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(54) **Title:** DELTA-8 DESATURASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

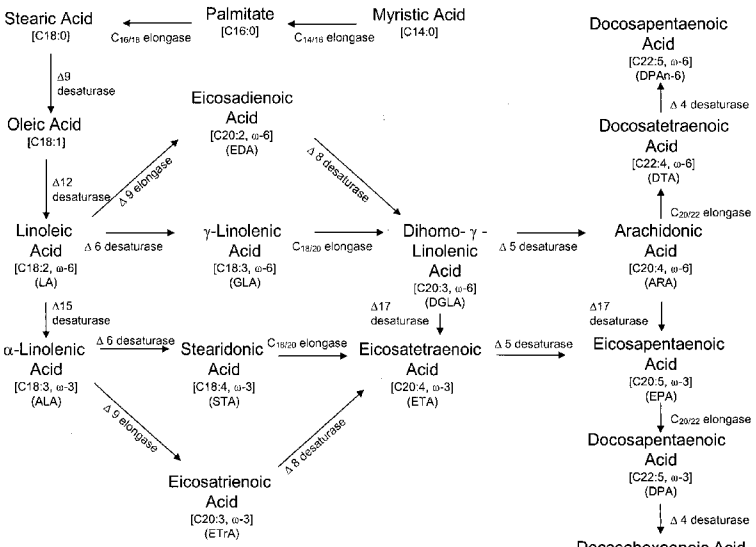

FIG. 1

(57) **Abstract:** The present invention relates to Δ8 desaturase genes, which have the ability to convert eicosadienoic acid (EDA; 20:2 ω-6) to dihomo-γ-linolenic acid (DGLA; 20:3 ω-6) and/or eicosatrienoic acid (ETrA; 20:3 ω-3) to eicosatetraenoic acid (ETA; (20:3 ω-3). Isolated nucleic acid fragments and recombinant DNA constructs comprising such fragments encoding Δ8 desaturases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) using these Δ8 desaturases in oleaginous yeast are disclosed.

WO 2008/124194 A1

TITLE

Δ8 DESATURASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

This application claims the benefit of United States Provisional Patent Application No. 60/910,831, filed April 10, 2007, which is incorporated herein in its entirety by reference.

FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this invention pertains to the identification of polynucleotide sequences encoding Δ8 fatty acid desaturases and the use of these desaturases in making long-chain polyunsaturated fatty acids (PUFAs).

BACKGROUND OF THE INVENTION

Today, a variety of different hosts including plants, algae, fungi, stramenopiles and yeast are being investigated as means for commercial PUFA production. Genetic engineering has demonstrated that the natural abilities of some hosts (even those natively limited to linoleic acid (LA; 18:2 ω-6) or α-linolenic acid (ALA; 18:3 ω-3) fatty acid production) can be substantially altered to result in high-level production of various long-chain ω-3/ω-6 PUFAs. Accordingly, production of arachidonic acid (ARA; 20:4 ω-6), eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) may require expression of a Δ8 desaturase.

The Δ8 desaturase enzymes identified thus far have the ability to convert both eicosadienoic acid (EDA; 20:2 ω-6) to dihomo-γ–linolenic acid (DGLA; 20:3 ω-6) and eicosatrienoic acid (ETrA; 20:3 ω-3) to eicosatetraenoic acid (ETA; 20:4 ω-3) (wherein ARA are EPA are subsequently synthesized from DGLA and ETA, respectively, following reaction with a Δ5 desaturase, while DHA synthesis requires subsequent expression of an additional $C_{20/22}$ elongase and a Δ4 desaturase).

Based on the role Δ8 desaturase enzymes play in the synthesis of e.g., ARA, EPA and DHA, considerable effort has been made to identify and characterize these enzymes from various sources. Initial efforts have focused on the isolation and characterization of Δ8 desaturases from *Euglena gracilis;* and, several sequence variations within the *Euglena gracilis* Δ8 desaturase have been reported (see, e.g., Wallis et al., *Arch. Biochem. and Biophys.*, 365(2):307-316 (1999); PCT Publication

No. WO 2000/34439; U.S. Patent No. 6,825,017; PCT Publication No. WO 2004/057001). Additionally, commonly owned, co-pending U.S. Application No. 11/166,003 and U.S. Patent 7,256,033 disclose amino acid and nucleic acid sequences for a *Euglena gracilis* Δ8 desaturase. In other work commonly owned, co-pending applications U.S. Patent Applications No. 11/635258 and No. 11/951697 describe a synthetically engineered mutant Δ8 desaturase, derived from *Euglena gracilis*.

U.S. Publication No. 2005/0273885 discloses amino acid and nucleic acid sequences for a Δ8 desaturase enzyme from *Pavlova salina* and commonly owned and co-pending application U.S. Patent Application No. 11/737772 discloses amino acid and nucleic acid sequences for a Δ8 desaturase enzyme from *Pavlova lutheri* (CCMP459), whereas U.S. Patent Application No. 11/876115 discloses amino acid and nucleic acid sequences for Δ8 desaturase enzymes from *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594. Sayanova et al. (*FEBS Lett.,* 580:1946-1952 (2006)) describe the isolation and characterization of a cDNA from the free living soil amoeba *Acanthamoeba castellanii* that, when expressed in *Arabidopsis*, encodes a polypeptide having C_{20} Δ8 desaturase activity.

Despite the disclosures cited above, there is a need for additional genes encoding polypeptides having Δ8 desaturase activity as it is only through genetic variation that a wide variety of host cells may be optimized for PUFA production. Applicants address the stated need herein by reporting the isolation of genes encoding Δ8 fatty acid desaturases from *Euglena anabaena*.

SUMMARY OF THE INVENTION

The present invention relates to new genetic constructs encoding polypeptides having Δ8 desaturase activity, and their use in algae, bacteria, yeast, euglenoids, stramenopiles and fungi for the production of PUFAs. Accordingly the invention provides, a microbial host cell comprising an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid

sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24;

(b) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence selected from the group consisting of: SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:39;

(c) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence hybridizes under stringent conditions to a nucleotide sequence selected from the group consisting of: SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:39; or

(d) a complement of the nucleotide sequence of (a), (b) or (c), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

In another embodiment the invention provides a method for the production of dihomo-γ-linoleic acid comprising:

a) providing a microbial host cell comprising:

(i) a recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

(ii) a source of eicosadienoic acid;

b) growing the microbial host cell of step (a) under conditions wherein the nucleic acid fragment encoding the Δ8 desaturase polypeptide is expressed and the eicosadienoic acid is converted to dihomo-γ-linoleic acid; and,

optionally recovering the dihomo-γ-linoleic acid of step (b).

Similarly the invention provides a method for the production of eicosatetraenoic acid comprising:

a) providing a microbial host cell comprising:

(i) a recombinant nucleotide molecule encoding a Δ5

desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

(ii) a source of eicosatrienoic acid;

b) growing the microbial host cell of step (a) under conditions wherein the nucleic acid fragment encoding the Δ8 desaturase polypeptide is expressed and the eicosatrienoic acid is converted to eicosatetraenoic acid; and,

c) optionally recovering the eicosatetraenoic acid of step (b).

In another embodiment the invention provides an isolated nucleic acid molecule which encodes a Δ8 desaturase as set forth in SEQ ID NO:39 wherein at least 208 codons are codon-optimized for expression in *Yarrowia* sp.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

FIG. 1 is a representative ω-3 and ω-6 fatty acid biosynthetic pathway providing for the conversion of myristic acid through various intermediates to DHA.

FIG. 2A diagrams the development of *Yarrowia lipolytica* strain Y4001U, producing about 17% EDA in the total lipid fraction. FIG. 2B provides the plasmid map for pZKLeuN-29E3, while FIG. 2C provides the plasmid map for pY116.

FIG. 3 shows a chromatogram of the lipid profile of an *Euglena anabaena* cell extract as described in Example 1.

FIGs. 4A, 4B and 4C show a Clustal V alignment of the Δ8 desaturase sequences for EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) and EaD8Des4 (SEQ ID NO:24), and a functional variant *Euglena gracilis* Δ8 desaturase amino acid sequence (EgD8; SEQ ID NO:25; described as Eg5 in PCT Application No. WO 2006/012325).

FIG. 5 provides plasmid maps for the following: (A) pY115 (SEQ ID NO:34); and, (B) pY175 (SEQ ID NO:35).

FIG. 6 provides the fatty acid profiles for *Yarrowia lipolytica* expressing pY175, pY176, pY177 and pY178 (see Example 5).

FIGs. 7A and 7B show a comparison of the nucleotide sequences of EaD8Des3 (SEQ ID NO:19) and EaD8S (SEQ ID NO:39).

FIG. 8 provides plasmid maps for the following: (A) pEaD8S (SEQ ID NO:41); and, (B) pZUFmEaD8S (SEQ ID NO:51).

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions, which form a part of this application.

The following sequences comply with 37 C.F.R. §1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NOs:1, 2, 10, 11, 13-25, and 28-51 are ORFs encoding genes or proteins (or portions thereof), or plasmids, as identified in Table 1.

Table 1

Summary Of Nucleic Acid And Protein SEQ ID Numbers

Description and Abbreviation	**Nucleic acid SEQ ID NO.**	**Protein SEQ ID NO.**
Euglena anabaena Δ8 desaturase partial sequence	1 (604 bp)	--
Euglena gracilis Δ8 desaturase CDS ("Eg5")	2 (1263 bp)	--
Plasmid pHD23-1	10 (4116 bp)	--
Euglena gracilis Δ8 desaturase (NCBI Accession No. AAD45877)	--	11 (419 AA)
Plasmid pLF118-1	13 (4363 bp)	--
Plasmid pLF118-2	14 (4307 bp)	--
Plasmid pLF118-3	15 (4307 bp)	--
Plasmid pLF118-4	16 (4297 bp)	--
Euglena anabaena Δ8 desaturase 1 coding sequence ("EaD8Des1")	17 (1260 bp)	21 (420 AA)
Euglena anabaena Δ8 desaturase 2 coding sequence ("EaD8Des2")	18 (1260 bp)	22 (420 AA)
Euglena anabaena Δ8 desaturase 3 coding	19	23

sequence ("EaD8Des3")	(1260 bp)	(420 AA)
Euglena anabaena Δ8 desaturase 4 coding sequence ("EaD8Des4")	20 (1260 bp)	24 (420 AA)
Euglena gracilis Δ8 desaturase ("EgD8") (U.S. Patent 7,256,033)	--	25 (421 AA)
Plasmid pLF120-1	28 (4794 bp)	--
Plasmid pLF120-2	29 (4794 bp)	--
Plasmid pLF120-3	30 (4794 bp)	--
Plasmid pLF120-4	31 (4794 bp)	--
plasmid pDMW263	32 (9472 bp)	--
plasmid pDMW237	33 (7879 bp)	--
plasmid pY115	34 (7783 bp)	--
plasmid pY175	35 (8254 bp)	--
plasmid pY176	36 (8254 bp)	--
plasmid pY177	37 (8254 bp)	
plasmid pY178	38 (8254 bp)	--
Synthetic Δ8 desaturase, derived from *Euglena anabaena,* codon-optimized for expression in *Yarrowia lipolytica* ("EaD8S")	39 (1260 bp)	40 (420 AA)
Plasmid pEaD8S	41 (3983 bp)	--
Plasmid pZKLeuN-29E3	42 (14,688 bp)	--
Fusarium moniliforme Δ12 desaturase ("FmD12")	43 (1434 bp)	44 (477 AA)
Synthetic Δ9 elongase derived from *Euglena gracilis*, codon-optimized for expression in *Yarrowia lipolytica* ("EgD9eS")	45 (777 bp)	46 (258 AA)
Escherichia coli LoxP recombination site, recognized by a Cre recombinase enzyme	47 (34 bp)	--
Synthetic $C_{16/18}$ elongase derived from *Mortierella alpina* ELO3, codon-optimized for expression in *Yarrowia lipolytica* ("ME3S")	48 (828 bp)	49 (275 AA)
Plasmid pY116	50 (8739 bp)	--
Plasmid pZUFmEaD8S	51 (8255 bp)	--

SEQ ID NO:3 is the nucleotide sequence of the vector-specific primer pDonor222Eg5-1.

SEQ ID NOs:4-7 correspond to degenerate primers D8DEG3-1, D8DEG3-2, D8DEG3-3 and D8DEG3-4, respectively, used to amplify a portion of the Δ8 desaturase genes from *Euglena anabaena* UTEX 373.

SEQ ID NOs:8 and 9 correspond to the T7 primer and primer M13-28Rev, respectively, used for sequencing a partial putative Δ8 desaturase cDNA fragment.

SEQ ID NO:12 is the nucleotide sequence of primer EaD8seq-1, used for full insert sequencing of eug1c Δ8 desaturase clones.

SEQ ID NOs:26 and 27 correspond to primers EaD8-5 and EaD8-3, respectively, used to amplify the EaD8Des1, EaD8Des2, EaD8Des3 and EaD8Des4 coding sequences.

DETAILED DESCRIPTION OF THE INVENTION

New *Euglena anabaena* Δ8 desaturase enzymes and genes encoding the same that may be used for the manipulation of biochemical pathways for the production of healthful PUFAs are disclosed herein. PUFAs, or derivatives thereof, are used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Alternatively, the purified PUFAs (or derivatives thereof) may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount for dietary supplementation. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products and may find use as anti-inflammatory or cholesterol lowering agents. Optionally, the compositions may be used for pharmaceutical use (human or veterinary).

Definitions

In the context of this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

"American Type Culture Collection" is abbreviated ATCC.

"Polyunsaturated fatty acid(s)" is abbreviated PUFA(s).

"Triacylglycerols" are abbreviated TAGs.

The term "invention" or "present invention" as used herein is not meant to be limiting to any one specific embodiment of the invention but applies generally to any and all embodiments of the invention as described in the claims and specification.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes a plurality of such plants, reference to "a cell" includes one or more cells and equivalents thereof known to those skilled in the art, and so forth.

The term "fatty acids" refers to long-chain aliphatic acids (alkanoic acids) of varying chain lengths, from about C_{12} to C_{22} (although both longer and shorter chain-length acids are known). The predominant chain lengths are between C_{16} and C_{22}. Additional details concerning the differentiation between "saturated fatty acids" versus "unsaturated fatty acids", "monounsaturated fatty acids" versus "polyunsaturated fatty acids" (or "PUFAs"), and "omega-6 fatty acids" (ω-6 or *n*-6) versus "omega-3 fatty acids" (ω-3 or *n*-3) are provided in U.S. Patent 7,238,482.

Fatty acids are described herein by a simple notation system of "X:Y", where X is the total number of carbon (C) atoms in the particular fatty acid and Y is the number of double bonds. The number following the fatty acid designation indicates the position of the double bond from the carboxyl end of the fatty acid with the "c" affix for the *cis*-configuration of the double bond (e.g., palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, 9c), petroselinic acid (18:1, 6c), LA (18:2, 9c,12c), GLA (18:3, 6c,9c,12c) and ALA (18:3, 9c,12c,15c)). Unless otherwise specified, 18:1, 18:2 and 18:3 refer to oleic, LA and ALA fatty acids, respectively. If not specifically written as otherwise, double bonds are assumed to be of the *cis* configuration. For instance, the double bonds in 18:2 (9,12) would be assumed to be in the *cis* configuration.

Nomenclature used to describe PUFAs in the present disclosure is shown below in Table 2. In the column titled "Shorthand Notation", the omega-reference system is used to indicate the number of carbons, the number of double bonds and the position of the double bond closest to the omega carbon, counting from the omega carbon (which is numbered 1 for this purpose). The remainder of the Table summarizes the common names of ω-3 and ω-6 fatty acids and their precursors, the abbreviations that will be used throughout the remainder of the specification, and each compounds' chemical name.

Table 2

Nomenclature of Polyunsaturated Fatty Acids and Precursors

Common Name	Abbreviation	Chemical Name	Shorthand Notation
Myristic	--	tetradecanoic	14:0
Palmitic	PA or Palmitate	hexadecanoic	16:0
Palmitoleic	--	9-hexadecenoic	16:1
Stearic	--	octadecanoic	18:0
Oleic	--	*cis*-9-octadecenoic	18:1
Linoleic	LA	*cis*-9,12-octadecadienoic	18:2 ω-6
γ-Linolenic	GLA	*cis*-6,9,12-octadecatrienoic	18:3 ω-6
Eicosadienoic	EDA	*cis*-11,14-eicosadienoic	20:2 ω-6
Dihomo-γ-linolenic	DGLA	*cis*-8,11,14-eicosatrienoic	20:3 ω-6
Sciadonic	SCI	*cis*-5,11,14-eicosatrienoic	20:3b ω-6
Arachidonic	ARA	*cis*-5,8,11,14-eicosatetraenoic	20:4 ω-6
α-Linolenic	ALA	*cis*-9,12,15-octadecatrienoic	18:3 ω-3
Stearidonic	STA	*cis*-6,9,12,15-octadecatetraenoic	18:4 ω-3
Eicosatrienoic	ETrA or ERA	*cis*-11,14,17-eicosatrienoic	20:3 ω-3
Eicosa-tetraenoic	ETA	*cis*-8,11,14,17-eicosatetraenoic	20:4 ω-3
Juniperonic	JUP	*cis*-5,11,14,17-eicosatrienoic	20:4b ω-3
Docosatrienoic	DRA	*cis*-10,13,16-docosatrienoic	22:3 ω-6
Docosa-tetraenoic	DTA	*cis*-7,10,13,16-docosatetraenoic	22:4 ω-6
Docosa-pentaenoic	DPAn-6	*cis*-4,7,10,13,16-docosapentaenoic	22:5 ω-6
Eicosa-pentaenoic	EPA	*cis*-5,8,11,14,17-eicosapentaenoic	20:5 ω-3
Docosa-pentaenoic	DPA	*cis*-7,10,13,16,19-docosapentaenoic	22:5 ω-3
Docosa-hexaenoic	DHA	*cis*-4,7,10,13,16,19-docosahexaenoic	22:6 ω-3

The terms "triacylglycerol", "oil" and "TAGs" refer to neutral lipids composed of three fatty acyl residues esterified to a glycerol molecule (and such terms will be used interchangeably throughout the present disclosure herein). Such oils can contain long chain PUFAs, as well as shorter saturated and unsaturated fatty acids and longer chain saturated fatty acids. Thus, "oil biosynthesis" generically refers to the synthesis of TAGs in the cell.

"Percent (%) PUFAs in the total lipid and oil fractions" refers to the percent of PUFAs relative to the total fatty acids in those fractions. The term "total lipid fraction" or "lipid fraction" both refer to the sum of all lipids (i.e., neutral and polar) within an oleaginous organism, thus including those lipids that are located in the phosphatidylcholine (PC) fraction, phosphatidyletanolamine (PE) fraction and triacylglycerol (TAG or oil) fraction. However, the terms "lipid" and "oil" will be used interchangeably throughout the specification.

A metabolic pathway, or biosynthetic pathway, in a biochemical sense, can be regarded as a series of chemical reactions occurring within a cell, catalyzed by enzymes, to achieve either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many of these pathways are elaborate, and involve a step by step modification of the initial substance to shape it into a product having the exact chemical structure desired.

The term "PUFA biosynthetic pathway" refers to a metabolic process that converts oleic acid to ω-6 fatty acids such as LA, EDA, GLA, DGLA, ARA, DRA, DTA and DPAn-6 and ω-3 fatty acids such as ALA, STA, ETrA, ETA, EPA, DPA and DHA. This process is well described in the literature (e.g., see PCT Publication No. WO 2006/052870). Briefly, this process involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds, via a series of special desaturation and elongation enzymes (i.e., "PUFA biosynthetic pathway enzymes") present in the endoplasmic reticulim membrane. More specifically, "PUFA biosynthetic pathway enzyme" refers to any of the following enzymes (and genes which encode said enzymes) associated with the biosynthesis of a PUFA, including: a Δ9 elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase, a $C_{20/22}$ elongase, a Δ4 desaturase,

a Δ5 desaturase, a Δ6 desaturase, a Δ12 desaturase, a Δ15 desaturase, a Δ17 desaturase, a Δ9 desaturase and/or a Δ8 desaturase.

The term "ω-3/ω-6 fatty acid biosynthetic pathway" refers to a set of genes which, when expressed under the appropriate conditions encode enzymes that catalyze the production of either or both ω-3 and ω-6 fatty acids. Typically the genes involved in the ω-3/ω-6 fatty acid biosynthetic pathway encode PUFA biosynthetic pathway enzymes. A representative pathway is illustrated in FIG. 1, providing for the conversion of myristic acid through various intermediates to DHA, which demonstrates how both ω-3 and ω-6 fatty acids may be produced from a common source. The pathway is naturally divided into two portions where one portion will generate ω-3 fatty acids and the other portion, ω-6 fatty acids.

The term "functional" as used herein in context with the ω-3/ω-6 fatty acid biosynthetic pathway means that some (or all) of the genes in the pathway express active enzymes, resulting in *in vivo* catalysis or substrate conversion. It should be understood that "ω-3/ω-6 fatty acid biosynthetic pathway" or "functional ω-3/ω-6 fatty acid biosynthetic pathway" does not imply that all the PUFA biosynthetic pathway enzyme genes are required, as a number of fatty acid products will only require the expression of a subset of the genes of this pathway.

The term "Δ6 desaturase/ Δ6 elongase pathway" will refer to a PUFA biosynthetic pathway that minimally includes at least one Δ6 desaturase and at least one $C_{18/20}$ elongase (also referred to as a Δ6 elongase), thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively, with GLA and/or STA as intermediate fatty acids. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized.

The term "Δ9 elongase/Δ8 desaturase pathway" will refer to a PUFA biosynthetic pathway that minimally includes at least one Δ9 elongase and at least one Δ8 desaturase, thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively, with EDA and/or ETrA as intermediate fatty acids. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized.

The term "intermediate fatty acid" refers to any fatty acid produced in a fatty acid metabolic pathway that can be further converted to an intended product fatty

acid in this pathway by the action of other metabolic pathway enzymes. For instance, when EPA is produced using the Δ9 elongase/Δ8 desaturase pathway, EDA, ETrA, DGLA, ETA and ARA can be produced and are considered "intermediate fatty acids" since these fatty acids can be further converted to EPA via action of other metabolic pathway enzymes.

The term "by-product fatty acid" refers to any fatty acid produced in a fatty acid metabolic pathway that is not the intended fatty acid product of the pathway nor an "intermediate fatty acid" of the pathway. For instance, when EPA is produced using the Δ9 elongase/Δ8 desaturase pathway, sciadonic acid (SCI) and juniperonic acid (JUP) also can be produced by the action of a Δ5 desaturase on either EDA or ETrA, respectively. They are considered to be "by-product fatty acids" since neither can be further converted to EPA by the action of other metabolic pathway enzymes.

The term "desaturase" refers to a polypeptide that can desaturate, i.e., introduce a double bond, in one or more fatty acids to produce a fatty acid or precursor of interest. Despite use of the omega-reference system throughout the specification to refer to specific fatty acids, it is more convenient to indicate the activity of a desaturase by counting from the carboxyl end of the substrate using the delta-system. Of particular interest herein are Δ8 desaturases that desaturate a fatty acid between the eighth and ninth carbon atom numbered from the carboxyl-terminal end of the molecule and that can, for example, catalyze the conversion of EDA to DGLA and/or ETrA to ETA. Other fatty acid desaturases include, for example: (1) Δ5 desaturases that catalyze the conversion of DGLA to ARA and/or ETA to EPA; (2) Δ6 desaturases that catalyze the conversion of LA to GLA and/or ALA to STA; (3) Δ4 desaturases that catalyze the conversion of DPA to DHA and/or DTA to DPAn-6; (4) Δ12 desaturases that catalyze the conversion of oleic acid to LA; (5) Δ15 desaturases that catalyze the conversion of LA to ALA and/or GLA to STA; (6) Δ17 desaturases that catalyze the conversion of ARA to EPA and/or DGLA to ETA; and, (7) Δ9 desaturases that catalyze the conversion of palmitic acid to palmitoleic acid (16:1) and/or stearic acid to oleic acid (18:1). In the art, Δ15 and Δ17 desaturases are also occasionally referred to as "omega-3 desaturases", "w-3 desaturases" and/or "ω-3 desaturases", based on their ability to convert ω-6 fatty acids into their ω-3 counterparts (e.g., conversion of LA into ALA and ARA into EPA, respectively). In some embodiments, it may be most desirable to empirically

determine the specificity of a particular fatty acid desaturase by transforming a suitable host with the gene for the fatty acid desaturase and determining its effect on the fatty acid profile of the host.

The term "EaD8Des1" refers to a Δ8 desaturase enzyme (SEQ ID NO:21) isolated from *Euglena anabaena,* encoded by SEQ ID NO:17 herein. The term "EaD8Des2" refers to a Δ8 desaturase enzyme (SEQ ID NO:22) isolated from *E. anabaena,* encoded by SEQ ID NO:18 herein. Likewise, the term "EaD8Des3" refers to a Δ8 desaturase enzyme (SEQ ID NO:23) isolated from *E. anabaena,* encoded by SEQ ID NO:19 herein. The term "EaD8Des4" refers to a Δ8 desaturase enzyme (SEQ ID NO:24) isolated from *E. anabaena,* encoded by SEQ ID NO:20 herein. Similarly, the term "EaD8S" refers to a synthetic Δ8 desaturase derived from *E. anabaena* that is codon-optimized for expression in *Yarrowia lipolytica* (i.e., SEQ ID NOs:39 and 40) .

The term "EgD8" refers to a Δ8 desaturase enzyme (encoded by the nucleotide sequence set forth as SEQ ID NO:2) isolated from *Euglena gracilis.* EgD8 is 100% identical and functionally equivalent to "Eg5", as described in PCT Publication Nos. WO 2006/012325 and WO 2006/012326 (i.e., SEQ ID NO:2 of U.S. Patent 7,256,033).

The terms "conversion efficiency" and "percent substrate conversion" refer to the efficiency by which a particular enzyme (e.g., a desaturase) can convert substrate to product. The conversion efficiency is measured according to the following formula: ([product]/[substrate + product])*100, where 'product' includes the immediate product and all products in the pathway derived from it.

The term "elongase" refers to a polypeptide that can elongate a fatty acid carbon chain to produce an acid that is 2 carbons longer than the fatty acid substrate that the elongase acts upon. This process of elongation occurs in a multi-step mechanism in association with fatty acid synthase, as described in U.S. Patent Publication No. 2005/0132442. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA, LA to EDA, ALA to ETrA, ARA to DTA and EPA to DPA.

In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree of unsaturation. For example, a $C_{14/16}$ elongase will utilize a C_{14} substrate (e.g., myristic acid), a $C_{16/18}$ elongase will

utilize a C_{16} substrate (e.g., palmitate), a $C_{18/20}$ elongase (also known as a Δ6 elongase as the terms can be used interchangeably) will utilize a C_{18} substrate (e.g., GLA, STA) and a $C_{20/22}$ elongase will utilize a C_{20} substrate (e.g., ARA, EPA). Similarily, a "Δ9 elongase" is able to catalyze the conversion of LA to EDA and/or ALA to ETrA. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions (e.g., thereby acting as both a $C_{16/18}$ elongase and a $C_{18/20}$ elongase). It may be desirable to empirically determine the specificity of a fatty acid elongase by transforming a suitable host with the gene for the fatty acid elongase and determining its effect on the fatty acid profile of the host.

The term "oleaginous" refers to those organisms that tend to store their energy source in the form of lipid (Weete, In: Fungal Lipid Biochemistry, 2nd Ed., Plenum, 1980). Within oleaginous microorganisms the cellular oil or TAG content generally follows a sigmoid curve, wherein the concentration of lipid increases until it reaches a maximum at the late logarithmic or early stationary growth phase and then gradually decreases during the late stationary and death phases (Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)). It is not uncommon for oleaginous microorganisms to accumulate in excess of about 25% of their dry cell weight as oil.

The term "oleaginous yeast" refers to those microorganisms classified as yeasts that can make oil. Examples of oleaginous yeast include, but are no means limited to, the following genera: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces.* Preferred for use in the present invention are oleaginous strains of *Yarrowia lipolytica.*

The term "Euglenophyceae" refers to a group of unicellular colorless or photosynthetic flagellates ("euglenoids") found living in freshwater, marine, soil and parasitic environments. The class is characterized by solitary unicells, wherein most are free-swimming and have two flagella (one of which may be nonemergent) arising from an anterior invagination known as a reservoir. Photosynthetic euglenoids contain one to many chloroplasts, which vary from minute disks to expanded plates or ribbons. Colorless euglenoids depend on osmotrophy or phagotrophy for nutrient assimilation. About 1000 species have been described and classified into about 40 genera and 6 orders. Examples of Euglenophyceae include,

but are no means limited to, the following genera: *Euglena, Eutreptiella* and *Tetruetreptia.*

As used herein, "nucleic acid" means a polynucleotide and includes single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence" or "nucleic acid fragment" are used interchangeably and refer to a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deosycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridlate, "T" for deosythymidylate, "R" for purines (A or G), "Y" for pyrimidiens (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

The term "conserved domain" or "motif" means a set of amino acids conserved at specific positions along an aligned sequence of evolutionarily related proteins. While amino acids at other positions can vary between homologous proteins, amino acids that are highly conserved at specific positions indicate amino acids that are essential in the structure, the stability, or the activity of a protein. Because they are identified by their high degree of conservation in aligned sequences of a family of protein homologues, they can be used as identifiers, or "signatures", to determine if a protein with a newly determined sequence belongs to a previously identified protein family.

The terms "homology", "homologous", "substantially similar" and "corresponding substantially" are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those

skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize (under moderately stringent conditions, e.g., 0.5X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 90% sequence identity, up to and including 100% sequence identity (i.e., fully complementary) with each other.

The term "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will selectively hybridize to its target sequence. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the

addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 to 65 °C.

Specificity is typically the function of post-hybridization washes, the important factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth et al., *Anal. Biochem.,* 138:267-284 (1984): T_m = 81.5 °C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); and, low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An

extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Hybridization and/or wash conditions can be applied for at least 10, 30, 60, 90, 120 or 240 minutes.

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

Thus, "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. Useful examples of percent sequence identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer percentage from 50% to 100%. These identities can be determined using any of the programs described herein.

Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values"

will mean any set of values or parameters that originally load with the software when first initialized.

The "Clustal V method of alignment" corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS,* 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

"BLASTN method of alignment" is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare nucleotide sequences using default parameters.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar function or activity. Useful examples of percent identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 50% to 100%. Indeed, any integer amino acid identity from 50% to 100% may be useful in describing the present invention, such as 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Also, of interest is any full-length or partial complement of this isolated nucleotide fragment.

"Codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the instant euglenoid polypeptides as set forth in SEQ ID NOs:21, 22, 23

and 24. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell, where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, and that may refer to the coding region alone or may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. A "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" 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, but are not limited to: promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

"Promoter" refers 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 that 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 some variation may have identical promoter activity.

A promoter sequence may consist of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter.

"Translation leader sequence" refers to a polynucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D., *Mol. Biotechnol.*, 3:225-236 (1995)).

The terms "3' non-coding sequences", "transcription terminator" and "termination sequences" refer to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other

sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript. A RNA transcript is referred to as the mature RNA when it is a RNA sequence derived from post-transcriptional processing of the primary transcript. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to, and synthesized from, a mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into double-stranded form using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that blocks the expression of a target gene (U.S. Patent 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

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., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. The term "recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the

manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragments of the invention. Expression may also refer to translation of mRNA into a protein (either precursor or mature).

"Mature" protein refers to a post-translationally processed polypeptide (i.e., one from which any pre- or propeptides present in the primary translation product have been removed). "Precursor" protein refers to the primary product of translation of mRNA (i.e., with pre- and propeptides still present). Pre- and propeptides may be but are not limited to intracellular localization signals.

The terms "plasmid" and "vector" refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. 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 an expression cassette(s) into a cell.

The term "expression cassette" refers to a fragment of DNA comprising the coding sequence of a selected gene and regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence that are required for expression of the selected gene product. Thus, an expression cassette is typically composed of: (1) a promoter sequence; (2) a coding sequence (i.e., ORF); and, (3) a 3' untranslated region (i.e., a terminator) that, in eukaryotes, usually contains a polyadenylation site. The expression cassette(s) is usually included within a vector, to facilitate cloning and transformation. Different expression cassettes can be transformed into different organisms including bacteria, yeast, plants and mammalian cells, as long as the correct regulatory sequences are used for each host.

A "recombinant DNA construct" (also referred to interchangeably herein as a "expression construct" and "construct") comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not found together in

nature. For example, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., *EMBO J.*, 4:2411-2418 (1985); De Almeida et al., *Mol. Gen. Genetics,* 218:78-86 (1989)), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

The term "introduced" means providing a nucleic acid (e.g., expression cassette) or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. Introduced includes reference to stable or transient transformation methods, as well as sexually crossing. Thus, "introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct or expression cassette) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

"Stable transformation" refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in genetically stable inheritance. In contrast, "transient transformation"

refers to the transfer of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host organism resulting in gene expression without integration or stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms or "recombinant" or "transformed" organisms.

As used herein, "transgenic" refers to a cell which comprises within its genome a heterologous polynucleotide. Preferably, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of an expression cassette. Transgenic is used herein to include any cell or cell line, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989); by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987). Transformation methods are well known to those skilled in the art and are described *infra*.An Overview: Microbial Biosynthesis Of Fatty Acids And Triacylglycerols

In general, lipid accumulation in oleaginous microorganisms is triggered in response to the overall carbon to nitrogen ratio present in the growth medium. This process, leading to the *de novo* synthesis of free palmitate (16:0) in oleaginous microorganisms, is described in detail in U.S. Patent 7,238,482. Palmitate is the

precursor of longer-chain saturated and unsaturated fatty acid derivates, which are formed through the action of elongases and desaturases (FIG. 1).

TAGs (the primary storage unit for fatty acids) are formed by a series of reactions that involve: (1) the esterification of one molecule of acyl-CoA to glycerol-3-phosphate via an acyltransferase to produce lysophosphatidic acid; (2) the esterification of a second molecule of acyl-CoA via an acyltransferase to yield 1,2-diacylglycerol phosphate (commonly identified as phosphatidic acid); (3) removal of a phosphate by phosphatidic acid phosphatase to yield 1,2-diacylglycerol (DAG); and, (4) the addition of a third fatty acid by the action of an acyltransferase to form TAG. A wide spectrum of fatty acids can be incorporated into TAGs, including saturated and unsaturated fatty acids and short-chain and long-chain fatty acids.

Biosynthesis Of Omega Fatty Acids

The metabolic process wherein oleic acid is converted to ω-3/ω-6 fatty acids involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds. This requires a series of special desaturation and elongation enzymes present in the endoplasmic reticulum membrane. However, as seen in FIG. 1 and as described below, there are often multiple alternate pathways for production of a specific ω-3/ω-6 fatty acid.

Specifically, all pathways require the initial conversion of oleic acid to LA, the first of the ω-6 fatty acids, by a Δ12 desaturase. Then, using the "Δ9 elongase/Δ8 desaturase pathway" and LA as substrate, long chain ω-6 fatty acids are formed as follows: (1) LA is converted to EDA by a Δ9 elongase; (2) EDA is converted to DGLA by a Δ8 desaturase; (3) DGLA is converted to ARA by a Δ5 desaturase; (4) ARA is converted to DTA by a $C_{20/22}$ elongase; and, (5) DTA is converted to DPAn-6 by a Δ4 desaturase. Alternatively, the "Δ9 elongase/Δ8 desaturase pathway" can use ALA as substrate to produce long chain ω-3 fatty acids as follows: (1) LA is converted to ALA, the first of the ω-3 fatty acids, by a Δ15 desaturase; (2) ALA is converted to ETrA by a Δ9 elongase; (3) ETrA is converted to ETA by a Δ8 desaturase; (4) ETA is converted to EPA by a Δ5 desaturase; (5) EPA is converted to DPA by a $C_{20/22}$ elongase; and, (6) DPA is converted to DHA by a Δ4 desaturase. Optionally, ω-6 fatty acids may be converted to ω-3 fatty acids; for example, ALA is produced from LA by Δ15 desaturase activity; ETA and EPA are produced from DGLA and ARA, respectively, by Δ17 desaturase activity.

Alternate pathways for the biosynthesis of ω-3/ω-6 fatty acids utilize a Δ6 desaturase and $C_{18/20}$ elongase (i.e., the "Δ6 desaturase/Δ6 elongase pathway"). More specifically, LA and ALA may be converted to GLA and STA, respectively, by a Δ6 desaturase; then, a $C_{18/20}$ elongase converts GLA to DGLA and/or STA to ETA. Downstream PUFAs are subsequently formed as described above.

It is contemplated that the particular functionalities required to be introduced into a specific host organism for production of ω-3/ω-6 fatty acids will depend on the host cell (and its native PUFA profile and/or desaturase/elongase profile), the availability of substrate, and the desired end product(s). For example, expression of the Δ9 elongase/Δ8 desaturase pathway may be preferred in some embodiments, as opposed to expression of the Δ6 desaturase/Δ6 elongase pathway, since PUFAs produced via the former pathway are devoid of GLA and/or STA.

One skilled in the art will be able to identify various candidate genes encoding each of the enzymes desired for ω-3/ω-6 fatty acid biosynthesis. Useful desaturase and elongase sequences may be derived from any source, e.g., isolated from a natural source (from bacteria, algae, fungi, plants, animals, etc.), produced via a semi-synthetic route or synthesized *de novo*. Although the particular source of the desaturase and elongase genes introduced into the host is not critical, considerations for choosing a specific polypeptide having desaturase or elongase activity include: (1) the substrate specificity of the polypeptide; (2) whether the polypeptide or a component thereof is a rate-limiting enzyme; (3) whether the desaturase or elongase is essential for synthesis of a desired PUFA; (4) co-factors required by the polypeptide; and/or, (5) whether the polypeptide was modified after its production (e.g., by a kinase or a prenyltransferase). The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell (see U.S. Patent 7,238,482).

It will also be useful to consider the conversion efficiency of each particular desaturase and/or elongase. Since each enzyme rarely functions with 100% efficiency to convert substrate to product, the final lipid profile of unpurified oils produced in a host cell will typically be a mixture of various PUFAs consisting of the desired ω-3/ω-6 fatty acid, as well as various upstream intermediary PUFAs. Thus,

each enzyme's conversion efficiency must be considered when optimizing biosynthesis of a desired fatty acid.

With each of the considerations above in mind, candidate genes having the appropriate desaturase and elongase activities (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ5 desaturases, Δ17 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ8 desaturases, Δ4 desaturases and $C_{20/22}$ elongases) can be identified according to publicly available literature (e.g., GenBank), the patent literature, and experimental analysis of organisms having the ability to produce PUFAs. These genes will be suitable for introduction into a specific host organism, to enable or enhance the organism's synthesis of PUFAs.

Sequence Identification of Novel Δ8 Desaturases

In the present invention, nucleotide sequences encoding Δ8 desaturases have been isolated from *Euglena anabaena,* as summarized below in Table 3.

Table 3

Summary Of *Euglena anabaena* Δ8 Desaturases

Abbreviation	Nucleotide SEQ ID NO	Amino Acid SEQ ID NO
EaD8Des1	17	21
EaD8Des2	18	22
EaD8Des3	19	23
EaD8Des4	20	24
EaD8S	39	40

*Note: SEQ ID NO:40 is identical in sequence to SEQ ID NO:23.

Thus, the present invention concerns an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 or SEQ ID NO:24;

(b) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a

nucleotide sequence as set forth in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or SEQ ID NO:39; or,

(c) a complement of the nucleotide sequence of (a) or (b), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary, and host cells comprising the same.

In still another aspect, the invention concerns an isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence has at least 90% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or SEQ ID NO:39.

More preferred amino acid fragments that are at least about 80%-90% identical are particularly suitable and those sequences that are at least about 90%-95% identical are most preferred. Similarly, preferred Δ8 desaturase encoding nucleic acid sequences corresponding to the instant ORFs are those encoding active proteins and which are at least about 80%-90% identical; those sequences that are at least about 90%-95% identical are most preferred.

In alternate embodiments, the instant EaD8Des1, EaD8Des2, EaD8Des3 and/or EaD8Des4 desaturase sequences can be codon-optimized for expression in a particular host organism. In general, host-preferred codons can be determined within a particular host species of interest by examining codon usage in proteins (preferably those expressed in the largest amount) and determining which codons are used with highest frequency. Then, the coding sequence for a polypeptide of interest having e.g., desaturase activity can be synthesized in whole or in part using the codons preferred in the host species. All (or portions) of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure that would be present in the transcribed mRNA. All (or portions) of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell.

In one embodiment of the invention, EaD8Des3 (SEQ ID NO:19) was codon-optimized for expression in *Yarrowia lipolytica*. This was possible based on previous determination of the *Y. lipolytica* codon usage profile, identification of those codons that were preferred, and determination of the consensus sequence around

the 'ATG' initiation codon (see U.S. Patent 7,238,482 and U.S. Patent 7,125,672, incorporated herein by reference). The resultant synthetic gene is referred to as EaD8S (SEQ ID NO:39). The protein sequence encoded by the codon-optimized Δ8 desaturase gene (i.e., SEQ ID NO:40) is identical to that of the wildtype protein sequence (i.e., SEQ ID NO:23). Similar techniques could be utilized to produce a synthetic Δ8 desaturase derived from EaD8Des1, EaD8Des2 and/or EaD8Des4 for expression in *Y. lipolytica*.

One skilled in the art would be able to use the teachings herein to create various other codon-optimized Δ8 desaturase proteins suitable for optimal expression in alternate hosts, based on the wildtype EaD8Des1, EaD8Des2, EaD8Des3 and/or EaD8Des4 sequences. Accordingly, the instant invention relates to any codon-optimized Δ8 desaturase protein that is derived from the wildtype nucleotide sequences of EaD8Des1 (SEQ ID NO:17), EaD8Des2 (SEQ ID NO:18), EaD8Des3 (SEQ ID NO:19) or EaD8Des4 (SEQ ID NO:20). This includes, but is not limited to, the nucleotide sequence set forth in SEQ ID NO:39, which encodes a synthetic Δ8 desaturase protein (i.e., EaD8S) that was codon-optimized for expression in *Yarrowia lipolytica*. In alternate embodiments, it may be desirable to modify a portion of the codons encoding EaD8Des1, EaD8Des2, EaD8Des3 and/or EaD8Des4 to enhance expression of the gene in a host organism including, but not limited to, a plant or plant part.

Identification and Isolation of Homologs

Any of the instant desaturase sequences (i.e., EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4, EaD8S) or portions thereof may be used to search for Δ8 desaturase homologs in the same or other bacterial, algal, fungal, euglenoid or plant species using sequence analysis software. In general, such computer software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

Alternatively, any of the instant desaturase sequences or portions thereof may also be employed as hybridization reagents for the identification of Δ8 desaturase homologs. The basic components of a nucleic acid hybridization test include a probe, a sample suspected of containing the gene or gene fragment of interest and a specific hybridization method. Probes of the present invention are typically single-stranded nucleic acid sequences that are complementary to the

nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected. Although the probe length can vary from 5 bases to tens of thousands of bases, typically a probe length of about 15 bases to about 30 bases is suitable. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base.

Hybridization methods are well defined. Typically the probe and sample must be mixed under conditions that will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added (e.g., guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, cesium trifluoroacetate). If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1 M sodium chloride, about 0.05 to 0.1 M buffers (e.g., sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9)), about 0.05 to 0.2% detergent (e.g., sodium dodecylsulfate), or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kdal), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA (e.g., calf thymus or salmon sperm DNA, or yeast RNA), and optionally from about 0.5 to 2% wt/vol glycine. Other additives may also be included, such as volume exclusion agents that include a variety of polar water-soluble or swellable agents (e.g., polyethylene glycol),

anionic polymers (e.g., polyacrylate or polymethylacrylate) and anionic saccharidic polymers (e.g., dextran sulfate).

Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the sequence.

In additional embodiments, any of the Δ8 desaturase nucleic acid fragments described herein (or any homologs identified thereof) may be used to isolate genes encoding homologous proteins from the same or other bacterial, algal, fungal, euglenoid or plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to: (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Patent 4,683,202; ligase chain reaction (LCR), Tabor et al., *Proc. Acad. Sci. U.S.A.*, 82:1074 (1985); or strand displacement amplification (SDA), Walker et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89:392 (1992)]; and, (3) methods of library construction and screening by complementation.

For example, genes encoding similar proteins or polypeptides to the Δ8 desaturases described herein could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from e.g., any desired yeast or fungus using methodology well known to those skilled in the art (wherein those organisms producing DGLA and/or ETA would be preferred). Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis, *supra*). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan (e.g., random primers DNA labeling, nick translation or end-labeling techniques), or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of (or full-length of) the instant sequences. The resulting amplification products can be labeled directly during amplification reactions

or labeled after amplification reactions, and used as probes to isolate full-length DNA fragments under conditions of appropriate stringency.

Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending on the desired test conditions, the sequences of the primers should be designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art (Thein and Wallace, "The use of oligonucleotide as specific hybridization probes in the Diagnosis of Genetic Disorders", in *Human Genetic Diseases: A Practical Approach*, K. E. Davis Ed., (1986) pp 33-50, IRL: Herndon, VA; and Rychlik, W., In Methods in Molecular Biology, White, B. A. Ed., (1993) Vol. 15, pp 31-39, PCR Protocols: Current Methods and Applications. Humania: Totowa, NJ).

Generally two short segments of the instant sequences may be used in PCR protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. PCR may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding eukaryotic genes.

Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., *Proc. Natl Acad. Sci. U.S.A.,* 85:8998 (1988)) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (Gibco/BRL, Gaithersburg, MD), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., *Proc. Natl Acad. Sci. U.S.A.,* 86:5673 (1989); Loh et al., *Science,* 243:217 (1989)).

In other embodiments, any of the Δ8 desaturase nucleic acid fragments described herein (or any homologs identified thereof) may be used for creation of new and/or improved fatty acid desaturases. As is well known in the art, *in vitro* mutagenesis and selection, chemical mutagenesis, "gene shuffling" methods or other means can be employed to obtain mutations of naturally occurring desaturase

genes (wherein such mutations may include deletions, insertions and point mutations, or combinations thereof). This would permit production of a polypeptide having desaturase activity, respectively, *in vivo* with more desirable physical and kinetic parameters for function in the host cell such as a longer half-life or a higher rate of production of a desired PUFA. If desired, the regions of a polypeptide of interest (i.e., a Δ8 desaturase) important for enzymatic activity can be determined through routine mutagenesis, expression of the resulting mutant polypeptides and determination of their activities. An overview of these techniques is described in U.S. Patent 7,238,482. All such mutant proteins and nucleotide sequences encoding them that are derived from EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4 and EaD8S are within the scope of the present invention.

Alternatively, improved fatty acids may be synthesized by domain swapping, wherein a functional domain from any of the Δ8 desaturase nucleic acid fragments described herein are exchanged with a functional domain in an alternate desaturase gene to thereby result in a novel protein. As used herein, "domain" or "functional domain" refer to nucleic acid sequence(s) that are capable of eliciting a biological response in plants or yeast.

Methods for Production of Various Omega-3 and/or Omega-6 Fatty Acids

It is expected that introduction of chimeric genes encoding the Δ8 desaturases described herein (i.e., EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4, EaD8S or other mutant enzymes, codon-optimized enzymes or homologs thereof), under the control of the appropriate promoters will result in increased production of DGLA and/or ETA in the transformed host organism. As such, the present invention encompasses a method for the direct production of PUFAs comprising exposing a fatty acid substrate (i.e., EDA and/or ETrA) to the desaturase enzymes described herein (e.g., EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4 or EaD8S), such that the substrate is converted to the desired fatty acid product (i.e., DGLA and/or ETA, respectively).

More specifically, it is an object of the present invention to provide a method for the production of DGLA in a microbial host cell (e.g., yeast, algae, bacteria, euglenoids, stramenopiles and fungi), wherein the microbial host cell comprises:

a) a recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of

alignment, when compared to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

b) a source of EDA;

wherein the microbial host cell is grown under conditions such that the nucleic acid fragment encoding the Δ8 desaturase is expressed and the EDA is converted to DGLA, and wherein the DGLA is optionally recovered.

In alternate embodiments of the present invention, the Δ8 desaturase may be used for the conversion of ETrA to ETA. Accordingly the invention provides a method for the production of ETA, wherein the microbial host cell comprises:

a) a recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

b) a source of ETrA;

wherein the microbial host cell is grown under conditions such that the nucleic acid fragment encoding the Δ8 desaturase is expressed and the ETrA is converted to ETA, and wherein the ETA is optionally recovered.

Alternatively, each Δ8 desaturase gene and its corresponding enzyme product described herein can be used indirectly for the production of various ω-6 and ω-3 PUFAs (see FIG. 1 and U.S. Patent 7,238,482). Indirect production of ω-3/ω-6 PUFAs occurs wherein the fatty acid substrate is converted indirectly into the desired fatty acid product, via means of an intermediate step(s) or pathway intermediate(s). Thus, it is contemplated that the Δ8 desaturases described herein (i.e., EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4, EaD8S or other mutant enzymes, codon-optimized enzymes or homologs thereof) may be expressed in conjunction with additional genes encoding enzymes of the PUFA biosynthetic pathway (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ17 desaturases, Δ8 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ5 desaturases, Δ4 desaturases, $C_{20/22}$ elongases) to result in higher levels of production of longer-chain ω-3/ω-6 fatty acids (e.g., ARA, EPA, DTA, DPAn-6, DPA and/or DHA).

In preferred embodiments, the Δ8 desaturases of the present invention will minimally be expressed in conjunction with a Δ9 elongase (e.g., from *Isochrysis galbana* [PCT Publication No. WO 2002/077213]; from *Euglena gracilis* [PCT Publication No. WO 2007/061845]; and from *Eutreptiella sp.* CCMP389 [PCT Publication No. WO 2007/061742]. However, the particular genes included within a particular expression cassette will depend on the host cell (and its PUFA profile and/or desaturase/elongase profile), the availability of substrate and the desired end product(s).

In some embodiments, it may be useful to express more than one Δ8 desaturase (i.e., the same or different Δ8 desaturase), to minimize by-product fatty acids. The relative abundance of by-product fatty acids could be decreased by increasing total Δ8 desaturase activity. One approach to minimize by-product fatty acids would be to express more than one Δ8 desaturase. For instance, the presence of sciadonic acid (SCI) and/or juniperonic acid (JUP) [commonly found in the seed lipids of gymnosperms (Wolff et al., *Lipids,* 35(1):1-22 (2000)), such as those in the *Pinaceae* family (pine)] might be considered by-product fatty acids of a Δ6 desaturase/Δ6 elongase pathway or Δ9 elongase/Δ8 desaturase pathway. Although these fatty acids are considered to have various health-enhancing properties themselves (Nakane et al., *Biol. Pharm. Bull.,* 23: 758-761 (2000)), their presence as by-product fatty acids in an engineered PUFA pathway, such as in an oilseed crop, may not be desirable depending on the application.

Occasionally, a Δ6 elongase may elongate fatty acids other than the intended fatty acid. For instance, Δ6 elongases generally convert GLA to DGLA but some Δ6 elongases may also convert unintended substrates such as LA or ALA to EDA or ETrA, respectively. In a Δ6 desaturase/Δ6 elongase pathway, EDA and ETrA would be considered "by-product fatty acids". Addition of a Δ8 desaturase to a Δ6 desaturase/Δ6 elongase pathway would provide a means to convert the "by-product fatty acids" EDA and ETrA back into the "intermediate fatty acids" DGLA and ETA, respectively.

Microbial Expression Systems, Cassettes And Vectors

The Δ8 desaturase genes and gene products described herein (i.e., EaD8Des1, EaD8Des2, EaD8Des3, EaD8Des4, EaD8S or other mutant enzymes, codon-optimized enzymes or homologs thereof) may be expressed in heterologous

microbial host cells, particularly in the cells of oleaginous yeasts (e.g., *Yarrowia lipolytica*).

Microbial 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 chimeric genes for production of any of the gene products of the instant sequences. These chimeric genes could then be introduced into appropriate microorganisms via transformation to provide high-level expression of the encoded enzymes.

Vectors (e.g., constructs, plasmids) and DNA expression cassettes useful for the transformation of suitable microbial host cells are well known in the art. The specific choice of sequences present in the construct is dependent upon the desired expression products (*supra*), the nature of the host cell and the proposed means of separating transformed cells versus non-transformed cells. Typically, however, the vector contains at least one expression cassette, a selectable marker and sequences allowing autonomous replication or chromosomal integration. Suitable expression cassettes comprise a region 5' of the gene that controls transcription (e.g., a promoter), the gene coding sequence, and a region 3' of the DNA fragment that controls transcriptional termination (i.e., a terminator). It is most preferred when both control regions are derived from genes from the transformed microbial 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 production host.

Transcriptional control regions (also initiation control regions or promoters) which are useful to drive expression of the instant Δ8 desaturase ORFs in the desired microbial host cell are numerous and familiar to those skilled in the art. Virtually any promoter (i.e., native, synthetic, or chimeric) capable of directing expression of these genes in the selected host cell is suitable for the present invention, although transcriptional and translational regions from the host species are particularly useful. Expression in a microbial host cell can be accomplished in an induced or constitutive fashion. Induced expression can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, while constitutive expression can be achieved by the use of a constitutive promoter operably linked to the gene of interest. As an example, when the host cell is yeast, transcriptional and translational regions functional in yeast cells are

provided, particularly from the host species (e.g., see Patent Publication No. US-2006-0115881-A1, for preferred transcriptional initiation regulatory regions for use in *Yarrowia lipolytica*). Any one of a number of regulatory sequences can be used, depending upon whether constitutive or induced transcription is desired, the efficiency of the promoter in expressing the ORF of interest, the ease of construction and the like.

Nucleotide sequences surrounding the translational initiation codon 'ATG' have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. For expression in yeast, this can be done by site-directed mutagenesis of an inefficiently expressed gene by fusing it in-frame to an endogenous yeast gene, preferably a highly expressed gene. Alternatively, one can determine the consensus translation initiation sequence in the host and engineer this sequence into heterologous genes for their optimal expression in the host of interest.

The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and function satisfactorily in a variety of hosts (when utilized both in the same and different genera and species from where they were derived). The termination region usually is selected more as a matter of convenience rather than because of any particular property. Termination control regions may also be derived from various genes native to the preferred hosts. In alternate embodiments, the 3'-region can also be synthetic, as one of skill in the art can utilize available information to design and synthesize a 3'-region sequence that functions as a transcription terminator. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

As one of skill in the art is aware, merely inserting a gene into a cloning vector does not ensure that it will be successfully expressed at the level needed. In response to the need for a high expression rate, many specialized expression vectors have been created by manipulating a number of different genetic elements that control aspects of transcription, translation, protein stability, oxygen limitation and secretion from the microbial host cell. More specifically, some of the molecular

features that have been manipulated to control gene expression include: the nature of the relevant transcriptional promoter and terminator sequences; the number of copies of the cloned gene (wherein additional copies may be cloned within a single expression construct and/or additional copies may be introduced into the host cell by increasing the plasmid copy number or by multiple integration of the cloned gene into the genome); whether the gene is plasmid-borne or integrated into the genome of the host cell; the final cellular location of the synthesized foreign protein; the efficiency of translation and correct folding of the protein in the host organism; the intrinsic stability of the mRNA and protein of the cloned gene within the host cell; and, the codon usage within the cloned gene, such that its frequency approaches the frequency of preferred codon usage of the host cell. Each of these types of modifications are encompassed in the present invention, as means to further optimize expression of the Δ8 desaturases described herein.

Transformation Of Microbial Host Cells

Once a DNA cassette that is suitable for expression in an appropriate microbial host cell has been obtained (e.g., a chimeric gene comprising a promoter, ORF and terminator), it is placed in a plasmid vector capable of autonomous replication in a host cell, or it is directly integrated into the genome of the host cell. Integration of expression cassettes can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination within the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of selection and should lack homology to the other construct(s) to maintain stable expression and prevent reassortment of elements among constructs. Judicious choice of regulatory regions, selection means and method of propagation of the introduced construct(s) can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

Constructs comprising the gene(s) of interest may be introduced into a microbial host cell by any standard technique. These techniques include transformation (e.g., lithium acetate transformation [*Methods in Enzymology*,

194:186-187 (1991)]), protoplast fusion, bolistic impact, electroporation, microinjection, or any other method that introduces the gene(s) of interest into the host cell.

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence (e.g., an expression cassette) will be referred to as "transformed", "transformant" or "recombinant" herein. The transformed host will have at least one copy of the expression construct and may have two or more, depending upon whether the expression cassette is integrated into the genome or is present on an extrachromosomal element having multiple copy numbers.

The transformed host cell can be identified by various selection techniques, as described in U.S. Patents 7,238,482 and 7,259,255 and PCT Publication No. WO 2006/052870.

Following transformation, substrates suitable for the instant Δ8 desaturases (and, optionally other PUFA enzymes that are co-expressed within the host cell) may be produced by the host either naturally or transgenically, or they may be provided exogenously.

Preferred Microbial Host Cells For Recombinant Expression

Microbial host cells for expression of the instant genes and nucleic acid fragments may include hosts that grow on a variety of feedstocks, including simple or complex carbohydrates, fatty acids, organic acids, oils and alcohols, and/or hydrocarbons over a wide range of temperature and pH values. The genes described in the instant invention have been expressed in an oleaginous yeast (an oleaginous stain of *Yarrowia lipolytica*); however, it is contemplated that because transcription, translation and the protein biosynthetic apparatus are highly conserved, any bacteria, yeast, algae, euglenoid, stramenopiles and/or fungus will be a suitable microbial host for expression of the present nucleic acid fragments.

Preferred microbial hosts are oleaginous organisms, such as oleaginous yeasts. These organisms are naturally capable of oil synthesis and accumulation, wherein the oil can comprise greater than about 25% of the cellular dry weight, more preferably greater than about 30% of the cellular dry weight, and most preferably greater than about 40% of the cellular dry weight. Genera typically identified as comprising strains that may be oleaginous include, but are not limited to: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and

Lipomyces. More specifically, illustrative oil-synthesizing yeasts include: *Rhodosporidium toruloides, Lipomyces starkeyii, L. lipoferus, Candida revkaufi, C. pulcherrima, C. tropicalis, C. utilis, Trichosporon pullans, T. cutaneum, Rhodotorula glutinus, R. graminis,* and *Yarrowia lipolytica* (formerly classified as *Candida lipolytica*). In alternate embodiments, oil biosynthesis may be genetically engineered such that the microbial host cell (e.g., a yeast) can produce more than 25% oil of the cellular dry weight, and thereby be considered oleaginous.

Preferred oleaginous yeasts are oleaginous strains of *Yarrowia lipolytica* where particularly preferred are the *Y. lipolytica* strains designated as ATCC #20362, ATCC #8862, ATCC #18944, ATCC #76982 and/or LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.*, 82(1):43-9 (2002)).

Specific teachings applicable for transformation of oleaginous yeasts (i.e., *Yarrowia lipolytica*) include U.S. Patent 4,880,741, U.S. Patent 5,071,764 and Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)). Specific teachings applicable for engineering ARA, EPA and DHA production in *Y. lipolytica* are provided in U.S. Patent Application No. 11/264784, U.S. Patent Application No. 11/265761, and U.S. Patent Application No. 11/264737 respectively.

The preferred method of expressing genes in this yeast is by integration of linear DNA into the genome of the host; and, integration into multiple locations within the genome can be particularly useful when high level expression of genes are desired [e.g., in the *Ura3* locus (GenBank Accession No. AJ306421), the *Leu2* gene locus (GenBank Accession No. AF260230), the *Lys5* gene locus (GenBank Accession No. M34929), the *Aco2* gene locus (GenBank Accession No. AJ001300), the *Pox3* gene locus (Pox3: GenBank Accession No. XP_503244; or, Aco3: GenBank Accession No. AJ001301), the Δ12 desaturase gene locus (U.S. Patent 7,214,491), the *Lip1* gene locus (GenBank Accession No. Z50020), the *Lip2* gene locus (GenBank Accession No. AJ012632), and/or the Pex10 gene locus (GenBank Accession No. CAG81606.

Preferred selection methods for use in *Yarrowia lipolytica* are resistance to kanamycin, hygromycin and the amino glycoside G418, as well as ability to grow on media lacking uracil, leucine, lysine, tryptophan or histidine. Additionally 5-fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; "5-FOA") may be used for selection of yeast *Ura*$^-$ mutants. The compound is toxic to yeast cells that

possess a functioning URA3 gene encoding orotidine 5'-monophosphate decarboxylase (OMP decarboxylase) and, because of this toxicity, 5-FOA is especially useful for the selection and identification of *Ura*⁻ mutant yeast strains (Bartel, P.L. and Fields, S., Yeast 2-Hybrid System, Oxford University: New York, v. 7, pp 109-147, 1997; see also PCT Publication No. WO 2006/052870 for 5-FOA use in *Yarrowia*).

An alternate preferred selection method for use in *Yarrowia* relies on a dominant, non-antibiotic marker for *Yarrowia lipolytica* based on sulfonylurea (chlorimuron ethyl; E. I. duPont de Nemours & Co., Inc., Wilmington, DE) resistance. More specifically, the marker gene is a native acetohydroxyacid synthase (AHAS or acetolactate synthase; E.C. 4.1.3.18) that has a single amino acid change (W497L) that confers sulfonyl urea herbicide resistance (PCT Publication No. WO 2006/052870). AHAS is the first common enzyme in the pathway for the biosynthesis of branched-chain amino acids (i.e., valine, leucine, isoleucine) and it is the target of the sulfonylurea and imidazolinone herbicides.

Other preferred microbial hosts include oleaginous bacteria, algae, euglenoids, stramenopiles and other fungi, may of which may be genetically engineered for the production of omega-3 fatty acids. Thus, for example, transformation of *Mortierella alpina* (which is commercially used for production of ARA) with any of the present Δ8 desaturase genes under the control of inducible or regulated promoters could yield a transformant capable of synthesizing increased quantities of DGLA. The method of transformation of *M. alpina* is described by Mackenzie et al. (*Appl. Environ. Microbiol.*, 66:4655 (2000)). Similarly, methods for transformation of Thraustochytriales microorganisms (e.g., *Thraustochytrium, Schizochytrium*) are disclosed in U.S. 7,001,772.

Irrespective of the host selected for expression of the Δ8 desaturases described herein, it may be necessary to screen multiple transformants to obtain a strain displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA blots (Southern, *J. Mol. Biol.*, 98:503 (1975)), Northern analysis of mRNA expression (Kroczek, *J. Chromatogr. Biomed. Appl.*, 618(1-2):133-145 (1993)), Western and/or Elisa analyses of protein expression, phenotypic analysis or GC analysis of the PUFA products.

Accordingly the scope of the present invention includes a method of producing either DGLA or ETA, respectively, comprising:

(a) providing an oleaginous yeast (e.g., *Yarrowia lipolytica*) comprising:

(i) a first recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,

(ii) a source of desaturase substrate consisting of EDA and/or ETrA, respectively; and,

(b) growing the yeast of step (a) in the presence of a suitable fermentable carbon source wherein the gene encoding the Δ8 desaturase polypeptide is expressed and EDA is converted to DGLA and/or ETrA is converted to ETA, respectively; and,

(c) optionally recovering the DGLA and/or ETA, respectively, of step (b).

Substrate feeding may be required.

The nucleotide sequence of the gene encoding a Δ8 desurase may be selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20. In alternate embodiments, the nucleotide sequence of the gene encoding a Δ8 desaturase polypeptide is set forth in SEQ ID NO:39 (wherein at least 208 codons have been optimized for expression in *Yarrowia* relative to SEQ ID NO:19).

Since naturally produced PUFAs in oleaginous yeast are limited to 18:2 fatty acids (i.e., LA), and less commonly, 18:3 fatty acids (i.e., ALA), the oleaginous yeast will be genetically engineered to express multiple enzymes necessary for long-chain PUFA biosynthesis (thereby enabling production of e.g., ARA, EPA, DPA and DHA), in addition to the Δ8 desaturases described herein. Specifically, in one embodiment this invention concerns an oleaginous yeast comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding a Δ8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of: a Δ4 desaturase, a

Δ5 desaturase, a Δ6 desaturase, a Δ9 desaturase, a Δ12 desaturase, a Δ15 desaturase, a Δ17 desaturase, a Δ9 elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase and a $C_{20/22}$ elongase.

In particularly preferred embodiments, the at least one additional recombinant DNA construct encodes a polypeptide having delta-9 elongase activity.

Metabolic Engineering Of Omega-3 And/Or Omega-6 Fatty Acid Biosynthesis In Microbes

Knowledge of the sequences of the present Δ8 desaturases will be useful for manipulating ω-3 and/or ω-6 fatty acid biosynthesis in various host cells. Methods for manipulating biochemical pathways are well known to those skilled in the art; and, it is expected that numerous manipulations will be possible to maximize ω-3 and/or ω-6 fatty acid biosynthesis in oleaginous yeasts, and particularly, in *Yarrowia lipolytica*. This manipulation may require metabolic engineering directly within the PUFA biosynthetic pathway or additional manipulation of pathways that contribute carbon to the PUFA biosynthetic pathway. Methods useful for up-regulating desirable biochemical pathways and down-regulating undesirable biochemical pathways are well known to those skilled in the art. For example, biochemical pathways competing with the ω-3 and/or ω-6 fatty acid biosynthetic pathways for energy or carbon, or native PUFA biosynthetic pathway enzymes that interfere with production of a particular PUFA end-product, may be eliminated by gene disruption or down-regulated by other means (e.g., antisense mRNA).

Detailed discussion of manipulations within the PUFA biosynthetic pathway as a means to increase ARA, EPA or DHA (and associated techniques thereof) are presented in U.S. Patent Publication No. 2006-0094092-A1, U.S. Patent Publication No. 2006-0115881-A1, andU.S. Patent Publication No. 2006-0110806-A1, respectively, as are desirable manipulations in the TAG biosynthetic pathway and the TAG degradation pathway (and associated techniques thereof).

Within the context of the present invention, it may be useful to modulate the expression of the fatty acid biosynthetic pathway by any one of the strategies described above. For example, the present invention provides methods whereby genes encoding key enzymes in the Δ9 elongase/Δ8 desaturase biosynthetic pathway are introduced into oleaginous yeasts for the production of ω-3 and/or ω-6 fatty acids. It will be particularly useful to express the present the Δ8 desaturase

genes in oleaginous yeasts that do not naturally possess ω-3 and/or ω-6 fatty acid biosynthetic pathways and coordinate the expression of these genes, to maximize production of preferred PUFA products using various means for metabolic engineering of the host organism.

Microbial Fermentation Processes For PUFA Production

The transformed microbial host cell is grown under conditions that optimize expression of chimeric desaturase and elongase genes and produce the greatest and most economical yield of desired PUFAs. In general, media conditions that may be optimized include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the amount of different mineral ions, the oxygen level, growth temperature, pH, length of the biomass production phase, length of the oil accumulation phase and the time and method of cell harvest. Microorganisms of interest, such as oleaginous yeast (e.g., *Yarrowia lipolytica*) are generally grown in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)) or a defined minimal media that lacks a component necessary for growth and thereby forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI)).

Fermentation media in the present invention must contain a suitable carbon source. Suitable carbon sources are taught in U.S. Patent 7,238,482. Although it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing sources, preferred carbon sources are sugars, glycerol, and/or fatty acids. Most preferred is glucose and/or fatty acids containing between 10-22 carbons.

Nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source. In addition to appropriate carbon and nitrogen sources, the fermentation media must also contain suitable minerals, salts, cofactors, buffers, vitamins and other components known to those skilled in the art suitable for the growth of the oleaginous host and promotion of the enzymatic pathways necessary for PUFA production. Particular attention is given to several metal ions (e.g., Fe^{+2}, Cu^{+2}, Mn^{+2}, Co^{+2}, Zn^{+2}, Mg^{+2}) that promote synthesis of lipids and PUFAs (Nakahara, T. et al., *Ind. Appl. Single Cell Oils,* D. J. Kyle and R. Colin, eds. pp 61-97 (1992)).

Preferred growth media in the present invention are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the transformant host cells will be known by one skilled in the art of microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to pH 8.0, wherein pH 5.5 to pH 7.5 is preferred as the range for the initial growth conditions. The fermentation may be conducted under aerobic or anaerobic conditions, wherein microaerobic conditions are preferred.

Typically, accumulation of high levels of PUFAs in oleaginous yeast cells requires a two-stage process, since the metabolic state must be "balanced" between growth and synthesis/storage of fats. Thus, most preferably, a two-stage fermentation process is necessary for the production of PUFAs in oleaginous yeast (e.g., *Yarrowia lipolytica*). This approach is described in U.S. Patent 7,238,482, as are various suitable fermentation process designs (i.e., batch, fed-batch and continuous) and considerations during growth.

Purification And Processing Of PUFA Oils

PUFAs may be found in the host microorganisms as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cells through a variety of means well-known in the art. One review of extraction techniques, quality analysis and acceptability standards for yeast lipids is that of Z. Jacobs (*Critical Reviews in Biotechnology,* 12(5/6):463-491 (1992)). A brief review of downstream processing is also available by A. Singh and O. Ward (*Adv. Appl. Microbiol.*, 45:271-312 (1997)).

In general, means for the purification of PUFAs may include extraction (e.g., U.S. Patent 6,797,303 and U.S. Patent 5,648,564) with organic solvents, sonication, supercritical fluid extraction (e.g., using carbon dioxide), saponification and physical means such as presses, or combinations thereof. One is referred to the teachings of U.S. Patent 7,238,482 for additional details.

PUFA-Containing Oils For Use In Foodstuffs, Health Food Products, Pharmaceuticals And Animal Feeds

The market place currently supports a large variety of food and feed products, incorporating ω-3 and/or ω-6 fatty acids (particularly e.g., ALA, GLA, ARA,

EPA, DPA and DHA). It is contemplated that the microbial biomass comprising long-chain PUFAs, partially purified microbial biomass comprising PUFAs, purified microbial oil comprising PUFAs, and/or purified PUFAs will function in food and feed products to impart the health benefits of current formulations. More specifically, oils of the invention containing ω-3 and/or ω-6 fatty acids will be suitable for use in a variety of food and feed products including, but not limited to: food analogs, meat products, cereal products, baked foods, snack foods and dairy products (see Patent Publication No. US-2006-0094092 for details).

Additionally, the present compositions may be used in formulations to impart health benefit in medical foods including medical nutritionals, dietary supplements, infant formula as well as pharmaceutical products. One of skill in the art of food processing and food formulation will understand how the amount and composition of the present oils may be added to the food or feed product. Such an amount will be referred to herein as an "effective" amount and will depend on the food or feed product, the diet that the product is intended to supplement or the medical condition that the medical food or medical nutritional is intended to correct or treat.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, 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 this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by: 1.) Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (Maniatis); 2.) T. J. Silhavy, M. L.

Bennan, and L. W. Enquist, Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and, 3.) Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, Eds), American Society for Microbiology: Washington, D.C. (1994)); or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of microbial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified. *E. coli* strains were typically grown at 37 °C on Luria Bertani (LB) plates.

General molecular cloning was performed according to standard methods (Sambrook et al., *supra*). DNA sequence was generated on an ABI Automatic sequencer using dye terminator technology (U.S. Patent 5,366,860; EP 272,007) using a combination of vector and insert-specific primers. Sequence editing was performed in Sequencher (Gene Codes Corporation, Ann Arbor, MI). All sequences represent coverage at least two times in both directions. Comparisons of genetic sequences were accomplished using DNASTAR software (DNASTAR Inc., Madison, WI).

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" or "hr" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" mean micromole(s), "g" means gram(s), "µg" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s) and "kB" means kilobase(s).

Nomenclature For Expression Cassettes:

The structure of an expression cassette will be represented by a simple notation system of "X::Y::Z", wherein X describes the promoter fragment, Y

describes the gene fragment, and Z describes the terminator fragment, which are all operably linked to one another.

Transformation And Cultivation Of *Yarrowia lipolytica*:

Yarrowia lipolytica strains with ATCC Accession Nos. #20362, #76982 and #90812 were purchased from the American Type Culture Collection (Rockville, MD). *Yarrowia lipolytica* strains were typically grown at 28-30 °C in several media, according to the recipes shown below. Agar plates were prepared as required by addition of 20 g/L agar to each liquid media, according to standard methodology.

YPD agar medium (per liter): 10 g of yeast extract [Difco], 20 g of Bacto peptone [Difco]; and 20 g of glucose.

Basic Minimal Media (MM) (per liter): 20 g glucose; 1.7 g yeast nitrogen base without amino acids; 1.0 g proline; and pH 6.1 (not adjusted).

Minimal Media + Leucine (MM+leucine or MMLeu) (per liter): Prepare MM media as above and add 0.1 g leucine.

Minimal Media + Leucine + Uracil (MMLeuUra) (per liter): Prepare MM media as above and add 0.1 g leucine, 0.1 g uracil and 0.1 g uridine.

Minimal Media + 5-Fluoroorotic Acid (MM + 5-FOA) (per liter): 20 g glucose, 6.7 g Yeast Nitrogen base, 75 mg uracil, 75 mg uridine and appropriate amount of FOA (Zymo Research Corp., Orange, CA), based on FOA activity testing against a range of concentrations from 100 mg/L to 1000 mg/L (since variation occurs within each batch received from the supplier).

High Glucose Media (HGM) (per liter): 80 glucose, 2.58 g KH_2PO_4 and 5.36 g K_2HPO_4, pH 7.5 (do not need to adjust).

Transformation of *Yarrowia lipolytica* was performed according to the method of Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)), unless otherwise noted. Briefly, *Yarrowia* was streaked onto a YPD plate and grown at 30 °C for approximately 18 h. Several large loopfuls of cells were scraped from the

plate and resuspended in 1 mL of transformation buffer, comprising: 2.25 mL of 50% PEG, average MW 3350; 0.125 mL of 2 M lithium acetate, pH 6.0; 0.125 mL of 2 M DTT; and (optionally) 50 μg sheared salmon sperm DNA. Then, approximately 500 ng of linearized plasmid DNA (or 100 ng circular plasmid) was incubated in 100 μL of resuspended cells, and maintained at 39 °C for 1 h with vortex mixing at 15 min intervals. The cells were plated onto selection media plates and maintained at 30 °C for 2 to 3 days.

Fatty Acid Analysis Of *Yarrowia lipolytica*:

Unless otherwise stated, for fatty acid analysis cells were collected by centrifugation and lipids were extracted as described in Bligh, E. G. & Dyer, W. J. (*Can. J. Biochem. Physiol.*, 37:911-917 (1959)). Fatty acid methyl esters were prepared by transesterification of the lipid extract with sodium methoxide (Roughan, G. and Nishida I., *Arch Biochem Biophys.*, 276(1):38-46 (1990)) and subsequently analyzed with a Hewlett-Packard 6890 GC fitted with a 30 m X 0.25 mm (i.d.) HP-INNOWAX (Hewlett-Packard) column. The oven temperature was from 170 °C (25 min hold) to 185 °C at 3.5 °C/min.

For direct base transesterification, *Yarrowia* culture (3 mL) was harvested, washed once in distilled water, and dried under vacuum in a Speed-Vac for 5-10 min. Sodium methoxide (100 μL of 1%) was added to the sample, and then the sample was vortexed and rocked for 20 min. After adding 3 drops of 1 M NaCl and 400 μL hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.

Construction Of *Yarrowia lipolytica* Strain Y4001U:

Y. lipolytica strain Y4001U was used as the host in Example 7, *infra*. The following description is a summary of the construction of strain Y4001U, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing about 17% EDA relative to the total lipids via expression of a Δ9 elongase/Δ8 desaturase pathway and having a *Leu-* and *Ura-* phenotype (FIG. 2A).

The development of strain Y4001U required the construction of strain Y2224 (a FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362) and strain Y4001 (producing 17% EDA with a *Leu-* phenotype).

Generation Of Strain Y2224: Strain Y2224 was isolated in the following manner: *Yarrowia lipolytica* ATCC #20362 cells from a YPD agar plate were streaked onto a MM plate (75 mg/L each of uracil and uridine, 6.7 g/L YNB with ammonia sulfate, without amino acids, and 20 g/L glucose) containing 250 mg/L 5-FOA (Zymo Research). Plates were incubated at 28 °C and four of the resulting colonies were patched separately onto MM plates containing 200 mg/mL 5-FOA and MM plates lacking uracil and uridine to confirm uracil *Ura3* auxotrophy.

Generation Of Strain Y4001 To Produce About 17% EDA Of Total Lipids: Strain Y4001 was created via integration of construct pZKLeuN-29E3 (FIG. 2B). This construct, comprising four chimeric genes (i.e., a Δ12 desaturase, a $C_{16/18}$ elongase and two Δ9 elongases), was integrated into the *Leu2* loci of strain Y2224 to thereby enable production of EDA.

Construct pZKLeuN-29E3 contained the components shown below in Table 4.

Table 4

Description of Plasmid pZKLeuN-29E3 (SEQ ID NO:42)

RE Sites And Nucleotides Within SEQ ID NO:42	Description Of Fragment And Chimeric Gene Components
BsiW I/*Asc* I (7797-7002)	788 bp 3' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)
Sph I/*Pac* I (4302-3591)	703 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)
Swa I/*BsiW* I (10533-7797)	GPD::FmD12::Pex20, comprising: • GPD: *Yarrowia lipolytica* GPD promoter (U.S. Patent 7,259,255); • FmD12: *Fusarium moniliforme* Δ12 desaturase gene (SEQ ID NO:43) (labeled as "F.D12" in Figure; PCT Publication No. WO 2005/047485); • Pex20: Pex20 terminator sequence from *Yarrowia* Pex20 gene (GenBank Accession No. AF054613)
Bgl II/*Swa* I (12559-10533)	EXP1::EgD9eS::Lip1, comprising: • EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp pro" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761); • EgD9eS: codon-optimized Δ9 elongase (SEQ ID NO:45), derived from *Euglena gracilis* (labeled as "EgD9E" in Figure; PCT Publication No. WO 2007/061742);

	• Lip1: Lip1 terminator sequence from *Yarrowia* Lip1 gene (GenBank Accession No. Z50020)
Pme I/*Cla* I (12577-1)	FBAINm::EgD9eS::Lip2, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (U.S. Patent 7,202,356); • EgD9eS: codon-optimized Δ9 elongase gene (SEQ ID NO:45), derived from *Euglena gracilis* (labeled as "EgD9ES" in Figure; PCT Publication No. WO 2007/061742); • Lip2: Lip2 terminator sequence from *Yarrowia* Lip2 gene (GenBank Accession No. AJ012632)
Cla I/*EcoR* I (1-1736)	LoxP::*Ura3*::LoxP, comprising: • LoxP sequence (SEQ ID NO:47); • *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421); • LoxP sequence (SEQ ID NO:47)
EcoR I/*Pac* I (1736-3591)	YAT1::ME3S::Pex16, comprising: • YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication No. U.S. 2006/0094102-A1); • ME3S: codon-optimized $C_{16/18}$ elongase gene (SEQ ID NO:48), derived from *M. alpina* (PCT Publication No. WO 2007/046817); • Pex16: Pex16 terminator sequence of *Yarrowia Pex 16* gene (GenBank Accession No. U75433)

Plasmid pZKLeuN-29E3 was digested with *Asc*I/*Sph*I, and then used for transformation of *Y. lipolytica* strain Y2224 (i.e., ATCC #20362 *Ura3*-) according to the General Methods. The transformant cells were plated onto MMLeu media plates and maintained at 30 °C for 2 to 3 days. The colonies were picked and streaked onto MM and MMLeu selection plates. The colonies that could grow on MMLeu plates but not on MM plates were selected as *Leu*- strains. Single colonies of *Leu*- strains were then inoculated into liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of EDA in the transformants containing the 4 chimeric genes of pZKLeuN-29E3, but not in the *Yarrowia* Y2224 control strain. Most of the selected 36 *Leu*- strains produced about 12 to 16.9% EDA of total lipids. There were 3 strains (i.e., strains #11, #30 and #34) that produced

about 17.4%, 17% and 17.5% EDA of total lipids; they were designated as strains Y4001, Y4002 and Y4003, respectively.

Single colonies of Y4001, Y4002 and Y4003 strains were inoculated in liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, resuspended in High Glucose Media and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC. GC analyses showed that the Y4001, Y4002 and Y4003 strains produced about 24% EDA of total lipids.

Generation Of Strain Y4001U (*Leu-, Ura-*): Strain Y4001U was created via temporary expression of the *Cre* recombinase enzyme in plasmid pY116 (FIG. 2C) within strain Y4001 to produce a *Leu-* and *Ura-* phenotype. Construct pY116 contained the following components:

Table 5

Description of Plasmid pY116 (SEQ ID NO:50)

RE Sites And Nucleotides Within SEQ ID NO:50	Description Of Fragment And Chimeric Gene Components
1328-448	ColE1 plasmid origin of replication
2258-1398	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
3157-4461	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
SwaI/PacI 6667-4504	*Yarrowia* Leu2 gene (GenBank Accession No. AF260230)
Swa I/Pme I (6667-218)	GPAT::Cre::XPR2, comprising: • GPAT: *Yarrowia lipolytica* GPAT promoter (U.S. Patent 7,264,949); • Cre: Enterobacteria phage P1 Cre gene for recombinase protein (GenBank Accession No. X03453); • XPR2: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)

Plasmid pY116 was used for transformation of freshly grown Y4001 cells according to the General Methods. The transformant cells were plated onto MMLeuUra plates containing 280 μg/mL sulfonylurea (chlorimuron ethyl, E. I.

duPont de Nemours & Co., Inc., Wilmington, DE) and maintained at 30 °C for 3 to 4 days. Four colonies were picked, inoculated into 3 mL liquid YPD media at 30 °C and shaken at 250 rpm/min for 1 day. The cultures were diluted to 1:50,000 with liquid MMLeuUra media, and 100 μL was plated onto new YPD plates and maintained at 30 °C for 2 days. Colonies were picked and streaked onto MMLeu and MMLeuUra selection plates. The colonies that could grow on MMLeuUra plates but not on MMLeu plates were selected and analyzed by GC to confirm the presence of C20:2 (EDA). Several strains, having a *Leu-* and *Ura-* phenotype, produced about 17% EDA of total lipids and were designated collectively as Y4001U; one of these strains was designated as Y4001U1.

EXAMPLE 1

Synthesis Of A cDNA Library From *Euglena anabaena* UTEX 373

The present Example describes the synthesis of a cDNA library from *Euglena anabaena* UTEX 373. This work included preparation of RNA, synthesis of cDNA, and generation of a cDNA library.

Growth Of *Euglena anabaena* UTEX 373 And Preparation Of RNA

Euglena anabaena UTEX 373 was obtained from Dr. Richard Triemer's lab at Michigan State University (East Lansing, MI). Approximately 2 mL of culture was removed for lipid analysis and centrifuged at 1,800 x g for 5 min. The pellet was washed once with water and re-centrifuged. The resulting pellet was dried for 5 min under vacuum, resuspended in 100 μL of trimethylsulfonium hydroxide (TMSH) and incubated at room temperature for 15 min with shaking. After this step, 0.5 mL of hexane was added and the vials were incubated for 15 min at room temperature with shaking. Fatty acid methyl esters (5 μL injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Supelco Inc., Catalog No. 24152). The oven temperature was programmed to hold at 170 °C for 1.0 min, increase to 240 °C at 5 °C /min and then hold for an additional 1.0 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc. Catalog No. U-99-A) and the resulting chromatogram is shown in FIG. 3. The presence of EDA, ERA, EPA and DHA in the fatty acid profile, with the absence of GLA and STA, suggested that *Euglena anabaena* uses the Δ9 elongase/Δ8

desaturase pathway for long-chain (LC) PUFA biosynthesis and would be a good source for LC-PUFA biosynthetic genes such as, but not limited to, Δ8 desaturases.

The remaining 5 mL of an actively growing culture was transferred into 25 mL of AF-6 Medium (Watanabe & Hiroki, NIES-Collection List of Strains, 5th ed., National Institute for Environmental Studies, Tsukuba, 127 pp (2004)) in a 125 mL glass flask. *Euglena anabaena* cultures were grown at 22 °C with a 16 h light, 8 h dark cycle for 2 weeks with very gentle agitation.

After 2 weeks, the culture (25 mL) was transferred to 100 mL of AF-6 medium in a 500 mL glass bottle and the culture was grown for 1 month as described above. After this time, two 50 mL aliquots were transferred into two separate 500 mL glass bottles containing 250 mL of AF-6 medium and the cultures were grown for two months as described above (giving a total of ~600 mL of culture). Next, the cultures were pelleted by centrifugation at 1,800 x g for 10 min, washed once with water and re-centrifuged. Total RNA was extracted from one of the resulting pellets using the RNA STAT-60™ reagent (TEL-TEST, Inc., Friendswood, TX) and following the manufacturer's protocol provided (use 5 mL of reagent, dissolved RNA in 0.5 mL of water). In this way, 340 μg of total RNA (680 ug/mL) was obtained from the pellet. The remaining pellet was frozen in liquid nitrogen and stored at -80 °C. The mRNA was isolated from all 340 μg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol provided. In this way, 9.0 μg of mRNA was obtained.

Preparation Of *Euglena anabaena* cDNA And Generation Of cDNA Library eug1c

A cDNA library was generated using the Cloneminer™ cDNA Library Construction Kit (Catalog No.18249-029, Invitrogen Corporation, Carlsbad, CA) and following the manufacturer's protocol provided (Version B, 25-0608). Using the non-radiolabeling method, cDNA was synthesized from 5.12 μg of mRNA (described above) using the Biotin-*att*B2-Oligo(dT) primer. After synthesis of the first and second strand, the *att*B1 adapter was added, ligated and the cDNA was size fractionated using column chromatography. DNA from fractions were concentrated, recombined into pDONR™222 and transformed into *E. coli* ElectroMAX™ DH10B™ T1 Phage-Resistant cells (Invitrogen Corporation). The *Euglena anabaena* library was named eug1c.

The cDNA library eug1c was plated onto LB +Kanamycin plates (approx. 100,000 colonies), the colonies were scraped off and DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. In this way, a plasmid DNA sub-library from eug1c was obtained.

EXAMPLE 2

Isolation Of A cDNA Fragment Encoding A Partial Δ8 Desaturase From *Euglena anabaena* UTEX 373

The present Example describes the identification of a cDNA fragment (SEQ ID NO:1) encoding a partial Δ8 desaturase from *Euglena anabaena* UTEX 373, based on PCR amplification of the cDNA library using degenerate oligonucleotides based on a *Euglena gracilis* Δ8 desaturase sequence (SEQ ID NO:2).

Identification Of cDNA Fragments Encoding Partial Putative Δ8 Desaturases

The plasmid DNA sub-library described in Example 1 was used as template for degenerate PCR using degenerate primers based on a nucleotide sequence of the *Euglena gracilis* Δ8 fatty acid desaturase (SEQ ID NO:2; described as Eg5 in PCT Publication No. WO 2006/012325) and the vector-specific primer pDonor222Eg5-1 (SEQ ID NO:3). The 4 degenerate primers used are shown in Table 6.

Table 6

Degenerate Oligonucleotides Used To Amplify A Portion Of The Δ8 Desaturase Genes From *Euglena anabaena* UTEX 373

Primer	Nucleotide Sequence	SEQ ID NO:
D8DEG3-1	RTTRTGNCKATCTTTCCACCA	SEQ ID NO:4
D8DEG3-2	RTTRTGNCKGTCTTTCCACCA	SEQ ID NO:5
D8DEG3-3	RTTRTGNCKATCCTTCCACCA	SEQ ID NO:6
D8DEG3-4	RTTRTGNCKGTCCTTCCACCA	SEQ ID NO:7

A total of 5 reactions were set up for the cDNA sample. The reaction mixture contained 1 µL of cDNA, 1 µL each of the vector-specific and degenerate primers (20 µM) and Phusion™ High-Fidelity DNA Polymerase (Catalog No. F553S, Finnzymes Oy, Finland). The PCR was carried out following the manufacturer's protocol. The resulting DNA fragments were cloned into the pCR-Blunt® cloning

vector using the Zero Blunt® PCR Cloning Kit (Invitrogen Corporation), following the manufacturer's protocol.

Plasmid DNA from the resulting clones was purified using the QIAprep® Spin Miniprep Kit (Qiagen Inc.) following the manufacturer's protocol and DNA inserts were end-sequenced in 384-well plates, using vector-primed T7 primer (SEQ ID NO:8) and M13rev-28 primer (SEQ ID NO:9) with the ABI BigDye version 3 Prism sequencing kit. For the sequencing reaction, 100-200 ng of template and 6.4 pmol of primer were used, and the following reaction conditions were repeated 25 times: 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. After ethanol-based cleanup, cycle sequencing reaction products were resolved and detected on Perkin-Elmer ABI 3700 automated sequencers.

A consensus sequence was assembled from the individual sequences obtained and one representative clone, designated as pHD23-1 (SEQ ID NO:10) having a sequence identical to the consensus was choosen for further study.

Identification of the partial cDNA insert in pHD23-1 (SEQ ID NO:1) as a partial Δ8 desaturase was confirmed using BLAST (Basic Local Alignment Search Tool; Altschul et al., *J. Mol. Biol.*, 215:403-410 (1993)) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL and DDBJ databases). The partial cDNA sequence obtained (SEQ ID NO:1) was translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States, *Nat. Genet.*, 3:266-272 (1993)) provided by the NCBI with the default parameter and the filter turned off. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as a "pLog" value, which represents the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

The BLASTX search using the nucleotide sequence insert from pHD23-1 revealed similarity of the protein encoded by the partial cDNA (SEQ ID NO:1) to the

Euglena gracilis Δ8 desaturase amino acid sequence (SEQ ID NO:11) (NCBI Accession No. AAD45877(GI 5639724), locus AAD45877, CDS AF139720; Wallis and Browse, *Arch. Biochem. Biophys.*, 365:307-316 (1999)) and yielded a pLog value of 63.4 (E value of 4e-63).

EXAMPLE 3

Isolation Of Full-length Δ8 Desaturases From *Euglena anabaena* UTEX 373

Approximately 17,000 clones of cDNA library eug1c were plated onto three large square (24 cm x 24 cm) petri plates (Corning, Corning, NY) each containing LB + 50 μg/mL kanamycin agar media. Cells were grown overnight at 37 °C and plates were then cooled to room temperature.

Colony Lifts

Biodyne B 0.45 μm membrane (Catalog No. 60207, Pall Corporation, Pensacola, FL) was trimmed to approximately 22 cm x 22 cm and the membrane was carefully layed on top of the agar to avoid air bubbles. After incubation for 2 min at room temperature, the membrane was marked for orientation, lifted off with tweezers and placed colony-side up on filter paper soaked with 0.5 M sodium hydroxide and 1.5 M sodium chloride. After denaturation for 4 min, the sodium hydroxide was neutralized by placing the membrane on filter paper soaked with 0.5 M Tris-HCL (pH 7.5) and 1.5 M sodium chloride for 4 min. This step was repeated and the membrane was rinsed briefly in 2X SSC buffer (20X SSC is 3 M sodium chloride, 0.3 M sodium citrate; pH 7.0) and air dried on filter paper.

Hybridization

Membranes were pre-hybridized at 65 °C in 200 mL hybridization solution for 2 hr. Hybridization solution contained 6X SSPE (20X SSPE is 3 M sodium chloride, 0.2 M sodium phosphate, 20 mM EDTA; pH 7.4), 5X Denhardt's reagent (100X Denhardt's reagent is 2%(w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) acetylated bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 100 μg/mL sheared salmon sperm DNA and 5% dextran sulfate.

A DNA probe was made using an agarose gel purified *Eco*RI DNA fragment, containing the *Euglena anabaena* Δ8 desaturase partial DNA fragment, from pHD23-1 (Example 2) labeled with P^{32} dCTP using the RadPrime DNA Labeling System (Catalog No. 18428-011, Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Unincorporated P^{32} dCTP was separated using a

NICK column (Catalog No. 17-0855-02, Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions. The probe was denatured for 5 min at 100 °C, placed on ice for 3 min and half was added to the hybridization solution.

The membrane was hybridized with the probe overnight at 65 °C with gentle shaking and then washed the following day twice with 2X SSC containing 0.5% SDS (5 min each) and twice with 0.2X SSC containing 0.1% SDS (15 min each). After washing, hyperfilm (Catalog No. RPN30K, Amersham Biosciences, Piscataway, NJ) was exposed to the membrane overnight at -80 °C.

Based on alignment of plates with the exposed hyperfilm, positive colonies were picked using the blunt end of a Pasteur pipette into 1 mL of water and vortexed. Several dilutions were made and plated onto small round Petri dishes (82 mm) containing LB media plus 50 µg/mL kanamycin to obtain around 100 well isolated colonies on a single plate. Lifts were done as described above except NytranN membrane circles (Catalog No. 10416116, Schleicher & Schuell, Keene, NH) were used and hybridization was carried out in 100 mL using the remaining radiolabeled probe. In this way, positive clones were confirmed.

Individual positive clones were grown at 37 °C in LB + 50 µg/mL kanamycin liquid media and plasmid was purified using the QIAprep® Spin Miniprep Kit (Qiagen Inc.) following the manufacturer's protocol.

The plasmid insert was sequenced as described in Example 2 with the ABI BigDye version 3 Prism sequencing kit using vector-primed T7 primer (SEQ ID NO:8), vector-primed M13rev-28 primer (SEQ ID NO:9) and the poly(A) tail-primed WobbleT oligonucleotides. Briefly, the WobbleT primer is an equimolar mix of 21mer poly(T)A, poly(T)C and poly(T)G, used to sequence the 3' end of cDNA clones. Based on initial sequence data, additional internal fragment sequence was obtained in a similar way using oligonucleotide EaD8seq-1 (SEQ ID NO:12). In this way, the full insert sequences of the eug1c Δ8 desaturase clones were obtained.

Sequences were aligned and compared using Sequencher™ (Version 4.2, Gene Codes Corporation, Ann Arbor, MI) and in this way, the clones could be categorized into one of four distinct groups based on insert sequence (designated as EaD8Des1, EaD8Des2, EaD8Des3 or EaD8Des4). Representative clones containing the cDNA for each class of sequence were chosen for further study and sequences for each representative plasmid (i.e., pLF118-1, pLF118-2, pLF118-3

and pLF118-4) are shown in SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16, respectively. The sequence shown by a string of NNNN's represents a region of the polyA tail which was not sequenced. The coding sequences for EaD8Des1, EaD8Des2, EaD8Des3 and EaD8Des4 are shown in SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, respectively. The corresponding amino acid sequences for EaD8Des1, EaD8Des2, EaD8Des3 and EaD8Des4 are shown in SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24, respectively. EaD8Des1, EaD8Des2, EaD8Des3 and EaD8Des4 are collectively referred to as EaD8Des genes.

EXAMPLE 4

Primary Sequence Analysis Of The Δ8 Desaturase Sequences Of *Euglena anabaena* UTEX 373 And Comparison To A Δ8 Desaturase Sequence Of *Euglena gracilis*

The amino acid sequences for EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) and EaD8Des4 (SEQ ID NO:24) were compared using the Clustal W method (using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.) with the default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB).

Compared to EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22) has 3 amino acid substitutions (i.e., T110S, M223I and K251T; based on numbering for EaD8Des1), EaD8Des3 (SEQ ID NO:23) has 2 amino acid substitutions (i.e., T110S and K251T) and EaD8Des4 (SEQ ID NO:24) has 1 amino acid substitution (i.e., T110S).

The amino acid sequences for EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) and EaD8Des4 (SEQ ID NO:24) were evaluated by BLASTP (Basic Local Alignment Search Tool; Altschul et al., *J. Mol. Biol.*, 215:403-410 (1993)) searches for similarity to sequences contained in the BLAST "nr" database (*supra*, Example 2) using default parameters and the filter off. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as

calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value.

All four sequences yielded a pLog value of 177 (P value of e-177) versus the *Euglena gracilis* Δ8 desaturase amino acid sequence (SEQ ID NO:11) (NCBI Accession No. AAD45877(GI 5639724), locus AAD45877, CDS AF139720; Wallis and Browse, *Arch. Biochem. Biophys.,* 365:307-316 (1999)) when compared to the "nr" database. BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire *Euglena anabaena* Δ8 fatty acid desaturases.

The amino acid sequences for EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) and EaD8Des4 (SEQ ID NO:24) were then compared to a functional variant *Euglena gracilis* Δ8 desaturase amino acid sequence (identified herein as EgD8 and set forth as SEQ ID NO:25; described as Eg5 in PCT Application No. WO 2006/012325) using BlastP (default parameters, filter off), Clustal V and the Jotun Hein methods of sequence comparison and the % identity using each method is shown in Table 7.

Sequence percent identity calculations performed by the Clustal V method (Higgins, D.G. and Sharp, P.M., *Comput. Appl. Biosci.,* 5:151-153 (1989); Higgins et al., *Comput. Appl. Biosci.,* 8:189-191 (1992)) were done using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5 and GAP LENGTH PENALTY=10). Sequence percent identity calculations performed by the Jotun Hein method (Hein, J. J., *Meth. Enz.,* 183:626-645 (1990)) were done using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=2).

Table 7

Sequence Comparison Of EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) And EaD8Des4 (SEQ ID NO:24) To EgD8 (SEQ ID NO:25)

Desaturase	% Identity to EgD8 (SEQ ID NO:25) by BLASTP	% Identity to EgD8 (SEQ ID NO:25) by the Jotun Hein Method	% Identity to EgD8 (SEQ ID NO:25) by the Clustal V

			Method
EaD8Des1	73%	74.4%	72.1%
EaD8Des2	73%	74.2%	71.9%
EaD8Des3	73%	74.2%	71.9%
EaD8Des4	73%	74.2%	71.9%

The Clustal V alignment of these five amino acid sequences can be seen in FIGs. 4A, 4B and 4C. Table 8 below sets forth a comparison of the percent identity (shown in the upper half triangle) and percent divergence (shown in the lower half triangle), among the five Δ8 desaturase sequences aligned in FIGs. 4A, 4B and 4C.

Table 8

Percent Identity And Percent Divergence Among EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23), EaD8Des4 (SEQ ID NO:24) And EgD8 (SEQ ID NO:25)

	EaD8Des1	EaD8Des2	EaD8Des3	EaD8Des4	EgD8
EaD8Des1	--	99.3	99.5	99.8	72.1
EaD8Des2	0.7	--	99.8	99.5	71.9
EaD8Des3	0.5	0.2	--	99.8	71.9
EaD8Des4	0.2	0.5	0.2	--	71.9
EgD8	31.4	31.8	31.8	31.8	--

EXAMPLE 5

Functional Analysis Of The *Euglena anabaena* UTEX 373 Δ8 Desaturases In *Yarrowia lipolytica*

The present Example describes functional analysis of EaD8Des1 (SEQ ID NO:21), EaD8Des2 (SEQ ID NO:22), EaD8Des3 (SEQ ID NO:23) and EaD8Des4 (SEQ ID NO:24) in *Yarrowia lipolytica*. This work included the following steps: (1) PCR amplification of each Δ8 desaturase with appropriate restriction sites for cloning from the plasmids described in Example 2; (2) cloning of the EaD8Des PCR products into cloning vector pCR-Blunt® (Invitrogen Corporation) to produce pY120-1, pY120-2, pY120-3 and pY120-4; (3) cloning of the EaD8Des genes into *Yarrowia* expression vector pY115 to produce pY175, pY176, pY177 and pY178; and, (4) comparison of lipid profiles within transformant organisms comprising pY175, pY176, pY177 and pY178, after substrate feeding.

PCR Amplification Of The *Euglena anabaena* Δ8 Desaturase Genes

In order to introduce *Not*I and *Nco*I restriction sites at the 5' end of the coding sequences and a *Not*I site at the 3' end of the coding sequences, each of the EaD8Des genes were PCR amplified. The coding sequences for EaD8Des1 (SEQ ID NO:17), EaD8Des2 (SEQ ID NO:18), EaD8Des3 (SEQ ID NO:19) and EaD8Des4 (SEQ ID NO:20) were amplified from pLF118-1 (SEQ ID NO:13), pLF118-2 (SEQ ID NO:14), pLF118-3 (SEQ ID NO:15) and pLF118-4 (SEQ ID NO:16), respectively, with oligonucleotide primers EaD8-5 (SEQ ID NO:26) and EaD8-3 (SEQ ID NO:27) using the Phusion™ High-Fidelity DNA Polymerase (Catalog No. F553S, Finnzymes Oy, Finland) following the manufacturer's protocol. The resulting DNA fragments were cloned into the pCR-Blunt® cloning vector using the Zero Blunt® PCR Cloning Kit (Invitrogen Corporation), following the manufacturer's protocol, to produce pLF120-1 (SEQ ID NO:28), pLF120-2 (SEQ ID NO:29), pLF120-3 (SEQ ID NO:30) and pLF120-4 (SEQ ID NO:31), respectively.

Construction Of *Yarrowia* Expression Vectors pY115, pY175, pY176, pY177 And pY178

Plasmid pY5-30 (described in U.S. Patent 7,259,255) is a shuttle plasmid that can replicate both in *E. coli* and *Yarrowia lipolytica*. Plasmid pY5-30 contains the following: a *Yarrowia* autonomous replication sequence (ARS18); a ColE1 plasmid origin of replication; an ampicillin-resistance gene (Amp^R), for selection in *E. coli*; a *Yarrowia* LEU2 gene, for selection in *Yarrowia*; and a chimeric TEF::GUS::XPR gene. Plasmid pDMW263 (SEQ ID NO:32) was created from pY5-30, by replacing the TEF promoter with the *Yarrowia lipolytica* FBAINm promoter (U.S. Patent 7,202,356) using techniques well known to one skilled in the art. Briefly, this promoter refers to a modified promoter which is located in the 5' upstream untranslated region in front of the 'ATG' translation initiation codon of the fructose-bisphosphate aldolase enzyme (E.C. 4.1.2.13) encoded by the *fba1* gene and that is necessary for expression, plus a portion of 5' coding region that has an intron, wherein FBAINm has a 52 bp deletion between the ATG translation initiation codon and the intron of the FBAIN promoter (thereby including only 22 amino acids of the N-terminus) and a new translation consensus motif after the intron. Table 9 summarizes the components of pDMW263 (SEQ ID NO:32).

Table 9

Components Of Plasmid pDMW263

RE Sites and Nucleotides Within SEQ ID NO:32	Description of Fragment and Chimeric Gene Components
4992-4296	ARS18 sequence (GenBank Accession No. A17608)
SalI/SacII (8505-2014)	FBAINm::GUS::XPR, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (U.S. Patent 7,202,356); • GUS: *E. coli* gene encoding β-glucuronidase (Jefferson, R.A. *Nature,* 14:342:837-838 (1989); • XPR: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)
6303-8505	*Yarrowia Leu2* gene (GenBank Accession No. AF260230)

The *NcoI/SalI* DNA fragment from pDMW263 (SEQ ID NO:32), containing the *Yarrowia lipolytica* FBAINm promoter, was cloned into the *NcoI/SalI* DNA fragment of pDMW237 (SEQ ID NO:33), previously described in PCT Publication No. WO 2006/012325 (the contents of which are hereby incorporated by reference), containing a synthetic Δ9 elongase gene derived from *Isochrysis galbana* and codon-optimized for expression in *Yarrowia lipolytica*, to produce pY115 (SEQ ID NO:34; FIG. 5A). In FIG. 5A and FIG. 5B, the modified FBAINm promoter is labeled as FBA1 + Intron.

The *NcoI/NotI* DNA fragments from pLF120-1 (SEQ ID NO:28), pLF120-2 (SEQ ID NO:29), pLF120-3 (SEQ ID NO:30) and pLF120-4 (SEQ ID NO:31), containing each EaD8Des, were cloned into the *NcoI/NotI* DNA fragment from pY115, containing the *Yarrowia lipolytica* FBAINm promoter, to produce pY175 (SEQ ID NO:35; FIG. 5B), pY176 (SEQ ID NO:36), pY177 (SEQ ID NO:37) and pY178 (SEQ ID NO:38), respectively.

Functional Analysis Of The *Euglena anabaena* Δ8 Desaturase Genes In *Yarrowia lipolytica* Strain Y2224

Strain Y2224 (see General Methods) was transformed with pY175 (SEQ ID NO:35), pY176 (SEQ ID NO:36), pY177 (SEQ ID NO:37) and pY178 (SEQ ID NO:38) as described in the General Methods.

Single colonies of transformant *Yarrowia lipolytica* containing pY175, pY176, pY177 and pY178 were grown in 3 mL minimal media lacking uracil supplemented with 0.2% tergitol at 30 °C for 1 day. After this, 0.1 mL was transferred to 3 mL of the same medium supplemented with EDA (20:2(11,14)) or ETrA (20:3(11,14,17)) to 0.175 mM. These were incubated for 16 hr at 30 °C, 250 rpm and then pellets were obtained by centrifugation. Cells were washed once with water, pelleted by centrifugation and air dried. Pellets were transesterified (Roughan, G. and Nishida, I., *Arch. Biochem. Biophys.*, 276(1):38-46 (1990)) with 500 µL of 1% sodium methoxide for 30 min at 50 °C after which 500 µL of 1 M sodium chloride and 100 µL of heptane were added. After thorough mixing and centrifugation, fatty acid methyl esters (FAMEs) were analyzed by GC. FAMEs (5 µL injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Catalog No. 24152, Supelco Inc.). The oven temperature was programmed to hold at 220 °C for 2.6 min, increase to 240 °C at 20 °C/min and then hold for an additional 2.4 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.).

The fatty acid profiles for *Yarrowia lipolytica* expressing pY175, pY176, pY177 and pY178 are shown in FIG. 6. Percent C20 desaturation ("C20 % delta-8 desat") was calculated either by dividing the weight percent (wt %) for DGLA by the sum of the wt % for EDA and DGLA and multiplying by 100 to express as a % or by dividing the wt % for ETA by the sum of the wt % for ERA and DTA and multiplying by 100 to express as a %, depending on which substrate was fed (i.e., EDA or ERA). Averages are indicated by Ave. followed by the appropriate header. The ratio of desaturation of EDA to ERA is calculated by dividing the Ave. C20 % delta-8 desat for EDA by that of ERA.

All of the *Euglena anabaena* Δ8 desaturases function similarly well in *Yarrowia* and convert approximately 50% of the fed EDA to DGLA. There appears to be a slight preference for the EDA over ERA with a EDA/ERA ratio of 1.1 to 1.2.

EXAMPLE 6

Synthesis Of A Codon-Optimized Δ8 Desaturase Gene For *Yarrowia lipolytica* (EaD8S)

The codon usage of the Δ8 desaturase gene (EaD8Des3; SEQ ID NO:19) of *Euglena anabaena* was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in PCT Publication No. WO 2004/101753 and U.S. Patent 7,125,672. Specifically, a codon-optimized Δ8 desaturase gene (designated "EaD8S", SEQ ID NO:39) was designed based on the coding sequence of EaD8Des3 (SEQ ID NOs:19 and 23), according to the *Yarrowia* codon usage pattern (PCT Publication No. WO 2004/101753), the consensus sequence around the 'ATG' translation initiation codon, and the general rules of RNA stability (Guhaniyogi, G. and J. Brewer, *Gene*, 265(1-2):11-23 (2001)). In addition to modification of the translation initiation site, 231 bp of the 1260 bp coding region were modified (18.3%) and 208 codons were optimized (49.5%). The GC content was reduced from 56.8% within the wild type gene (i.e., EaD8Des3) to 54.8% within the synthetic gene (i.e., EaD8S). A *Nco*I site and *Not*I sites were incorporated around the translation initiation codon and after the stop codon of EaD8S (SEQ ID NO:39), respectively. FIGs. 7A and 7B show a comparison of the nucleotide sequences of EaD8Des3 (SEQ ID NO:19) and EaD8S (SEQ ID NO:39). The protein sequence encoded by the codon-optimized gene (i.e., SEQ ID NO:40) is identical to that of the wildtype EaD8Des3 protein sequence (i.e., SEQ ID NO:23). The designed EaD8S gene was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pEaD8S (SEQ ID NO:41; FIG. 8A).

EXAMPLE 7

Construction And Functional Analysis Of *Yarrowia lipolytica* Expression Vector pZUFmEaD8S, Comprising A Synthetic Δ8 Desaturase Gene (Derived From *Euglena anabaena*), Codon-Optimized For Expression In *Yarrowia lipolytica* (EaD8S)

The present Example describes the functional expression of *Yarrowia lipolytica* vector pZUFmEaD8S, comprising a chimeric FBAINm::EaD8S::Pex20 gene, wherein EaD8S is the synthetic Δ8 desaturase derived from *Euglena anabaena* and codon-optimized for expression in *Yarrowia* (Example 6). The plasmid pZUFmEaD8S (FIG. 8B) contained the following components:

Table 10

Components Of Plasmid pZUFmEaD8S (SEQ ID NO:51)

RE Sites And Nucleotides Within SEQ ID NO:51	Description Of Fragment And Chimeric Gene Components
Swa I/BsiW I (7333-1584)	FBAINm::EaD8S::Pex20, comprising: • FBAINm: *Yarrowia lipolytica* FBAIN promoter (U.S. Patent 7,202,356) • EaD8S: codon-optimized Δ8 desaturase (SEQ ID NO:39), derived from *Euglena anabaena* • Pex20: Pex20 terminator sequence of *Yarrowia* Pex20 gene (GenBank Accession No. AF054613)
2620-1740	ColE1 plasmid origin of replication
3550-2690	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
4449-5753	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
7297-5796	*Yarrowia Ura 3* gene (GenBank Accession No. AJ306421)

Functional Analysis Of *Yarrowia lipolytica* Transformants Comprising pZUFmEaD8S

Plasmid pZUFmEaD8S (SEQ ID NO:51; FIG. 8B) was transformed into strain Y4001U as described in the General Methods. The transformants were selected on MMLeu plates. After 2 days growth at 30 °C, transformants were picked and re-streaked onto fresh MMLeu plates. Once grown, these strains were individually inoculated into 3 mL liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that there were about 6.5% DGLA and 9.4% EDA of total lipids produced in all 7 transformants, wherein the conversion efficiency of EDA to DGLA in these 7 strains was determined to be about 41%.

CLAIMS

What is claimed is:

1. A microbial host cell comprising an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24;

(b) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence selected from the group consisting of: SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:39;

(c) a nucleotide sequence encoding a polypeptide having Δ8 desaturase activity, wherein the nucleotide sequence hybridizes under stringent conditions to a nucleotide sequence selected from the group consisting of: SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:39; or

(d) a complement of the nucleotide sequence of (a), (b) or (c), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

2. The microbial host cell of Claim 1 wherein the isolated polynucleotide encodes an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24.

3. The microbial host cell of Claim 1 wherein the microbial host cell is selected from the group consisting of yeast, algae, bacteria, euglenoids, stramenopiles and fungi.

4. The microbial host cell of Claim 3 wherein the cell is a fungus of the genus *Mortierella* sp.

5. The microbial host cell of Claim 3 wherein the cell is a stramenopiles selected from the group consisting of: *Thraustochytrium* sp., and *Schizochytrium* sp.

6. The microbial host cell of Claim 3 wherein the yeast is an oleaginous yeast.

7. The microbial host cell of Claim 6 wherein the oleaginous yeast is selected from the group consisting of: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces.*

8. A method for the production of dihomo-γ-linoleic acid comprising:

a) providing a microbial host cell comprising:

(i) a recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence selected from the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

(ii) a source of eicosadienoic acid;

c) growing the microbial host cell of step (a) under conditions wherein the nucleic acid fragment encoding the Δ8 desaturase polypeptide is expressed and the eicosadienoic acid is converted to dihomo-γ-linoleic acid; and,

d) optionally recovering the dihomo-γ-linoleic acid of step (b).

9. A method for the production of eicosatetraenoic acid comprising:

a) providing a microbial host cell comprising:

(i) a recombinant nucleotide molecule encoding a Δ5 desaturase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence selected from

the group consisting of: SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 and SEQ ID NO:24; and,

(ii) a source of eicosatrienoic acid;

b) growing the microbial host cell of step (a) under conditions wherein the nucleic acid fragment encoding the Δ8 desaturase polypeptide is expressed and the eicosatrienoic acid is converted to eicosatetraenoic acid; and,

c) optionally recovering the eicosatetraenoic acid of step (b).

10. The method of either of Claims 8 or 9 wherein the microbial host cell is a *Yarrowia sp.,* comprising a recombinant nucleotide molecule encoding a Δ8 desaturase polypeptide as set forth in SEQ ID NO:39 wherein the recombinant nucleotide molecule comprises at least 208 codons which are optimized for expression in *Yarrowia*.

11. A method according to either of Claims 8 or 9 wherein:

a.) the recombinant nucleic acid molecule has a nucleic acid sequence selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:39; and,

b.) the host cell is *Yarrowia lipolytica*.

12. An isolated nucleic acid molecule which encodes a Δ8 desaturase as set forth in SEQ ID NO:39 wherein at least 208 codons are codon-optimized for expression in *Yarrowia* sp.

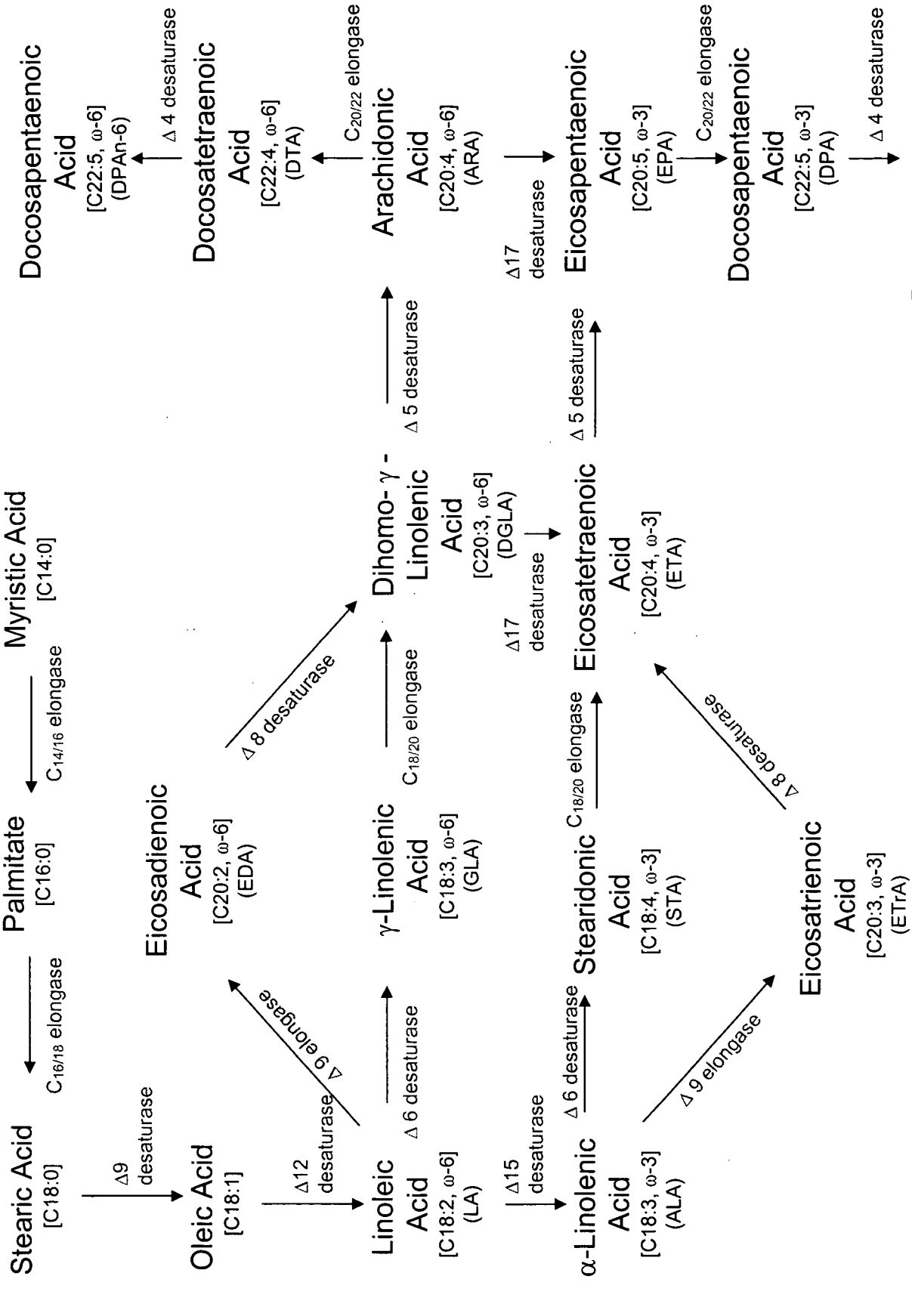
Stearic Acid [C18:0]
Δ9 desaturase
Oleic Acid [C18:1]
Δ12 desaturase
Linoleic Acid [C18:2, ω-6] (LA)
Δ15 desaturase
α-Linolenic Acid [C18:3, ω-3] (ALA)
C16/18 elongase
Palmitate [C16:0]
C14/16 elongase
Myristic Acid [C14:0]
Δ 9 elongase
Eicosadienoic Acid [C20:2, ω-6] (EDA)
Δ 8 desaturase
Dihomo- γ - Linolenic Acid [C20:3, ω-6] (DGLA)
Δ 5 desaturase
Arachidonic Acid [C20:4, ω-6] (ARA)
Δ 4 desaturase
Docosapentaenoic Acid [C22:5, ω-6] (DPAn-6)
C20/22 elongase
Docosatetraenoic Acid [C22:4, ω-6] (DTA)
Δ 6 desaturase
γ-Linolenic Acid [C18:3, ω-6] (GLA)
C18/20 elongase
Stearidonic Acid [C18:4, ω-3] (STA)
Δ 6 desaturase
Eicosatrienoic Acid [C20:3, ω-3] (ETrA)
Δ 9 elongase
Eicosatetraenoic Acid [C20:4, ω-3] (ETA)
Δ 8 desaturase
Eicosapentaenoic Acid [C20:5, ω-3] (EPA)
Δ17 desaturase
Docosapentaenoic Acid [C22:5, ω-3] (DPA)
C20/22 elongase
Docosahexaenoic Acid [C22:6, ω-3] (DHA)
Δ 4 desaturase

FIG. 1

FIG. 2

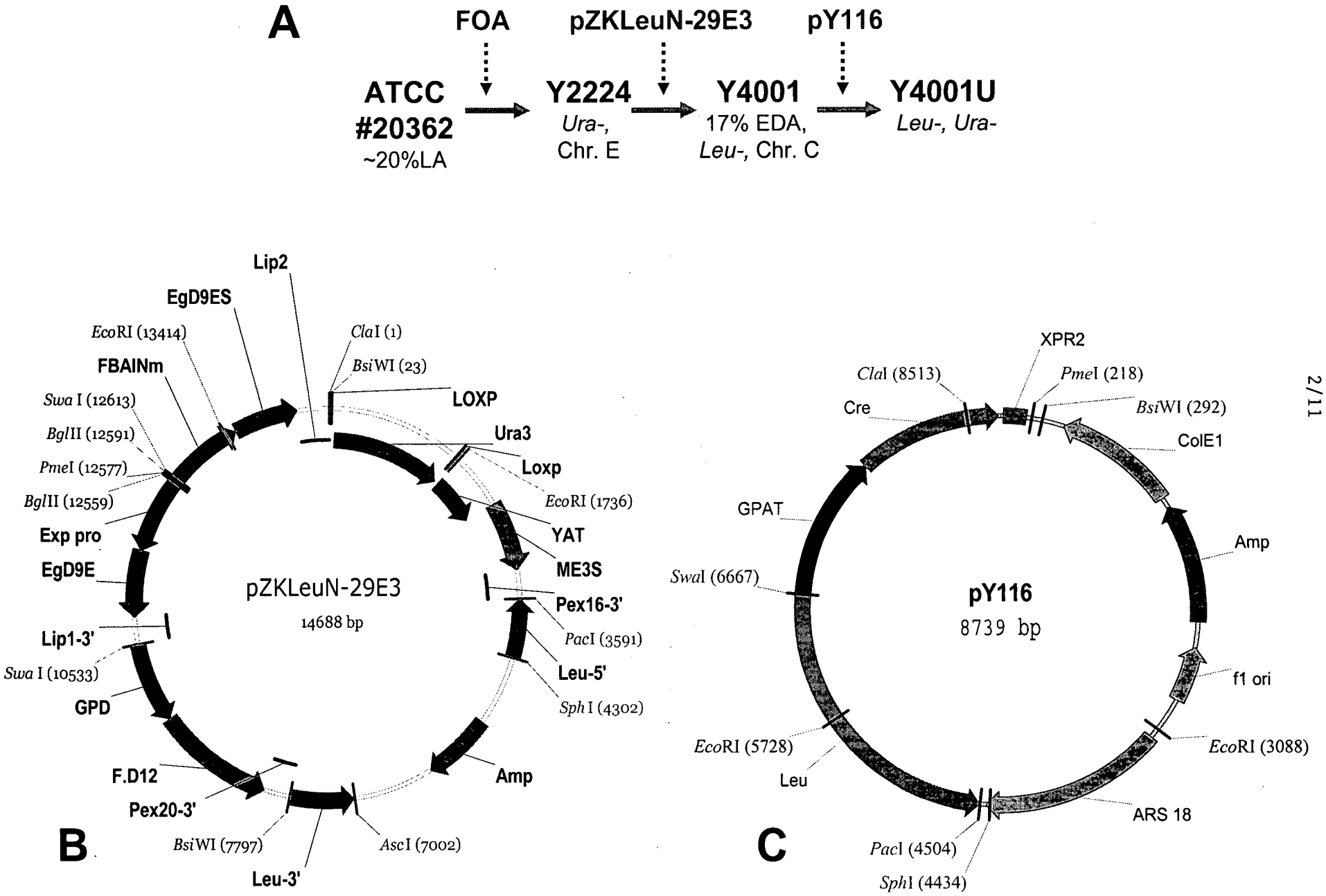
A
FOA
pZKLeuN-29E3
pY116
ATCC #20362
~20%LA
Y2224
Ura-, Chr. E
Y4001
17% EDA, Leu-, Chr. C
Y4001U
Leu-, Ura-
B
pZKLeuN-29E3
14688 bp
Lip2
EgD9ES
EcoRI (13414)
ClaI (1)
BsiWI (23)
FBAINm
LOXP
Swa I (12613)
Ura3
BglII (12591)
Loxp
PmeI (12577)
EcoRI (1736)
BglII (12559)
YAT
Exp pro
ME3S
EgD9E
Pex16-3'
Lip1-3'
PacI (3591)
Swa I (10533)
Leu-5'
GPD
SphI (4302)
F.D12
Amp
Pex20-3'
BsiWI (7797)
AscI (7002)
Leu-3'
C
pY116
8739 bp
XPR2
ClaI (8513)
PmeI (218)
Cre
BsiWI (292)
ColE1
GPAT
Amp
SwaI (6667)
f1 ori
EcoRI (5728)
EcoRI (3088)
Leu
ARS 18
PacI (4504)
SphI (4434)

FIG. 3

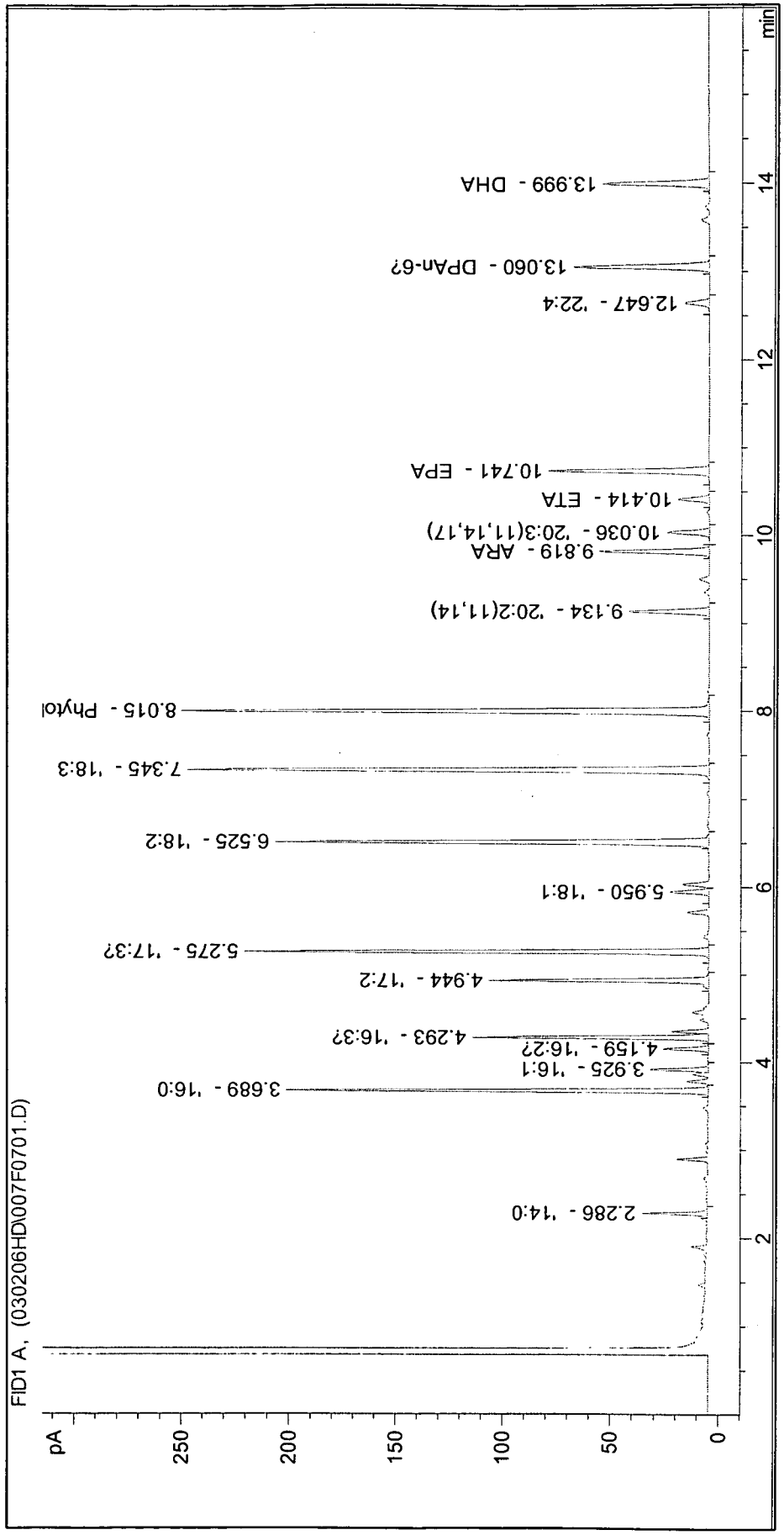
FID1 A, (030206HD\007F0701.D)
pA
250
200
150
100
50
0
min
2
4
6
8
10
12
14
2.286 - 14:0
3.689 - 16:0
3.925 - 16:1
4.159 - 16:2?
4.293 - 16:3?
4.944 - 17:2
5.275 - 17:3?
5.950 - 18:1
6.525 - 18:2
7.345 - 18:3
8.015 - Phytol
9.134 - 20:2(11,14)
9.819 - ARA
10.036 - 20:3(11,14,17)
10.414 - ETA
10.741 - EPA
12.647 - 22:4
13.060 - DPAn-6?
13.999 - DHA

FIG. 4A

```
    M..KR.ALPLT.DG.TYDVSAW.N.HPGGA.IIENY.GRDATD.FMVMHS Consensus #1
             10        20        30        40        50
1   M-VKRPALPLTVDGVTYDVSAWLNHHPGGADIIENYRGRDATDVFMVMHS EaD8Des1 (SEQ ID NO21).pro
1   M-VKRPALPLTVDGVTYDVSAWLNHHPGGADIIENYRGRDATDVFMVMHS EaD8Des2 (SEQ ID NO22).pro
1   M-VKRPALPLTVDGVTYDVSAWLNHHPGGADIIENYRGRDATDVFMVMHS EaD8Des3 (SEQ ID NO23).pro
1   M-VKRPALPLTVDGVTYDVSAWLNHHPGGADIIENYRGRDATDVFMVMHS EaD8Des4 (SEQ ID NO24).pro
1   MKSKRQALPLTIDGTTYDVSAWVNFHPGGAEIIENYQGRDATDAFMVMHS corrected EgD8 (SEQ ID NO25).pro

    ..A..KL.RMP...PSS.L...PP.....E.QEDFRKLR.ELIA.GMFDA Consensus #1
             60        70        80        90       100
50  ENAVSKLRRMPIMEPSSPLTPTPPKPNSDEPQEDFRKLRDELIAAGMFDA EaD8Des1 (SEQ ID NO21).pro
50  ENAVSKLRRMPIMEPSSPLTPTPPKPNSDEPQEDFRKLRDELIAAGMFDA EaD8Des2 (SEQ ID NO22).pro
50  ENAVSKLRRMPIMEPSSPLTPTPPKPNSDEPQEDFRKLRDELIAAGMFDA EaD8Des3 (SEQ ID NO23).pro
50  ENAVSKLRRMPIMEPSSPLTPTPPKPNSDEPQEDFRKLRDELIAAGMFDA EaD8Des4 (SEQ ID NO24).pro
51  QEAFDKLKRMPKINPSSEL---PPQAAVNEAQEDFRKLREELIATGMFDA corrected EgD8 (SEQ ID NO25).pro

    SP.WY.YK...TLGLGVL...LM.Q...Y..GA..LG.H.QQMGWLSHDI Consensus #1
            110       120       130       140       150
100 SPMWYAYKTLTTLGLGVLAVLLMTQWHWYLVGAIVLGIHFQQMGWLSHDI EaD8Des1 (SEQ ID NO21).pro
100 SPMWYAYKTLSTLGLGVLAVLLMTQWHWYLVGAIVLGIHFQQMGWLSHDI EaD8Des2 (SEQ ID NO22).pro
100 SPMWYAYKTLSTLGLGVLAVLLMTQWHWYLVGAIVLGIHFQQMGWLSHDI EaD8Des3 (SEQ ID NO23).pro
100 SPMWYAYKTLSTLGLGVLAVLLMTQWHWYLVGAIVLGIHFQQMGWLSHDI EaD8Des4 (SEQ ID NO24).pro
98  SPLWYSYKISTTLGLGVLGYFLMVQYQMYFIGAVLLGMHYQQMGWLSHDI corrected EgD8 (SEQ ID NO25).pro
```

FIG. 4B

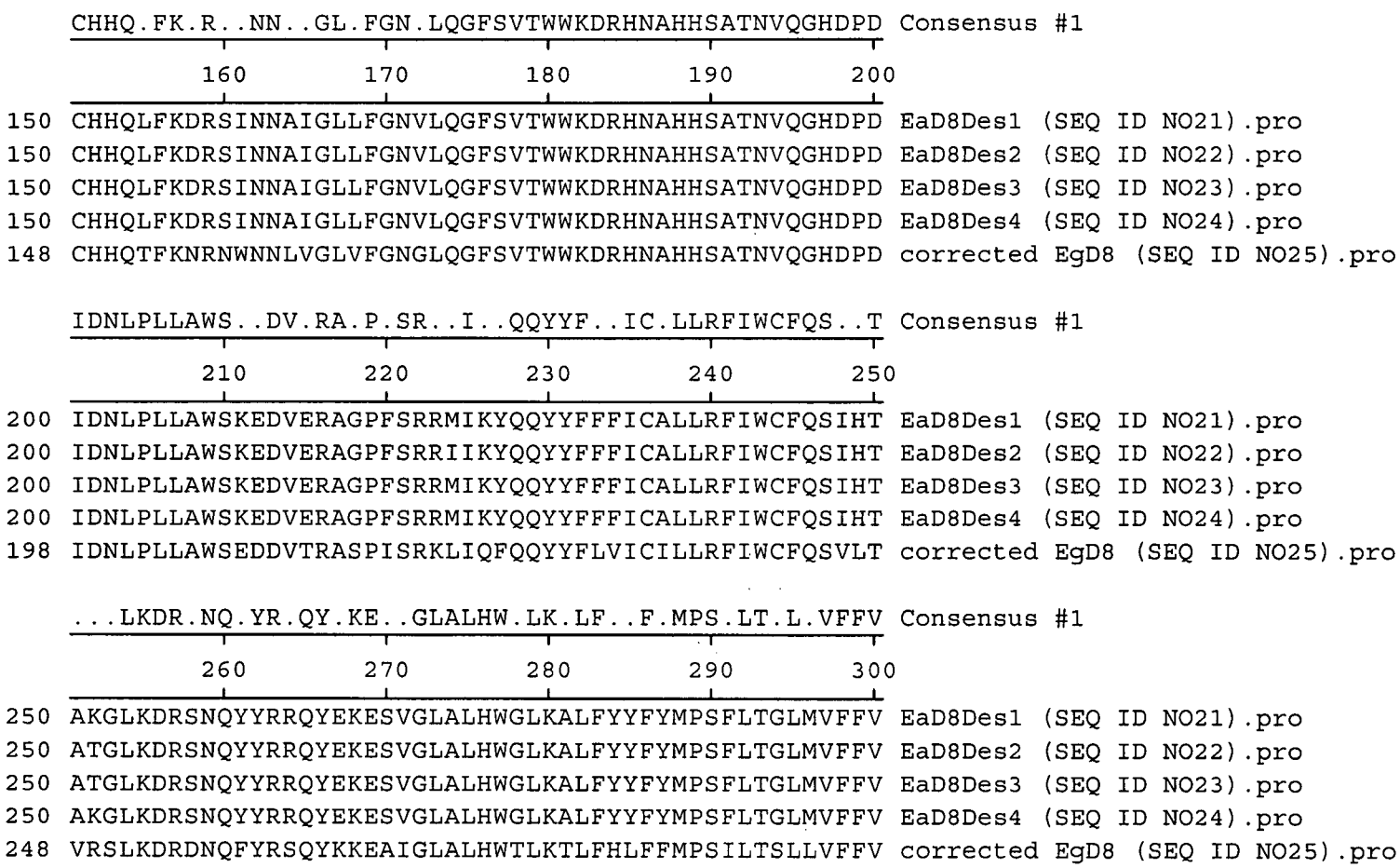
CHHQ.FK.R..NN..GL.FGN.LQGFSVTWWKDRHNAHHSATNVQGHDPD Consensus #1
160 170 180 190 200
150 CHHQLFKDRSINNAIGLLFGNVLQGFSVTWWKDRHNAHHSATNVQGHDPD EaD8Des1 (SEQ ID NO21).pro
150 CHHQLFKDRSINNAIGLLFGNVLQGFSVTWWKDRHNAHHSATNVQGHDPD EaD8Des2 (SEQ ID NO22).pro
150 CHHQLFKDRSINNAIGLLFGNVLQGFSVTWWKDRHNAHHSATNVQGHDPD EaD8Des3 (SEQ ID NO23).pro
150 CHHQLFKDRSINNAIGLLFGNVLQGFSVTWWKDRHNAHHSATNVQGHDPD EaD8Des4 (SEQ ID NO24).pro
148 CHHQTFKNRNWNNLVGLVFGNGLQGFSVTWWKDRHNAHHSATNVQGHDPD corrected EgD8 (SEQ ID NO25).pro
IDNLPLLAWS..DV.RA.P.SR..I..QQYYF..IC.LLRFIWCFQS..T Consensus #1
210 220 230 240 250
200 IDNLPLLAWSKEDVERAGPFSRRMIKYQQYYFFFICALLRFIWCFQSIHT EaD8Des1 (SEQ ID NO21).pro
200 IDNLPLLAWSKEDVERAGPFSRRIIKYQQYYFFFICALLRFIWCFQSIHT EaD8Des2 (SEQ ID NO22).pro
200 IDNLPLLAWSKEDVERAGPFSRRMIKYQQYYFFFICALLRFIWCFQSIHT EaD8Des3 (SEQ ID NO23).pro
200 IDNLPLLAWSKEDVERAGPFSRRMIKYQQYYFFFICALLRFIWCFQSIHT EaD8Des4 (SEQ ID NO24).pro
198 IDNLPLLAWSEDDVTRASPISRKLIQFQQYYFLVICILLRFIWCFQSVLT corrected EgD8 (SEQ ID NO25).pro
...LKDR.NQ.YR.QY.KE..GLALHW.LK.LF..F.MPS.LT.L.VFFV Consensus #1
260 270 280 290 300
250 AKGLKDRSNQYYRRQYEKESVGLALHWGLKALFYYFYMPSFLTGLMVFFV EaD8Des1 (SEQ ID NO21).pro
250 ATGLKDRSNQYYRRQYEKESVGLALHWGLKALFYYFYMPSFLTGLMVFFV EaD8Des2 (SEQ ID NO22).pro
250 ATGLKDRSNQYYRRQYEKESVGLALHWGLKALFYYFYMPSFLTGLMVFFV EaD8Des3 (SEQ ID NO23).pro
250 AKGLKDRSNQYYRRQYEKESVGLALHWGLKALFYYFYMPSFLTGLMVFFV EaD8Des4 (SEQ ID NO24).pro
248 VRSLKDRDNQFYRSQYKKEAIGLALHWTLKTLFHLFFMPSILTSLLVFFV corrected EgD8 (SEQ ID NO25).pro

FIG. 4C

```
    SEL.GGFGIAIVVFMNHYPLEKI.DSVWDGHGF..GQIHETMN..RG..T Consensus #1
             310        320        330        340        350
300 SELLGGFGIAIVVFMNHYPLEKIQDSVWDGHGFCAGQIHETMNVQRGLVT EaD8Des1 (SEQ ID NO21).pro
300 SELLGGFGIAIVVFMNHYPLEKIQDSVWDGHGFCAGQIHETMNVQRGLVT EaD8Des2 (SEQ ID NO22).pro
300 SELLGGFGIAIVVFMNHYPLEKIQDSVWDGHGFCAGQIHETMNVQRGLVT EaD8Des3 (SEQ ID NO23).pro
300 SELLGGFGIAIVVFMNHYPLEKIQDSVWDGHGFCAGQIHETMNVQRGLVT EaD8Des4 (SEQ ID NO24).pro
298 SELVGGFGIAIVVFMNHYPLEKIGDSVWDGHGFSVGQIHETMNIRRGIIT corrected EgD8 (SEQ ID NO25).pro

    DWFFGGLNYQIEHHLWPTLPRHNLTA.S..VEQLC.KHNLPYR.P...EG Consensus #1
             360        370        380        390        400
350 DWFFGGLNYQIEHHLWPTLPRHNLTAASIKVEQLCKKHNLPYRSPPMLEG EaD8Des1 (SEQ ID NO21).pro
350 DWFFGGLNYQIEHHLWPTLPRHNLTAASIKVEQLCKKHNLPYRSPPMLEG EaD8Des2 (SEQ ID NO22).pro
350 DWFFGGLNYQIEHHLWPTLPRHNLTAASIKVEQLCKKHNLPYRSPPMLEG EaD8Des3 (SEQ ID NO23).pro
350 DWFFGGLNYQIEHHLWPTLPRHNLTAASIKVEQLCKKHNLPYRSPPMLEG EaD8Des4 (SEQ ID NO24).pro
348 DWFFGGLNYQIEHHLWPTLPRHNLTAVSYQVEQLCQKHNLPYRNPLPHEG corrected EgD8 (SEQ ID NO25).pro

    ..IL..YL..FARM..K..A.KA.                           Consensus #1
             410        420
400 VGILISYLGTFARMVAK--ADKA                             EaD8Des1 (SEQ ID NO21).pro
400 VGILISYLGTFARMVAK--ADKA                             EaD8Des2 (SEQ ID NO22).pro
400 VGILISYLGTFARMVAK--ADKA                             EaD8Des3 (SEQ ID NO23).pro
400 VGILISYLGTFARMVAK--ADKA                             EaD8Des4 (SEQ ID NO24).pro
398 LVILLRYLAVFARMAEKQPAGKAL                            corrected EgD8 (SEQ ID NO25).pro
```

FIG. 5

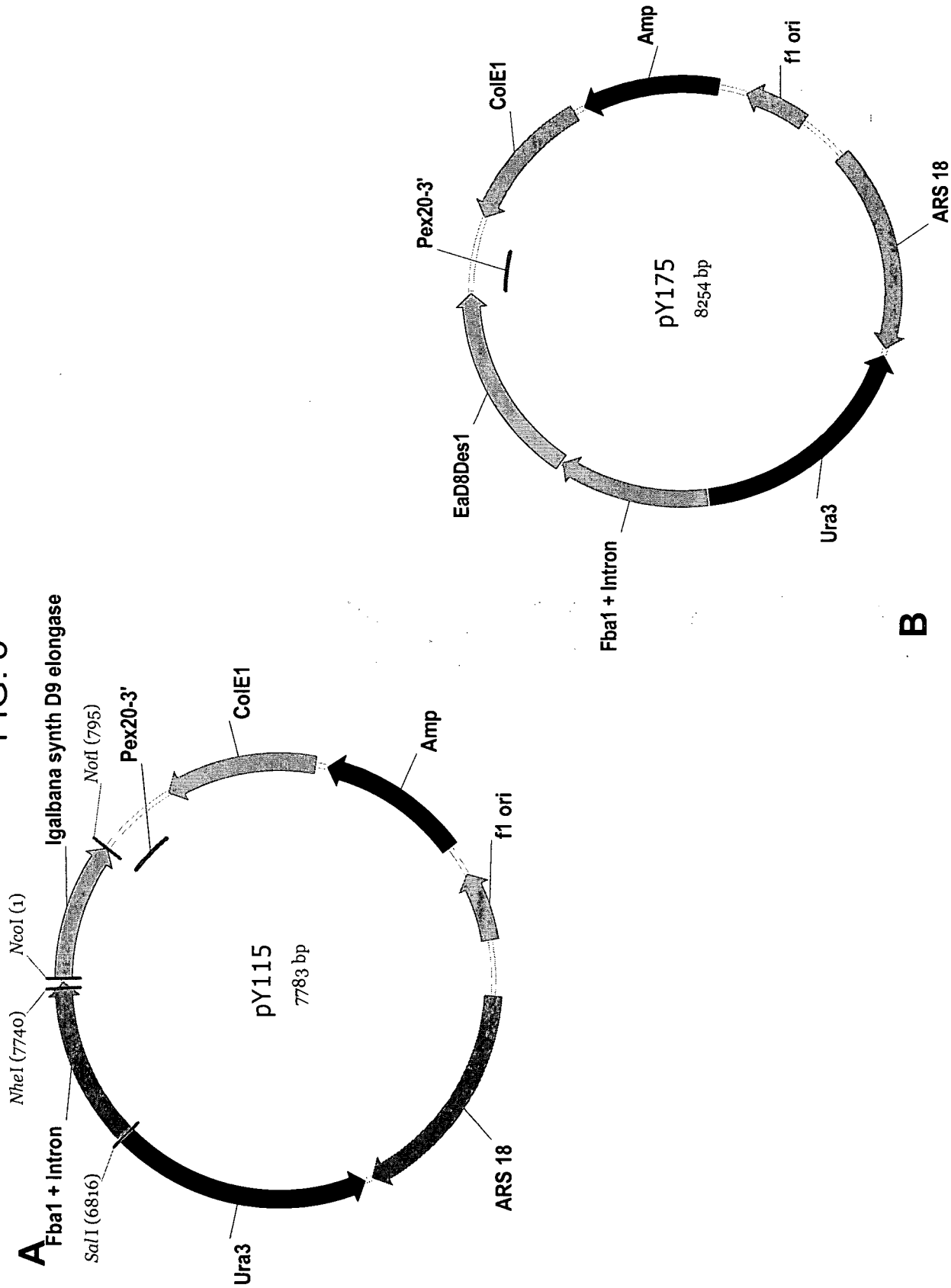
A
pY115
7783 bp
Fba1 + Intron
Igalbana synth D9 elongase
Pex20-3'
ColE1
Amp
f1 ori
ARS 18
Ura3
NheI (7740)
NcoI (1)
NotI (795)
SalI (6816)
B
pY175
8254 bp
Fba1 + Intron
EaD8Des1
Pex20-3'
ColE1
Amp
f1 ori
ARS 18
Ura3

FIG. 6

Event	Fatty Acid	Fatty acid composition (wt.%)													C20 % delta-8 desat	Ave. C20 % delta-8 desat	Ratio (EDA/ERA) % desat
		16:0	16:1	18:0	18:1	LA	ALA	20:1	EDA	DGLA	ERA	ETA	24:0	24:1			
pY175-1	EDA	13.1	10.0	1.2	20.4	50.8	0.1	0.2	1.5	1.6	0.0	0.0	0.2	1.0	51.4	52.2	1.2
Y175-2	EDA	13.5	9.8	1.2	21.0	50.7	0.1	0.1	1.3	1.5	0.0	0.0	0.3	0.5	52.6		
Y175-2	EDA	13.5	9.7	1.2	21.1	50.8	0.1	0.2	1.3	1.4	0.0	0.0	0.2	0.4	52.6		
Y176-1	EDA	13.6	9.5	1.1	18.3	52.6	0.1	0.1	1.8	1.8	0.0	0.0	0.2	0.9	50.7	52.2	1.2
Y176-2	EDA	13.3	10.6	1.1	20.7	50.8	0.1	0.1	1.1	1.3	0.0	0.0	0.2	0.6	53.0		
Y176-3	EDA	13.2	10.6	1.1	21.1	50.5	0.1	0.1	1.1	1.3	0.0	0.0	0.2	0.7	53.0		
Y177-1	EDA	13.3	10.3	1.1	19.2	52.3	0.1	0.1	1.4	1.6	0.0	0.0	0.1	0.3	52.6	52.3	1.1
Y177-2	EDA	13.3	10.1	1.2	21.5	50.4	0.1	0.1	1.2	1.3	0.0	0.0	0.2	0.6	52.7		
Y177-3	EDA	13.3	10.2	1.1	22.6	49.7	0.1	0.1	1.2	1.2	0.0	0.0	0.2	0.2	51.5		
Y178-1	EDA	13.5	9.6	1.2	21.8	50.5	0.1	0.2	1.4	1.3	0.0	0.0	0.1	0.3	47.5	49.2	1.1
Y178-2	EDA	13.7	9.2	1.2	19.5	51.9	0.1	0.2	1.9	1.8	0.0	0.0	0.1	0.3	48.4		
Y178-3	EDA	13.6	9.8	1.2	22.2	49.8	0.1	0.2	1.3	1.4	0.0	0.0	0.1	0.3	51.5		
Y175-1	ERA	12.2	8.8	1.3	21.9	40.8	7.2	0.1	0.1	0.1	3.8	3.1	0.1	0.5	44.3	44.3	
Y175-2	ERA	12.1	9.2	1.3	21.4	40.9	7.2	0.1	0.1	0.1	3.9	3.1	0.1	0.4	44.1		
Y175-3	ERA	12.1	9.1	1.2	21.2	41.1	7.3	0.1	0.1	0.1	3.9	3.2	0.1	0.3	44.5		
Y176-1	ERA	12.1	8.8	1.2	20.1	41.1	8.1	0.1	0.1	0.1	4.3	3.3	0.1	0.5	43.6	44.7	
Y176-2	ERA	12.3	9.6	1.3	23.0	40.5	6.3	0.1	0.1	0.1	3.3	2.7	0.2	0.3	45.2		
Y176-3	ERA	12.0	9.6	1.3	21.0	41.3	7.4	0.1	0.1	0.1	3.7	3.0	0.0	0.4	45.3		
Y177-1	ERA	12.1	9.5	1.2	22.4	40.4	7.1	0.1	0.1	0.1	3.5	3.0	0.1	0.3	45.7	45.5	
Y177-2	ERA	12.0	9.9	1.2	21.3	40.3	7.6	0.1	0.1	0.1	3.6	3.0	0.1	0.6	45.1		
Y177-3	ERA	12.0	9.8	1.2	20.1	40.6	7.7	0.1	0.1	0.1	3.8	3.2	0.1	1.0	45.9		
Y178-1	ERA	11.7	9.8	1.0	19.5	42.6	8.3	0.1	0.1	0.1	3.7	2.7	0.1	0.4	42.3	44.1	
Y178-2	ERA	12.0	9.8	1.2	20.8	40.9	7.8	0.1	0.1	0.1	3.7	3.0	0.1	0.3	44.6		
Y178-3	ERA	12.2	10.2	1.2	24.4	40.4	5.6	0.1	0.1	0.1	2.8	2.3	0.2	0.4	45.5		

FIG. 7A

```
1    ATGGTGAAAAGGCCAGCACTTCCGCTGACCGTTGATGGTGTCACCTATGA  (SEQ ID NO:19)
1    ATGGTCAAGCGACCCGCTCTGCCTCTCACCGTGGACGGTGTCACCTACGA  (SEQ ID NO:39)

51   TGTGTCTGCCTGGTTGAACCATCATCCAGGGGGGTGCTGACATCATTGAGA  (SEQ ID NO:19)
51   CGTTTCTGCCTGGCTCAACCACCATCCCGGAGGTGCCGACATTATCGAGA  (SEQ ID NO:39)

101  ACTACCGCGGTCGTGATGCCACTGATGTCTTTATGGTTATGCACTCTGAA  (SEQ ID NO:19)
101  ACTACCGAGGTCGGGATGCTACCGACGTCTTCATGGTTATGCACTCCGAG  (SEQ ID NO:39)

151  AATGCTGTGAGTAAACTAAGAAGGATGCCTATCATGGAACCATCATCTCC  (SEQ ID NO:19)
151  AACGCCGTGTCCAAACTCAGACGAATGCCCATCATGGAACCTTCCTCTCC  (SEQ ID NO:39)

201  ACTGACGCCTACGCCACCGAAACCCAACTCAGACGAACCGCAGGAGGATT  (SEQ ID NO:19)
201  CCTGACTCCAACACCTCCCAAGCCAAACTCCGACGAACCTCAGGAGGATT  (SEQ ID NO:39)

251  TCCGCAAGCTCCGAGATGAGCTCATCGCAGCAGGAATGTTCGACGCATCA  (SEQ ID NO:19)
251  TCCGAAAGCTGCGAGACGAGCTCATTGCTGCAGGCATGTTCGATGCCTCT  (SEQ ID NO:39)

301  CCGATGTGGTACGCATATAAGACGCTCAGTACGCTGGGCCTCGGGGTCCT  (SEQ ID NO:19)
301  CCCATGTGGTACGCTTACAAGACCCTGTCGACTCTCGGACTGGGTGTCCT  (SEQ ID NO:39)

351  CGCGGTGCTATTGATGACCCAGTGGCACTGGTACCTCGTCGGGGCAATCG  (SEQ ID NO:19)
351  TGCCGTGCTGTTGATGACCCAGTGGCACTGGTACCTGGTTGGTGCTATCG  (SEQ ID NO:39)

401  TGTTGGGCATTCACTTCCAACAAATGGGTTGGTTGTCGCACGATATCTGC  (SEQ ID NO:19)
401  TCCTCGGCATTCACTTTCAACAGATGGGATGGCTCTCGCACGACATTTGC  (SEQ ID NO:39)

451  CACCATCAGCTGTTCAAGGACCGATCGATCAACAACGCCATCGGCTTGCT  (SEQ ID NO:19)
451  CATCACCAGCTGTTCAAGGACCGATCCATCAACAATGCCATTGGCCTGCT  (SEQ ID NO:39)

501  TTTCGGGAACGTCTTGCAAGGGTTCTCTGTGACCTGGTGGAAGGACAGGC  (SEQ ID NO:19)
501  CTTCGGAAACGTGCTTCAGGGCTTTTCTGTCACTTGGTGGAAGGACCGAC  (SEQ ID NO:39)

551  ACAATGCACACCACTCCGCCACCAACGTGCAAGGCCACGACCCCGACATT  (SEQ ID NO:19)
551  ACAACGCTCATCACTCCGCCACCAACGTGCAGGGTCACGATCCCGACATC  (SEQ ID NO:39)

601  GACAACCTGCCGCTGCTGGCATGGTCCAAGGAGGACGTGGAGAGGGCCGG  (SEQ ID NO:19)
601  GACAACCTGCCTCTCCTGGCGTGGTCCAAGGAGGACGTCGAGCGAGCTGG  (SEQ ID NO:39)
```

FIG. 7B

```
651  CCCGTTCTCACGGCGGATGATCAAGTACCAGCAATACTACTTCTTCTTCA  (SEQ ID NO:19)
651  CCCGTTTTCTCGACGGATGATCAAGTACCAACAGTATTACTTCTTTTTCA  (SEQ ID NO:39)

701  TCTGTGCCCTCCTGAGGTTCATCTGGTGCTTCCAGAGCATCCACACAGCC  (SEQ ID NO:19)
701  TCTGTGCCCTTCTGCGATTCATCTGGTGCTTTCAGTCCATTCATACTGCC  (SEQ ID NO:39)

751  ACGGGCCTGAAGGATCGCAGCAACCAGTACTACCGCAGGCAGTACGAGAA  (SEQ ID NO:19)
751  ACGGGTCTCAAGGATCGAAGCAATCAGTACTATCGAAGACAGTACGAGAA  (SEQ ID NO:39)

801  AGAGAGCGTGGGCCTGGCCCTCCACTGGGGCCTGAAGGCGTTGTTCTACT  (SEQ ID NO:19)
801  GGAGTCCGTCGGTCTGGCACTCCACTGGGGTCTCAAGGCCTTGTTCTACT  (SEQ ID NO:39)

851  ACTTTTATATGCCAAGCTTCTTGACCGGACTCATGGTGTTTTTCGTGTCC  (SEQ ID NO:19)
851  ATTTCTACATGCCCTCGTTTCTCACCGGACTCATGGTGTTCTTTGTCTCC  (SEQ ID NO:39)

901  GAGTTGCTTGGGGGCTTCGGCATCGCCATCGTGGTGTTCATGAACCACTA  (SEQ ID NO:19)
901  GAGCTGCTTGGTGGCTTCGGAATTGCCATCGTTGTCTTCATGAACCACTA  (SEQ ID NO:39)

951  CCCCCTGGAGAAGATCCAGGACTCGGTGTGGGACGGCCACGGCTTTTGCG  (SEQ ID NO:19)
951  CCCTCTGGAGAAGATTCAGGACTCCGTGTGGGATGGTCATGGCTTCTGTG  (SEQ ID NO:39)

1001 CCGGCCAGATTCACGAAACGATGAACGTCCAGCGGGGACTCGTCACGGAC  (SEQ ID NO:19)
1001 CTGGACAGATTCACGAGACCATGAACGTTCAGCGAGGCCTCGTCACAGAC  (SEQ ID NO:39)

1051 TGGTTCTTCGGTGGGCTGAATTACCAAATCGAGCACCACCTGTGGCCGAC  (SEQ ID NO:19)
1051 TGGTTTTTCGGTGGCCTCAACTACCAGATCGAACATCACCTGTGGCCTAC  (SEQ ID NO:39)

1101 GCTGCCCCGGCACAACCTGACGGCGGCCAGCATCAAAGTGGAGCAGTTGT  (SEQ ID NO:19)
1101 TCTTCCCAGACACAACCTCACCGCTGCCTCCATCAAAGTGGAGCAGCTGT  (SEQ ID NO:39)

1151 GCAAGAAGCACAACTTGCCGTATCGCAGCCCCCAATGCTGGAGGGGGTG  (SEQ ID NO:19)
1151 GCAAGAAGCACAACCTGCCCTACCGATCTCCTCCCATGCTCGAAGGTGTC  (SEQ ID NO:39)

1201 GGCATCCTGATCAGCTACCTGGGCACCTTTGCCCGCATGGTGGCAAAGGC  (SEQ ID NO:19)
1201 GGCATTCTTATCTCCTACCTGGGCACCTTCGCTCGAATGGTTGCCAAGGC  (SEQ ID NO:39)

1251 CGACAAGGCG                                          (SEQ ID NO:19)
1251 AGACAAGGCC                                          (SEQ ID NO:39)
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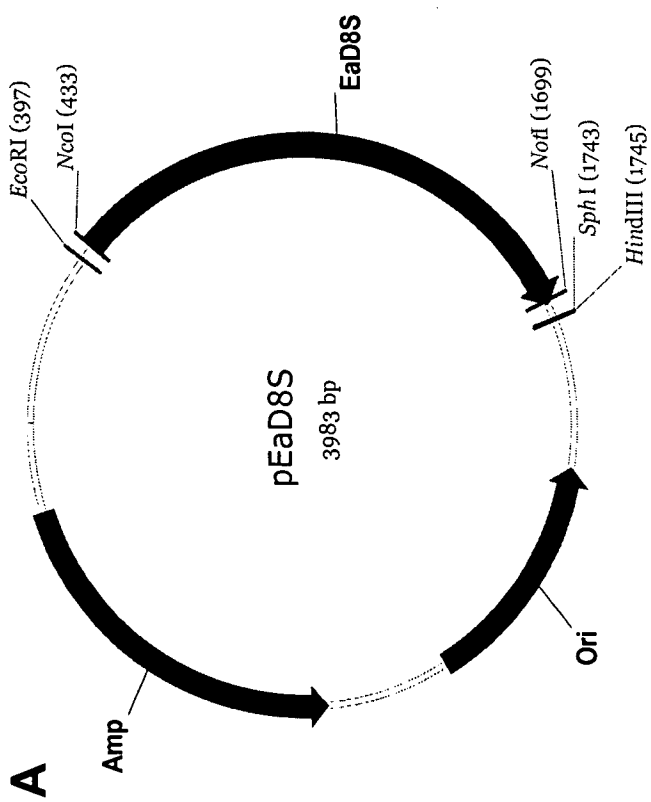
FIG. 8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NotI (1699)
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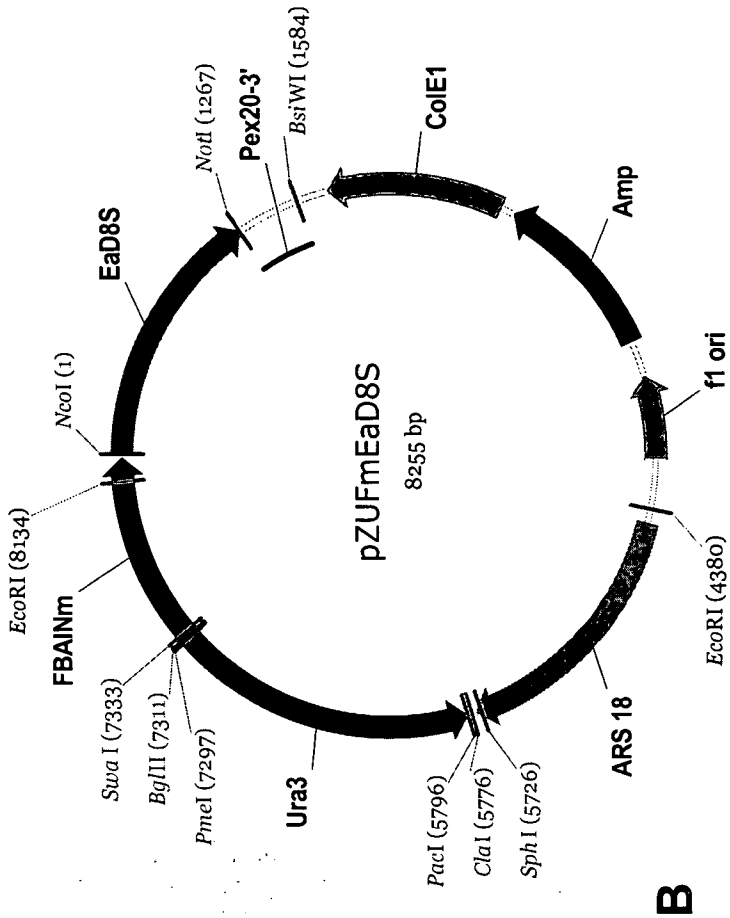
HindIII (1745)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EcoRI (4380)
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SwaI (7333)
BglII (7311)
PmeI (7297)
PacI (5796)
ClaI (5776)
SphI (5726)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/004700

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/63 C12N15/80 C12N9/02

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

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 practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/34439 A (UNIV WASHINGTON [US]; BROWSE JOHN A [US]; WALLIS JAMES G [US]; WATTS J) 15 June 2000 (2000-06-15) claims 1-27; figures 1b,3	1-9
X	US 2006/115881 A1 (DAMUDE HOWARD G [US] ET AL) 1 June 2006 (2006-06-01) see SEQ ID No. 59	1-9

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2008	19/09/2008

Name and mailing address of the ISA/	Authorized officer
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4

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/004700

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALLIS J G ET AL: "The DELTA8-desaturase of Euglena gracilis: An alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, ACADEMIC PRESS, US, vol. 365, no. 2, 15 May 1999 (1999-05-15), pages 307-316, XP002291433 ISSN: 0003-9861 figures 1,3	1-9
A	WARUDE DNYANESHWAR ET AL: "POLYUNSATURATED FATTY ACIDS: BIOTECHNOLOGY" CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, CRC PRESS, BOCA RATON, FL, US, vol. 26, no. 2, 1 January 2006 (2006-01-01), pages 83-93, XP008070892 ISSN: 0738-8551 page 90; table 1	1-12

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