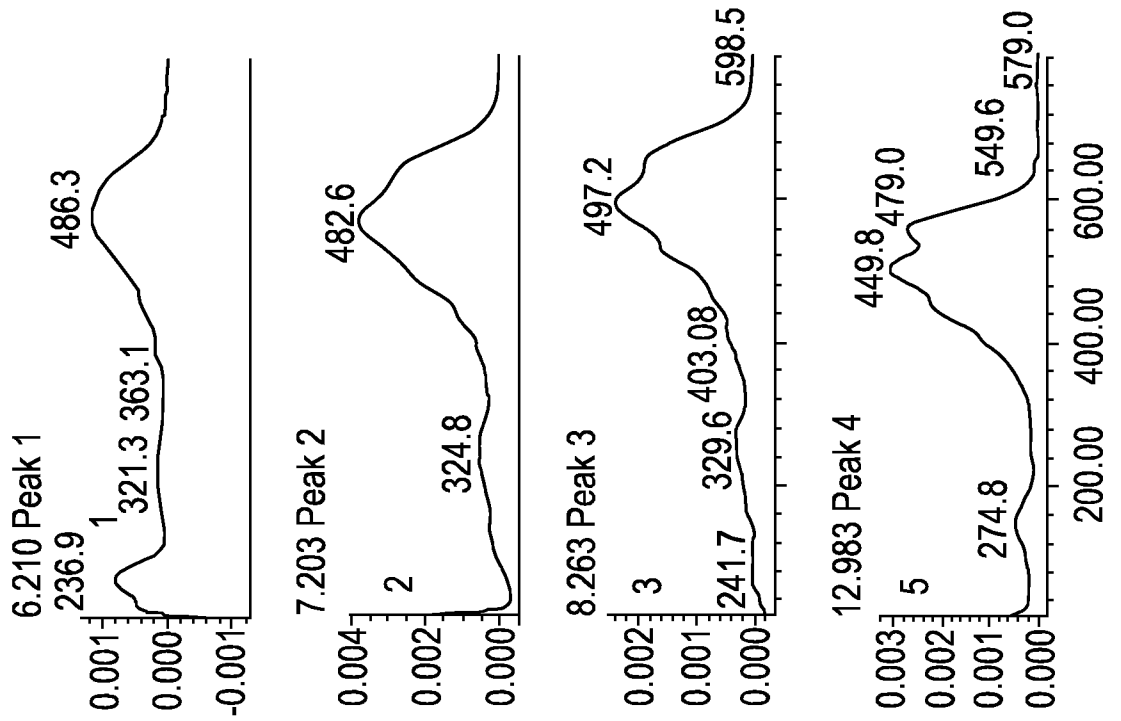
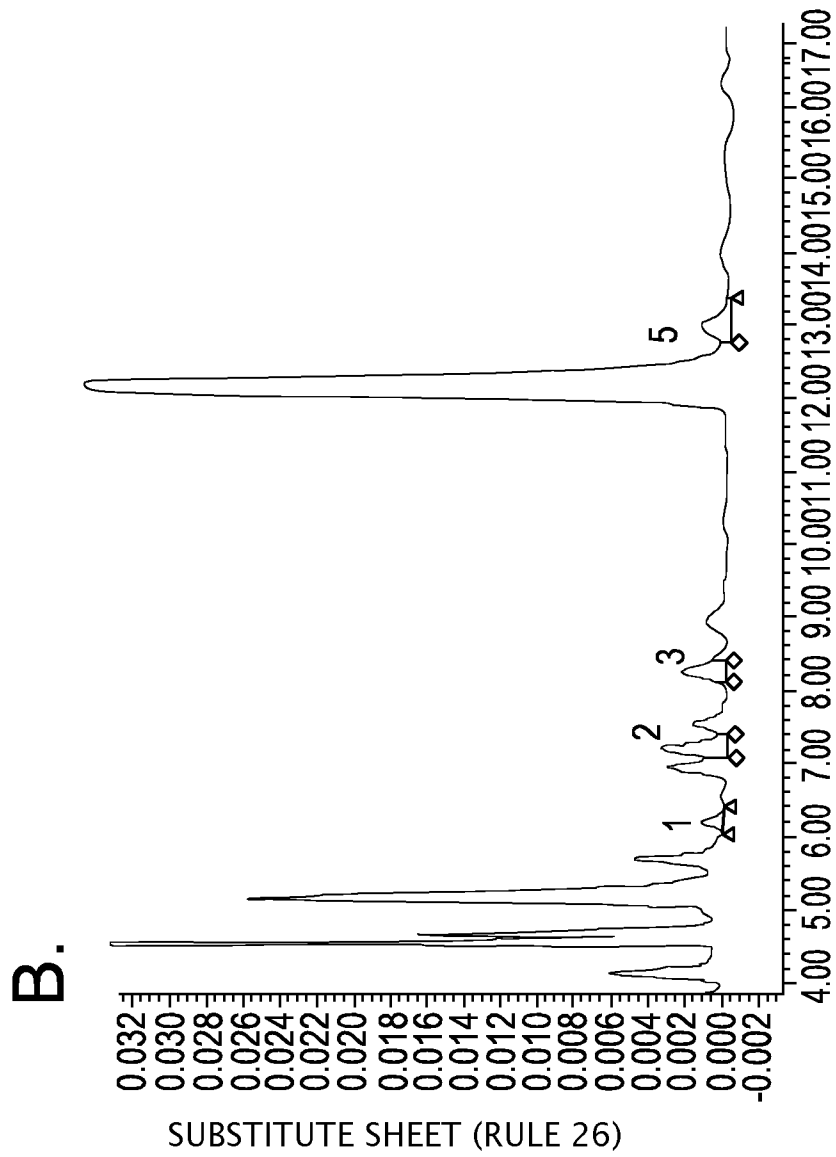


Figure 3

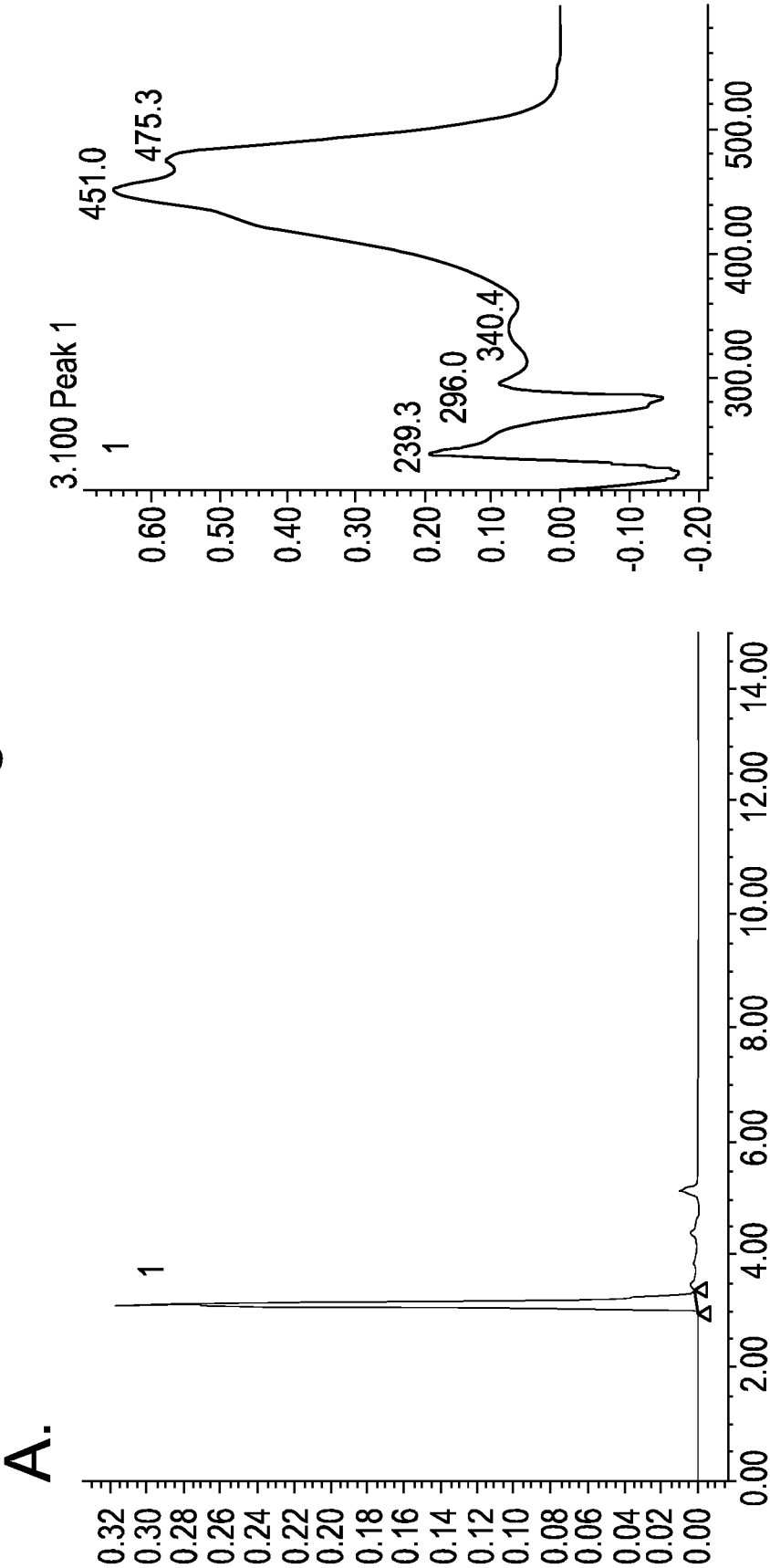
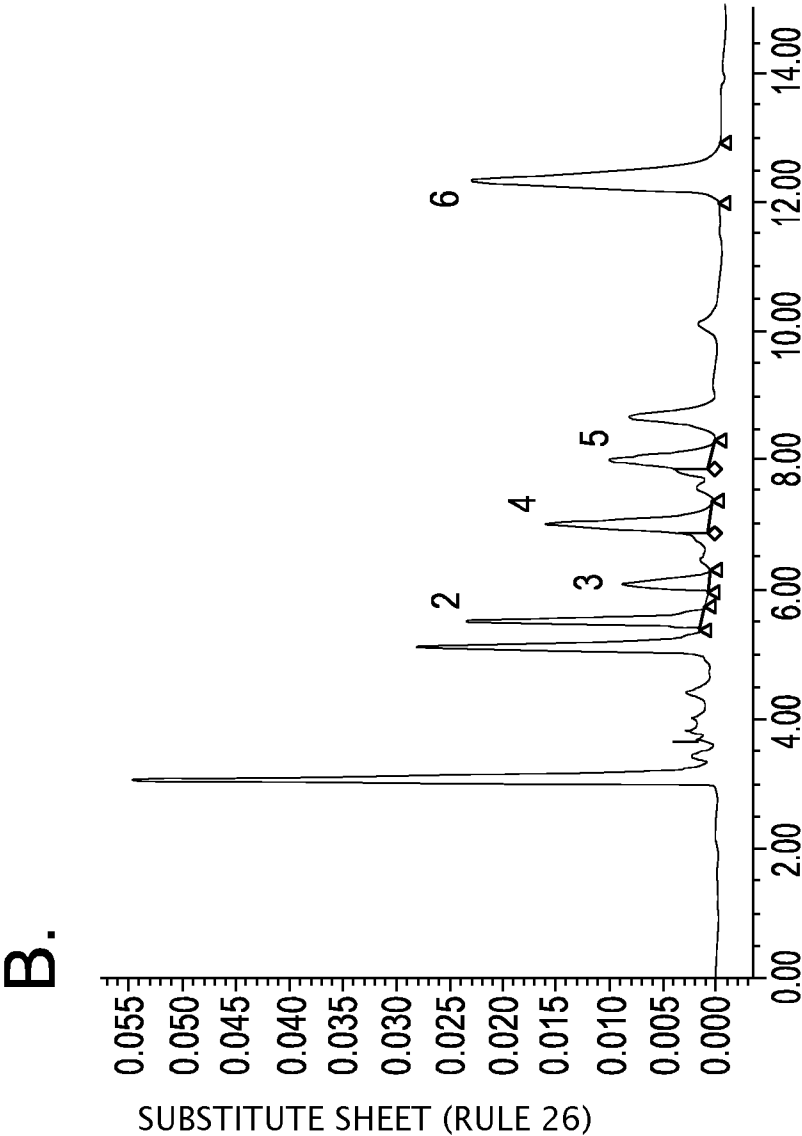
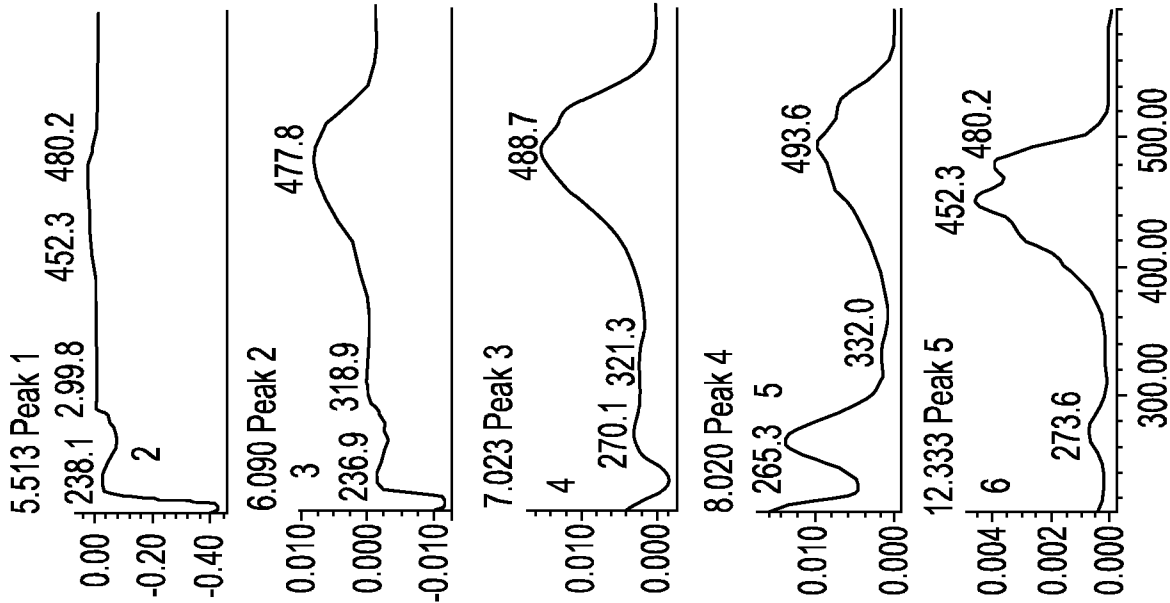


Figure 3



7/15

Figure 4

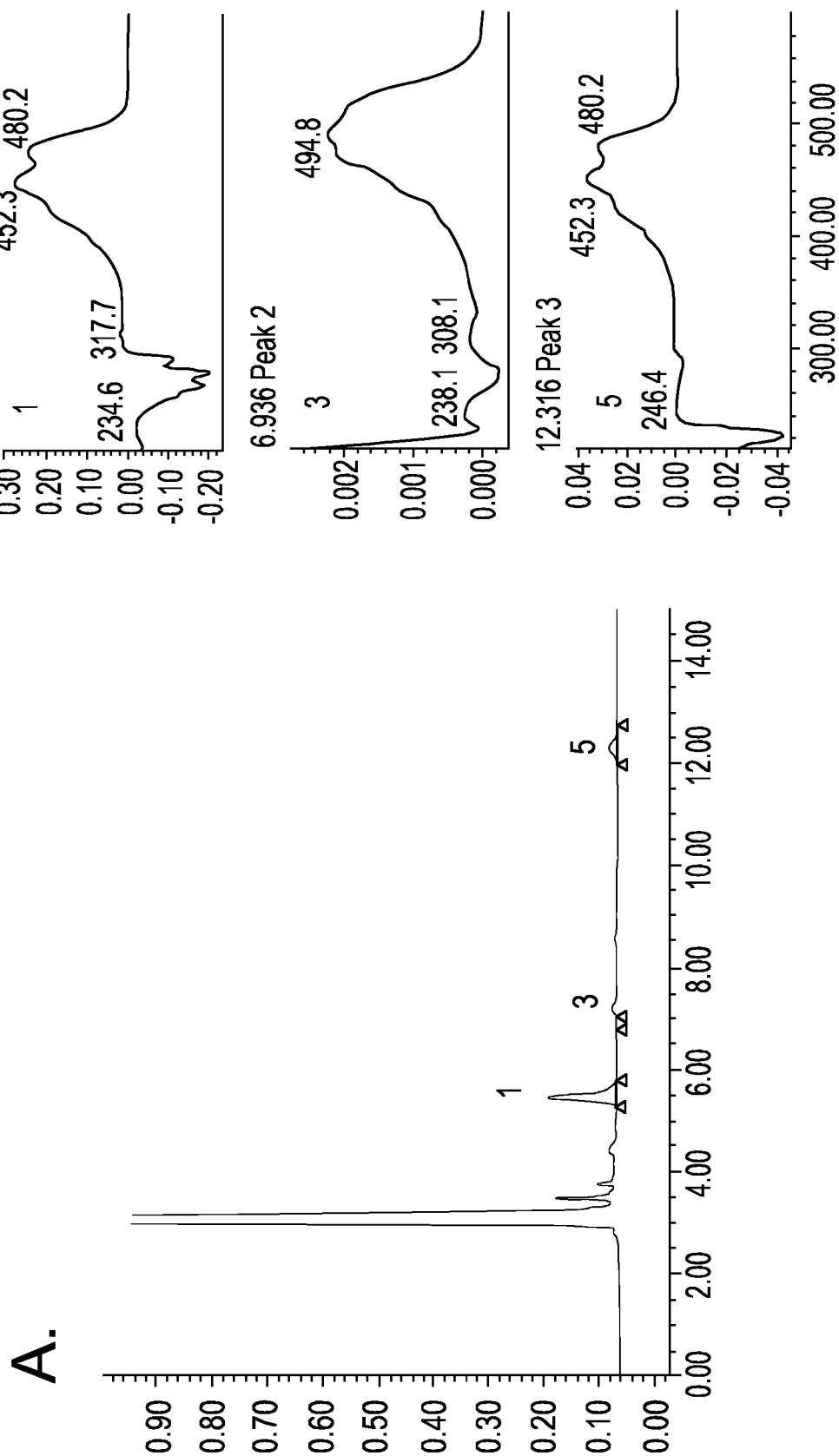
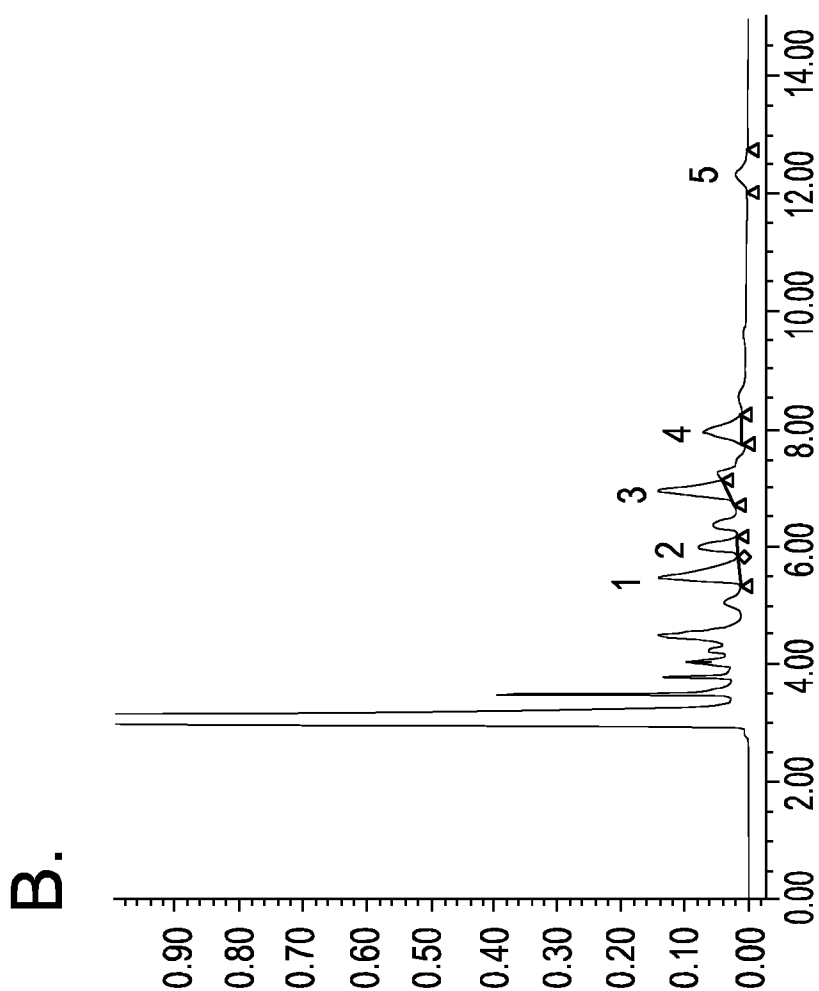
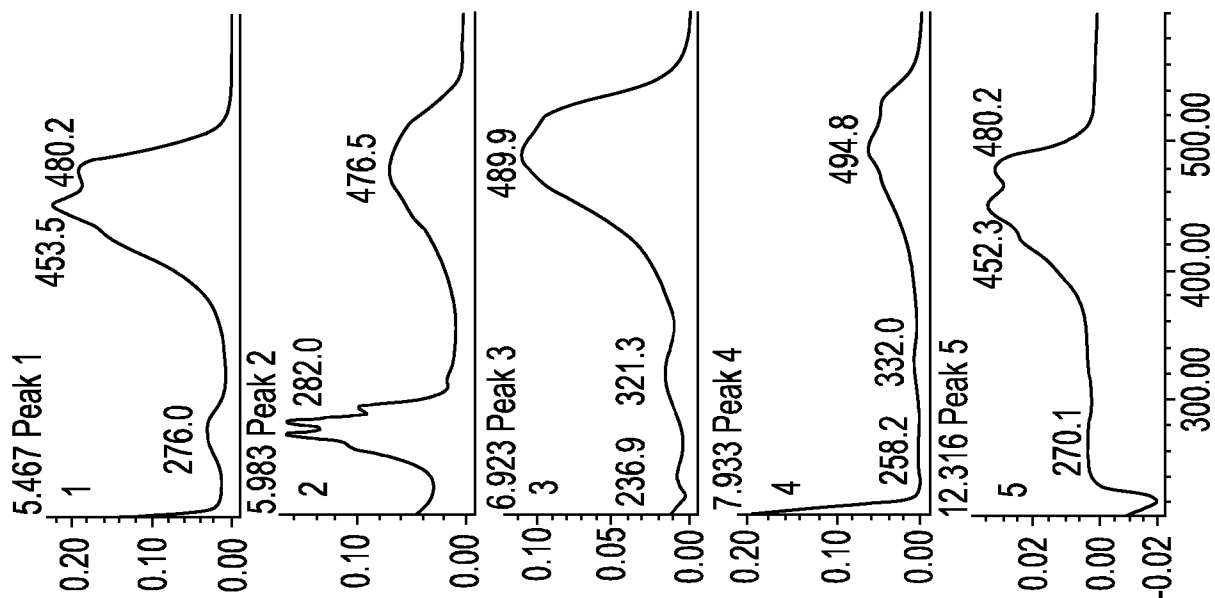


Figure 4



9/15

Figure 5

SEQ ID NO:1_BHYr full length, Lonicera DNA

ATGGCAACCGGAGTTCCGGCTTGTTGTACCACCACGGTAAGCCATATTTTCAGTGAAAATTC
TTGCGTGACTCGCAAACCTGGCTCCGAAAGAAACCAGAGGAGCTCAATTTCTCACCCACCA
AACAGGGGCGTGGACAATTTATTTCTTTAGGGAGAAAGAGGATTGTGGTTTATGGGAAAATG
GGACCGTCGGATGATAATAAGGAAGTGAGAATAATCAAGGGGTCTAAATTACGAGCGCAAGA
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GTTGCAGCTGCCACCTTCTTCATCATTAGATAGATTTAATGGAGTTCCATTTGGGTTACT
CTTGGGACCTAAGGAAGTAGAAGAAATAGGTGCAGGTGAAAAAGAGGAT

SEQ ID NO:2_BHYr full length, protein

MATGVPAACCTTTVSHIFSENSCVTRKPGSERNQRSSIFSPTKQGRGQFISLGRKRIVVYGKM
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VAAHLLHHSDFRNGVPFGLLLGPKELEEIGAGEKED

SEQ ID NO:3_BHYr trunc plus ATG start, for E. coli expression, Lonicera DNA (11209-1)

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GTGGCACATGCATGAGTCTCACCATAGGCCTGGGGACGGTCCATTCGAGCTTAATGATGTGA
TCCCCATCATCAATGCCATTCCAGCCATTGCTCTTCTCTCCCTTGATTCTTGAACAAGGGT
TTTTTTCCTGATCTTTGTTTTGGTGCTGGTCTAGGAATAACAGTATTTGGAATAGCATACAT
ACTCGTACACGACGGCCTCGTACACCGTCGATTTCTGTGGGACCCATTGCCACGTCCCTT
ATTTACAAAAAGTTGCAGCTGCCACCTTCTTCATCATTAGATAGATTTAATGGAGTTCCA
TTTGGGTTACTCTTGGGACCTAAGGAAGTAGAAGAAATAGGTGCAGGTGAAAAAGAGGAT

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Figure 5 Continued

SEQ ID NO:4_ BHYr trunc plus ATG start, for E. coli expression, protein (11209-1)

MAQERTERKRKERYTYLVAAIMSSLGITSLVVVSVYYRFSCQFEGGQIPVSEMLGTFALSMG
TAVGMEFWARWTHKELWHASLWHMHESHHRPGDGPPELNDVIPIINAI PAIALLSLGFLNKG
FFPDLFCGAGLGITVFGIAYILVHDGLVHRRFPVGPIAHVPYLQKVAAHLLHHS DRFNGVP
FGLLLGPKELEEIGAGEKED

SEQ ID NO:5_ BHYr trunc plus ATG start, codon optimized for expression in Yarrowia, DNA

ATGGAGCGAACTGAGCGAAAGCGAAAGGAGCGATACACTTACCTTGTTGCTGCTATTATGTC
TTCTCTTGGTATCACTTCTCTTGTGTTGTGTCCGTTTACTACCGATTTTCTTGTCAGTTTG
AGGGTGGACAGATTCTCTGTTTCTGAGATGCTTGGTACCTTTGCTCTTTCTATGGGTACCGCT
GTGGGTATGGAGTTTGGGCTCGATGGACTCACAAGGAGCTTTGGCACGCTTCTCTTTGGCA
CATGCACGAGTCTCACCACCGACCTGGCGACGGTCCTTTCGAGCTTAACGATGTCATCCCCA
TCATTAACGCCATTCTGCCATTGCTCTTCTCTCTCGGTTTTCTCAACAAGGGTTTTTTC
CCTGACCTTTGTTTTGGTGCTGGTCTTGGTATTACTGTTTTTGGTATTGCTTACATTCTCGT
TCACGACGGCCTCGTTCACCGACGATTTCCCGTTGGACCCATCGCTCACGTCCCTTACCTTC
AGAAGGTTGCTGCCGCTCACCTCCTTCACCACTCTGATCGATTTAACGGAGTTCCCTTTGGT
CTTCTCCTTGCTCCTAAGGAGCTTGAGGAGATTGGTGCTGGTGAGAAGGAGGAT

SEQ ID NO:6_ BHYr trunc plus ATG start, codon optimized for expression in Yarrowia, protein

MERTERKRKERYTYLVAAIMSSLGITSLVVVSVYYRFSCQFEGGQIPVSEMLGTFALSMGTA
VGMEFWARWTHKELWHASLWHMHESHHRPGDGPPELNDVIPIINAI PAIALLSLGFLNKGFF
PDLFCGAGLGITVFGIAYILVHDGLVHRRFPVGPIAHVPYLQKVAAHLLHHS DRFNGVPFG
LLLGPKELEEIGAGEKED

SEQ ID NO:7_ Aloe Ferredoxin Fd2 (c435_YLopt) codon optimized for Yarrowia expression, DNA

ATGGCCTCCCTCTCCACCCCTGCTACCCTCAACAACCCTTTCACCCGAGGCTCCCTCGCCCT
GAAGTCCCGACCTTCTGTGGCCTCCTCCTTCCGAGTGAAGCTCCGACGAGGCCGAGTGCAGG
CCATGGCTACCCACAAGGTGAAGCTGATTACCCCTGAGGGCACCCGAGAGATTAAGTGCAT
GAGGACACCTACATTCTGGACGCCGCGAGGACGCCGGCCTCGATCTCCCCTACTCCTGCCG
AGCCGGAGCTTGCTCTTCTGTGTGGGCAAGGTCGTGGAGGGTACCGTGAACCAGGATGACC
AGTCCTTCCTTGACTCCGAGCAGATCGATGCCGGCTGGGTGCTCACCTGCGTCGCTTACCCC
ACCTCTGACGTCGTCATCGAGACTAACAAGGAGGCTGAGCTGGCTGGGTGCTTAACATTCT
TTGTATGAAC

SEQ ID NO:8_ Aloe Ferredoxin Fds (c435_YLopt), protein

MASLSTPATLNNPFTRGSLALKSRPSVASSFRVKLRGRVQAMATHKVKLITPEGTREIKCD
EDTYILDAEDAGLDLPYSCRAGACSSCVGKVVEGTVNQDDQSFLDSEQIDAGWVLT CVAYP
TSDVVIETNKEAELAWVNNILCMN

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Figure 5 Continued

SEQ ID NO:9_Aloe Ferredoxin reductase c29069YLopt_Full length, codon optimized for Yarrowia expression, DNA

ATGGCCTCTGCTGCCCTTTCTGCTGCTGTCTCTCTCCCTTCTCCAAGTCCTCTTCTCTCAT
CCTCCCCTCTCGAACCTCCATCTCTTCCACTGAGAAGCTCACCTTCAACAACAAGACTGGCC
TCTACCTGAAGAACGTGTCTGTGACCTCCCGATCTGTTACTGTTGAGCTCAGGTCACCCCT
GAGGCTCCTGCTAAGGTCAAGAAGGAGTCCAAGAAGGACGAGGAGGGAGTTGTGACCAACAA
GTACGAGCCCAAGGATCCCTACATCGGACGATGCCTCCTCAACACCAAGATCACCGGTGATG
ACGCCCCCTGGCGAGACTTGGCACATGGTTTTTCTCCACTGAGGGCGAGATTCCCTACCGAGAG
GGCCAGTCCATTGGAGTTATCCCTGACGGTGTTGATAAGAACGGAAAGCCTCACAACCCCTG
CCTTTACTCCATCGCCTCTTCTGCTCTCGGTGACTTCGGCGATTCCAAGACTGTTTCCCTCT
GTGTGAAGCGACTGATTTACACCAACGATCAGGGAGAGATCGTCCGAGGTGTGTGCTCTAAC
TTCCTCTGTGATCTCAAGCCTGGTGCCGAGGTGAAGATTACCGGTCTGTGGAAGGAGAT
GCTTATGCCCAAGGACCCTAACGCTACTATCATTATGCTTGCTACTGGCACCGGAATTGCTC
CTTTCCGAGGCTTTCTCTGGAAGATGTTCTTTGAGAAGCACGAGGATTACAAGTTCAACGGA
CTCGCTTGGCTCTTCCTCGGAGTTCCTACCTCCTCTTCCCTCCTTTACAAGGAGGAGTTCGA
GAAGATGAAGGAGAAGTCCCCTGACAAGTTCGCACTTGACTTCGCCGTGTCTCGAGAGCAGA
CTAACGAGAAGGGAGAGAAGATGTACATTCAGACTCGAATGGCTCAGTACGCCGAGGAGCTG
TGGGAGCTGCTCAAGAAGGATGACACCTACGTCTACATGTGTGGACTCAAGGGAATGGAGAA
GGGAATTGACGATATCATGGAGTCTCTCGCTGCCAAGGATGGCATTGATTGGCTCACTTACA
AGAAGGAGCTGAAGAAGGGAGAGCAGTGAACGTGAGGTGTAC

SEQ ID NO:10_Aloe Ferredoxin reductase c29069YLopt_Full length, codon optimized for Yarrowia expression, protein

MASAALSAAVSLPFSKSSSLILPSRTSISSTEKLTFNNKTGLYLKNVSVTSRSVTVRAQVTP
EAPAKVKKESKKDEEGVVTNKYEPKDPYIGRCLLNTKITGDDAPGETWHMVFSTEGEIPYRE
GQSIGVIPDGVDKNGKPHNPCLYSIASSALGDFGDSKTVSLCVKRLIYTNDQGEIVRGVCSN
FLCDLKPGA EVKITGPVGKEMLPKDPNATIIMLATGTGIAPFRGFLWKMFFEKHEDYKFNG
LAWLFLGVPTSSSLLYKEEFEKMEKSPDKFRLLDFAVSREQTNEKGEKMYIQTRMAQYAEEL
WELLKKDDTYVYMCGLKGMEKGIDDIMESLAAKDGIDWLTYKKELKKGEQWNVEVY

SEQ ID NO:11_Pisum sativum CPE Q40983 Truncated and codon optimized for expression in Yarrowia, DNA

ATGGCTACTGTTGGTCCCGATGAGCCTCACGCCGCTTCTACCACTTGGCAGGAGGGCGTTGC
TGAGAAGCAGGACCTTTCCCTTTTCGATTCTGAGCTGGAGCGACTTGAGGGTTTCCTTG GTT
CTGAGCTCCCTTCTCACCCCTAAGCTCCACCGAGGTCAGCTCAAGAACGGTATTCGATACCTT
ATTCTGCCCAACAAGGTTCCCTCCCACCCGATTTGAGGCTCACATGGAGGTTACGTTGGATC
TATTGATGAGGAGGATGACGAGCAGGGAATTGCTCACATGATTGAGCACGTTGCTTTCCTCG
GATCTAAGAAGCGAGAGAAGCTGCTCGGAACCGGAGCCCGATCTAACGCTTACACCGATTTT
CACCACACCGTTTTTTCACATCCACTCTCCTACCTCTACCAAGGATTCTGATGACCTTCTCCC
TTCTGTTCTCGATGCCCTGAACGAGATTACCTTCCACCCCTAACTTCCTTGCTTCCCGAATTG
AGAAGGAGCGACGAGCTATTCTCTCTGAGCTTCAGATGATGAACACCATTTGAGTACCGAGTT

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Figure 5 Continued

GATTGCCAGCTCCTTCAGCACCTTCACTCTGAGAACAAGCTGTCTAAGCGATTTCCCATTTGG
TCTTGAGGAGCAGATTAAGAAGTGGGATGCCGATAAGATTGAAAGTTTCACGAGCGATGGT
ACTTCCCTGCTAACGCTACCCTCTACATTGTTGGTGATATTGGTAACATTCCCAAGACTGTT
AACCAGATTGAGGCTGTTTTTCGGACAGACTGGTGTGACAACGAGAAGGGTTCTGTTGCCAC
TTCTTCTGCTTTTTGGTGCTATGGCTTCTTTTCTCGTTCCTAAGCTCTCTGTTGGTCTTGGTG
GAAACTCTATTGAGCGACCCACCAACACTACCGATCAGTCTAAGGTTTTTAAGAAGGAGCGA
CACGCTGTTGACCTCCCGTGAAGCACACTTGGTCCCTTCCCGGATCTTCTGCTAACCTTAA
GCCCCCTCAGATTTTTTCAGCACGAGCTTCTCCAGAACTTTTTCTATTAACATGTTCTGCAAGA
TTCCCGTGAACAAGGTTGAGACTTACCGAGATCTCCGAATTGTCCTGATGAAGCGAATTTTT
CTCTCTGCTCTTCACTTTTGAATTAACACCCGATACAAGTCTCTAACCCCTCCCTTCACTTC
TGTTGAGCTCGATCACTCTGATTCTGGACGAGAGGGATGTACTGTTACCACTCTCACCATTA
CTGCCGAGCCTAAGAACTGGCAGAACGCTATTGAGTTGCTGTTACGAGGTTTCGACGACTT
AAGGAGTTTGGTGTTACTCAGGGTGAGCTCACTCGATACCTGGACGCCCTCCTTCGAGATTC
TGAGCACCTTGCTGCCATGATTGATAACGTTTCTTCTGTTGACAACCTTGACTTTATCATGG
AGTCTGATGCTCTTGGCCACAAGGTTATGGACCAGTCTCAGGGTCACGAGTCCCTCATTGCT
GTTGCTGGAACCGTTACCCCTGACGAGGTTAACTCTGTTGGTGCTCAGGTTCTTGAGTTTAT
TGCTGATTTCCGAAAGCTCTCTGCTCCCTCCCTGCTGCTATTGTTGCTTGTTGTTCCCTAAGA
AGGTTACATCGAGGGAGCTGGTGAGACTGAGTTCAAGATCTCCTCTACTGAGATTACCGAT
GCTATGAAGGCTGGACTCGATGAGCCTATTGAGCCCGAGCCCGAGCTCGAGGTTCCCTAAGGA
GCTTGTCAGTCTTCTACCCTGCAGGAGCTGAAGAACCAGCGAAAGCCTGCCTTTATTCCCG
TTTCTCCTGAGATTGAGGCTAAGAAGCTCCACGATGAGGAGACCGGAATCACCCGACTCCGA
CTTGCCAACGGAATCCCGTCAACTACAAGATTTCTAAGTCCGAGACTCAGTCCGGCGTGAT
GCGACTGATTGTTGGTGCGGACGAGCTGCTGAGGGTTCTGATTCCCGAGGATCTGTGATTG
TGGGTGTTTGAACCCCTTTCCGAGGGAGGTCGAGTTGGCAACTTCTCTCGAGAGCAGGTTGAG
CTTTTCTGCGTTAACAACCAGATTAAGTCTCCCTTGAGTCTACTGAGGAGTTTCAATTTCTCT
CGAGTTTCGATTCACTCTCCGAAACAACGGAATGCGAGCTGCCTTTTCAGCTCCTTCACATGG
TTCTTGAGCACTCTGTCTGGTCTGACGATGCTCTGGACCGAGCTCGACAGGTGTACCTGTCT
TACTACCGATCCATCCCCAAGTCCCTTGAGCGATCTACTGCTCACAAGCTCATGGTTGCCAT
GCTCGATGGTGATGAGCGATTTACCGAGCCCACTCCCTCTTCTCTTGAGAACCTTACCCTGC
AGTCTGTTAAGGATGCTGTGATGAACCAGTTTGTGTTGGAACAACATGGAGGTTTCCATTGTT
GGTGATTTCACTGAGGAGGAGATTGAGTCTGTATTCTGGACTACCTTGGCACTGCTCAGGC
CACTGGTAACCTTTAAGAACCAGCAGCAGATTATCCCTCCCACTTTCCGACTTTCTCCCTCCT
CTCTTCAGTCTCAGGAGGTTTTCTGAACGACACCGATGAGCGAGCTTGCGCTTACATTGCT
GGTCCCTGCTCCTAACCGATGGGGTTTTTACCGCTGATGGTAACGACCTCCTTGAGACTATTGA
TAACGCTTCTCTGTCAACAACAACGGCACTAAGTCTGATGCTCTTCAGACCGAGGGTGCTC
CTCGACGATCTCTCCGATCTCACCCCTTTTTCTTTGGTATTACTATGGGCCTGCTCTCTGAG
ATTATTAACCTCTCGACTCTTCACTACCGTCCGAGATTCCCTGGGCCTTACCTACGACGTGTC
CTTTGAGCTTAACCTCTTCGATCGACTTAAGCTGGGTGGTACGTTGTCTCTGTCACTTCTA
CTCCCTCTAAGGTGCACAAGGCTGTTGATGCTTGCAAGAACGTTCTTCGAGGTCTGCACTCC
AACGGAATTACTGTCCGAGAGCTTGACCGAGCTAAGCGAACCCCTCCTTATGCGACACGAGGC
TGAGATTAAGTCTAACGCTTACTGGCTTGGTCTTCTCGCTCACCTTCAGTCTTCTTCTGTTC
CTCGAAAGGACCTTTCTTGATCAAGGATCTCACTTCTCTGTACGAGGCTGCTACTATTGAG
GACACTTGCCCTTGCTTACGAGCAGCTTAAGGTTGATGAGGATTCTCTTTACTCTTGCAATTGG
AGTTTCTGGTGCTCAGGCTGCTCAGGATATTGCTGCTCCTGTTGAGGAGGAGGAGGCTGGTG
AGGGTTACCCTGGTGTTCTCCCTATGGGACGAGGTCTTCTACTATGACTCGACCCACTACC

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Figure 5 Continued

SEQ ID NO:12_ *Pisum sativum* CPE Q40983 Truncated and codon optimized for expression in *Yarrowia*, protein

MATVGPDEPHAASTTWQEGVAEKQDLSLFDSELERLEGFLGSELPSHPKLHRGQLKNGIRYL
ILPNKVPPTREFAHMEVHVGS IDEEDDEQGIAHMI EHVAFLGSKKREKLLGTGARSNAYTDF
HHTVVFHIHSPTSTKDSDDLPSVLDALNEITFHPNFLASRIEKERRAILSELQMMNTIEYRV
DCQLLQHLHSENKLSKRFP IGLEEQI KKWDADKIRKFHERWYFPANATLYIVGDIGNIPKTV
NQIEAVFGQTGVDNEKGSVATSSAFGAMASFLVPKLSVGLGGNSIERPTNTTDDQSKVFKKER
HAVRPPVKHTWSLPGSSANLKPPQIFQHELLQNFSINMFCKIPVNKVQTYRDLRIVLMKRIF
LSALHFRINTRYKSSNPPFTSVELDHSDSGREGCTVTTLTITAEPKNWQNAIRVAVHEVRRL
KEFGVTQGELTRYLDALLRDSEHLAAMIDNVSSVDNLD FIMESDALGHKVMQDSQGHESLIA
VAGTVTLDEVNSVGAQVLEFIADFGKLSAPLPAAI VACVPKKVHIEGAGETEFKISSTEITD
AMKAGLDEPIEPEPELEV PKELVQSSTLQELKNQRKPAFIPVSPEIEAKKLHDEETGITRLR
LANGIPVNYKISKSETQSGVMRLIVGGGRAAEGSDSRGSVIVGVRTLSEGGRVGNFSREQVE
LFCVNNQINCSLESTEEFISLEFRFTLRNNGMRAAFQLLMVLEHSVWSDDALDRARQVYLS
YYRSIPKSLERSTAHKLMVAMLDGDERFTEPTPSSLENLTLSVKDAVMNQFVGNMMEVSIV
GDFTEEEIESCILDYLGTAQATGNFKNQOQIIPPTFRLSPSSLQSQEVFLNDTDERACAYIA
GPAPNRWGFTADGNDLLETIDNASSVNNNGTKSDALQTEGAPRRSLRSHPLFFGITMGLLSE
IINSRLFTTVRDSLGLTYDVSFELNLFDRCLKGWYVVSVTSTPSKVHKAVDACKNVLRLGLHS
NGITVRELDRAKRTLMLRHEAEIKSNAYWLGLLAHLQSSSVPRKDLSCIKDLTSLYEAATIE
DTCLAYEQLKVDEDSLYSCIGVSGAQAAQDIAAPVEEEEEAGEGYPGVLP MGRGLSTMTRPTT

SEQ ID NO:13_ *Lonicera beta* carotene cyclase, full length, *Lonicera* DNA

ATGGCAACTCTCACAAGGCCACCACTATGCCCTCTTCTCTCAATTTCCCACACCTCTTCTTC
ACCATTACCTCCCAATTTCTGCTCCCAAAACCCACAAAAAAGGTCTCTCCAAGATTCACCTC
ACCACAAAATCTTTAGCAAACATGGCAACTTTCTTGATCTTAAACCTGAATTTACCCCCGAA
CCCATCAAATTTGACCTCAATTGGCTCGATCCAACCTCAAGATTTGCTCCGATGTGATCAT
AATTGGGACTGTTCCGATCTCCGGGCTTCGCCTCGCAGAGCGAATTTCCGGGTTTGGGATCC
GGGTCTGTTGCGTTGACCCGGATCCCCTATCTATGTGGCCTAATAATTATGGTGCTTGGATT
GATGAATTGGAGGCGATTGGGCTTGATGATTGTTTTGATAAAAAATGGGGCATGGCAACTGT
TTGTATAAATGACTCAAAAACAAAGTATTTGGATAGGGCTTATGGGAGGATTAATAGGAAGT
TTTTGAAGTTGAAGTTGTTGGAAAGTTGTGTTAATAATGGGGTTAAGTTCATAAGGCAAAG
GTTTGGGAATGTTGTGCACAACGAGTTTGATAGTATGATTGTGTGTGATGATGGGGATGAGTT
AAAGGCAAGTTTGGTCGTTGATGCAAGTGGGTTTGCTAGTACTTTTGTGGAGTATGATAAGC
CAAGAAACCATGGGTATCAAATTGCCCATGGGATTCTTGCTGAAGTGAAGAACACCCCTTT
GATTTGGACAAGATGCTTTTGTATGGATTGGAGAGATTCTCATTTGGGTAATGAGCCTTATTT
GAGGGCTAGTAATTCAAGAACACCGACTTTTTTTTGTACGCGATGCCGTT

SEQ ID NO:14_ *Lonicera beta* carotene cyclase, full length, protein

MATLTRPPLCPLLSISHTSSSPFTSQFLLPKPTKKVSPRFTHHKIFSKHGNFLDLKPEFHPE
PIKFDLNLWDPTSRFRSDVIIIGTVPIISGLRLAERISGFGIRVCCVDPDPLSMWPNNYGAWI
DELEAIGLDDCFDKKWGMATVCINDSKTKYLDGRAYGRINRKFLKLKLLLESCVNNGVKFHKAK
VWNVVHNEFDSMIVCDDGDELKASLVVDASGFASTFVEYDKPRNHGYQIAHGILAEVEEHPF
DLDKMLLMDWRDShLGNepYLRASNSRTPTFVVRDAV

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Figure 5 Continued

SEQ ID NO:15_Lonicera beta carotene cyclase, truncated Lonicera DNA plus ATG start codon, DNA

ATGCCACAAAAAAGGTCTCTCCAAGATTCACCTACCACAAAATCTTTAGCAAACATGGCAA
CTTTCTTGATCTTAAACCTGAATTTACCCCGAACCCATCAAATTTGACCTCAATTGGCTCG
ATCCAACCTCAAGATTTTCGCTCCGATGTGATCATAATTGGGACTGTTCCGATCTCCGGGCTT
CGCCTCGCAGAGCGAATTTCCGGGTTTGGGATCCGGGTCTGTTGCGTTGACCCGGATCCCCT
ATCTATGTGGCCTAATAATTATGGTGTCTGGATTGATGAATTGGAGGCGATTGGGCTTGATG
ATTGTTTTTGATAAAAAATGGGGCATGGCAACTGTTTGTATAAATGACTCAAAAACAAAGTAT
TTGGATAGGGCTTATGGGAGGATTAATAGGAAGTTTTTGAAGTTGAAGTTGTTGGAAAGTTG
TGTTAATAATGGGGTTAAGTTCATAAGGCAAAGGTTTGAATGTTGTGCACAACGAGTTTG
ATAGTATGATTGTGTGTGATGATGGGGATGAGTTAAAGGCAAGTTTGGTCGTTGATGCAAGT
GGGTTTGCTAGTACTTTTGTGGAGTATGATAAGCCAAGAAACCATGGGTATCAAATTGCCCA
TGGGATTCTTGCTGAAGTGGAAGAACACCCCTTTGATTTGGACAAGATGCTTTTGATGGATT
GGAGAGATTCTCATTTGGGTAATGAGCCTTATTTGAGGGCTAGTAATTCAAGAACACCGACT
TTTTTTGTACGCGATGCCGTT

SEQ ID NO:16_Lonicera beta carotene cyclase, truncated plus ATG start codon, protein

MPTKKVSPRFTHHKIFSKHGNFLDLKPEFHPEPIKFDLNWLDPTSRFRSDV I I I GTVPI SGL
RLAERISGFGIRVCCVDPDPLSMWPNNYGAWIDELEAIGLDDCFDKKWGMATVCINDSKTKY
LDRAYGRINRKFLKLKLESCVNNGVKFHKAKVWNVVHNEFDSMIVCDDGDELKASLVVDAS
GFASTFVEYDKPRNHGYQIAHGILAEVEEHPFDLDKMLLMDWRDSHLGNEPYLRASNSRTPT
FFVRDAV

SEQ ID NO:17_ Aloe Ferredoxin Fd2 aloerefcl_c435_FD2_ATG1_Full length, Aloe sequence

ATGGCCTCCCTCTCCACCCCAGCAACCCTCAACAACCCATTACCCGGGGCTCCCTCGCGCT
GAAGAGCCGCCCCTCTGTGGCCTCCTCCTTCCGGGTGAAGCTCAGGCGCGGAGAGTGCAGG
CCATGGCGACCCACAAGGTGAAGCTGATAACCCCTGAAGGGACGCGGGAGATTAAGTGCAGT
GAGGACACCTACATACTGGACGCGGCCGAGGACGCGGGGCTCGATCTGCCCTACTCGTGCAG
GGCCGGGGCGTGCTCTTCCTGTGTGGGCAAGGTCGTGGAGGGGACGGTGAACCAGGATGACC
AGAGCTTCTTGACTCCGAGCAGATCGATGCCGGCTGGGTGCTCACCTGCGTCGCTTACCCC
ACCTCTGACGTCGTCATCGAGACTAACAAGGAGGCAGAGCTGGCGTGGGTAGTTAATATATT
ATGTATGAAT

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Figure 5 Continued

SEQ ID NO:18_Aloe Ferredoxin reductase aloerefcl_c29069_PETH_ATG1_Full length, Aloe sequence

ATGGCCTCTGCTGCACTTTCTGCTGCAGTCTCTCTCCCCTTCTCCAAGTCCTCTTCTCTAAT
CCTCCCCCTCAAGAACCTCCATCTCTTCCACTGAGAAGCTCACATTCAACAACAAGACAGGCT
TGTACCTGAAGAATGTGTCTGTGACTTCGAGATCAGTGACTGTGAGAGCTCAAGTCACCCCT
GAGGCTCCTGCGAAAGTCAAGAAAGAATCGAAGAAGGACGAAGAGGGAGTTGTGACCAACAA
GTACGAGCCCCAAGGATCCTTACATCGGGAGGTGCCTCCTCAACACGAAGATCACCGGTGATG
ATGCCCCCTGGAGAGACTTGGCATATGGTTTTTCAGCACTGAGGGGGGAAATTCCATATAGAGAA
GGCCAATCCATTGGAGTGATCCCTGATGGGGTTGATAAGAATGGGAAGCCTCACAACCCGTG
CCTATATTCGATCGCCAGCAGCGCTCTCGGTGACTTCGGCGATTCCAAAAGTGTTCCTAT
GTGTGAAGAGGCTGATTTACACCAATGATCAGGGAGAAATAGTCAGAGGGGTGTGCTCAAAT
TTCTTATGTGATTTGAAGCCTGGGGCAGAAGTGAAGATTACAGGTCCAGTTGGAAAGGAAAT
GCTTATGCCCAAAGATCCAAATGCTACTATCATAATGCTTGCAACAGGCACTGGAATTGCTC
CTTTCCGCGGCTTTTTTATGGAAGATGTTCTTTGAGAAGCACGAAGATTACAAGTTCAATGGA
TTGGCATGGCTCTTCTTGGGAGTTCCTACAAGCAGCTCATTGCTTTACAAGGAGGAATTCTGA
GAAGATGAAGGAGAAATCACCTGATAAGTTCAGGCTAGACTTCGCAGTGAGCAGAGAGCAGA
CAAATGAGAAAGGAGAGAAGATGTACATTCAGACTCGAATGGCTCAGTACGCCGAGGAGCTG
TGGGAGCTACTCAAGAAAGATGACACCTATGTATATATGTGTGGACTCAAAGGAATGGAGAA
GGGAATTGATGATATAATGGAGTCTTTGGCTGCAAAAGATGGCATTGATTGGCTAACTTACA
AGAAGGAGCTGAAGAAAGGAGAGCAATGGAATGTAGAAGTGTAC

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/069803

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/02 C12P23/00 C12N15/63 C12N15/82
ADD.

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)

C12N C12P

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/068946 A2 (THOMPSON BOYCE PLANT RES [US]; US AGRICULTURE [US]; VAN ECK JOYCE [US]) 29 June 2006 (2006-06-29)	1-5
A	beta-carotene hydroxylase 62% sequence identity to SEQ ID , 77.3% to Seq ID No. 4, 77.6% to SEQ ID No. 6; claims 1-42 ----- -/--	6-14



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

24 September 2018

Date of mailing of the international search report

04/10/2018

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Vollbach, Silke

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/069803

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LI XI-RAN ET AL: "Metabolic engineering of Escherichia colito produce zeaxanthin", JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, BASINGSTOKE, GB, vol. 42, no. 4, 23 December 2014 (2014-12-23), pages 627-636, XP035470735, ISSN: 1367-5435, DOI: 10.1007/S10295-014-1565-6 [retrieved on 2014-12-23] page 635 -----	1-14
Y	MISAWA N ET AL: "Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 59, no. 3, 3 January 1998 (1998-01-03), pages 169-181, XP004113748, ISSN: 0168-1656, DOI: 10.1016/S0168-1656(97)00154-5 page 177 - page 180; figure 2; table 1 -----	1-14
Y	WO 2005/044975 A2 (DU PONT [US]; CHENG QIONG [US]; SEDKOVA NATALIA [US]; TAO LUAN [US]) 19 May 2005 (2005-05-19) claims 1-36; figures 1,5 -----	1-14
Y	ZHOU PINGPING ET AL: "Highly efficient biosynthesis of astaxanthin in Saccharomyces cerevisiae by integration and tuning of algal crtZ and bkt", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, DE, vol. 99, no. 20, 9 July 2015 (2015-07-09), pages 8419-8428, XP035547321, ISSN: 0175-7598, DOI: 10.1007/S00253-015-6791-Y [retrieved on 2015-07-09] page 635 -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/069803

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006068946 A2	29-06-2006	US 2008276331 A1 WO 2006068946 A2	06-11-2008 29-06-2006
WO 2005044975 A2	19-05-2005	CA 2528574 A1 EP 1631660 A2 JP 2007516697 A US 2004253663 A1 WO 2005044975 A2	19-05-2005 08-03-2006 28-06-2007 16-12-2004 19-05-2005