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(54) **OMEGA-3 DESATURASE USED IN THE BIOSYNTHESIS OF POLYUNSATURATED FATTY ACIDS**

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USPC **435/134**; 536/23.2; 435/320.1; 435/189

(57) **ABSTRACT**

The present invention provides novel fatty acid desaturases genes used for synthesis of polyunsaturated fatty acids, especially omega-3 desaturases (FADS15). The present invention also provides nucleic acid sequence coding the above-described desaturases, expression vector of the above-described desaturases and recombinant microorganism expressing above-described desaturases.

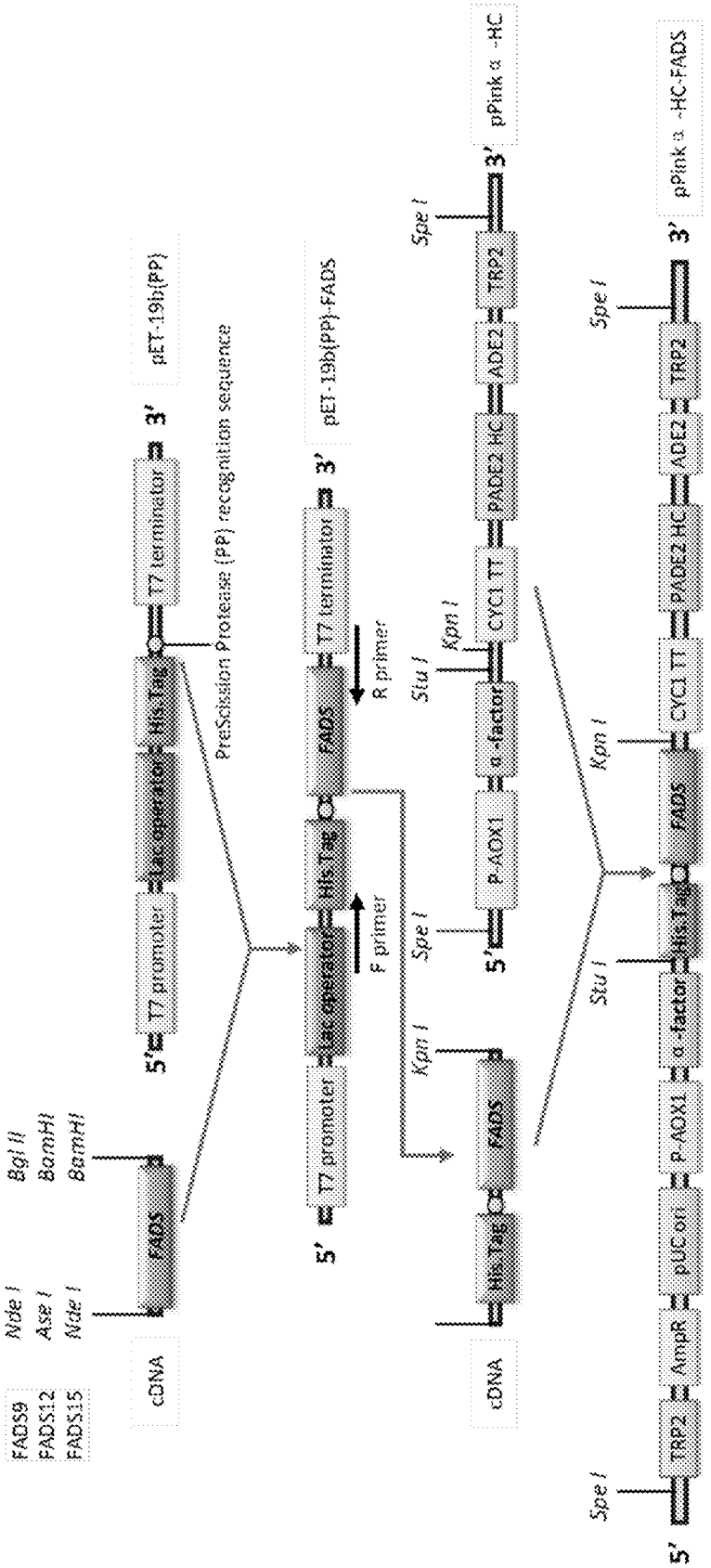
FADS9
FADS12
FADS15
Nde I
Ase I
Nde I
Bgl II
BamHI
BamHI
cDNA
FADS
5'
T7 promoter
Lac operator
His Tag
T7 terminator
3'
pET-19b(PP)
PreScission Protease (PP) recognition sequence
F primer
R primer
pET-19b(PP)-FADS
Kpn I
Spe I
Stu I
α-factor
P-AOX1
CYC1 TT
PADE2 HC
ADE2
TRP2
pPink α -HC
pUC ori
AmpR
pPink α -HC-FADS

Fig. 1

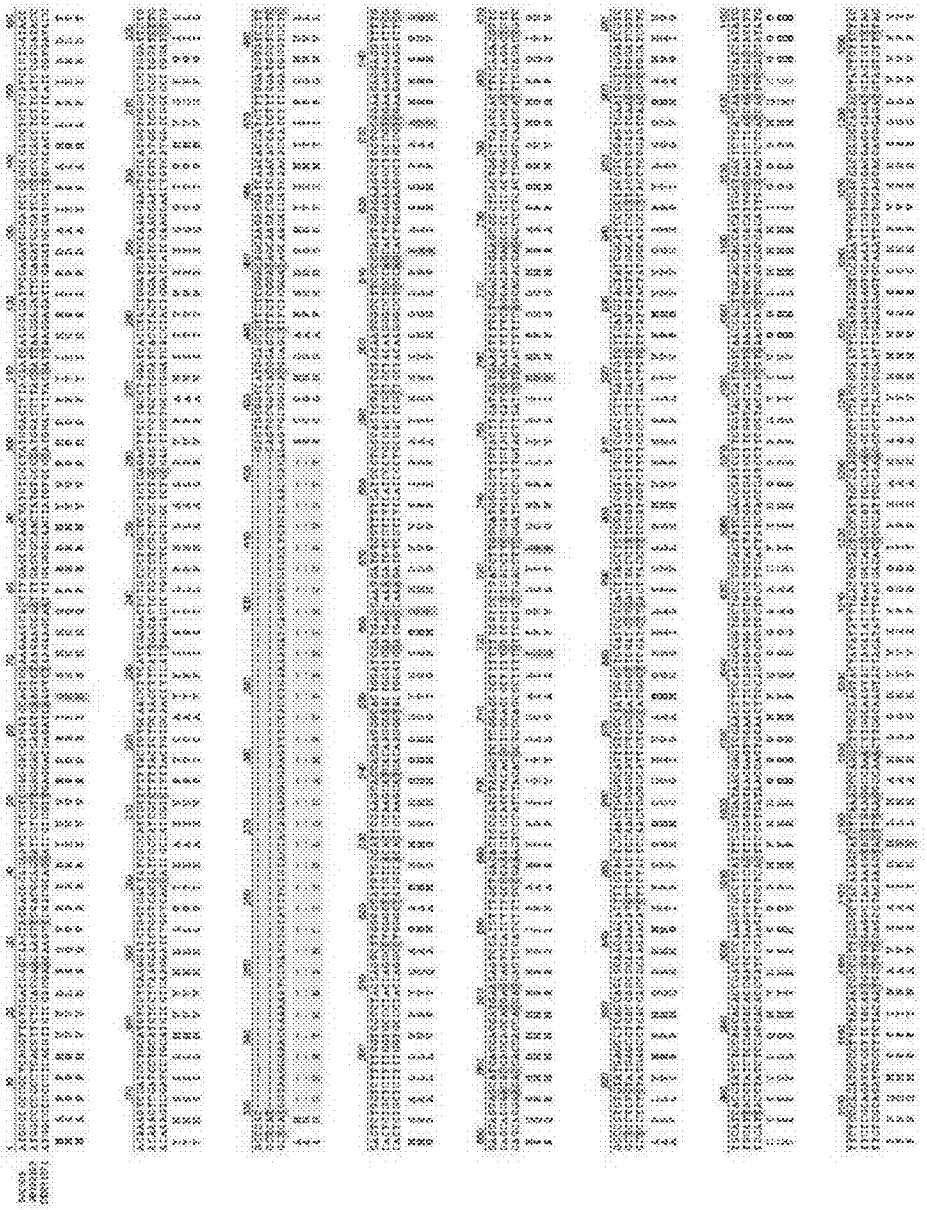

Fig. 2

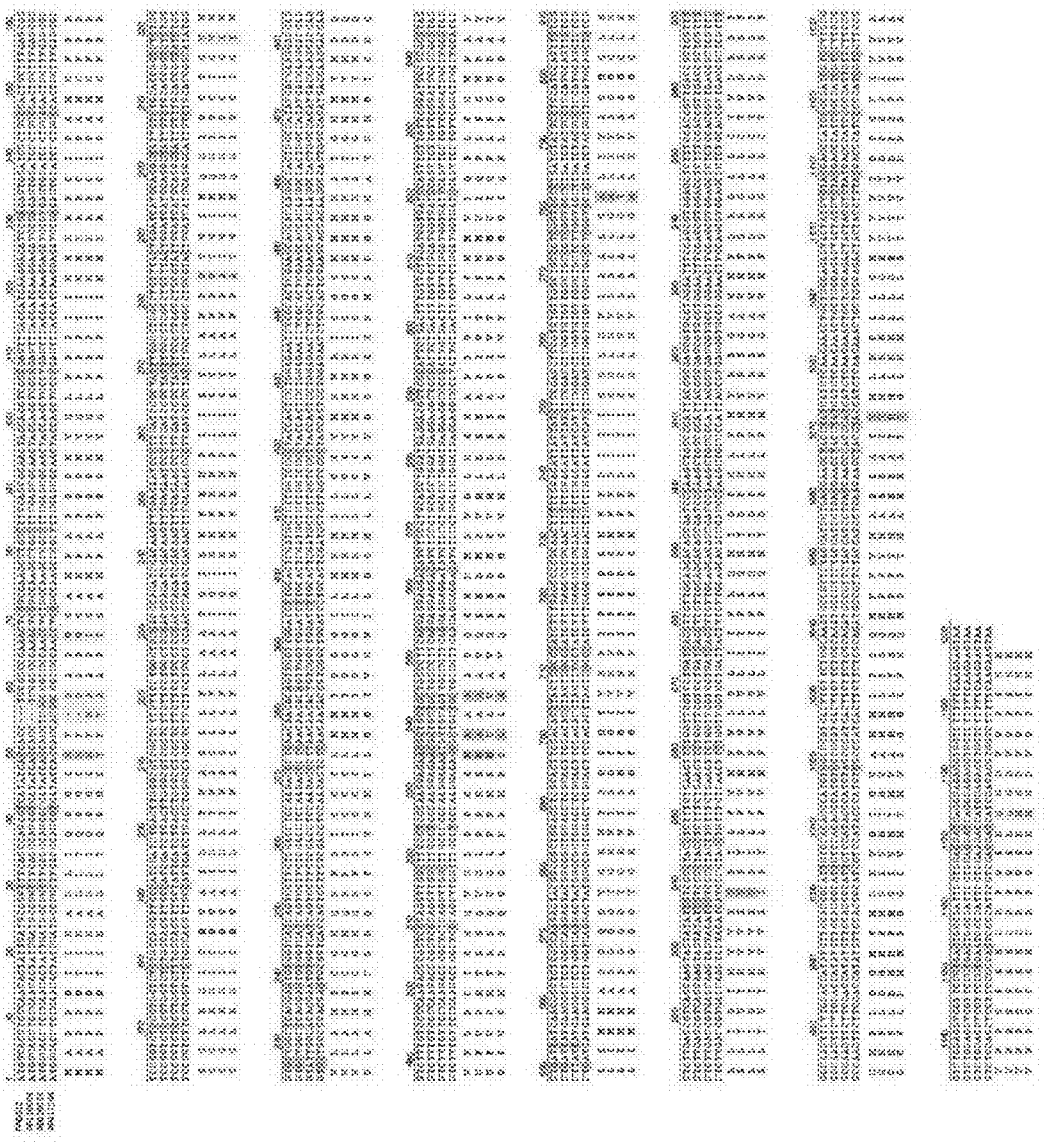

Fig. 3

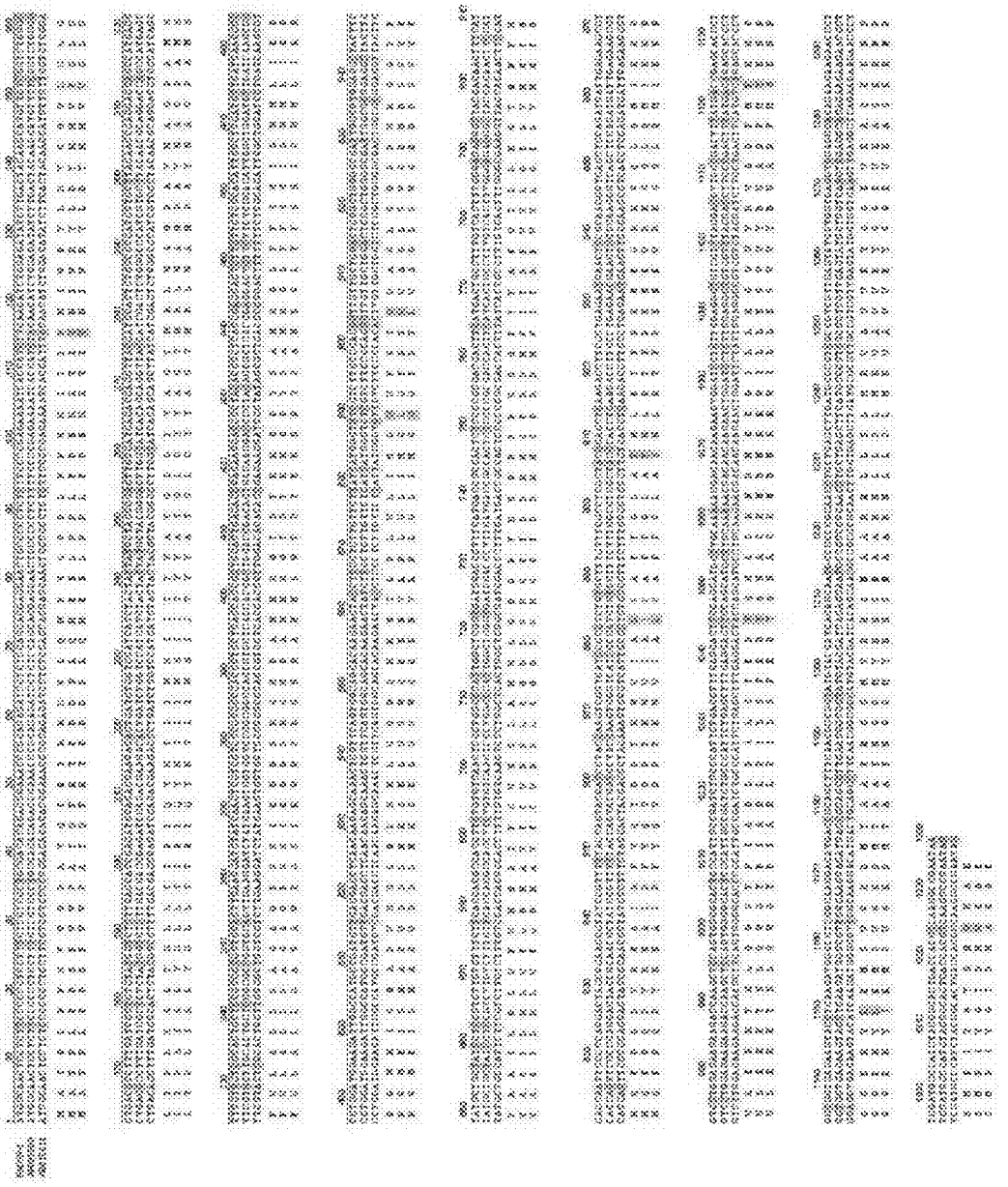

Fig. 4

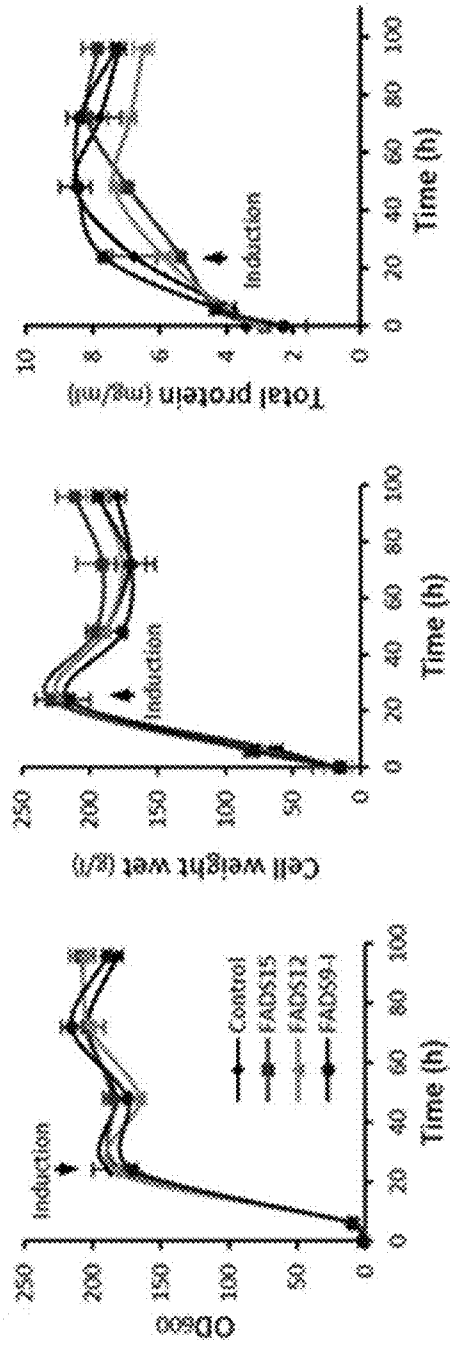
OD600
Induction
Time (h)
Control
FADS15
FADS12
FADS9-1
Cell weight wet (g/l)
Induction
Time (h)
Total protein (mg/ml)
Induction
Time (h)

Fig. 5A

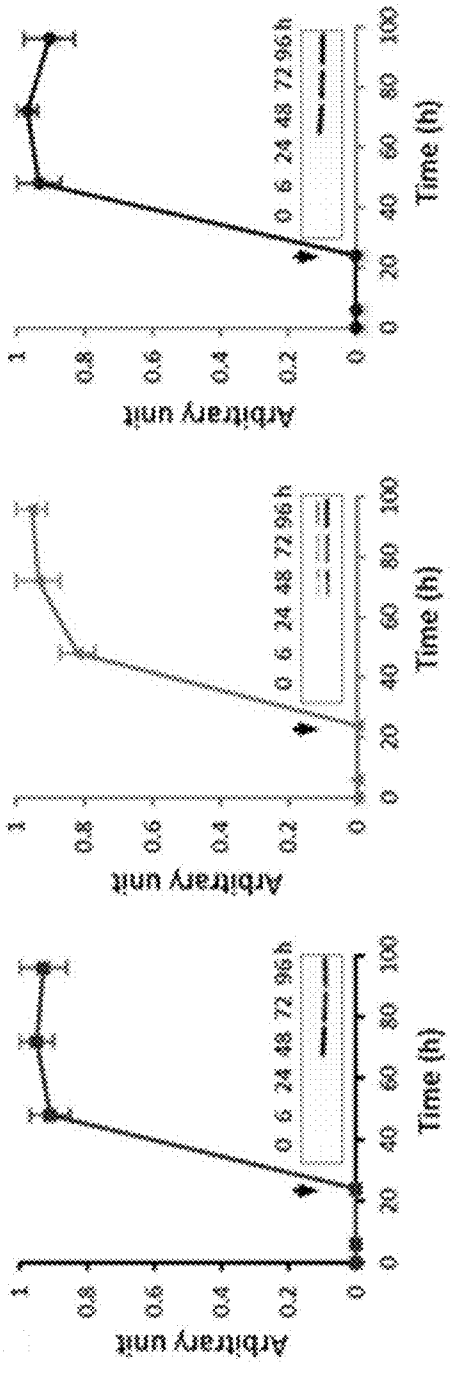
Arbitrary unit
0 6 24 48 72 96 h
Time (h)
Arbitrary unit
0 6 24 48 72 96 h
Time (h)
Arbitrary unit
0 6 24 48 72 96 h
Time (h)

Fig. 5B

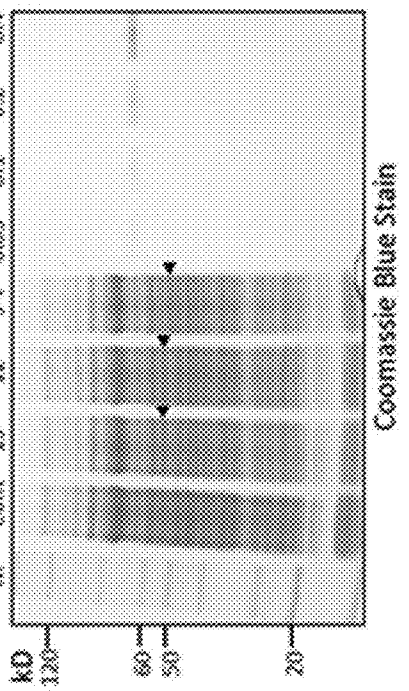
BSA
M
Cont
15
12
9-1
0.05
0.1
0.2
0.4
kD
120
80
50
20
Coomassie Blue Stain

Fig. 5C

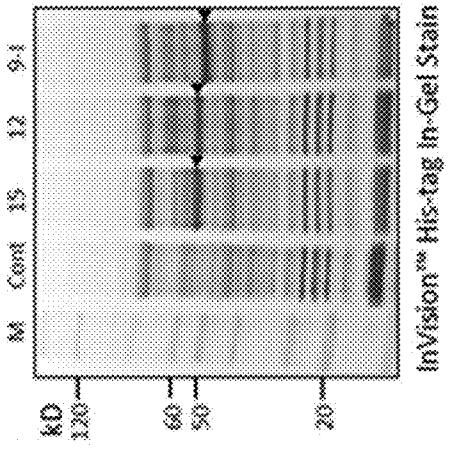
M
Cont
15
12
9-1
kD
120
80
50
20
InVision™ His-tag In-Gel Stain

Fig. 5D

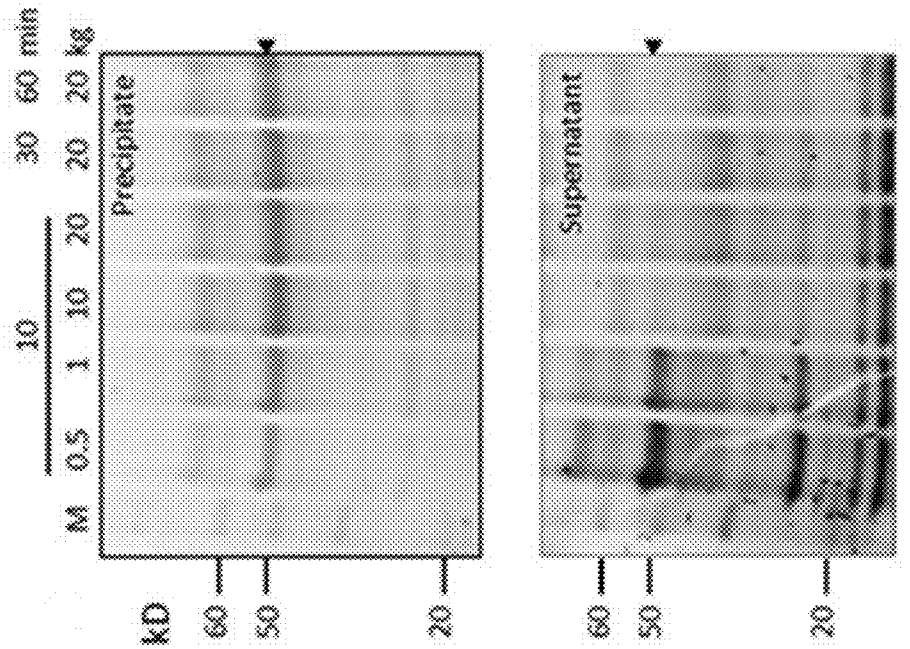
M
0.5
1
10
10
20
30
20
60 min
20 kg
Precipitate
Supernatant
kD
60
50
20
60
50
20

Fig. 6A

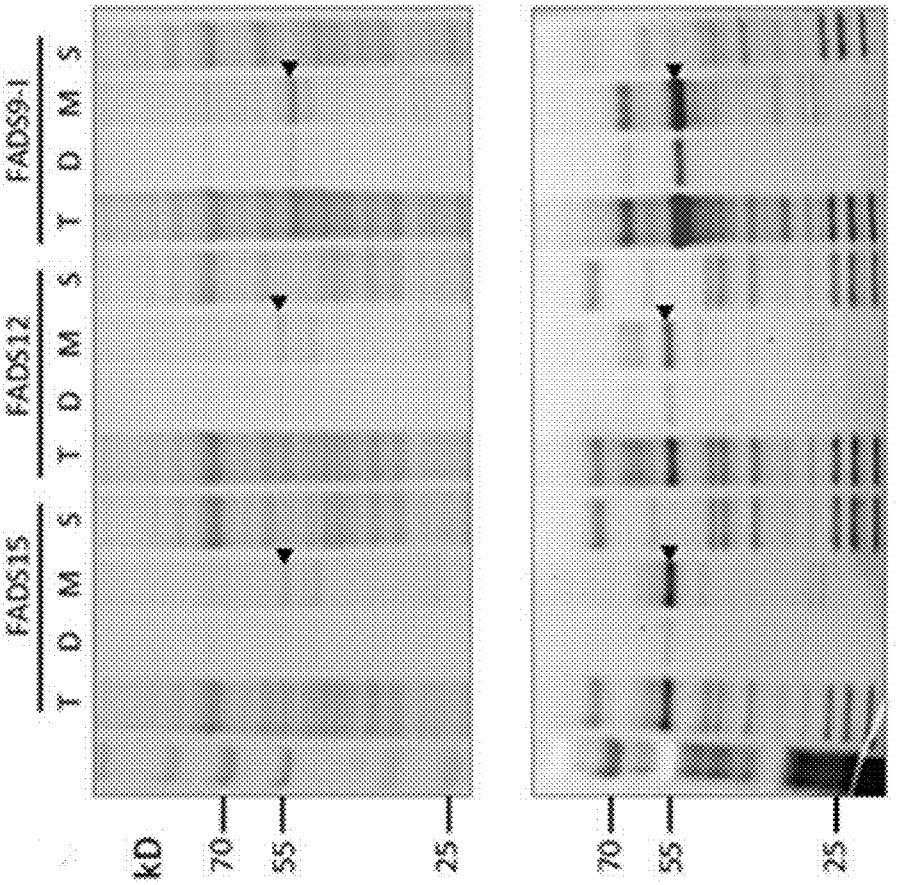

Fig. 6B

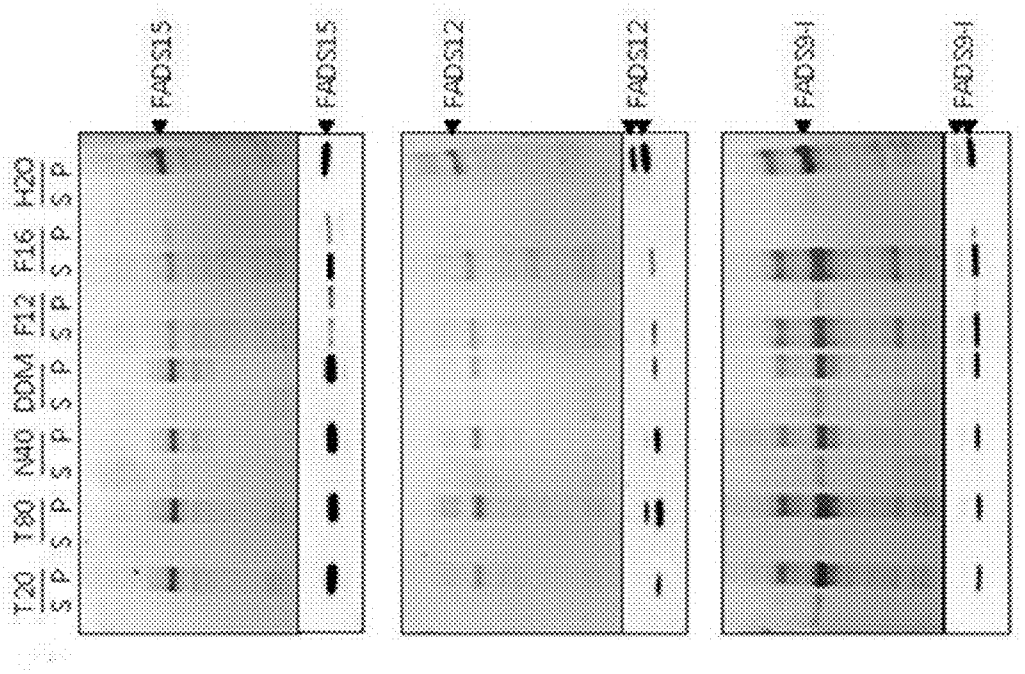
FADS15
FADS15
FADS12
FADS12
FADS9-1
FADS9-1
T20 S P
T80 S P
N40 S P
DDM S P
F12 S P
F16 S P
H20 S P

Fig. 7A

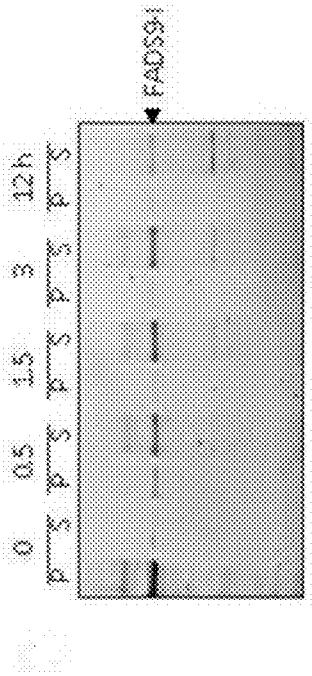
FADS9-1
0 0.5 1.5 3 12h
pS pS pS pS pS

Fig. 7B

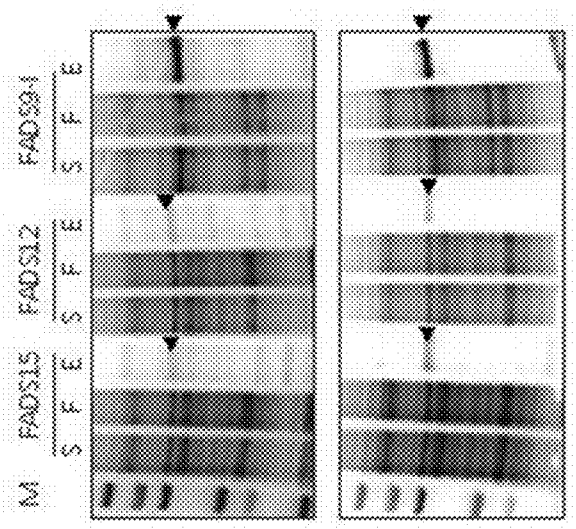
M
FADS1-3
S F E
FADS1-2
S F E
FADS9-1
S F E

Fig. 7C

OMEGA-3 DESATURASE USED IN THE BIOSYNTHESIS OF POLYUNSATURATED FATTY ACIDS

FIELD OF INVENTION

[0001] The present invention relates to the field of microbial manufacturing of polyunsaturated fatty acids (PUFAs), specifically to the fatty acid desaturases in the synthesis process of polyunsaturated fatty acids.

BACKGROUND OF THE INVENTION

[0002] Lipids are first synthesized as saturated fatty acids and double bonds are introduced post-synthetically by oxygen-dependent enzymes known as fatty acid desaturases, in a process that is initiated by abstraction of hydrogen from a methylene group. Fatty acid desaturases are divided into soluble and integral membrane classes, which may have been evolved independently (Shanklin J, Somerville C., "Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs", Proc Natl Acad Sci USA 1991; 88:2510-4). The acyl-ACP desaturases are soluble enzymes found in the plastids of higher plants, whereas the more widespread class of integral membrane acyl-CoA desaturases is found in endomembrane systems in prokaryotes and eukaryotes (Shanklin J, Cahoon E B., "Desaturation and Related Modifications of Fatty Acids1", Annu Rev Plant Physiol Plant Mol Biol 1998; 49:611-41). Fatty acid desaturases in each class are closely related homologs based on their amino acid sequences, and yet perform highly regio- and stereo-selective reactions on long-chain fatty acids composed of essentially equivalent methylene chains that lack distinguishing landmarks close to the site of desaturation. As pointed out by Nobel Laureate Dr. Konrad Bloch, this region- and stereo-specific removal of hydrogen "would seem to approach the limits of the discriminatory power of enzymes" (Bloch K., "Enzymatic synthesis of monounsaturated fatty acids", Accounts of Chemical Research 1969; 2:193-202).

[0003] The membrane class of desaturases consists of enzymes with c5, c6, c9, c12 or ω3-regio-selectivity. Mammalian cells possess c5, c6 and c9, but lack c12 and ω3 desaturases (Berquin I M, Edwards I J, Kridel S J, Chen Y Q, "Polyunsaturated fatty acid metabolism in prostate cancer", Cancer Metastasis Rev 2011; 30:295-309, and Chen Y Q, Edwards I J, Kridel S J, Thornburg T, Berquin I M., "Dietary fat-gene interactions in cancer", Cancer Metastasis Rev 2007; 26:535-51). *Mortierella alpina* belongs to the subphylum of *Mucoromycotina* (Hibbett D S, Binder M, Bischoff J F, et al., "A higher-level phylogenetic classification of the Fungi", Mycol Res 2007; 111:509-47). It can produce lipids up to 50% of its dry weight. We have recently characterized *M. alpina* genome (Wang L, Chen W, Feng Y, et al., "Genome Characterization of the Oleaginous Fungus *Mortierella* alpine", PLoS One 2011; 6:e28319) which encodes one c5, two c6, three c9, one c12 and one ω3 desaturase. Therefore, *M. alpina* has all known regio-selective groups of membrane desaturases.

[0004] We have expressed *M. alpina* c9, c12 and ω3 desaturases (FADS9-I, FADS12 and FADS15) in the methylotrophic yeast *Pichia pastoris*, purified the recombinant proteins and determined their enzymatic activities.

DETAILED DESCRIPTION OF THE INVENTION

[0005] The applicant has identified a novel ω3 desaturase and a Δ9 desaturase, successfully expressed, purified and characterized their enzymatic activities More specifically, the applicant designed primers of nucleotide aiming to three desaturases coding for Δ9-I Des, Δ12 Des and ω3 Des on the basis of whole-genome sequencing of *M. alpina*, the sequences of specific primers are listed in Table 1. *M. alpina* RNA was extracted and reverse transcribed to obtain cDNA. The amplified three sequences by PCR with three pairs of primer of FF1 and FR1, FF2 and FR2, FF3 and FR3 to PCR were inserted into pET19b (PP) and sequenced, and then subcloned into pPinkα-HC, an expression vector in *Pichia pastoris*. Expression vectors were linearized and transformed into PichiaPink strain 2. The recombinant strains thus obtained express the above-mentioned three desaturases. The enzyme activity analysis showed that the purified recombinant desaturases were functional

[0006] The present invention provides genes coding for *M. alpina* ω3 desaturase (FADS 15) and Δ9 desaturase (FADS9-I), whose nucleic acid sequences are shown as SEQ ID NO:1 and SEQ ID NO:3, respectively.

[0007] The present invention also provides expression vectors respectively containing SEQ ID NO:1 and SEQ ID NO:3 which can respectively express *M. alpina* ω3 desaturase (FADS 15) and Δ9 desaturase (FADS9-I). Preferably, the said expression vector is *Pichia pastoris* expression vector.

[0008] The present invention also provides a recombinant microorganism which can respectively express ω3 desaturase (FADS15) and *M. alpina* Δ9 desaturase (FADS9-I). Preferably, the said recombinant microorganism is recombinant *Pichia pastoris* PichiaPink strain 2, which contains *Pichia pastoris* expression vector carrying SEQ ID NO:1 or SEQ ID NO:3.

[0009] The present invention successfully expresses and purifies the novel membrane ω3 desaturase (FADS15) and Δ9 desaturase (FADS9-I), which play a key role in the polyunsaturated fatty acid biosynthetic pathway, and whose amino acid sequences are shown as SEQ ID NO:2 and SEQ ID NO:4. Furthermore, the present invention verifies the enzyme activity of the above two kinds of membrane desaturases. The present invention apply the above-mentioned novel membrane desaturases ω3 desaturase (FADS 15) in the polyunsaturated fatty acid biosynthesis, converting fatty acid C18:$1^{\Delta 9}$ into C18:$2^{\Delta 9,15}$, C18:$2^{\Delta 9,12}$ into C18:$3^{\Delta 9,12,15}$, C20:$4^{\Delta 5,8,11,14}$ into C20:$5^{\Delta 5,8,11,14,17}$. Omega-3 polyunsaturated fatty acids can be utilized as food supplementation to prevent human disorders, wherein said human disorders consisting of cancer, cardiovascular disease, inflammation, developmental disorders, psychiatric disorders, and cognitive aging.

TABLE 1

Primers' sequence table and its restriction enzyme cutting site:

Name	Sequence	Targeted vector
FF1(SEQ ID: 5)	atat*CATATG*ATGGCCCCCCCTCACGTTGTCGACGAGCA(Nde I)	pET19b-FADS15
FF2(SEQ ID: 6)	atat*ATTAAT*ATGGCACCTCCCAACACTATTGATGCCGG(Ase I)	pET19b-FADS12
FF3(SEQ ID: 7)	atat*CATATG*ATGGCAACTCCTCTTCCCCCCTCCTTTGT (Nde I)	pET19b-FADS9-I
FR1(SEQ ID: 8)	atat*GGATCC*TAATGCTTGTAGAACACTACGTC(BamH I)	pET19b-FADS15
FR2(SEQ ID: 9)	atat*GGATCC*TTACTTCTTGAAAAAGACCACGTC(BamH I)	pET19b-FADS12
FR3(SEQ ID: 10)	atat*AGATCT*TTATTCGGCCTTGACGTGGTCAGT(BgI II)	pET19b-FADS9-I
SF1(SEQ ID: 11)	atatatTGCGCACATCATCATCATCATCATCAT(Fsp I)	pPink-FADS
SR1(SEQ ID: 12)	atat*GAATTC*AT*ATTTAAAT*TAATGCTTGTAGAACACTACGTC(Eco RI, Swa I)	pPink-FADS15
SR2(SEQ ID: 13)	atatat*GGTACC*TTACTTCTTGAAAAAGACCACGTC(Kpn I)	pPink-FADS12
SR3(SEQ ID: 14)	atatat*GGTACC*TTATTCGGCCTTGACGTGGTCAGT(Kpn I)	pPink-FADS9-I

DESCRIPTION OF THE ATTACHED DRAWINGS

[0010] FIG. **1**: Diagram of the cloning strategy for desaturase expression vectors. FADS coding sequences were PCR amplified using primers listed in Table 1. PCR fragment were digested with indicated restriction enzymes, column purified and inserted into the pET-19b(PP) vector linearized with corresponding restriction enzymes. The FADS coding sequence plus His tag and Precision protease recognition sequence were PCR amplified and inserted into the pPinkalpha-HC vector. TRP2: TRP2 gene, AmpR: ampicillin resistance gene, pUC ori: oriental promoter of pUC, PAOX1: 5'AOX1 promoter region, α-factor: α-mating factor secretion signal, CYC1 TT: CCY1 transcription termination region, PADE2 HC: high-copy ADE2 promoter region, ADE2: ADE2 open reading frame.

[0011] FIG. **2**: the sequencing results of the clone sequence of ω3 desaturase (FADS 15), in which FADS15 nucleotide sequence from *M. alpina* ATCC#32222 shows 93.1% identity with AB182163 from *M. alpina* 1s-4.

[0012] FIG. **3**: the sequencing results of the clone sequence of Δ12 desaturase (FADS12), in which FADS 12 nucleotide sequence from *M. alpina* ATCC#32222 shows 99.9% identity with AF110509 from *M. alpina* 1s-4.

[0013] FIG. **4**: the sequencing results of the clone sequence of Δ9 desaturase (FADS9-I), in which FADS9-I nucleotide sequence from *M. alpina* ATCC#32222 shows 98.4% identity with AF085500 from *M. alpina* 1s-4.

[0014] FIG. **5**A: Growth curve of the recombinant *P. pastoris* measured by cell density, wet weight and total protein concentration.

[0015] FIG. **5**B: Kinetics of recombinant protein induction. Desaturase expression was determined by Western blotting using anti-His tag antibody. The normalized level of highest expression was set at one arbitrary unit. Three independent experiments were performed and bars represent standard deviations.

[0016] FIG. **5**C: Quantification of the recombinant desaturase proteins by Coomassie blue staining after SDS-PAGE. Known concentrations of BSA were used as quantification standard.

[0017] FIG. **5**D: InVision™ His-tag In-Gel Stain of recombinant FADS proteins. The arrow head indicates the addition of methanol for induction of recombinant protein expression. The triangles indicate the expressed recombinant proteins. M: protein marker, Cont: negative control which was PichiaPink™ harboring pPinkα-HC, 15: FADS15, 12: FADS12, 9-I: FADS9-I.

[0018] FIG. **6**A: Fractionation of recombinant desaturases. InVision™ His-tag In-Gel Staining after SDS-PAGE analysis of FADS9-I membrane (top panel) and supernatant fraction (bottom panel), using different speeds of centrifugation. The triangles indicate the recombinant FADS9-I.

[0019] FIG. **6**B: Coomassie blue Staining and InVision™ His-tag In-Gel Staining of recombinant desaturases after fractionation. T: total protein after grinded by glass beads, D: debris after centrifugation at 500 g for 10 min, M: membrane fraction after centrifugation at 10,000 g for 10 min, S: supernatant after the centrifugation. The triangles indicate the recombinant desaturase proteins.

[0020] FIG. **7**A: Solubilization of recombinant desaturases. Membrane fractions were suspended in 1% concentrations of various detergents and incubated at 4° C. for 2 hr. Proteins were visualized by InVision™ His-tag In-Gel Staining (upper panel) and Western blot (lower panel). T20: Tween-20, T80: Tween-80, N40: NP-40, DDM: n-Dodecyl-β-D-maltoside, F12: Fos-Choline 12, F16: Fos-Choline 16,S: supernatant, P: pellet.

[0021] FIG. **7**B: Membrane fractions of recombinant FADS9-I were suspended in 1% Fos-Choline 16 and incubated at 4° C. for various time (0, 0.5, 1.5, 3, 12 hr). Aliquots were analyzed by InVision™ His-tag In-Gel Staining. S: supernatant, P: pellet.

[0022] FIG. **7**C: One-step purification using His Mag Sepharose Ni beads under the high yield (upper panel) and high stringency conditions (lower panel). Proteins were analyzed by SDS-PAGE and Coomassie blue staining. M: protein marker, S: supernatant, F: flow through, E: eluate.

SPECIFIC EMBODIMENTS

Example 1

Mortierella Alpina Culture

[0023] *Mortierella alpina* (#32222, American Type Culture Collection, Manassas, Va., USA) was inoculated on Potato Dextrose Agar (PDA) plates (BD Difco™ Potato Dextrose Agar cat#213400) and incubated for 20-30 days at 25° C. 5 mL broth (20 g/L Glucose, 5 g/L Bacto yeast extract BD Biosciences cat#212750, 1 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 10 g/L KNO_3) were added to three plates. Spores were gently scraped off the surface with a sterile loop, and then filtrated through a 40 micron cell strainer. Spores were concentrated by centrifuging at 12,000×g for 15 min, suspended in a small volume of broth, enumerated using a hemocytometer, and kept at −80° C. in 30% glycerol at a density of approximately 10^7 spores/mL. Alternatively, 3 mL of unconcentrated spore suspension were directly added into 45 mL broth without KNO_3 in a 250-mL flask covered with 8 layers of cheese cloth, and shaken at 200 rpm, 25° C. for 5 days. Cultures were blended using a Braun hand blender for 5 sec/pulse, 8 pulses, then 0.3 g wet mycelia were inoculated into 45 mL broth without KNO_3 in a 250-ml flask and shaken at 200 rpm, 25° C. for 24 h. The above step was repeated once, by which time the whole fungal culture was in proliferative phase and ready for experiments. Mycelia were collected by filtration and weighed. Samples were snap-frozen in liquid nitrogen, pulverized and kept at −80° C. for RNA extraction.

Example 2

Expression Vector Construction

[0024] *M. alpina* RNA extraction was performed using Trizol Reagent (Invitrogen, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed with SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) following the manufacturer's instructions. Using both C- and N-terminal sequences as primers (Table 1), desaturase coding sequences were PCR amplified as follows: denaturation at 95° C. for 30 sec, annealing at 55° C. for 45 sec and extension at 72° C. for 1 min for 25 cycles. The amplified products were cloned into a modified pET19 vector (Novagen) derivative containing a PreScission protease cleavage site (GE Healthcare) between the multiple cloning site and N-terminal His tag (Jonsson T J, Johnson L C, Lowther W T (2009) Protein engineering of the quaternary sulfuredoxin.peroxiredoxin enzyme.substrate complex reveals the molecular basis for cysteine sulfinic acid phosphorylation. J Biol Chem 284: 33305-33310.) to construct pET19b-FADS15, pET19b-FADS12 and pET19b-FADS94). The desaturase genes, including the His-Tag and PreScission protease cleavage site, were then PCR amplified using primers SF1 and SR1-SR3 (Table 1). The PCR conditions used were the same as the first step for cDNAs. The PCR fragments were then purified and inserted into pPinkα-HC to generate the expression vectors pPinkα-HC-FADS15, pPinkα-HC-FADS12 and pPinkα-HC-FADS9-I. The presence of the inserts in the plasmids was confirmed by restriction digestion analysis and sequencing. The strategy used for constructing desaturase expression vectors is shown in FIG. **1**. Sequencing results from the amplified fragment of these desaturases are in FIG. **2-4**. The FADS12 and FADS9-I genes from *M. alpina* ATCC#32222 are 99.9% and 98.4% identical, respectively, to the corresponding genes from *M. alpina* 1s-4. The FADS12 and FADS9-I proteins from *M. alpina ATCC*#32222 are 100% and 99.6% identical, respectively, to these proteins from *M. alpina* 1s-4. The high similarity of FADS12 and FADS9-I genes between two strains indicates that these genes are highly conserved in *M. alpina. Interestingly, the FADS* 15 gene is much less conserved at both DNA (93.1% identity) and protein (97.9%) levels.

Example 3

Protein Expression

[0025] Desaturase expression vectors and pPinkα-HC (negative control vector) were linearized with restriction enzyme Spe I and transformed into *P. pastoris* strains (PichiaPink strain 1, 2, 3 and 4) using the MicroPulser Electroporator (Bio-Rad Laboratories, Hercules, Calif.) according to the User Manual of PichiaPink Expression System (Invitrogen). *P. pastoris* were incubated with YPDS media (YPD with 1 M sorbitol) in the Gene Pulser Cuvettes at 28° C. for 2 hr without shaking, spread onto PAD (Pichia Adenine Dropout) agar selection plates, and then incubated at 28° C. for 4 days until distinct colonies were formed. Eight white colonies for each transformation were picked and plasmid integration in the yeast genome was confirmed by PCR.

[0026] Isolated clones were individually inoculated into 10 mL of BMGY medium (Buffered Glycerol-complex Medium, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% YNB-Yeast Nitrogen Base; 0.0004% biotin; 1% glycerol) in 50 mL conical tubes. The cells were grown for 48 hr at 28° C. with vigorous shaking at 250 rpm. Then, the cultures were centrifuged at 1,500 g for 5 min at room temperature, the cell pellets were resuspended in 2 mL of BMMY medium (Buffered Methanol-complex Medium, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% YNB; 0.0004% biotin; 0.5% methanol) and cultured at 28° C. with shaking at 250 rpm to induce the expression. After continuous cultivation for 72 hr with daily addition of 0.5% methanol, cells were harvested by centrifuging for 10 min at 1500 g. Supernatant was transferred to a separate tube and both the supernatant and cell pellet were stored at −80° C. until ready for assay. Supernatants and cell pellets were analyzed for protein expression by SDS-PAGE Coomassie blue staining and Western blot. Our data showed that PichiaPink strain 2(ade2, pep4) supported the highest level of expression for FADS15, 12 and 9-I.

Example 4

Expression Condition Optimization and Protein Analysis

[0027] Individual colonies of *P. pastoris*-FADS15, FADS12 and FADS9-I were inoculated into 10 mL of BMGY medium in 50 mL conical tubes and cultured for 48 hr at 28° C. at shaking speed of 250 rpm. Then, 2.5 mL of culture were inoculated into 50 mL of BMGY medium in 250-mL volume shaker flasks and grown at 28° C. for 24 hr at 250 rpm. The cells were collected by centrifugation at 1500 g for 10 min, and resuspended in 10 mL induction medium (BMMY medium with 0.5% methanol) in a 100-mL shaker flask. The induction of protein expression was performed for 96 hr at 28° C. with 250 rpm agitation and daily addition of 0.5% methanol. Samples were collected at 0, 6, 24, 48, 72 and 96 hr

for measuring cell density at OD_{600}, wet cell weight and total protein concentration, and for Western blot analysis of desaturase expression levels.

[0028] The cell pellets and supernatants were collected by centrifuging 100 μL cell culture at 1500 g for 10 min Cell pellets were resuspended in 100 μL lysis buffer (20 mM Tris. Cl pH7.9, 1 mM EDTA, 5% Glycerol) with an equal volume of 0.5 mm Glass Beads (Biospec products, Inc.), and vortexed for 10 min at 4° C. Cell lysates were mixed with 4×SDS sample buffer and heated for 5 min at 95° C. About 5 μl sample was loaded onto Mini-Protein Precast Gels (4-15%, Bio-Rad Laboratories, Cat #456-1086), and ran for 40 min at 150 V. Then, the SDS-PAGE gels were used for Coomassie blue stain, Invision His-Tag in-gel stain (Invitrogen) or Western blot.

[0029] For Western blot analysis, protein gels were transferred onto a nitrocellulose transfer membrane (Schleicher & Schuell GmbH, Germany) by electroblotting (100 V, 2 hr) using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). The membrane was blocked with 3% BSA in TBST (150 mM NaCl, 10 mM Tris-Cl pH 7.5, 0.05% Tween20), and probed with mouse Penta.His antibody (Invitrogen) followed by HRP-conjugated goat anti-mouse IgG (GE Healthcare). Blots were then incubated with enhanced chemiluminescence reagent (ECL, GE healthcare) and analyzed using Fluorchem E (Cell Biosciences, Inc.).

[0030] The total protein concentration was determined with Pierce BCA protein assay kit (Thermo Scientific). The quantification of target protein on Coomassie blue stained gel was performed using known concentrations of BSA as standard, and analyzed with the AlphaView SA software (Cell Biosciences, Inc.).

[0031] To determine potential toxicity of recombinant proteins, we first examined cell growth density, weight and total protein synthesis of the PichiaPink pPinkα-HC-FADS clones. The recombinant PichiaPink pPinkα-HC-FADS cells had growth characteristics similar to the control (FIG. **5**A). A time course experiment showed that desaturase expression was detectable after 24 hr induction with 0.5% methanol and remained high for at least 72 hr post-induction (FIG. **5**B). There were no significant differences in protein expression when cells were induced at different temperatures (16° C., 22° C., 28° C.) or with a different concentration of methanol (0.5%, 1%). Therefore, we used an optimized procedure as described in the Materials and Methods for the expression of recombinant desaturase. Under this condition, expression levels of recombinant desaturase proteins reached approximately 130 mg/L of culture for FADS15, 110 mg/L for FADS12 and 350 mg/L for FADS9-I (FIG. **5**C).

Example 5

Protein Purification

[0032] All purification procedures were performed at 4° C. Cells harvested from 800 μL of culture were suspended in 800 μL of lysis buffer. After addition of 0.5 mm glass beads to the cell suspension, *P. pastoris* cells were disrupted by vortexing at 4° C. for 10 min Cell lysis efficiency was usually more than 95% evaluated using a light microscope. Intact cells and cell debris were removed from the membrane suspension by low speed centrifugation (500 g, 10 min at 4° C.). Then various centrifugation speeds and time (1,000 g for 10 min; 10,000 g for 10 min; 10,000 g for 20 min; 20,000 g for 10 min; 20,000 g for 20 min) were used to determine the best centrifugation conditions for collecting the membrane fraction.

[0033] Fractions containing recombinant desaturases were solubilized in buffer, containing 20 mM Tris.Cl, pH 7.9, 500 mM NaCl, 10% glycerol, 0.1 mM EDTA, and different concentrations (0.5%, 1%, 2%) of various detergents (Tween 20, Tween 80, Nonidet P-40, DDM, Fos-Choline 12, Fos-Choline 16) at 4° C. for different times (0.5, 1, 1.5, 2 hr and overnight). The insoluble materials were removed by centrifugation at 25,000 g for 30 min at 4° C.

[0034] Optimized culture and protein solubilization conditions were used for the subsequent purification process. His Mag Sepharose™ Ni affinity beads (GE Healthcare) were washed with binding buffer (20 mM Tris.Cl, pH 7.9, 500 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.5% Fos-Choline 16, 5 or 20 mM imidazole) and added to the solubilized fractions after detergent incubation. The bead-protein sample mixtures were incubated for 45 min at 4° C. with end-over-end mixing. After washing three times with binding buffer containing 5 mM or 20 mM imidazole, desaturase enzymes were eluted with elution buffer (20 mM Tris.Cl, pH 7.9, 500 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.5% Fos-Choline 16, 500 mM imidazole). The purified FADS15, FADS12 and FADS9-I proteins were stored at −80° C. in aliquots. The quantity and quality of these purified enzymes were analyzed by SDS-PAGE and desaturase activity assay.

[0035] In order to solubilize and purify the recombinant desaturases from cell membrane for in vitro enzymatic activity, we first tested conditions to enrich the cell membrane containing recombinant FADS15, FADS12 and FADS9-I. Different centrifugation speeds and times were examined for the separation of the membrane fractions containing target proteins. Efficient recovery of each recombinant desaturase produced in *P. pastoris* was achieved by centrifuging the cell homogenates at 500 g for 10 min to remove cell debris, then at 10,000 g for 10 min to collect membrane fractions (FIG. **6**).

[0036] Solubilization of membrane proteins requires the presence of detergents. Therefore, we tested the conditions for solubilization of the recombinant FADS 15, FADS 12 and FADS9-I from enriched cell membrane fractions using a panel of detergents: Tween-20, Tween-80, NP-40, n-Dodecyl-β-D-maltoside (DDM), Fos-Choline 12 or Fos-Choline 16. After treatment with 1% (w/v) of Fos-Choline 12 or Fos-Choline 16, FADS9-I and FADS12 were totally solubilized, and approximately 50% and 80% of FADS 15 was solubilized with Fos-Choline 12 and Fos-Choline 16, respectively (FIG. **7**A). Tween-20, Tween-80, NP-40 and DDM had little effect on extracting these desaturase enzymes from the membrane. In addition, we noticed that FADS9-I protein degradation occurred during protein solubilization. This phenomenon was visible for proteins solubilized by both Fos-Choline 12 and 16. Thus, we investigated detergent incubation time during solubilization to optimize for the least protein degradation. Our results showed that the solubilization of FADS9-I protein reached its maximum level after incubation with detergent for 1.5 hr. Degradation of desaturase protein increased after more than 3 hr of incubation (FIG. **7**B). To maximize the ratio of intact vs. degraded proteins, we used 1.5 hr as our standard detergent incubation time for protein solubilization. We also compared the effect of detergent concentrations on protein solubilization efficiency and found that 0.5%, 1% or 2% of Fos-Choline 16 had similar effects. Taken together, our results indicate that all three recombinant

desaturase enzymes can be solubilized efficiently from the cell membrane with 0.5% Fos-Choline 16 for 1.5 hr at 4° C.

[0037] Solubilized FADS 15, FADS 12 and FADS9-I were affinity-purified on His Mag Sepharose Ni beads (GE healthcare) with aims of high purity or high yield. High purity (>95%) was achieved after one step purification using the His Mag Sepharose Ni beads with high stringency wash before elution (FIG. 7C). High yield (2-fold higher than that in the high purity process) was achieved with low stringency wash. Yield and quantity of each desaturase enzyme are summarized in Table 2. Our estimated yields of desaturases with purity >95% are approximately 22.5 mg/L for FADS15, 12 mg/L for FADS12 and 188 mg/L for FADS9-I.

TABLE 2

Purification of *M. alpina* desaturases

		FADS15 protein			FADS12 protein			FADS9-I protein		
Process	Vol (μL)	Con. (mg/L)	Total (μg)	Yield (%)	Con. (mg/L)	Total (μg)	Yield (%)	Con. (mg/L)	Total (μg)	Yield (%)
Cell lysates	200	130.0	26.0	100.0	110.0	22.0	100.0	350.0	70.0	100.0
Centrifugal collections (500-10 kg)	200	112.0	22.4	86.2	82.0	16.0	72.7	254.0	50.8	72.6
Detergent treatment extracts	200	76.0	15.2	58.5	74.8	15.0	68.2	223.0	44.6	63.7
Ni-NTA (20 mM ID)	40	22.5	0.9	3.5	12.0	0.5	2.3	188.0	7.5	10.7
Ni-NTA (5 mM ID)	100	10.5	1.1	4.2	7.5	0.8	3.6	185.0	18.5	26.4

ID: imidazole

Example 6

Desaturase Activity Assay

[0038] Approximately 20 mg of *P. pastoris* cell pellets were collected and used for each lipid extraction with the method of Bligh and Dyer (Bligh E G, Dyer W J (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917). under acidified conditions with pentadecanoic acid and heneicosanoic acid added as internal standards. The solvent from the extract was removed under a stream of nitrogen. Lipids were saponified in 1 mL of freshly prepared 5% ethanolic potassium hydroxide at 60° C. for 1 hr under an argon atmosphere. After cooling, 1 mL of water was added to the samples and non-saponifiable lipids were extracted into 3 mL of hexane. The aqueous layer was acidified with 220 μL of 6 M hydrochloric acid and the fatty acids extracted into 3 mL of hexane. After removing the hexane in a stream of nitrogen, fatty acids were converted to methyl esters by first treating with 1 mL of 0.5 M methanolic sodium hydroxide at 100° C. for 5 min under argon followed by 1 mL of 14% methanolic boron trifluoride at 100° C. for 5 min under argon (Metcalfe L D, Schmitz A A, Pelka J R (1966) Rapid preparation of fatty acids esters from lipids for gas chromatographic analysis. Analytical Chemistry 38: 514-515.). After cooling, the sample was mixed with 2 mL of hexane followed by 4 mL of saturated aqueous sodium chloride. After separating the phases, aliquots of the hexane layers were diluted 24-fold with hexane and then analyzed by GC/MS. One μL was injected in the splitless mode onto a 30 m×250 μm DB-WAXETR column (Agilent Technologies, Santa Clara, Calif.) with 0.25 μm film thickness. The temperature program was as follows: 100° C. for 2 min, ramp to 200° C. at 16° C. per min, hold for one min, ramp to 220° C. at 4° C. per min, hold one min, ramp to 260° C. at 10° C. per min, and hold for 11 min Helium was the carrier gas at a constant flow of 1.5 mL/min. The mass spectrometer was operated in positive-ion electron impact mode with interface temperature 260° C., source temperature 200° C., and filament emission 250 μA. Spectra were acquired from m/z 50 to 450 with a scan time of 0.433 s. Lower-boiling fatty acid methyl esters were quantified using the pentadecanoic acid internal standard, whereas higher-boiling methyl esters were quantified using the heneicosanoic acid internal standard.

[0039] In Vivo Desaturase Activity Analysis:

[0040] Individual colonies of *P. pastoris*-FADS15, FADS 12 and FADS9-I were cultured as described in the Recombinant protein expression section. Protein expression was induced for 72 hr with 0.5% methanol. Cell pellets were collected by centrifugation and stored at −80° C. for fatty acid analysis.

[0041] In Vitro Desaturase Activity Analysis:

[0042] 20 μL of the purified protein was added to 200 μL of yeast EGY49 cell homogenate, prepared by breaking cells with 0.5 mm glass beads in lysis buffer (20 mM Tris-HCl pH7.9, 1 mM EDTA, 5% Glycerol). The enzyme reactions were performed at 28° C. for 3 h with shaking (250 rpm), and the assay mixture (220 μL) were stored at −80° C. for fatty acid analysis.

[0043] To determine the functional activity of the recombinant *M. alpina* desaturase in vivo, PichiaPink cells were cultured and induced to express desaturases. Fatty acid methyl esters (FAME) analysis of cell pellets showed that expression of recombinant desaturases in PichiaPink cells altered their fatty acid contents compared to the control. Table 3 shows the percentage increase of C16:$1^{\Delta 9}$, C18:$1^{\Delta 9}$, C18:$2^{\Delta 9,12}$ and C18:$2^{\Delta 9,12,15}$ compared to the negative control. The C16:$1^{\Delta 9}$ and C18:$1^{\Delta 9}$ were increased 40% and 20%, respectively, in PichiaPink cells expressing FADS9-I, suggesting that FAD9-I can insert the first double bond into both C16:0 and C18:0 with a preference for C16:0 as substrate. The C18:$2^{\Delta 9,12}$ content was 27% higher in cells expressing FADS12, suggesting that FADS12 can desaturate C18:$1^{\Delta 9}$ at the c12-position to produce C18:$2^{\Delta 9,12}$. There was a 5% increase in C18:$3^{\Delta 9,12,15}$ in cells expressing FADS15, suggesting that FADS15 can desaturate C18:$2^{\Delta 9,12}$ at the c15-position to produce C18:$3^{\Delta 9,12,15}$. These results suggest that the recombi-

nant desaturases, FADS9-I, FADS 12 and FADS 15, were active in *P. pastoris*.

[0044] We used yeast EGY49 cell homogenate for our in vitro assay of recombinant desaturase activity. Our results showed that purified recombinant FADS12 converted C18:$1^{\Delta 9}$ to C18:$2^{\Delta 9,12}$ in vitro, and C18:$2^{\Delta 9,12}$ level was increased 116% compared to the control (Table 3). Activities of purified FADS9-I and FADS 15 were relatively low in vitro.

TABLE 3

M. alpina desaturases in-vivo and in-vitro activities

In vivo				In vitro			
FADS9-I		FADS12	FADS15	FADS9-I		FADS12	FADS15
C16:$1^{\Delta 9}$ (%[a])	C18:$1^{\Delta 9}$ (%)	C18:$2^{\Delta 9,12}$ (%)	C18:$3^{\Delta 9,12,15}$ (%)	C16:$1^{\Delta 9}$ (%)	C18:$1^{\Delta 9}$ (%)	C18:$2^{\Delta 9,12}$ (%)	C18:$3^{\Delta 9,12,15}$ (%)
40 ± 6	20 ± 7	27 ± 4	5 ± 3	6 ± 8	7 ± 7	116 ± 40	8 ± 4
60 ± 7[b]				13 ± 8			

[a]Percent increase over control;

[b]Sum of two products

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Ser Pro Leu Thr Val Phe Met Tyr Tyr Gly Ile Pro Tyr Leu Gly Val
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Asn Ala Trp Ile Val Cys Ile Thr Tyr Leu Gln His Thr Asp Pro Lys
    290                 295                 300

Val Pro His Phe Arg Asp Asn Glu Trp Asn Phe Gln Arg Gly Ala Ala
305                 310                 315                 320

Cys Thr Ile Asp Arg Ser Phe Gly Thr Ile Val Asn His Leu His His
                325                 330                 335

His Ile Gly Asp Ser His Gln Cys His His Met Phe Ser Gln Met Pro
            340                 345                 350

Phe Tyr Asn Ala Val Glu Ala Thr Lys His Leu Lys Ala Lys Leu Gly
        355                 360                 365

Lys Tyr Tyr Ile Phe Asp Asp Thr Pro Ile Ala Lys Ala Leu Tyr Arg
    370                 375                 380

Asn Trp Arg Glu Cys Lys Phe Val Glu Asp Glu Gly Asp Val Val Phe
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Tyr Lys His

<210> SEQ ID NO 3
<211> LENGTH: 1338
<212> TYPE: DNA
<213> ORGANISM: Mortierella alpina

<400> SEQUENCE: 3

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gatcctctcc agcacgagga actgcccccc ctcttccccg agaaaatcac catcttcaac 120

atctggagat atcttgacta caagcatgtt ctcggtctgg ggctgacgcc tttgatcgct 180

ctctatggtc tcttgacgac cgagatccag acgaagacac tgatctggtc catcatctac 240

tattatgcta cgggacttgg catcacagca ggttaccatc gactctgggc ccatcgtgct 300

tacaacgcag gaccagccat gagcttcgtg ctcgcactgc tcggcgccgg tgcagttgaa 360

ggatctatca agtggtggtc ccgcggccac cgtgctcacc accgctggac tgacaccgag 420

aaggacccct acagcgctca ccgaggactc ttcttctcgc acattggctg gatgttgatc 480

aagcgccctg gatggaagat tggccatgcc gatgttgacg acctcaacaa gagcaaactc 540

gttcagtggc agcacaagaa ctaccttcct cttgttctta tcatgggtgt cgtcttcccc 600

acggttgttg ctggactcgg ctggggcgat tggcgtggag gctacttcta tgctgctatc 660

ctccgtcttg tctttgttca ccacgccacc ttctgtgtca actccctggc ccattggctc 720

ggtgatggac cctttgatga ccgccactct ccccgcgacc acttcatcac tgcctttgtc 780

actttgggag agggctacca caactttcat caccaattcc cccaggacta ccgcaacgct 840

atccgttttt accagtacga tcctaccaag tgggtcatcg ccctctgtgc tttctttggc 900

ctcgctacgc acctcaagac cttccctgag aatgaagtcc gcaagggtca gctccagatg 960

attgagaagc gtgtcctgga gaagaagacc aagctccagt ggggcactcc cattgccgat 1020

ctgcccattc tgagctttga ggacttccag catgcttgca aaaacgacaa caagaagtgg 1080

atcctcttgg agggtgtcgt ttacgacgtt gccgacttta tgaccgagca ccctggtggt 1140

gagaagtacc tcaagatggg cgtcggcaag gacatgaccg cagctttcaa cggcggtatg 1200

tacgatcaca gcaatgccgc ccgcaacctg ctgagcttga tgcgcgtcgc cgtcgttgag 1260

tatggtggtg aggtggaggc acagaagaag aacccttcga tgcccatcta cggcactgac 1320

cacgtcaagg ccgaataa 1338

<210> SEQ ID NO 4
<211> LENGTH: 445
<212> TYPE: PRT
<213> ORGANISM: Mortierella alpina

<400> SEQUENCE: 4

Met Ala Thr Pro Leu Pro Pro Ser Phe Val Val Pro Ala Thr Gln Thr
1 5 10 15

Glu Thr Arg Arg Asp Pro Leu Gln His Glu Glu Leu Pro Pro Leu Phe
20 25 30

Pro Glu Lys Ile Thr Val Tyr Asn Ile Trp Arg Tyr Leu Asp Tyr Lys
35 40 45

His Val Phe Gly Leu Gly Leu Thr Pro Leu Ile Ala Leu Tyr Gly Leu
50 55 60

Leu Thr Thr Glu Ile Gln Thr Lys Thr Leu Ile Trp Ser Ile Ile Tyr

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65                  70                  75                  80

Tyr Tyr Ala Thr Gly Leu Gly Ile Thr Ala Gly Tyr His Arg Leu Trp
                85                  90                  95

Ala His Arg Ala Tyr Asn Ala Gly Pro Ala Met Ser Phe Val Leu Ala
            100                 105                 110

Leu Leu Gly Ala Gly Ala Val Glu Gly Ser Ile Lys Trp Trp Ser Arg
        115                 120                 125

Gly His Arg Ala His His Arg Trp Thr Asp Thr Glu Lys Asp Pro Tyr
    130                 135                 140

Ser Ala His Arg Gly Leu Phe Phe Ser His Ile Gly Trp Met Leu Ile
145                 150                 155                 160

Lys Arg Pro Gly Trp Lys Ile Gly His Ala Asp Val Asp Asp Leu Asn
                165                 170                 175

Lys Ser Lys Leu Val Gln Trp Gln His Lys Asn Tyr Leu Pro Leu Val
            180                 185                 190

Leu Ile Met Gly Val Val Phe Pro Thr Val Val Ala Gly Leu Gly Trp
        195                 200                 205

Gly Asp Trp Arg Gly Gly Tyr Phe Phe Ala Ala Ile Leu Arg Leu Val
    210                 215                 220

Phe Val His His Ala Thr Phe Cys Val Asn Ser Leu Ala His Trp Leu
225                 230                 235                 240

Gly Asp Gly Pro Phe Asp Asp Arg His Ser Pro Arg Asp His Phe Ile
                245                 250                 255

Thr Ala Phe Val Thr Leu Gly Glu Gly Tyr His Asn Phe His His Gln
            260                 265                 270

Phe Pro Gln Asp Tyr Arg Asn Ala Ile Arg Phe Tyr Gln Tyr Asp Pro
        275                 280                 285

Thr Lys Trp Val Ile Ala Leu Cys Ala Phe Phe Gly Leu Ala Thr His
    290                 295                 300

Leu Lys Thr Phe Pro Glu Asn Glu Val Arg Lys Gly Gln Leu Gln Met
305                 310                 315                 320

Ile Glu Lys Arg Val Leu Glu Lys Lys Thr Lys Leu Gln Trp Gly Thr
                325                 330                 335

Pro Ile Ala Asp Leu Pro Ile Leu Ser Phe Glu Asp Phe Gln His Ala
            340                 345                 350

Cys Lys Asn Asp Asn Lys Lys Trp Ile Leu Leu Glu Gly Val Val Tyr
        355                 360                 365

Asp Val Ala Asp Phe Met Thr Glu His Pro Gly Gly Glu Lys Tyr Ile
    370                 375                 380

Lys Met Gly Val Gly Lys Asp Met Thr Ala Ala Phe Asn Gly Gly Met
385                 390                 395                 400

Tyr Asp His Ser Asn Ala Ala Arg Asn Leu Leu Ser Leu Met Arg Val
                405                 410                 415

Ala Val Val Glu Phe Gly Gly Glu Val Glu Ala Gln Lys Lys Asn Pro
            420                 425                 430

Ser Met Pro Ile Tyr Gly Thr Asp His Val Lys Ala Glu
        435                 440                 445


<210> SEQ ID NO 5
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 5

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<210> SEQ ID NO 6
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 6

atatattaat atggcacctc ccaacactat tgatgccgg 39

<210> SEQ ID NO 7
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 7

atatcatatg atggcaactc ctcttccccc ctcctttgt 39

<210> SEQ ID NO 8
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 8

atatggatcc taatgcttgt agaacactac gtc 33

<210> SEQ ID NO 9
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 9

atatggatcc ttacttcttg aaaaagacca cgtc 34

<210> SEQ ID NO 10
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 10

atatagatct ttattcggcc ttgacgtggt cagt 34

<210> SEQ ID NO 11
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 11

atatattgcg cacatcatca tcatcatcat cat 33

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<210> SEQ ID NO 12
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 12

atatgaattc atatttaaat taatgcttgt agaacactac gtc 43

<210> SEQ ID NO 13
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 13

atatatggta ccttacttct tgaaaaagac cacgtc 36

<210> SEQ ID NO 14
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer sequences for PCR

<400> SEQUENCE: 14

atatatggta ccttattcgg ccttgacgtg gtcagt 36

1. (canceled)
2. (canceled)
3. (canceled)
4. (canceled)
5. (canceled)
6. (canceled)
7. (canceled)
8. (canceled)
9. (canceled)
10. (canceled)

11. An isolated cDNA molecule consists of a nucleotide sequence that encodes an omega-3 desaturase.

12. The isolated cDNA molecule of claim **11**, said nucleotide sequence is shown as SEQ ID NO:1, and said omega-3 desaturase is shown as SEQ ID NO:2.

13. A vector consists of a cDNA molecule encoding an omega-3 desaturase, wherein said vector expresses a sequence that encodes for said omega-3 desaturase in said cDNA molecule.

14. The vector of claim **13**, said cDNA molecule includes a nucleotide sequence shown as SEQ ID NO:1, said omega-3 desaturase is shown as SEQ ID NO:2.

15. The vector of claim **13**, said vector is constructed by:

(a) obtaining mRNA from a fungi;

(b) synthesizing primers;

(c) performing a polymerase chain reaction (PCR) on said mRNA and said primers to produce said nucleotide sequence that encodes said omega-3 desaturase; and

(d) cloning said nucleotide sequence into an expression vector.

16. The vector of claim **15**, said fungi is *Mortierella alpina*.

17. The vector of claim **15**, said primers are selected from a group of nucleotide primers consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14.

18. The vector of claim **15**, said expression vector is pPinkα-HC.

19. The vector of claim **13**, said vector is able to be utilized for treatment of fatty acid-associated disorders in a subject.

20. The vector of claim **19**, said subject includes human.

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