US012037630B2

(12) **United States Patent**
Moseley et al.

(10) **Patent No.: US 12,037,630 B2**
(45) **Date of Patent: Jul. 16, 2024**

(54) **PRODUCTION OF LIPIDS AND TERPENOIDS IN *AUXENOCHLORELLA PROTOTHECOIDES***

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 316 days.

(21) Appl. No.: **17/519,854**

(22) Filed: **Nov. 5, 2021**

(65) **Prior Publication Data**

US 2022/0154229 A1 May 19, 2022

Related U.S. Application Data

(60) Provisional application No. 63/109,901, filed on Nov. 5, 2020.

(51) **Int. Cl.**

C12N 15/52	(2006.01)
C12N 9/02	(2006.01)
C12N 9/16	(2006.01)
C12N 9/90	(2006.01)
C12P 7/6472	(2022.01)

(52) **U.S. Cl.**

CPC ***C12P 7/6472*** (2013.01); ***C12N 9/0071*** (2013.01); ***C12N 9/0083*** (2013.01); ***C12N 9/16*** (2013.01); ***C12N 9/90*** (2013.01); ***C12N 15/52*** (2013.01); ***C12Y 114/19*** (2013.01); ***C12Y 114/19002*** (2013.01); ***C12Y 301/02014*** (2013.01); ***C12Y 505/01018*** (2013.01)

(58) **Field of Classification Search**

CPC C12N 15/52; C12N 9/16; C12N 9/90

See application file for complete search history.

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(57) **ABSTRACT**

Methods to produce oils with modified profiles of fatty acid, carotenoids and/or terpenoids in microalgal mutants are provided. Microalgal mutants produce the oil containing fatty acids, carotenoids and/or terpenoids of a modified profile with a disruption or ablation of one or more alleles of an endogenous polynucleotide or comprising an exogeneous gene are also provided.

12 Claims, 36 Drawing Sheets

Specification includes a Sequence Listing.

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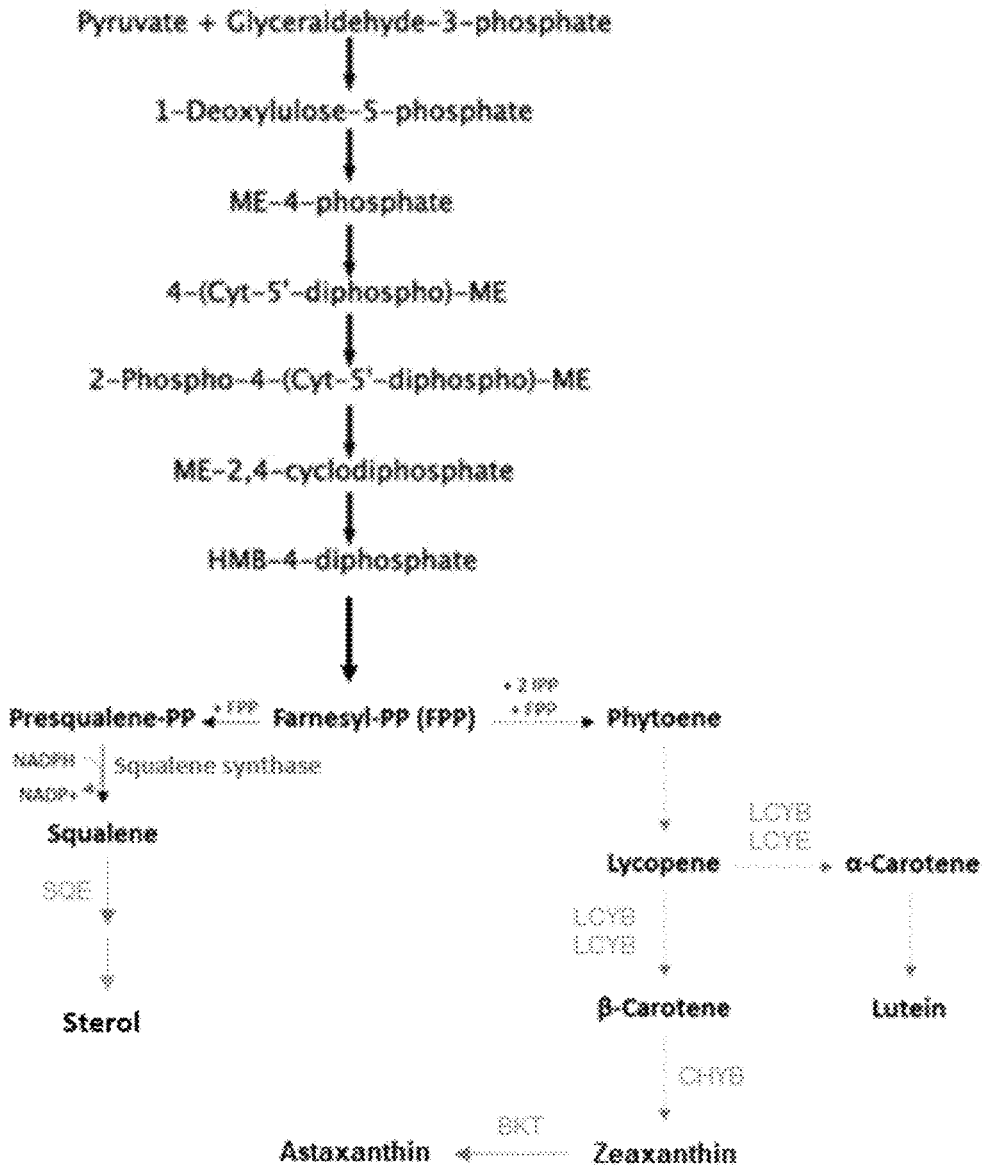
【FIG. 1】


Pyruvate + Glyceraldehyde-3-phosphate
1-Deoxylulose-5-phosphate
ME-4-phosphate
4-(Cyt-5'-diphospho)-ME
2-Phospho-4-(Cyt-5'-diphospho)-ME
ME-2,4-cyclodiphosphate
HMB-4-diphosphate
Presqualene-PP
Farnesyl-PP (FPP)
Phytoene
Squalene synthase
Squalene
SQE
Sterol
Lycopene
LCYB
LCYE
α-Carotene
LCYB
LCYB
β-Carotene
Lutein
CHYB
BKT
Astaxanthin
Zeaxanthin

【FIG. 2A】

aagcttCAAGTGCGTGCGTTACAGTGTTACCAACAACAGTCTAACCTACCCCTTTCGGTCATTCTGC
CCTTTGGCAAGAGTTCAGAATGAAGTGTGCTTGCACATCGAGCTAGTGCTGTGAGCGAAGACAA
GGAAGTCCCCACTCACCCACGTGGCCAGATTCTATCTTTTTTCAGATTGCAAGGGCCACGCCCAG
CGAACCCCGCGATGGGGCCGAGCCATGCCCGACATCTCGACATCTTCATATGATAAGGCGCTTCA
AAGTGCAATTTTTGTGCATGGCATCAATTAGGAGAGTGCTTGAACACCAGCCCATCTTCCACCGG
GGAAGGACCGTCGAAATGCCTCTGCAGACGGCCACCGTCTGATCGCTGCCTGTCCCGAGGTGA
CGGCGATGTCGTCCTTATCCCAAACAATCGTTCGAAGACCTTTCTTTTGTTCGCTCAACCCACCG
AGGAGACCGTCTGGATTCCATGCTGCTGTGACGCCTAGCCCCCTGAGACCCTCCAAGTGGGCG
GTCCCCTCCCTAGCCCCCAGCCTCTCTGACGTGGCAGATGCCTCCGCGGGAAGCAAATCAGGATC
GCAGGGAGGGCTCCTACGAGCAGCCCCTGGTCCAACGCCAGGTGCCTAGGGGGAAAGGAGGG
CAGAGGGGCTTGAGGCGAGCCTGGCCCAGGCAGGGCTTCCATGGTCAGTCGTGGCAGTGCCAT
GACAGCCGAAGCCCAACGCGACACCGTGGGTGCAGCATGCGTGGACGGAAACATTGGCAATGC
CTTGCCCCATTGGCCCCCCAGGCCCGGAAACGGGACGATCAGCAGGACCCCTTGTCCAGCCTC
CTCCCCAC**ggtacc**ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaaca
ccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggccc
ccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcg
cactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccgcaaad*ATGctgctgcaggccttcctgttcctgctggccggcttcgcc*
gccaagatcagcgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaacaagggctggatgaacgaccccaa
cggcctgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacgacacgtctgggggacgccttgttctgg
ggccacgccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgccttctccgg
ctccatggtggtggactacaacaacacctccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggccatctggacctacaaca
ccccggagtccgaggagcagtacatctcctacagcctggacggcggctacaccttcaccgagtaccagaagaaccccgtgctggccgcca
actccacccagttccgcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagat
cgagatctactcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaacgagggcttcctcggctaccagtacgagtgccccg
gcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatctccatcaaccccggcgcccccggccggcggctc
cttcaaccagtacttcgtcggcagcttcaacggcaccccacttcgaggccttcgacaaccagtcccgcgtggtggacttcggcaaggactacta
cgccctgcagaccttcttcaacaccgacccgacctacgggagcgccctgggcatcgcgtgggcctccaactgggagtactccgccttcgtgc

【FIG. 2B】

ccaccaaccctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccaggccaacccggagacggagctgatcaa
cctgaaggccgagccgatcctgaacatcagcaacgccggccccctggagccggttcgccaccaacaccacgttgacgaaggccaacagct
acaacgtcgacctgtccaacagcaccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttc
gcggacctctccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggacc
gcgggaacagcaaggtgaagttcgtgaaggagaaccctacttcaccaaccgcatgagcgtgaacaaccagccctttcaagagcgagaa
cgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtccaccaacaccta
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GTCTCACCACACCTTGAAGATTTTTGGGAGGGGGGGGGGAAGTCGGAATGGAAAC**gagctc**CGCGATTGTCAGAT
GGTGGAGTGGGTGGATGGCCCTGCTCCGAGGAGCTTTCTAGGGCGCGAACTTGGCCCTTCTCC
CCTCTGATGCAGTGTGGGGGGACGCGGTGTGCTATTTCTCCGAGGGCCGCCCAACTAGGGTGG
GGCGGGCATACGCCCGCGTCGACAGGTGGGTGTGGCCTCGAGGTGTTGAGAGGAGTGTTATGT
CGACAGCCAAAGTGGAGACTGATTGAACCCTACTCCAGGTGCTATCTTGGGAGCACACTGCGCC
CACCGTGGCTGGACTGCCCGAATTCCAACCTTGGTGCCCAGAAACAGGGCAAAGCCGGTCATC
AGTGCAGCATGAGACTCAAGCTCCCTAGCTCATGACCGTTGGCATAGGCAGAAGCTGCGGCAGC
ACCTGGTGGAGGCCTGCCAGGCAAACGGTGTCACCTTCCAGCCGGGGGAGGTAGTGGATGTGG
GCGTGAAGAACGGCACAGCCTCGGTCACCTGCCAAGATGGCTCCGTCCTGACTGCGAGGTGGG
CGCTGTGCATGGCATTTGTTGGCACGAGTCTGCATCTCTGAAGCTGCTGGGTAGCGTCAGAGCA
GTGGAGTCAACAGCACACAGCTCTGGCGGTGCTCAGGGAACATACATCGCACTGTTTCCTGGAG
TTGCTGGCCCTCTGTGGGGCAACCAGGACCCCCCGACGCATGCATGCCCCCTCGCACATCCCG
CACAGGCTGGTGACCCTGGCCTCCGGCGCGGCGGCGGGGCGCTTCCTCAAGTACGAGAG**aagc**
tt

【FIG. 3A】

aagcttCAAGTGCGTGCGTTACAGTGTTACCAACAACAGTCCAACCTAACCCTTTCGGTCATTCTGT
CCTTTGGCAAGGGCTCAGAATGAAGTGTGCCTGCACATCGAGCTAGTGCTGTGAGCGAAGACAA
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CGAACCCCGCGATGGGGCCGAGCCATGCCCGACATCTCGACATCTTCATATGACAAGGCGCTTC
AAAGTGCAATTTATGTGCATGGCATCGATTAGGAGAGTGGTTGAACACCAGCCCATCTTCCACCG
GGGAAGGACCGTCGAAATGCCTCTGCAGACGGCCACCGTCTGATCGCTGCCTGTCCCGAGGTG
ACGGCGATGTCGTCCTTATCCCAAACAATCGTTCGAAGACCTTTCTTTTGTTCGCTCAACCCACC
GAGGAGACCGTCTGGATTCCATGCCGCTGTGACGCCTAGCCCCCTGAGACCCTCCAAGTGGGC
GGTCCCCTCCCTAGCCCCCAGCCTCTCTGACGTGGCAGATGCCTCCGCGGAAGCAAATCAGGAT
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TCCTCCCCAC**ggtacc**tcccgcttttaattgagccccttcgtcgctgaatcagcgaaagcaccgcgaaacaatgcctgtcccgtccat
gcatctcaacagcctcatgcaaggtttgcacaagcaagaccattctgatctgggaacttgtaggtgttgtatgggggaggttgtgctcttgaatca
agtggtatcacgtttccggaacaccccgaaacgtgcatgggcttattgcgatgagagcatttcccaccgcgattgtctcacgcgcatttcggag
aaggtttgcagaacactccaggacatgaaatgccttgtcacgtatgaaccatctcccacggccttgaaaagatcgctcgacttccattctagat
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gcgccacgctgacgttcgaccccccacgaccaactccgagcgcgccaagcagcgcaagcacaccatcgaccctcctccccgacttcc
agcccatccctccttcgaggagtgcttccccaagtccacgaaggagcacaaggaggtggtgcacgaggagtccggccacgtcctgaagg
tgccttccgccgcgtgcacctgtccggcggcgagccgccttcgacaactacgacacgtccggcccccagaacgtcaacgcccacatcgg
cctggcgaagctgcgcaaggagtggatcgaccgccgcgagaagctgggcacgccccgctacacgcagatgtactacgcgaagcagggc
atcatcacggaggagatgctgtactgcgcgacgcgcgagaagctggaccccgagttcgtccgctccgaggtcgcgcggggccgccgccatc

【FIG. 3B】

atcccctccaacaagaagcacctggagctggagcccatgatcgtgggccgcaagttcctggtgaaggtgaacgcgaacatcggcaactcc
gccgtggcctcctccatcgaggaggaggtctacaaggtgcagtgggccaccatgtggggcgccgacaccatcatggacctgtccacgggc
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*ggcgagcagcacggcgaggtcggcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA*GTCCTGGCGACC
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ACAACACTTGCCTGACCGAGGGTCGGGTCGCGAAGTAAAAGCCACAATCAACACCCCAGGCACATTAAGAGTGCACA
GCATGACGCAGCATAGGGTTTGTGTCGGAGGAAGGGGGTCGAGTCGCGTTGGCGAGGGGGTGGTCACGATGACCA
CATCTGCGGGATAATTGAATCCTCAGGGGAAAATACCAGTCTCTGCTTCCAGGTGCTCCG**gagctc**CGCGATTGTC
AGATGGTGGAGTGGGTGGATGGCCCTGCTCCAGAGGAGCGTTCTAGGGCGCAAACTTGGCACT
TTCCCCCCTCTGATGCAGTGTGGGGGGACGCGGTGTGCTATTTCTCCGAGGGCCGCCCAACCA
GGGTGGGGCGGGCATATGCCCGCGTCGACAGGTGGGTGTGGCCTCGAGGTGTTGAGAGGAGG
GTTATGTCGACAGCCAAAGTGGAGACTGAGTGAACCCTACTCCAGGTGCTGTCGTGGGAGCGCA
CTGCGCCCACCGTGGCTGGACTGCTCGTATTCCAACCTTGGTGCCCAGAAACAGGGCAAAGCC
GGTCATCAGTGCAGCATGAGACTCAAGCTCCCTAACTCATGACCGTTGGCATAGGCAGAAGCTG
CGGCAGCACCTGGTGGAGGCCTGCCAGGCAAACGGTGTCACCTTCCAGCCGGGGGAGGTAGT
GGATGTGGGCGTGAAGAACGGCACAGCCTCGGTCACCTGCCGAGACGGCTCCGTCCTGACTGC
GAGGTGGGCGCTGTGCATGGCACTTGTTGGCACGAGTCTGCATCCCTGAAGCTGCTGGGTAGC
GTCAGAGCAGTGGCGTCAACAGCACACAGCTCTGGCGGTGCCCAGGGAACATACATCGCTCTG

【FIG. 3C】

TTTCCTGGAGTTGCGGGCCCTCTGTGTGGCAGCCAGGGCCCCCCGACGCATGCATGCCTCCTC
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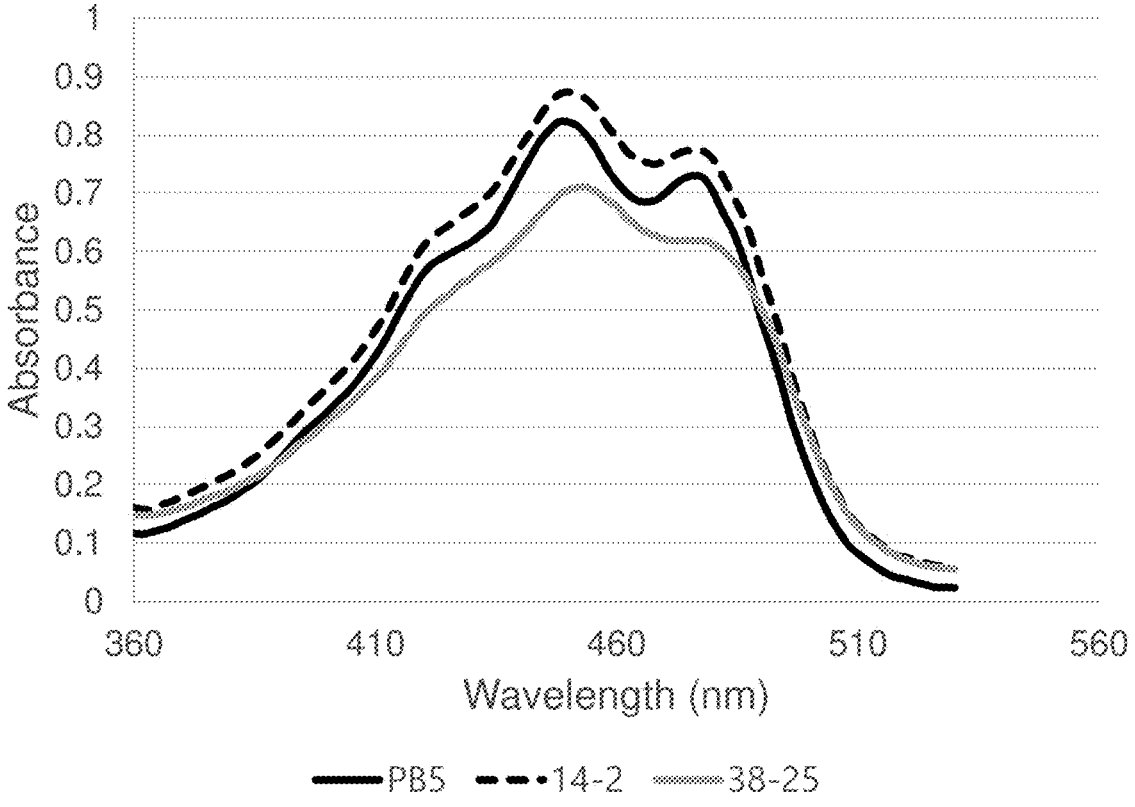
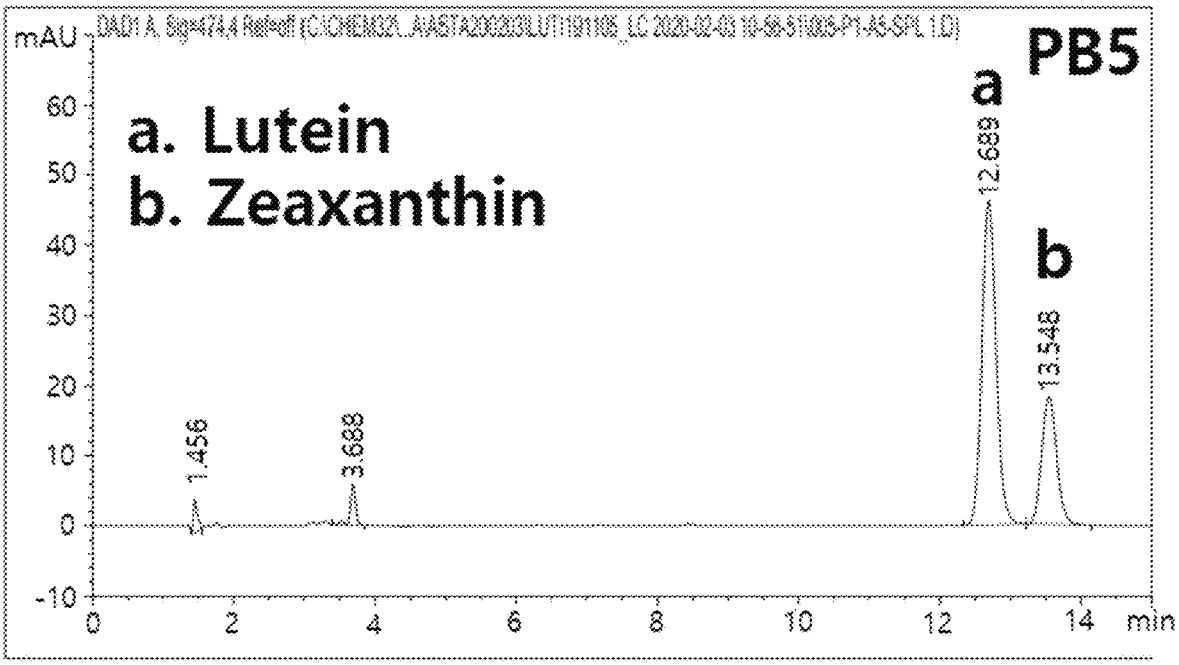
ACGAGAG**aagctt**

【FIG. 4A】



【FIG. 4B】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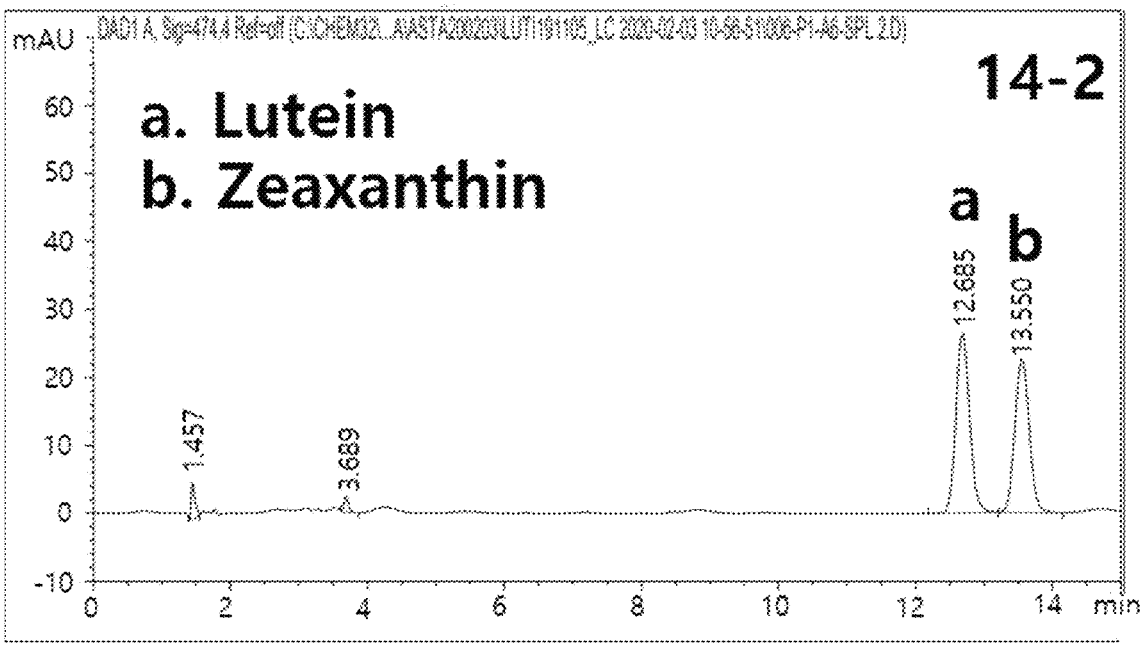
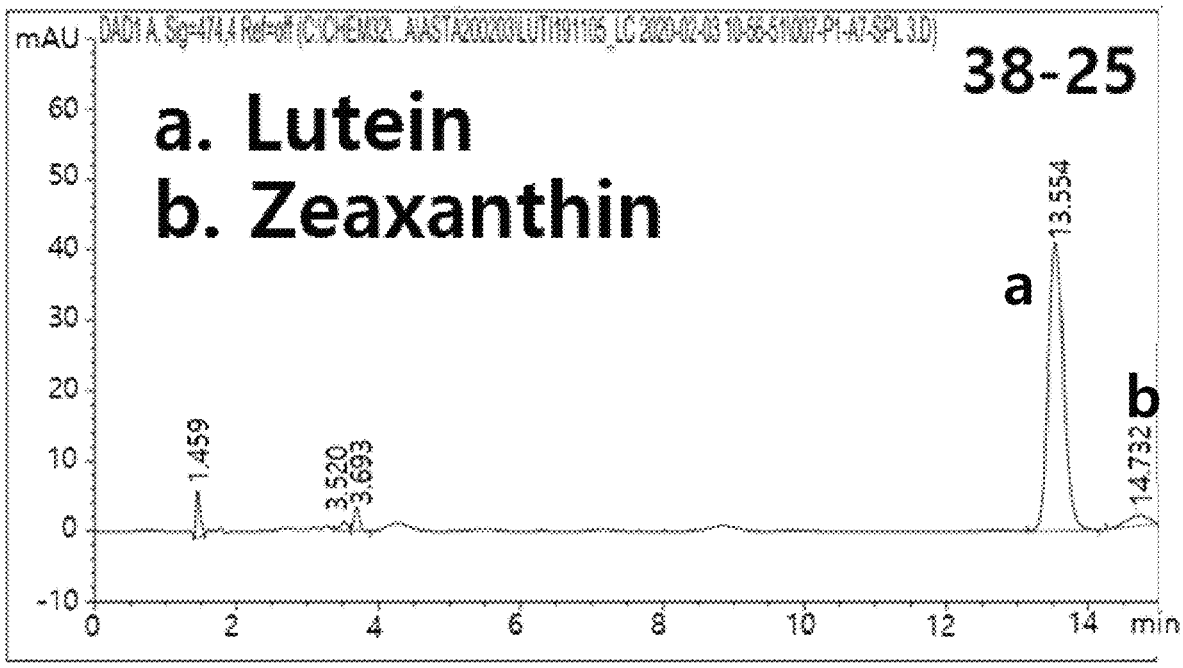
PB5
a. Lutein
b. Zeaxanthin
a
b
12.689
13.548
1.456
3.688
60
50
40
30
20
10
0
-10
0
2
4
6
8
10
12
14
min

【FIG. 4C】


mAU
14-2
a. Lutein
b. Zeaxanthin
a
b
12.685
13.550
1.457
3.689
60
50
40
30
20
10
0
-10
0
2
4
6
8
10
12
14
min

【FIG. 4D】


38-25
a. Lutein
b. Zeaxanthin
mAU
60
50
40
30
20
10
0
-10
0
2
4
6
8
10
12
14
min
1.459
3.520
3.693
13.554
14.732
a
b

【FIG. 5】

【FIG. 6】

ATGgccaccgcatccactttctcggcgttcaatgcccgctgcggcgacctgcgtcgctcggcgggctccgggccccggcgcc
cagcgaggcccctccccgtgcgcgctgccatccgctcccgccgcatggccgaggacatcctgaagctgtggcagcgccagt
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ccatcctgtccgccttccgcctgttctactacggcacctacgtgccccaccaccccgagaagggccacaccggcgccatgccc
tggcaggtgtcccgcacctcctccgcctcccgcctgcagtccttcctgacctgctaccacttcgacctccactgggagcaccacc
gctggccctacgccccctggtgggagctgcccaagtgccgccagatcgcccgcggcgccgccctggccTGA

【FIG. 7A】

MGPGIQPTSARPCSRTKHSRFALLAAALTARRVKQFTKQFRSRRMAEDILKLWQRQYHLP
REDSDKRTLRERVHLYRPPRSDLGGIAVAVTVIALWATLFVYGLWFVKLPWALKVGETATS
WATIAAVFFSLEFLYTGLFITTHDAMHGTIALRNRRLNDFLGQLAISLYAWFDYSVLHRKHW
EHHNHTGEPRVDPDFHRGNPNLAVWFAQFMVSYMTLSQFLKIAVWSNLLLLAGAPLANQL
LFMTAAPILSAFRLFYYGTYVPHHPEKGHTGAMPWQVSRTSSASRLQSFLTCYHFDLHWE
HHRWPYAPWWELPKCRQIARGAALA*

【FIG. 7B】

MATASTFSAFNARCGDLRRSAGSGPRRPARPLPVRAAIRSRRMAEDILKLWQRQYHLPRE
DSDKRTLRERVHLYRPPRSDLGGIAVAVTVIALWATLFVYGLWFVKLPWALKVGETATSWA
TIAAVFFSLEFLYTGLFITTHDAMHGTIALRNRRLNDFLGQLAISLYAWFDYSVLHRKHWEH
HNHTGEPRVDPDFHRGNPNLAVWFAQFMVSYMTLSQFLKIAVWSNLLLLAGAPLANQLLF
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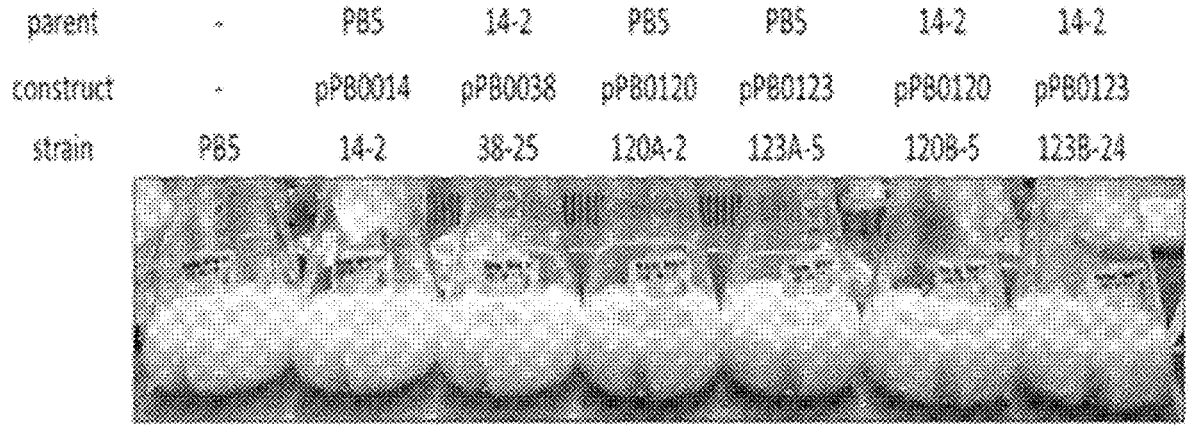
HRWPYAPWWELPKCRQIARGAALA*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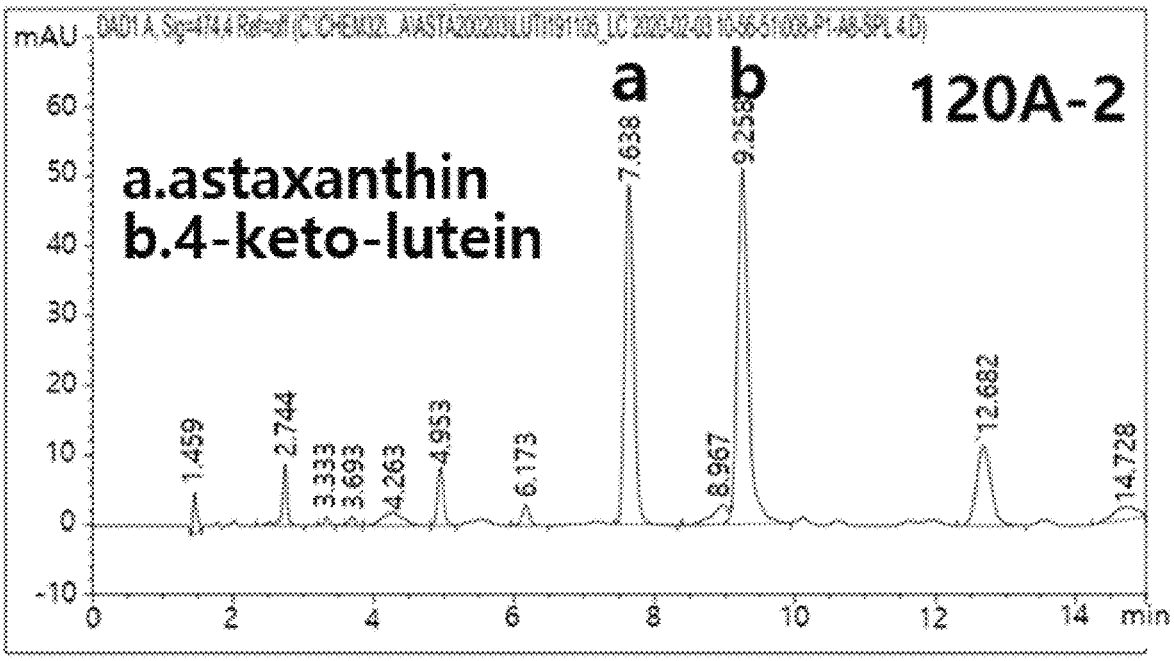
construct - pPB0014 pPB0038 pPB0120 pPB0123 pPB0120 pPB0123
strain PBS 14-2 38-25 120A-2 123A-5 120B-5 123B-24

FIG. 8A

【FIG. 8B】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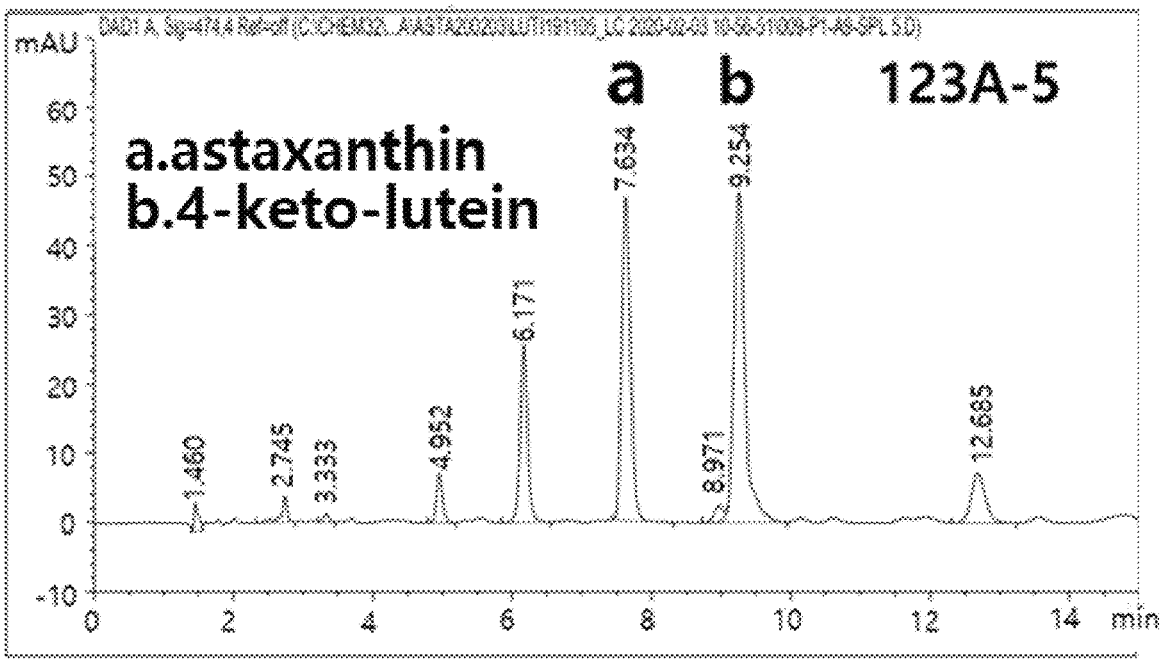
a.astaxanthin
b.4-keto-lutein
7.638
9.258
1.459
2.744
3.333
3.693
4.263
4.953
6.173
8.967
12.682
14.728
min

【FIG. 8C】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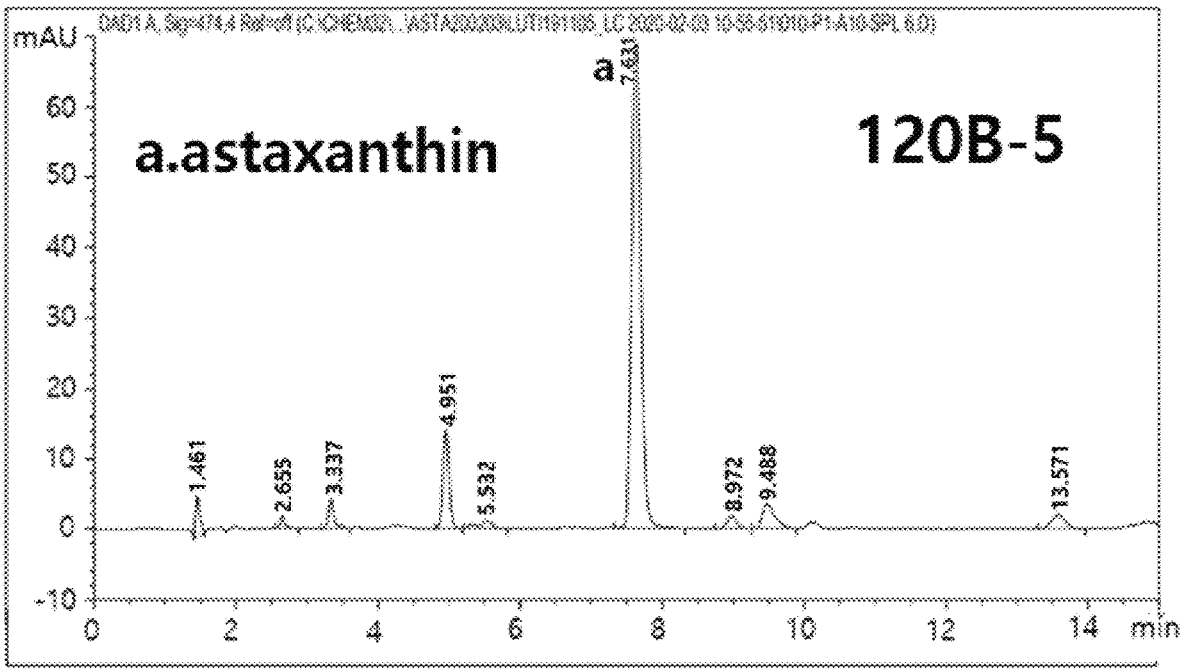
a
b
123A-5
a.astaxanthin
b.4-keto-lutein
7.634
9.254
1.460
2.745
3.333
4.952
6.171
8.971
12.665
min

【FIG. 8D】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40
30
20
10
0
-10
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120B-5
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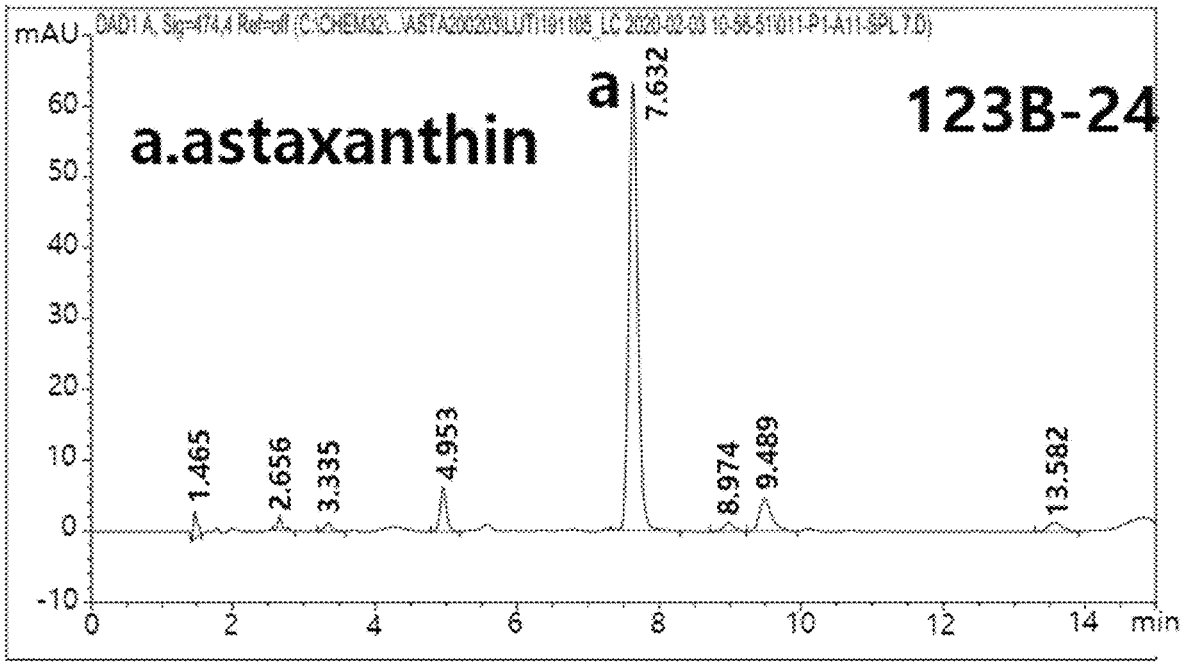
7.831
1.461
2.655
3.337
4.951
5.532
8.972
9.488
13.571
0
2
4
6
8
10
12
14
min

【FIG. 8E】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30
20
10
0
-10
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7.632
123B-24
1.465
2.656
3.335
4.953
8.974
9.489
13.582
0
2
4
6
8
10
12
14
min

【FIG. 9A】

aagcttTCACTGCTCCATTCACACCCAATTTCCCACCGCCGCACCCGCTCCCAGGTGGGAGGATGG
GAGGGAACCCAGCTGGGAGCCGGAGGAGCATGTGACCCCGGACCTGATCGAGCTATATGCCGC
CAGGGACGCTCGAGCACGGGATGTCAAGGCCGCGGCTGACAAGGCTGGCAAGGGTAGGACAC
CTGCGTTGAGGCAAACGCTGGAGACGGCCGGTCTCAGCATGTGATCCTGTACTTGCTGTGACAA
GGTGGACAATGCAGTACGAGGTGTACCAGGCTAAGTATTCCTAGTATACCGGCACGCCAGATCAT
CTGTACAGGAGTCTGTGCAGGAGTGAAAAGGTAAGAGCCAGAGCCATGACCGGCGACCATCCAT
GTCCCGTCACTCGGATGCACTGGCTGACATCGGCGGCAGGTCATCCAACGGTCCTGAATGATCG
AGAGGAAGCTGCCCGATTTCAAAACGCCCCCCCACGTCGCCCTCCATGGCCGCACAGCATGCT
CAGCACAGGTTGCTGCGTGTCCTCACACAAACTGCTCCTTTAAAGCGATCAACTTTCCAGGGCAT
GGGGCACTCGTACTGACAATCACCCACATTCGTATACCTTTGACGTCATTATTTTTTCGCCCCAAC
GCGGTTGCCATCCCGAGTTGTACCTCCGCGGCTACCATACCCCTGTCTTCTGGCCCTCACCGTC
GCTCGCAGGCGGGATCCAGCGCAGCCAGTCTGAATACTTTTACACAACATAGTACGTAACGCGCA
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gcttttaattgagccccttcgtcgctgaatcagcgaaagcaccgcgaaacaatgcctgtccgtccatgcatctcaacagcctcatgcaaggt
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ccgaaacgtgcatgggcttattgcgatgagagcatttcccaccgcgattgtctcacgcgcatttcggagaaggtttgcagaacactccaggac
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cggcggcgagccccgccttcgacaactacgacacgtccggcccccagaacgtcaacgcccacatcggcctggcgaagctgccgcaaggag
tggatcgaccgccgcgagaagctgggcacgccccgctacacgcagatgtactacgcgaagcagggcatcatcacggaggagatgctgta
ctgccgacgccgcgagaagctggaccccgagttcgtccgctccgaggtccgcgcggggccgccgccatcatccccctccaacaagaagcacct

【FIG. 9B】

ggagctggagccatgatcgtgggccgcaagttcctggtgaaggtgaacgcgaacatcggcaactccgccgtggcctcctccatcgaggag
gaggtctacaaggtgcagtgggccaccatgtggggcgccgacaccatcatggacctgtccacgggccgccacatccacgagacgcgcga
gtggatcctgcgcaactccgcggtcccgtgggcaccgtccccatctaccaggcgctggagaaggtggacggcatcgcggagaacctgaa
ctgggaggtgttccgcgagacgctgatcgagcaggccgagcagggcgtggactacttcacgatccacgcgggcgtgctgctgcgctacatc
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*gcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA*GTCCTGGCGACCCTGCTCCCCTGACCCCTGTTC
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AGATTGATCTGCCTGTTGCAGATCCCCCGACCCGCTGCCGGCCCCTCTGCCGTGCGACACCCCT
TGCCCTGGGGTGTGCCTCTTGTCCTGCATCGCACACCTCCTCCGCCGGACCTTCACCCCCTCCC
ACCTCGACACAAGCAGGTGTGGGACGTGATAGTGGTGGGCGCGGGCGTGGCCGGCGCGGCGC
TGGCGCATCAGCAGGGCTTGGACGGCCGACGCGTGCTGCTCCTCGAGCGGGATCTGGCCCAG
CCCGACCGCATCGTGGGCGAGCTGCTGCAGCCTGGCGGCGTGCTGGCCCTGGAGCGCCTGGG
CCTGGGCGGCGCCGTGGACGGCATCGACGCGCAGCCCGTGGTCGGGTACTGCATGTTCAAGG
GCGGGCGCGAGGCGTGCATCGCCTACCCCACCCCCGCCGAGCTGGGGGGTCCAGCGGCTGC
GGCTGCGGCATGCAGGGGCCCCACTGGAAGCGCCAGCGCCGCGCCCGCCGGCGACGCCCCC
GTCACGGGCTTCTCCTTCCACAACGGGCGATTCGTGCAGCGGCTGCGCGCCGCGGCGGCGGC
TGCGCCCGGGGTCACGCTGCGTCGCGGCACGGTGCGCGCGCTGGTGGATGACGCCGGCGCG

【FIG. 9C】

GACTGGGAGGAGGGGCGCGTGGTGACGGGCGTGCGGTACCGCGCGGGCGACGGCGGCGAGC
GCGTGGCACTGGGCCACCTCACCGTGGTCTGCGACGGCATGTACTCGGCCCTGCGGTCCAAGC
TGGCGGTGCCCGACCTGCGCACGCCCTCCCACTTCATC**aagctt**

【FIG. 10A】

aagcttTCACTGCTCCATTCACACCCAATTTCCCACCGCCGCACCCGCTCCCAGGTGGGAGGATGG
GAGGGAACCCAGCTGGGAGCCAGAGGAGCATGTGACCCCGGACCTGATCAAGCTATATGCCGC
CAGGGACGCTCGAGCACGGGATGTCAAGGCCGCGGCTGACAAGGCTGGCAAGGGCAGGACAC
CTGCGCCGAGGCAAACGCTGGAGACGGCCGGTCTCAGCATGTGATCCTGTACTTGCTGTGACAA
GGTGGACAATGCAGTACGAGTTGTACCAGGCTAAGTATCCCTAGTATACCGGCACGCCAGATCAT
CTGTACAGGAGTCTGTGCAGGAGTAAAAAGGCAACAGCCAGAGCCATGACCGGCGACTATCCAC
ATCCCGTCACTCCGATGCACTGGCTGACATCGGCGGCAGGTCGTCCAACGGTCCTGAATGATCG
AGAGGAAGCTGCCCGATTTCACCCCCCCCCCCCCACGTCGCCCTCCATGGCCGCACAGCATGCT
CAGCACAGGTTGCTGCGTGTCCTCACACAAACTGCTCCTTTAAAGCGATCAACTTTCCAGGGCAT
GGGGCACTCGTACTGACAATCACCCACATTCGTATACCTTTGACGTCATTTTTTTTTCGCCCCAAC
GCGGTTGCCATCCCGAGTTGTACCTCCGCGGCTACCATACCCCTGTCTTCTGGCCCTCACCGTC
GCTCGCAGGCGGGATCCAGCGCAGCCAGTCTGAATACTTTTACACAACATAGTACGTAACGCGCA
TTAGGCCCCCAATACCAGCAGCTGGCTCCAGCATGGGCAAGGGTGCGCGGCAGGAG**gaattc**ctttct
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cctcttcgtttcagtcacaacccgcaaac*ATGctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcagcgcctccatgac*
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tccctcgtgcgcaagttctccctcaacaccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaac

【FIG. 10B】

atcagcaacgccggccctggagccggttcgccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacagcac
cggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctctcctctggttcaaggg
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*gctccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag*TGATTGATTGGAACTCA
CAAAGCGGCCCACGGCTTCGAACGTCCCGTGTCAATTGCGCGGGGTGTGCCAGAGTTTCTGCGCCACCGATGCTCA
CCCTAGGGGGGGATGCCCTTTGACATTCATGTGTGCCTGCATGCACGTTTGTATCAGTCTCACCACACCTTGAAGATT
TTTGGGAGGGGGGGGGGAAGTCGGAATGGAAAC**gagctc**CCCAGGCGAGTCAATCAGTTGTGTCATGAGAT
TGATCTGCCTGTTGCAGATCCCCCCGACCCGCTGCCGGCCCCTCTGCCGTGCGACACCCCTTGC
CCTGGGGTGTGCCTCTTGTCCTGCATCGCACACCTCCTCCGCCGGACCTTCACCCCCTCCCACC
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CGCATCAGCAGGGCTTGGACGGCCGACGCGTGCTGCTCCTCGAGCGGGATCTGGCCCAGCCC
GACCGCATCGTGGGCGAGCTGCTGCAGCCTGGCGGCGTGCTGGCCCTGGAGCGCCTGGGCCT
GGGCGGCGCCGTGGACGGCATCGACGCGCAGCCCGTGGTCGGGTACTGCATGTTCAAGGGCG
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GCGGCATGCAGGGGCCCCACTGGAAGCGCCAGCGCCGCGCCCGCCGGCGACGCCCCCGTCA
CGGGCTTCTCCTTCCACAACGGGCGATTCGTGCAGCGGCTGCGCGCCGCGGCGGCGGCTGCG
CCCGGGGTCACGCTGCGTCGCGGCACGGTGCGCGCGCTGGTGGATGACGCCGGCGCGGACT
GGGAGGAGGGGCGCGTGGTGACGGGCGTGCGGTACCGCGCGGGCGACGGCGGCGAGCGCG
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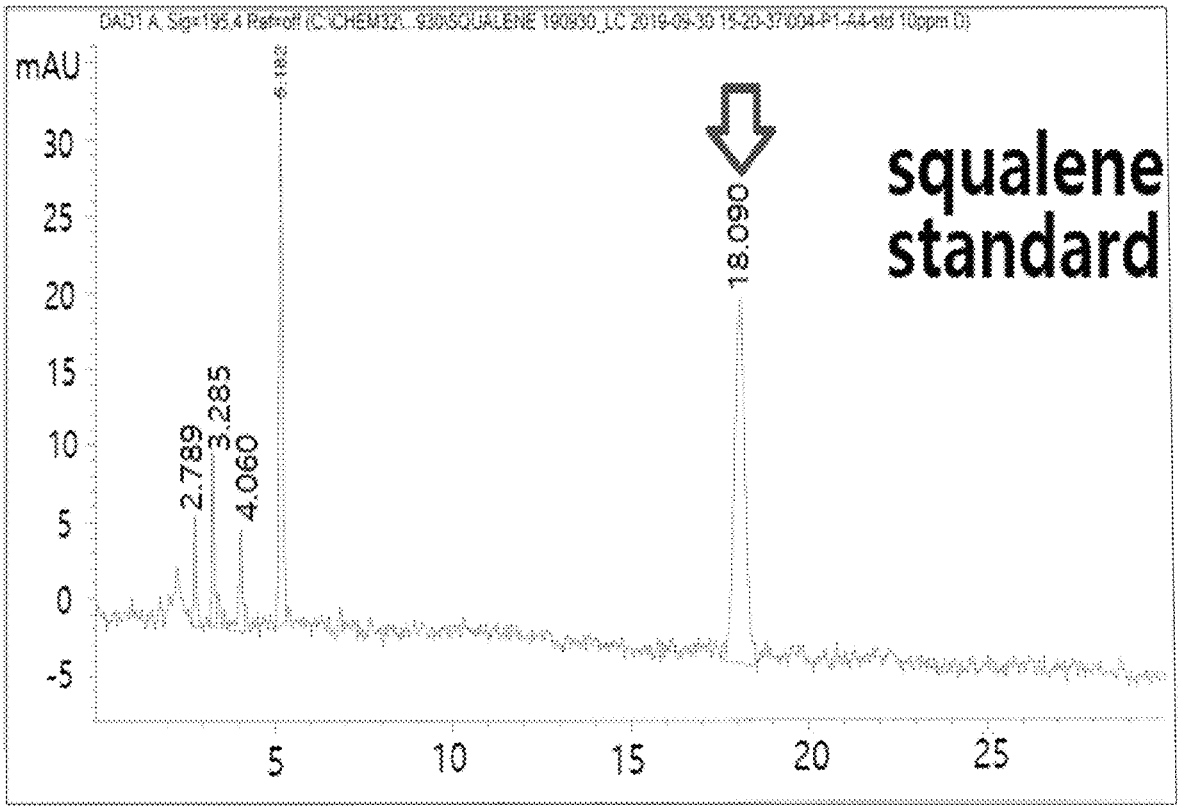
CGGTGCCCGACCTGCGCACGCCCTCCCACTTCATC**aagctt**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15
10
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-5
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3.285
4.060
18.090
squalene
standard
5
10
15
20
25

FIG. 11A

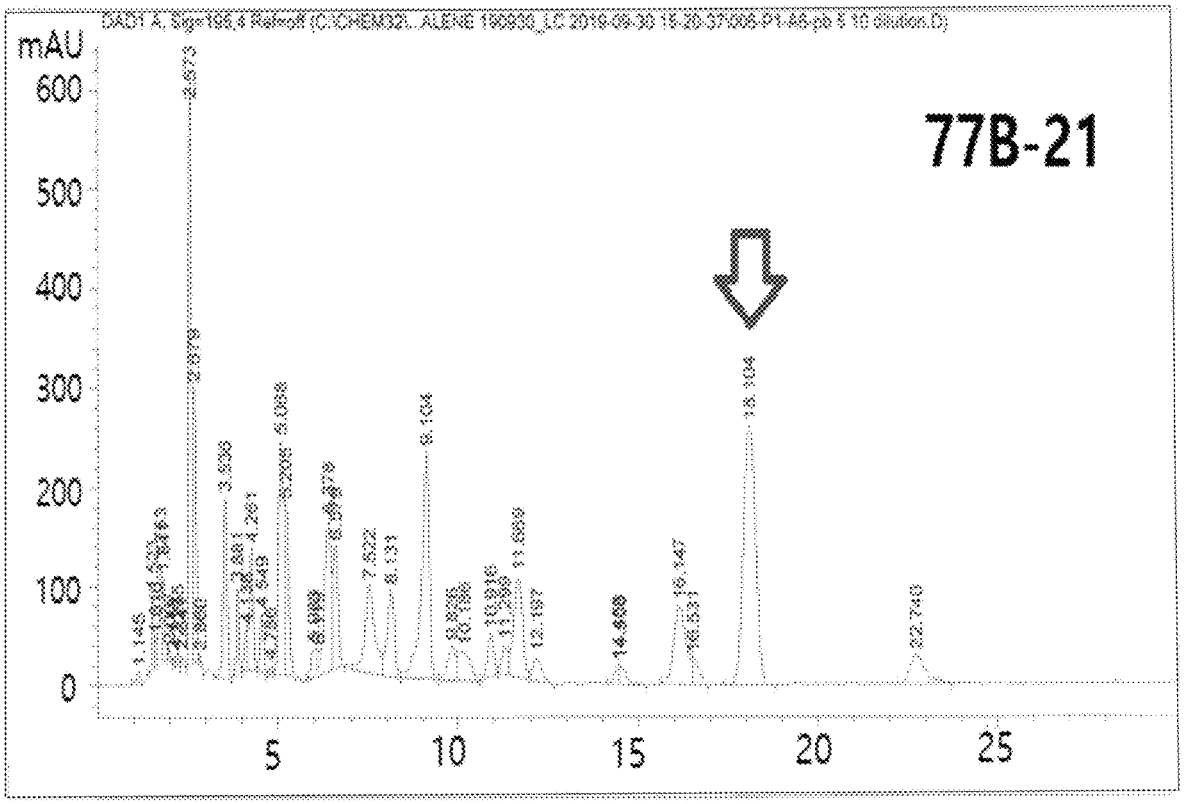


FIG. 11B

【FIG. 12】

MQFSLAGMNTRALQTGARPSLPAARPSRRVRPARRSAPCPVARTMGGGEEQPSSAEGV
AWDKISTDELADWAGAGPPTPLLDTVAFPVHIKNFNSRQLQQLCKELRADLIHTVAKTGGH
LGSSLGVVELTVALHHVFNTPEDRIVWDVGHQAYIHKMLTGRRARMHTIRQQGGLSGFTR
RAESVYDPFGAGHSSTSVSAALGMAVGRDRKGRANNCIAVIGDGAITGGMAYEAMNHAG
FLDTNMIVILNDNQQVSLPTQYNGKNQEPVGALSSALARLQANRQLRELREIAKGVTKQLP
DVIQNATAKIDEYARGMISGTGSTLFEELGFYYIGPVDGHNMQDLVDVLSEIKATETVGPVL
LHVVTQKGRGYTPAETASDRMHGVVQYDTLTGKQKKGSGGPQSYTNYFADALVAEAKRD
ARVLGIHAAMGGGTGMNRFEAAFPDRVFDTGIAEQHAVTFAAGLATEGLVPFVAIYSTFLQ
RGYDQIVHDVSLQSLPVRFALDRAGNVGADGATHAGAFDVTYLACLPNMVVMAPSNEAE
LVHAVATAAAIDDRPSAFRFPRGNGLGVDLAAAGVTDDLKGQPMEVGRGVVRRGGADVA
LLGYGTCVNACLAAADLLAAQGVSATVVDARFCKPLDTALVRRMAAEHPVMITVEEGSIGG
FAAHVMQFLALEGLLDGKLKFRPMTLPDRYIEHGTQAEQMAEAGLTASHIAGTALSVMGV
KRDAPSIFST*

【FIG. 13】

MRCSAQLNTRGPTLPNSARPRTCRVVSASAAPVPSAWPGRVVLPEKSASRTGPKKFSLL
GSTGSIGTQTLDIVAEHPDRFQVVSLAAGGNVALLAEQIARFSPSLVSVRDSGGARALEAA
LDAAGVDRRPEIQIGAAGIDAVAAHPEADACVTGIVGCAGLRPTMAAIEAGKDICLANKETLI
AGGPTVLPAAAKHGVSILPADSEHSAIFQCLQGLPEGGLRRIILTASGGAFRDLPVSELPKV
TVADALKHPNWAMGKKITIDSATLMNKGLEVIEAHYLFGASYDNIDIVIHPQSIVHSMIETQD
SSVLAQLGWPDMRLPILYTMSWPERVPCSEVTWPRLDFVKAGNLTFRQPDHAKYPAMEL
AYSAGRAGGTMTGVMSAANEAAVELFLEEAIGYLDIVPVVEAACEAHRVELVERPSLEEIV
HYDQWARRHVRESVAKRAPAAVPAL*

【FIG. 14】

MAAVVEAGHAASKQKTEAHQTKQEFLAVFEKLRDELLEDSILAGQPESSKDWLRTMLDYN
VPHGKLNRGMAVLDVLLAARGGDVTEKEREAANVLGWCIELLQAYFLVADDIMDSSLTRR
GQPCWYRQPHVGMVAINDGIILESCIYRLLKLHFRAHPAYVHLLELFHDTTHRTAHGQLLDT
TTAPPGGVDLTRYTEGTYLRIVTYKTAFYTIYLPVACGLALAGVTDEASLALAEDLSVRMGR
YFQIQDDVLDAFGEPEVIGKVGTDIQDSKCSWLVVRALAVASAEQREAIKANYGRDDAEAV
EAVKAVYRELDLPAAFAAYEQESYDGLVQAIEGQDKFPPAVFMGILAKIYKRTK

【FIG. 15】

MGKLGELLSHPDEIIPMAAMYLAARRAAVLPHDPDLAFCYSMLNKVSRSFAIVIQQLPEQL
RDAVCVFYLVLRALDTVEDDMAIDQAEKVPILLSFHEKTYEKDWSMKCGHGHYVELMEQY
PVVCAAFQGLEPQYQEVITDICRRMGAGMAEFIVKEVETVKDYDLYCHYVAGLVGVGLSN
LFAGSGLESEDFASLHELSNGMGLFLQKTNIIRDYLEDIMEEPAPRMFWPKEIWGKHGDSL
EDFKDPENAEAAVACLNDMIADALRHVDASLDYMQRLRNRPIFRFCAVPQIMAIGTLAACF
DNPSVFTGVVKMRRGQTAKIMHDVEDYADLLAYFRAFGQALAAKARAARGKGAESVGRA
AERVVAGCSAALADLSRAENARMAAAARRPLSLPARALLLVAALLYLFLAWRAEGVRRWL
GVDSPPAAHKLDYYNQIVASMFLGYSLFAVGTGRRP*

【FIG. 16A】

aagcttGCACAGTCAGTCGTCATCCACGAAGTCGCGCCCGTCTGTCCACCGGGGGTCTCCTGAACG
CAGCAATCTCCTCCTGAGTGTATGAGCCCGTGGCCGGGAGTTTGTATGCAGGGCGAGGCAAGGA
CGACCATGCCGGGAGAAACCCAAGGTGACGAAGTGACATTGTGCTCGATCACTCCATGCACTGC
CTCACTCGCCCATGTACCTTGGTCATGTACTCCCCAGTCTGCATCTTGGTGTTCCTGTTCAGCTC
GCGGAGCTCCTCCAGGCGCTGCTCGTCGCGGCGGTCCCGCGAGATGTAAAAGGCAGGGACAC
CCACCCCCAGGGCCACTCCCAGTAGAGCCCCCCCTACAGCAACCAGGGTGTCCGGATCGCTAA
AGTCGATGTTGATCGCGCGGCACGGTGGCATTATCGGGCGTGGATGGCCCCATGATCGGTGCAA
CGAAGGCGCCCTCATGGCAGGTCCGCATGGTCGTCCATTGCAGGGGATACCCGCTCGCACTTTC
GTTGACAATAACATCCTCGTATAGTTGGAGAAAGGATTTGTGATCTGTCTCTGGAGGCCCTTAAAG
TCCTGCCCCTCCTCTGCTGGAACCTGACCTCTCATGCCCCTGCGCCACGCCCCCGGATCTGATA
TGGCTCTGATATGGGTGGCTCGTACCTCTTGGCTAGGCGACCCCCCTAAGCACGCGTGCGGGCC
AGGGCACAACATTATATTTTGCCCTCTCCTTCGTCAACGCTCATTTTTTTGGAATACTAACGTTTAA
AAGCTCTCG**ggtacc**tcccgcttttaattgagccccttcgtcgctgaatcagcgaaagcaccgcgaaacaatgcctgtcccgtccatg
catctcaacagcctcatgcaaggtttgcacaagcaagaccattctgatctgggaacttgtaggtgttgtatgggggaggttgtgctcttgaatcaa
gtggtatcacgtttccggaacaccccgaaacgtgcatgggcttattgcgatgagagcatttcccaccgcgattgtctcacgcgcatttcggaga
aggtttgcagaacactccaggacatgaaatgccttgtcacgtatgaaccatctcccacggccttgaaaagatcgctcgacttccattctagatg
gtgcaaaaccctacgactcaagaaggtgccaccgactcaggcattgggcacggcgggcagggagaagagaggagttgatcaaaactgc
tcgatcacgttcccccatggcgatccgagcagcacatgatgcatcgaggtggcgccgttgcaaaggagttgcgcatgggtcgaagcaggga
gaaggaaacggcgaggcgtgccgcgggggtgaattcagagtcaaatctgcgcctgccccggcgctcctgacggggattaacccccacga
ctgtatccatcgacactcgtctcgggggaataaaagcggcgacccagctccagaggcgcaatccttctcacaatctgtttaacttcaacaaag
tataagtcaattcaacttgacaca*ATGgccgcgtccgtccactgcaccctgatgtccgtggtctgcaacaacaagaaccactccgcccgccc*
caagctgcccaactcctccctgctgccggcttcgacgtggtggtccaggccgcggccaccccgcttcaagaaggagacgacgaccaccccg
cgccacgctgacgttcgacccccccacgaccaactccgagcgcgccaagcagcgcaagcacaccatcgacccctcctcccccgacttcca
gcccatcccctcctttcgaggagtgcttcccaagtccacgaaggagcacaaggaggtggtgcacgaggagtccggccacgtcctgaaggt
gccccttccgccgcgtgcacctgtccggcggcgagccgccttcgacaactacgacacgtccggcccccagaacgtcaacgcccacatcgg
cctggcgaagctgcgcaaggagtggatcgaccgccgcgagaagctgggcacgccccgctacacgcagatgtactacgcgaagcagggc
atcatcacggaggagatgctgtactgcgcgacgcgcgagaagctggaccccgagttcgtccgctccgaggtgcgcgcggggccgcgccatc
atccctccaacaagaagcacctggagctggagcccatgatcgtgggccgcaagttcctggtgaaggtgaacgcgaacatcggcaactcc

【FIG. 16B】

gccgtggcctcctccatcgaggaggaggtctacaaggtgcagtgggccaccatgtggggcgccgacaccatcatggacctgtccacgggc
cgccacatccacgagacgcgcgagtggatcctgcgcaactccgcggtccccgtgggcaccgtccccatctaccaggcgctggagaaggtg
gacggcatcgcggagaacctgaactgggaggtgttccgcgagacgctgatcgagcaggccgagcagggcgtggactacttcacgatcca
cgcgggcgtgctgctgcgctacatccccctgaccgccaagcgcctgacgggcatcgtgtcccgcggcggctccatccacgcgaagtggtgc
ctggcctaccacaaggagaacttcgcctacgagcactgggacgacatcctggacatctgcaaccagtacgacgtcgccctgtccatcggcg
acggcctgcgccccggctccatctacgacgccaacgacacggcccagttcgccgagctgctgaccccagggcgagctgacgcgccgcgcg
tgggagaaggacgtgcaggtgatgaacgagggccccggccacgtgcccatgcacaagatccccgagaacatgcagaagcagctggagt
ggtgcaacgaggcgcccttctacaccctgggccccctgacgaccgacatcgcgcccggctacgaccacatcacctccgccatcggcgcgg
ccaacatcggcgccctgggcaccgccctgctgtgctacgtgacgcccaaggagcacctgggcctgcccaaccgcgacgacgtgaaggcg
ggcgtcatcgcctacaagatcgccgcccacgcggccgacctggccaagcagcacccccacgcccaggcgtgggacgacgcgctgtcca
aggcgcgcttcgagttccgctggatggaccagttcgcgctgtccctggaccccatgacggcgatgtccttccacgacgagacgctgcccgcg
gacggcgcgaaggtcgcccacttctgctccatgtgcggccccaagttctgctccatgaagatcacggaggacatccgcaagtacgccgagg
agaacggctacggctccgccgaggaggccatccgccagggcatggacgccatgtccgaggagttcaacatcgccaagaagacgatctcc
*ggcgagcagcacggcgaggtcggcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA*GTCCTGGCGACC
CTGCTCCCCTGACCCCTGTTCCCCTGCGCTGCTTCTCCCCGGTGACATCCGACCTGCTGCAAAATTCCCGTTCCTGC
ACAACACTTGCCTGACCGAGGGTCGGGTCGCGAAGTAAAAGCCACAATCAACACCCCAGGCACATTAAGAGTGCACA
GCATGACGCAGCATAGGGTTTGTGTCGGAGGAAGGGGGTCGAGTCGCGTTGGCGAGGGGGTGGTCACGATGACCA
CATCTGCGGGATAATTGAATCCTCAGGGGAAAATACCAGTCTCTGCTTCCAGGTGCTCCG**actagt**cttgcagtgccccaa
aaactggctaccacctaacaattctcacgcagtttatcctctgcactttgatgtcagcttttgattcgtctgcgtacattacagcgttgagtggcca
gcaggaaggagaccgcggtccgagacgagtctgagggcgcgctctcgcaacttggattccggatttcttaccctgcatcgacctcggcctgg
agtcgatcagaaattgtcattgccagattgcctggcgaggacgggtgatatactcaaggcgttgcatcgcccacaaaacacacacttatctgc
aagggagttactgcatcaggctctgctcaacagctcgtgacatcgatcgttcagctccccagcaggtgcgtgtccgcatggagcacccctccc
gagacacctgcgttgggtgtcggaggagctcacatgccagggaggtgcccacattgcaccacgcgaccgcgaaataggcagacttcggg
catcctgtcatcgcatgtccgctggccgggaatcatggcctccccaccaggcgtcacgcgctgcccacctccctccccttgctgcgcagggca
ccgcgttcctgtggagagccgaccacATGGTCCTGTGCGCTGGTAGTTTGAGCTGCAGGGCGCCGCCTTCA
GGCGGTGCTAGGTTGGAGCGGGGGTCCCCCTTCGTGCGCCTGGGCCACCATGCCCGCCCACA
AGCTCGCAGACGGATGTCAGACCTCGTAATAAGGTCCATCGCAGCCCCTGCTCCGCCCCCGCTG
CGACCTGCATCTCAGCCTCAAGCCCCACCTGCATCAGAGACCTTCACGGTGAGTCATGATCGAG

【FIG. 16C】

GTCGGCCCCTGCAGCGCTCGTGCTCCGGAAACGACCCGCTACTCAATCCCTGAACCATGAATAC
TTCAGGGGGGCCGCGAACTGGCCAACCGCCCTCCCTTCTCCCTCCAAGACATCCGCAACGCCA
TCCCGGCGGAGTGCTGGAAGAAGGACACCTTCAAGTCCTTCGCCTACCTGGCGCTAGATGTAGG
AATCGTTGCTGCCCTGGCCGTGACAGCACATGCCGTCAACTCGCCATGGCTCTGGCCCTTCTAC
TGGCTGGCGCAGGGGACCATGTTCTGGGCCCTGTTTGTCGTCGGCCACGACTGGTGCGGTTGG
AGGGGGTAATCTGGCGACCCTGCAGGGCATGCAGTGGGGACAAGAGCATCGCCAGGCTCCGCC
TTGCCTGCTGATCCCAGCCCGACTTGGCTGGACAATAGATGCTGTCGGGGACATGCCGCAGTGC
ATGCCACAATGGGCCCCTTCAACAACTGACCACCACTATGACCCATATTCCCTGCAGCGGCCACC
AGAGCTTCAGCACAAACAAGCAGCTGAATGACGTGG**aagctt**

【FIG. 17】


ggaatcccgcctccgagatgaagccgtggttggcacggaggaggccgctgcgggccagagtgttcttctgctgcacgtcctcc
ggctttggtggctcgctgggcttgggtgcggccatgagctgcagtgcaagtgtacatataggtcaatcttatgacccggcactac
caatgatgatcaacaccgagcggccctctgtgttgtgcttgcctctttaccttcactgcgtactgctgcaggagcttcatgaggatc
acactgacggtcaggggatcagcacccagtcccggacatcccgatccagtacgaggtcctggctgaccatgatggtaggt
gaagttgggccctgggaggagcgctagaggagcctcggggcaaagatcaccctactctgacgtggctggctcaatcaccca
tccctcccctttgaagtcggctctcagtttgcgttgtttcgaaatcgagccacaatcgaatatacactacctaaaggctctcaccac
ctggcgtacctcggaatgcccatcagcccaaacacatgagaaaaggcgcgcgcggttcgaccccagtccgtcgattgacgc
agtggggagctccattctgtcagctcttgggtggccaggtcgctgacagattggcacatacaggaccctgccgacccgttcctc
cagcactttgtgaatttaagcagcgcattagatcgtcgatggcttagagaaccccgcgcctgctcccccatctccctttcacacgt
ttgaacacccggaccggcc

【FIG. 18A】

aagcttAGCATACTCCTATTCTGACAATGTCACAGTCGGTCTGCCAGGCGATAGTGGCTTTGCTGTC
AGACTCGGCCCCGGACTCTCCCCTGAACTGCGACGCCGGGAATCTGTTGAGAGGAGGCGATCT
GCGAGGGTTCGCCTCCATGGCCCGCATGTACACCATCGAGTATGCCATGAAGCGATGATGTCTGT
GAAAATGATGTTCAGAATTCATTATATACTCATGTTTTTGTGTAAATGCTGTGTCGACTTAAGTTACC
GAGCTGGCTGACAGAGACAATCTTCAGGTCAAATGTTGGCACCAATGATCGCGACGATCGTTCA
GGGGTTATCAAGTCAGATCTGAACGAAAACCAGAAATCAAATTTGCCAAAGCGCATGTTTGTATGT
CGAGAATTATCATGCGGGTGACTGGCTCGCTAATTCTGGCATGGAAGGATGCCACATCGAATTGA
TCCGGGGAGACTAACACTTGTCAGAATTGCAATGTGCCATATTCCAGATATCCCAGCCGGCCCTT
CTATAAACCACCTGCGGGCTCAGATACCTACGAAGAGGCTCAGATAACTCAAGGACGTGCATTCG
AATTATCCCTGCCGCGCGGAAACATCAGACCAGGTGCGGATGCTGAGCGTCGAGTTGGGTGCTT
GATAGACCTTCACCTTGATCTGAGGTTCCCGTCCCCAGAGCACTCGAATCTCCGGCATCTTACAG
GCAAACCGCAAACAGTAAATAATGGCGAGCACCATCACCATG**ggtacc**cttgcagtgccccaaaaactggctac
cacctaacaattctcacgcagttttatcctctgcactttgatgtcagcttttgattcgtctgcgtacattacagcgttgagtggccagcaggaagga
gaccgcggtccgagacgagtctgagggcgcgctctcgcaacttggattccggatttcttaccctgcatcgacctcggcctggagtcgatcaga
aattgtcattgccagattgcctggcgaggacgggtgatatactcaaggcgttgcatcgcccacaaaacacacacttatctgcaagggagttact
gcatcaggctctgctcaacagctcgtgacatcgatcgttcagctccccagcaggtgcgtgtccgcatggagcacccctcccgagacacctgc
gttgggtgtcggaggagctcacatgccagggaggtgcccacattgcaccacgcgaccgcgaaataggcagacttcgggcatcctgtcatcg
catgtccgctggccgggaatcatggcctccccaccaggcgtcacgcgctgcccacctccctccccttgctgcgcagggcaccgcgttcctgtg
gagagccgaccac*ATGtccgccgccgccgccgagaccgacgtgtcctgcgccgccgctccaactccctgaacggcaaccacaccaa*
cggcgtggccatcgacggcacccctggacaacaacaaccgccgcgtgggcgacaccaacacccacatggacatctccgccaagaagac
cgacaacggctacgccaacggcgtgggcggcggcggctggcgctccaaggcctccttcaccacctggaccgccgcgacatcgtgtacgt
ggtgcgctaccactggatccccctgcatgttcgccgccggcctgctgttcttcatgggcgtggagtacaccctgcagatgatccccgcccgctccg
agccccttcgacctgggcttcgtggtgacccgctccctgaaccgcgtgctggcctcctccccgacctgaacaccgtgctggccgccctgaaca
ccgtgttcgtgggcatgcagaccacctacatcgtgtggacctggctggtggagggcgcgcccgcgccaccatcgccgccctgttcatgttca
cctgccgcggcatcctgggctactccacccagctgccccctgccccaggacttcctgggctccggcgtggacttccccgtgggcaacgtgtcctt
cttcctgttcttctccggccacgtggccggctccatgatcgcctccctggacatgcgccgcatgcagcgcctgcgcctggccatggtgttcgacat
cctgaacgtgctgcagtccatccgcctgctgggcacccgcggccactacaccatcgacctggccgtgggcgtgggcgccggcatcctgttcg
actccctggccggcaagtacgaggagatgatgtccaagcgccacctgggcaccggcttctccctgatctccaaggactccctggtgaacTG

【FIG. 18B】

AGCGGAGGCCTTGGAAATATTCGCGTCACGCGAGGAGTAGGCTCTGCTGGTCGGCCCTGGATACGCTGACTCTTCAA
GCAGTGGGGCACCACACCCACCTTTTGCCAAGGGCAAGGAGTCGGAAGGGGGCGGGGCTGCCATGCACCCCTGAC
GGGCATGGCCGTTCCGCGAGGGCGCCAACTGCGGCGGCCTGCCCGCTGGCTCGTGCCCCCCTACCCCCACCATTG
CCTGGAGCGTTTCCATCCCCAAATCACATTCCATCCAAGTTGTATCACTATGCCCCTTTGGCTCTATACACTCACGGCC
TGAGGTCCCTTCTCGGCCGTGGCGGCACACGCCCAACCCCCCCACCATACTCTTTCCATACACTGCAATGCTTCGAGC
CTGCCTGCCACCTGCTCTGCTTGTCTCCCCTCCCTTCCCTTGAGGTTTTCCAATGCAGTAAGAGAAGTCGACGTGCAT
GGACAGATGATTGAGAGATGAG**actagt**ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccgg
cgctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttta
aatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacacaggccact
cgagcttgtgatcgcactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccgcaaad*ATGctgctgcaggccttcctgttcctgct*
ggccggcttcgccgccaagatcagcgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcaccccctaacaagggctggat
gaacgaccccaacggcctgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggga
cgcccttgttctggggccacgccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccg
gcgccttctccggctccatggtggtggactacaacaacacctccggcttcttcaacgacaccatcgaccccgccagcgctgcgtggccatct
ggacctacaacaccccggagtccgaggagcagtacatctcctacagcctggacggcggctacaccttcaccgagtaccagaagaacccc
gtgctggccgccaactccacccagttccgcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgaccgcggccaagtccc
aggactacaagatcgagatctacctccgacgacctgaagtcctggaagctggagtccgcgttcgccaacgagggcttcctcggctaccag
tacgagtgccccggcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatctccatcaaccccggcgcccc
ggccggcggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcgaggccttcgacaaccagtccgcgtggtggacttcgg
caaggactactacgccctgcagaccttcttcaacaccgaccccgacctacgggagcgccctgggcatcgcgtgggcctccaactgggagtac
tccgccttcgtgcccaccaaccctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccaggccaaccccggagacg
gagctgatcaacctgaaggccgagccgatcctgaacatcagcaacgccggccctggagccggttcgccaccaacaccacgttgacgaa
ggccaacagctacaacgtcgacctgtccaacagcaccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctc
caagtccgtgttcgcggacctctccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctcc
ttcttcctggaccgcgggaacagcaaggtgaagttcgtgaaggagaaccccctacttcaccaaccgcatgagcgtgaacaaccagccctca
agagcgagaacgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtcc
accaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttcc
*aggtgcgcgaggtcaagTGA*TTGATTGGAACTCACAAAGCGGCCCACGGCTTCGAACGTCCCGTGTCAATTGCGCGGG
GTGTGCCAGAGTTTCTGCGCCACCGATGCTCACCCTAGGGGGGGATGCCCTTTGACATTCATGTGTGCCTGCATGCA

【FIG. 18C】

CGTTTGTATCAGTCTCACCACACCTTGAAGATTTTTGGGAGGGGGGGGGGAAGTCGGAATGGAAACctcgagCAACG
CTACGCAACTCCCTTCGATGGCTTCAAGTACGGAGATGTGGGCATCCAGGATTCGCATGTGCTGC
TTCAGCCCTCCTCATGCCACTAGCACTCATTTTTCGACTCCCGGATTGCCAGGTTCAAGGGCATC
AAGGAGTCGGAGATCAGCCGCGCCATGACCTCCCGCTACTTCGAGGACCTAAACGTCAATGCCG
AGGTGCTTTTGCATATATTTACAGCTAATTATGATGGGTGTGGTGCGCGATATGCTTGCAAGGTCTC
CGGTGAGCTAATGATCGGCACATCCCTTCCGCGCATCCGCAGGTCGATGTCGTCATTGTTGGGG
CTGGGTCTGCGGGCCTCTCGTGCGCCTATGAGCTGAGCAAGCACCCGGATGTCAAGGTATGGG
CTGAGCAGGGCACATCCTCAGATGATGTTGCTGTAATTGCAATTGAAACTTGCGGTTGTTCCCAG
CACAGCCTCAATCAATCATGTGTGCTGCGTTGGAAACGCTATGATACCCCAGCCTTCAACATGGG
GCAGGGATATCGTTTACACCTGCTTGAACCCCCCGCAACAGGTGGCCATCATCGAGCAGGGCGT
CGCCCCTGGGGGTGGAGCGTGGCTGGGGGGTCAGCTCTTCTCGGCTATGTGTGTGAGTCTAGG
CACGGGGACGGGTGGACTGAAGCAAGGGTTGGGCGCAGGGTGTTGATATCCATGTGTTGGACA
TTCTCGTTGGGAAAACAAGATGTGTGTATTTAGTGCTATCTCGGTGGCTGCATTCCaagctt

【FIG. 19A】

aagcttAGCATACTCCTATTCTGACAATGTCACAGTCGGTCTGCCAGGCGATAGTGGCTTTGCTGTC
AGACTCGGCCCCGGACTCTCCCCTGAACTGCGACGCCGGGAATCTGTTGAGAGGAGGCGATCT
GCGAGGGTTCGCCTCCATGGCCCGCATGTACACCATCGAGTATGCCATGAAGCGATGATGTCTGT
GAAAATGATGTTCAGAATTCATTATATACTCATGTTTTTGTGTAAATGCTGTGTCGACTTAAGTTACC
GAGCTGGCTGACAGAGACAATCTTCAGGTCAAATGTTGGCACCAATGATCGCGACGATCGTTCA
GGGGTTATCAAGTCAGATCTGAACGAAAACCAGAAATCAAATTTGCCAAAGCGCATGTTTGTATGT
CGAGAATTATCATGCGGGTGACTGGCTCGCTAATTCTGGCATGGAAGGATGCCACATCGAATTGA
TCCGGGGAGACTAACACTTGTCAGAATTGCAATGTGCCATATTCCAGATATCCCAGCCGGCCCTT
CTATAAACCACCTGCGGGCTCAGATACCTACGAAGAGGCTCAGATAACTCAAGGACGTGCATTCG
AATTATCCCTGCCGCGCGGAAACATCAGACCAGGTGCGGATGCTGAGCGTCGAGTTGGGTGCTT
GATAGACCTTCACCTTGATCTGAGGTTCCCGTCCCCAGAGCACTCGAATCTCCGGCATCTTACAG
GCAAACCGCAAACAGTAAATAATGGCGAGCACCATCACCATG**ggtacc**cttgcagtgccccaaaaactggctac
cacctaacaattctcacgcagttttatcctctgcactttgatgtcagctttttgattcgtctgcgtacattacagcgttgagtggccagcaggaaggga
gaccgcggtccgagacgagtctgagggcgcgctctcgcaacttggattccggatttcttaccctgcatcgacctcggcctggagtcgatcaga
aattgtcattgccagattgcctggcgaggacgggtgatatactcaaggcgttgcatcgcccacaaaacacacacttatctgcaagggagttact
gcatcaggctctgctcaacagctcgtgacatcgatcgttcagctccccagcaggtgcgtgtccgcatggagcacccctcccgagacacctgc
gttgggtgtcggaggagctcacatgccagggaggtgcccacattgcaccacgcgaccgcgaaataggcagacttcgggcatcctgtcatcg
catgtccgctggccgggaatcatggcctccccaccaggcgtcacgcgctgcccacctccctccccttgctgcgcagggcaccgcgttcctgtg
gagagccgaccac*ATGtcccccccccaactccatgtcccccgccaccaacggctccaccaacggcgtggccatcaacggcgccaagaa*
gctgctggacttcgaccccctccgccgcccccccccttcaagatcgccgacatccgcgccgccatcccccccccactgctgggtgaagaaccccct
ggcgctccctgtcctacgtgctgcgcgacctgctggtgatcctgtccttcgccgtggccgccaccaagctggactcctggaccgtgtggccccctg
tactggatcgcccagggcaccatgttctgggccgtgttcgtgctgggccacgactgcggccacggctccttctccgactcctggctgctgaaca
acgtgatgggccacatcctgcactcctccatcctggtgccctaccacggctggcgcatctcccacaagacccaccaccagaaccacggcaa
cgtggagaaggacgagtcctgggtgcccctgcccgagaaggtgtacaagtccctggacaccggcaccaagttcatgcgcttcaccatcccc
ctgcccatgttcgcctaccccatctacctgtggcgccgctcccccggcaagaagggctcccacttcaaccctactccgaccigttcgccccca
acgagcgcacctccgtgatgatctccaccctgtgctggaccgccatggccctgctgctgtgctactcctccttcatctacggcttcctgcccgtgtt
caagatctacggcgtgccctacctgatcttcgtggcctggctggacatggtgacctacctgcaccaccacggctacgagcagaagctgccctg
gtacaggggcaaggagtggtcctacctgcgcggcggcctgaccaccgtggaccgcgactacggcgtgatcaacaacatccaccacgaca

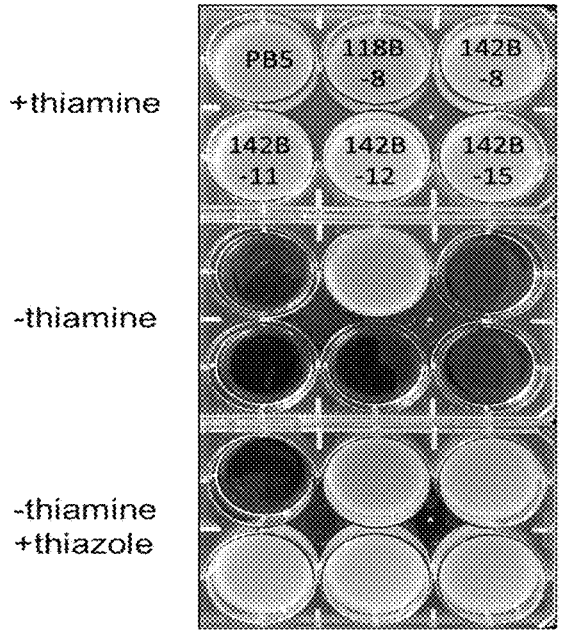
【FIG. 19B】


tcggcacccacgtgatccaccacctgttccccagatgccccactaccacctggtggaggccacccaggccgccaagcacgtgctgggca
agtactaccgcgagcccaagaagtccggccccttcccttccacctgttcggctacctggtgcgctccctgggcgaggaccactacgtgtccg
acaccggcgacgtggtgttctaccagtccgaccccccacatccccaagttccccacctccgccaccaccaagtccaagtcctcctgagTGAT
CCGGGAGGAGGGAGTGAGCGGGGAAGGGGGCAGCCACACGGGGCCCGTCTCGACCTGCCACCCCTCCCCTCGTC
GAGCCCTGCCCAGGGGGCGCCGCAACGAGCCATGCGTGTGCATGTGTCTGGAGGGCCCTTCCACCGGGCGATGTG
CGAGCCATCCTCGCCTATTTCAACACACCGCTGCCGGCATGCGCTCCACTCCCCCCAAAACCACCTCGACCCTCCCA
GGGCTCCTCCCCCGCCCCACCCTGCCTGCTGATATAGAAACCAGTGTTCTGTGAACGTTTGACATGCTCAACGAGGG
TACAGGGGTGCACCAACAGAGGAGGAGTGGTTCACACAGTCGGATAC**actagt**ccttcctgtcccacaatgcttggtgaatgca
gtgggttgatcaccgcggaggagctgtggcttactcgttctgatcaagggagcctctgcaccttaaccctgccaggatcgaaaccaaccttgtc
agtcccgtggtgggcaacatcatcctcgtgaagctgattgaccaggaaaacatgatgagtcggtatgaggacgagcatgagtggcccaaca
tcgatatgacacatcttggagtttacggcaaatgtatcacacttccatcctggcttgcaccacaatattagtggacccctccttgcagtggcacggt
gagaagctagtttgtagtaatcttcttaattgacgaaccagacgtgtgtaatggcctccttgagtgatggaaggatggaacctacccccccccctc
cccagtactctgcggtacatccgagtaaccctccattgatcagcccaaacgcaatatgcaacgactctacatacggccaccgagtgcttattc
cttcgctatcaccgcacaaaaatcccatccgcgaactcatccgaggtgatagattgcgatcggggttattcgggttaaggtgcgactagggatc
cctgaatcttttggggatttccccgggtctcgtcctgcatgcttatcatcagtctcgtgggttatttggatcgctgcgcatgccataacagagcgctca
taatatttgctgcggcggtggtgctggcaaaatcccctgcgtaccgggcgcctgtcaagccaaccccgccgtgcggcactcccctgcagatcc
atcacc*ATGatcgagcaggacggcctccacgccggctccccgccgcctgggtggagcgcctgttcggctacgactgggcccagcagac
catcggctgctccgacgccgccgtgttccgcctgtccgcccagggccgccccgtgctgttcgtgaagaccgacctgtccggcgcccctgaacg
agctgcaggacgaggccgcccgcctgtcctggctggccaccacccggcgtgccctgcgccgccgtgctggacgtggtgaccgaggccggcc
gcgactggctgctgctgggcgaggtgccccggccaggacctgctgtcctcccacctggccccgccgagaaggtgtccatcatggccgacgc
catgcgccgcctgcacaccctggaccccgccacctgccccttcgaccaccaggccaagcaccgcatcgagcgcgccccgcaccccgcatgg
aggccggcctggtggaccaggacgacctggacgaggagcaccagggcctggccccgccgagctgttcgccgcctgaaggcccgcat
gcccgacggcgaggacctggtggtgacccacggcgacgcctgcctgcccaacatcatggtggagaacggccgcttctccggcttcatcgac
tgcggccgcctgggcgtggccgaccgctaccaggacatcgccctggccacccgcgacatcgccgaggagctgggcggcgagtgggccg
accgcttcctggtgctgtacggcatcgccgcccccgactcccagcgcatcgccttctaccgcctgctggacgagttcttc*TGAGCTGTTCCT
AGGAACGTGGAGGAGGTGCAAGGAGGGTGATCTCACCCTGGTGTGTCTCTTCATGGAGCTCAGATCTTGAAAACTGT
GAGGTGCTTATCCGATACCTGCTTCGTGCATGGCTTGTGCGATATGTACACGCATTTGCAGATTGGTGGGAGCAGCAG
ATTGGTGGGAGCAGCATAGAGCTTTAGAAGGGGCTTAGGAGCGGGAATGTGAAACTCAGGCGGTTGGGCCAGATGA
GAGCGCAAAG**ggatcc**CAACGCTACGCAACTCCCTTCGATGGCTTCAAGTACGGAGATGTGGGCATC

【FIG. 19C】

CAGGATTCGCATGTGCTGCTTCAGCCCTCCTCATGCCACTAGCACTCATTTTTCGACTCCCGGAT
TGCCAGGTTCAAGGGCATCAAGGAGTCGGAGATCAGCCGCGCCATGACCTCCCGCTACTTCGAG
GACCTAAACGTCAATGCCGAGGTGCTTTTGCATATATTTACAGCTAATTATGATGGGTGTGGTGCG
CGATATGCTTGCAAGGTCTCCGGTGAGCTAATGATCGGCACATCCCTTCCGCGCATCCGCAGGTC
GATGTCGTCATTGTTGGGGCTGGGTCTGCGGGCCTCTCGTGCGCCTATGAGCTGAGCAAGCACC
CGGATGTCAAGGTATGGGCTGAGCAGGGCACATCCTCAGATGATGTTGCTGTAATTGCAATTGAA
ACTTGCGGTTGTTCCCAGCACAGCCTCAATCAATCATGTGTGCTGCGTTGGAAACGCTATGATAC
CCCAGCCTTCAACATGGGGCAGGGATATCGTTTACACCTGCTTGAACCCCCCGCAACAGGTGGC
CATCATCGAGCAGGGCGTCGCCCCTGGGGGTGGAGCGTGGCTGGGGGGGTCAGCTCTTCTCGG
CTATGTGTGTGAGTCTAGGCACGGGGACGGGTGGACTGAAGCAAGGGTTGGGCGCAGGGTGTT
GATATCCATGTGTTGGACATTCTCGTTGGGAAAACAAGATGTGTGTATTTAGTGCTATCTCGGTGG
CTGCATTCC**aagctt**

【FIG. 20】



【FIG. 21】

ATGgccaccgcctccctgcccgtgcaggtggccgtgacctccacccactgcttcggcctgcgcgagccccgccgcaagcg
ccagtgggcccgccagacccgctgccacgcctccgccgccggcaagcccaagcgccgcgtggtggtgaccggccagggc
gtggtgacctccctgggccagtccacccagcagttctacgaccagctgctggccggcgcctccggcatcacccacatcgagg
gcttcgacacctccgactactccaccaagatcgccggcgaggtgaagtccgtggacgccgccccctacgtggcccgcaagt
gggtgaagcgcatggacgaggtgatgaagttcatgttcgtggccggcaagcaggccctggaggacgccggcctgcccttcg
agggccccggcctggaggacctggaccgcaagctgtgcggcatcctgatcggcaccgccatgggcggcatgaccaccttcg
cctccggcgtggaggccctgaccctgtccggccaccgcaagatgaaccccttctgcatcccctttctccatcggcaacatgggc
ggcgccatgctggccatggacctgggcttcatgggccccaactactccatctccaccgcctgcgccaccggcaactactgcat
catctccgccgccgaccacatccgcaacggcgacgccgtgctgatgctggcgggcggcgccgacgccgccgtgatcccctc
cggcatcggcggcttcatcgcctgcaaggccctgtcccgccgcaacgacgcccccgagcgcgcctcccgcccctgggacgc
cggccgcgacggcttcgtgatgggcgagggcgccggcgtgctggtgctggaggagctggagcacgcccgcgcccgcggc
gccaccatcctggccgagttcatcggcggcgcggccacctgcgacgcccaccacatgaccgagcccgagccctccggccg
cggcgtgcgcctgtgcctggagcgcggcctggccgccgccggcgtggcccccgaggaggtgacctacgtgaacgcccacg
gcacctccacccccgccggcgacgtggccgagttccgcgccatccgcgccgtgctgggccacgacggcctgcgcatcaact
cctccaagggcgccatcggccacctgctgggcgcggcgggcgccgtggaggccgtggccaccatccaggccctgcgcacc
ggctggctgcaccccaacctgaacctggacgagcccgacaagggcgtggacgcctccgtgctggtgggcggcgtgaagga
gcaggccgacgtgaaggtggccctgtccaactccttcggcttcggcggccacaactcctgcgtgctgttccgcaagttcgagg
agTGA

【FIG. 22】

MATASLPVQVAVTSTHCFGLREPRRKRQWARQTRCHASAAGKPKRRVVVTGQGVVTSL
GQSTQQFYDQLLAGASGITHIEGFDTSDYSTKIAGEVKSVDAAPYVARKWVKRMDEVMKF
MFVAGKQALEDAGLPFEGPGLEDLDRKLCGILIGTAMGGMTTFASGVEALTLSGHRKMNP
FCIPFSIGNMGGAMLAMDLGFMGPNYSISTACATGNYCIISAADHIRNGDAVLMLAGGADA
AVIPSGIGGFIACKALSRRNDAPERASRPWDAGRDGFVMGEGAGVLVLEELEHARARGAT
ILAEFIGGAATCDAHHMTEPEPSGRGVRLCLERGLAAAGVAPEEVTYVNAHGTSTPAGDV
AEFRAIRAVLGHDGLRINSSKGAIGHLLGAAGAVEAVATIQALRTGWLHPNLNLDEPDKGV
DASVLVGGVKEQADVKVALSNSFGFGGHNSCVLFRKFEE

PRODUCTION OF LIPIDS AND TERPENOIDS IN *AUXENOCHLORELLA PROTOTHECOIDES*

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 63/109,901 filed on Nov. 5, 2020, the disclosures of which are herein incorporated by reference in their entirety.

TECHNICAL FIELD

The present invention describes microalgae having a functional oil-producing ability comprising an altered profile of fatty acids, carotenoids and/or terpenoids and a method of extracting oil using the microalgae. More specifically, it relates to a method for producing oil having modified fatty acids, carotenoids and/or terpenoids content compared to wild-type microalgae, such as Auxenochlorella protothecoides with inhouse strain designation as PB5.

BACKGROUND ART

Industrial production of lipids and fats from oleaginous microorganisms has been investigated since the late 19th century, but the promise of these "single cell oils" has only been realized in the last 20-30 years[1]. Oleaginous microorganisms trigger biosynthesis of storage lipids in response to imbalances between carbon (C) supply and other major nutrients such as nitrogen (N) or phosphorus (P) that are required for growth. This response allows accumulation of excess C under conditions where limitation for other nutrients prevents cell growth and division. Microbial storage lipids are largely composed of triacylglycerides (TAGs), which can be mobilized and used for rapid growth when the limiting nutrient or nutrients become available. Subsets of yeasts, fungi, and algae, but very few bacteria are oleaginous[1]. In the case of some algae, oil production can be stimulated by photosynthesis or by heterotrophic fermentation of sugars, but there is little economic incentive to make lipids which have similar composition or properties to commodity plant oils. In the early 2000s there was a flurry of interest and investment in photosynthetic production of biofuels from algae, but this has now largely faded due to the unfavorable energy return and economics of fuel production from aquatic microbes[2,3]. Heterotrophic oil productivity is substantially higher than is the case for photoautotrophic production, but the price of common sugar feedstocks is seldom any less that one quarter of the price of typical commodity oils, and the lipid yield for most heterotrophic processes is less than 25%. The economics can improve if the carbon source comes from a low value waste stream (e.g., lactose from cheese production, cellulosic sugars from agricultural waste).

On the other hand, heterotrophic production of very long chain polyunsaturated fatty acids (VLCPUFAs) from algae, thraustochytrids and yeasts provides a roadmap for successful commercialization of microbial oils. Docosahexaenoic acid (DHA) from the dinoflagellate algae *Crypthecodinium cohnii* and arachidonic acid (ARA) from the yeast *Mortierella alpina* are both important components of infant formula[1]. These fatty acids are important components of human breast milk and are vital for brain and nervous system development[4]. The microbial oils address a particular market need for which there are no appropriate substitutes, since

the only other significant sources of VLCPUFAs come from fish oils which are subject to contamination with toxins and heavy metals.

Other successful commodity products from aquatic microalgae follow the VLCPUFA model. Astaxanthin and beta-carotene are key carotenoids for human nutrition; the former is one of the most powerful antioxidants known, while beta-carotene also has strong antioxidant activity and is a pro-vitamin A supporting vision[5]. Synthetic astaxanthin derived from petrochemicals is used extensively as a colorant in fish farming but is not approved for human consumption in food or supplements. The major natural source of astaxanthin is the chlorophyte *Haematococcus lacustris* (also *H. pluvalis*), which is grown photoautotrophically in ponds or bioreactors. *Haematococcus* cultures can produce up to 5% of their biomass as astaxanthin when they are subjected to nutrient and high light stress. Astaxanthin is also produced commercially by the heterotrophic fermentation of the yeast *Phaffia rhodozyma*, but the carotenoid content of the biomass is much lower than *Haematococcus*[6,7]. Natural beta-carotene is extracted from another chlorophyte, the halophile *Dunaliella salina*, which is grown photoautotrophically in large salt ponds. Both *Haematococcus* and *Dunaliella* fetch high prices for the biomass, but photoautotrophic cell densities are too low and the production costs for harvesting and extraction are too high for the natural sources to reach the scale where they can compete with synthetic carotenoids.

Squalene is an important component of human sebum (12%), a fact that justifies its role in the physiology of skin[8], its action in skin hydration, repairing of the damaged skin, and rejuvenating the aging skin was demonstrated. The emollient and hydration properties of squalene and its biocompatibility with skin make squalene an important component in cosmetic formulations (moisturizing creams, makeup, lipstick, and nail and hair products).

Furthermore, squalene appears to play an essential role in protecting skin from free radical oxidative damage. Squalene acts in skin as a quencher of singlet oxygen, protecting by this mechanism the skin surface from lipid peroxidation due to exposure to UV light.

Squalene has been known to play diverse biological roles as an antioxidant[9,10], anti-cancer agent[10,11], age defyer[12,13] chemo preventive agent[14,15,16], antibacterial agent[13,17], adjuvant for vaccines and drug carrier[18,19], and detoxifier[11,20] among others.

Squalene has proved to be a well-tolerated, non-toxic cytoprotective agent that mitigates undesirable side effects of cancer chemotherapy. Many anticancer therapies damage normal healthy tissues, even to the point of organ toxicity. These toxic secondary effects can limit the anticancer drug dosage, and even lead to treatment failure. Squalene is effective at scavenging and detoxifying free radicals produced by chemotherapeutic or radiation therapy agents.

The role of squalene is not just confined to these applications, but it is also a precursor to thousands of bioactive molecules, including steroids and hopanoids. Consequently, many chemicals, food, cosmetic, and pharmaceutical industries have started to use squalene extensively. It also acts as a boosting agent, or adjuvant, that improves the immune system and makes vaccines more effective. Over the last decade, global squalene demand has increased and gained much public and scientific attention. In 2014, the global squalene market demand was about 2.67 kilotons[21], with a projected value of 241.9 million USD by 2022, with major revenues expected from the personal care and cosmetic products[22]. In order to fulfill this ever-increasing demand of

squalene, a pressing need has arisen to produce squalene in a renewable and sustainable manner.

Squalene is harvested from deep-sea sharks and exists in high concentration in shark liver. However, the intensive fishing of these sharks puts in danger the existence of these species, with many of them being close to extinction as their reproductive cycle is quite long and the growth is slow.

Most plant seed oils contain small amounts of squalene[23]. This phytosqualene has superior qualities compared to shark squalene, in that it is highly stable, generally free of heavy metal contamination, odorless, and colorless. In spite of containing significant amounts of squalene, plants oils are not ideal sources. Some plants are strictly seasonal, and squalene content varies greatly geographically. Oilseed crops require the appropriate climatic conditions, soil quality, scheduled irrigation or sufficient rainfall, fertilizer, and pest management. The process of cultivation is labor intensive and correspondingly the amount of squalene produced from plant sources is not sufficient to fulfill the increasing worldwide demand.

Several microalgae and other microorganisms accumulate comparable levels of squalene to plant oils. Short generation times and ease of genetic engineering make microalgae a better alternative for squalene production than plants.

Native biosynthetic pathways which include squalene as an intermediate are present in many microalgae, and these pathways can be transformed or extended to convert the organisms into "cellular factories" for squalene production.

Auxenochlorella protothecoides is one of the most oleaginous species of microalgae and can be cultivated heterotrophically with productivity in commercial scale[24]. Metabolic engineering using genetic tools on this organism has strong potential to produce variety products including carotenoids, terpenoids, and other active ingredients, which can be an important alternative source of currently depleted natural resources.

BRIEF SUMMARY OF THE INVENTION

The inventors of the present application have developed the Trebouxiophyte alga, Auxenochlorella protothecoides PB5, as a biotechnology platform for the heterotrophic production of valuable lipids, carotenoids, terpenoids, and other compounds. Efficient transformation and facile gene targeting by homologous recombination are features of this system. Targeted knockouts and knock-ins of fatty acid and lipid biosynthetic pathway genes enabled the improved synthesis of polyunsaturated fatty acids. Valuable ketocarotenoids were produced by disrupting endogenous carotenoid biosynthetic genes and expressing heterologous beta-carotene ketolase transgenes. Squalene accumulation was increased by blocking phytosterol biosynthesis. *A. protothecoides* PB5 can be employed as a general platform for the photoautotrophic, mixotrophic, or heterotrophic production of valuable biomolecules. Here the inventors of the present application demonstrate the utility of the system for the biosynthesis of modified fatty acids, lipids, carotenoids, and other terpenoids (FIG. **1**).

It is an object of the present invention to provide mutant microalgae having functional oil-producing ability comprising modified profiles of fatty acids, carotenoids and/or terpenoids as proposed to solve the above problems.

Another object of the present invention is to provide a method for producing oil using the microalgal mutant.

Another object of the present invention is to provide oil prepared by the above production method.

Advantageous Effects

The present application shows that when using a microalgal mutant in which Auxenochlorella protothecoides PB5 microalga genes are knocked out or knocked in by homologous recombination, an oil containing fatty acids, carotenoids and/or terpenoids of an altered profile compared to that of wild type thereof can be effectively extracted.

DESCRIPTION OF THE DRAWING

FIG. **1** shows a diagram of the Isoprenoid pathway, an essential metabolic pathway present in microalgae.

FIGS. **2**A-B show the sequence of the transforming DNA from the LCYE-1 disruption construct pPB0014. (SEQ ID NO: 1)

FIGS. **3**A-C show the sequence of the transforming DNA from the LCYE-2 disruption construct pPB0038. (SEQ ID NO: 2)

FIGS. **4**A-D show spectrophotometric and HPLC analysis of carotenoid pigments from wild-type *A. protothecoides* PB5, 14-2, and 38-25. Absorbance spectra of acetone-methanol-extracted pigments from wild-type *A. protothecoides* PB5, LCYE-1 disruption strain 14-2, and LCYE-1/LCYE-2 double knockout strain 38-25. Peak absorbances are indicated for each curve (FIG. **4**A). HPLC chromatograms showing carotenoid pigments present in oil extracted from the same strains as in A (FIG. **4**B: PB5, FIG. **4**C: 14-2, FIG. **4**D: 38-25).

FIG. **5** shows the sequence of the expression module cloned into the KpnI site of pPB0038 to generate construct pPB0120. (SEQ ID NO: 3)

FIG. **6**. Nucleotide sequence of the ApSAD2tp_CrBKT1 expression module from pPB0123. (SEQ ID NO: 4)

FIG. **7**A shows the amino acid sequence of native CrBKT1. The predicted plastid transit peptide is underlined. (SEQ ID NO:5)

FIG. **7**B shows the amino acid sequence of chimeric ApSAD2tp_CrBKT1. The predicted ApSAD2 plastid transit peptide is underlined. (SEQ ID NO:6)

FIGS. **8**A-E show shake flask cultures illustrating the color differences due to engineered alterations in the carotenoid profiles (FIG. **8**A). HPLC chromatograms showing carotenoid pigments present in oil extracted from the same strains as in A (FIG. **8**B: 120A-2, FIG. **8**C: 123A-5, FIG. **8**D: 120B-5, FIG. **8**E: 123B-24).

FIGS. **9**A-C show the sequence of the transforming construct pPB0065, targeting disruption of ApSQE-2. (SEQ ID NO: 7)

FIGS. **10**A-B show the sequence of the transforming DNA from pPB0077. (SEQ ID NO: 8)

FIGS. **11**A-B show the chromatograms of HPLC analysis of the standard squalene (FIG. **11**A), the accumulation of squalene in SQE double knockout strain 77B-21 (FIG. **11**B).

FIG. **12** shows the amino acid sequence of *A. protothecoides* 1-deoxy-D-xylulose 5-phosphate synthase (DXS). The predicted plastid transit peptide is underlined. (SEQ ID NO:9)

FIG. **13** shows the amino acid sequence of *A. protothecoides* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). (SEQ ID NO:10)

FIG. **14** shows the amino acid sequence of *A. protothecoides* farnesyl diphosphate synthase (FDPS). (SEQ ID NO:11)

FIG. **15** shows the amino acid sequence of *A. protothecoides* squalene synthase (SQS). (SEQ ID NO:12)

FIGS. **16**A-C show the sequence of the transforming construct pPB0039, targeting insertion of the ApSAD2 promoter upstream of the FAD3-1 coding sequence. (SEQ ID NO: 13)

FIG. **17** shows the nucleotide sequence of the ApFATA promoter in pPB0041 SEQ ID NO: 14)

FIGS. **18**A-C show the sequence of the transforming construct pPB0118, targeting AtPDCT and ScSUC2 expression to the THI4 locus. (SEQ ID NO: 15)

FIGS. **19**A-C show the sequence of the transforming construct pPB0142, targeting LuFAD3A and neoR expression to the THI4 locus. (SEQ ID NO: 16)

FIG. **20** shows the growth of strains targeting one or both alleles of THI4. Wild-type *A. protothecoides* PB5 is unable to grow without added thiamine. Thiamine prototrophy is observed in strain 118B-8, expressing AtTHIC, targeted to one allele of THI4. Disruption of the second THI4 allele by pPB0142 renders the transformants thiazole auxotrophs.

FIG. **21** shows the coding sequence of KASII from *A. protothecoides*, optimized for translation. (SEQ ID NO:17)

FIG. **22** shows the amino acid sequence of *A. protothecoides* beta-ketoacyl-ACP synthase II. (SEQ ID NO:18)

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner like a term "comprising." The transitional terms/phrases (and any grammatical variations thereof) "comprising," "comprises," "comprise," "consisting essentially of," "consists essentially of," "consisting," and "consists of" can be used interchangeably.

The phrases "consisting essentially of" or "consists essentially of" indicate that the claim encompasses embodiments containing the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claim.

The term "about" means within an acceptable error range for the value as determined by one of ordinary skills in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. Where values are described in the application and claims unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed. In the context of compositions containing amounts of ingredients where the term "about" is used, these compositions contain the stated amount of the ingredient with a variation (error range) of 0-10% around the value (X±10%).

An "allele" refers to a version of a gene at the same place on homologous chromosomes. An allele may encode the same or similar protein.

"Exogenous gene" shall mean a nucleic acid that codes for the expression of an RNA and/or protein that has been introduced into a cell (e.g., by transformation/transfection), and is also referred to as a "transgene". A cell comprising an exogenous gene may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. The exogenous gene may be from a different species (and so heterologous), or from the same species (and so homologous), relative to the cell being transformed. Thus, an exogenous gene can include a homologous gene that occupies a different location in the genome of the cell or is under different control, relative to the endogenous copy of the gene. An exogenous gene may be present in more than one copy in the cell. An exogenous gene may be maintained in a cell as an insertion into the genome (nuclear or plastid) or as an episomal molecule.

"Fatty acids" shall mean free fatty acids, fatty acid salts, or fatty acyl moieties in a glycerolipid. It will be understood that fatty acyl groups of glycerolipids can be described in terms of the carboxylic acid or anion of a carboxylic acid that is produced when the triglyceride is hydrolyzed or saponified.

"Fixed carbon source" is a molecule(s) containing carbon, typically an organic molecule that is present at ambient temperature and pressure in solid or liquid form in a culture media that can be utilized by a microorganism cultured therein. Accordingly, carbon dioxide is not a fixed carbon source.

"Microalgae" are eukaryotic microbial organisms that contain a chloroplast or other plastid, and optionally that can perform photosynthesis, or a prokaryotic microbial organism capable of performing photosynthesis. Microalgae include obligate photoautotrophs, which cannot metabolize a fixed carbon source as energy, as well as heterotrophs, which can live solely off a fixed carbon source. Microalgae include unicellular organisms that separate from sister cells shortly after cell division, such as *Chlamydomonas*, as well as microbes such as, for example, Volvox, which is a simple multicellular photosynthetic microbe of two distinct cell types. Microalgae include cells such as *Chlorella, Dunaliella*, and *Prototheca*. Microalgae also include other microbial photosynthetic organisms that exhibit cell-cell adhesion, such as *Agmenellum, Anabaena*, and *Pyrobotrys*. Microalgae also include obligate heterotrophic microorganisms that have lost the ability to perform photosynthesis.

In connection with a recombinant cell, the term "knockdown" refers to a gene that has been partially suppressed (e.g., by about 1-95%) in terms of the production or activity of a protein encoded by the gene.

Also, in connection with a recombinant cell, the term "knockout" refers to a gene that has been completely or nearly completely (e.g., >95%) suppressed in terms of the production or activity of a protein encoded by the gene. Knockouts can be prepared by ablating the gene by homologous recombination of a nucleic acid sequence into a coding sequence, gene deletion, mutation, or other methods. When homologous recombination is performed, the nucleic acid that is inserted ("knocked-in") can be a sequence that encodes an exogenous gene of interest or a sequence that does not encode for a gene of interest.

An "oleaginous" cell is a cell capable of producing at least 20% lipid by dry cell weight, naturally or through recombinant or classical strain improvement. An "oleaginous microbe" or "oleaginous microorganism" is a microbe, including a microalga that is oleaginous (especially eukaryotic microalgae that store lipid). An oleaginous cell also encompasses a cell that has had some or all its lipid or other content removed, and both live and dead cells.

In connection with a functional oil, a "profile" is the distribution of species or triglycerides or fatty acyl groups within the oil. A "fatty acid profile" is the distribution of fatty acyl groups in the triglycerides of the oil without reference to the attachment to a glycerol backbone. Fatty acid profiles are typically determined by conversion to a fatty acid methyl ester (FAME), followed by gas chroma-

tography (GC) analysis with flame ionization detection (FID). The fatty acid profile can be expressed as one or more percent of fatty acid in the total fatty acid signal determined from the area under the curve for that fatty acid.

"Recombinant" is a cell, nucleic acid, protein, or vector that has been modified due to the introduction of an exogenous nucleic acid or the alteration of a native nucleic acid. Thus, e.g., recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell or express native genes differently than those genes are expressed by a non-recombinant cell. Recombinant cells can, without limitation, include recombinant nucleic acids that encode for a gene product or for suppression elements such as mutations, knockouts, antisense, interfering RNA (RNAi), or dsRNA that reduce the levels of the active gene product in a cell. A "recombinant nucleic acid" is a nucleic acid originally formed in vitro, in general, by the manipulation of nucleic acid, e.g., using polymerases, ligases, exonucleases, and endonucleases, using chemical synthesis, or otherwise is in a form not normally found in nature. Recombinant nucleic acids may be produced, for example, to place two or more nucleic acids in operable linkage. Thus, an isolated nucleic acid or an expression vector formed in vitro by ligating DNA molecules that are not normally joined in nature, are both considered recombinant for the purposes of this invention. Once a recombinant nucleic acid is made and introduced into a host cell or organism, it may replicate using the in vivo cellular machinery of the host cell; however, such nucleic acids, once produced recombinantly, although subsequently replicated intracellularly, are still considered recombinant for purposes of this invention. Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid.

The terms "triglyceride", "triacyl glyceride" and "TAG" are used interchangeably as is known in the art.

In the present disclosure, ranges are stated in shorthand, to avoid having to set out at length and describe each value within the range. Any appropriate value within the range can be selected, where appropriate, as the upper value, lower value, or the terminus of the range. For example, a range of 0.1-1.0 represents the terminal values of 0.1 and 1.0, as well as the intermediate values of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and all intermediate ranges encompassed within 0.1-1.0, such as 0.2-0.5, 0.2-0.8, 0.7-1.0, etc. When ranges are used herein, combinations and sub-combinations of ranges (e.g., subranges within the disclosed range), specific embodiments therein are intended to be explicitly included.

According to one aspect of the present invention, a method for producing oil comprising a fatty acid, carotenoid, and/or terpenoid of a modified profile using a gene knockout or knock-in microalgal mutant by homologous recombination is provided.

According to one aspect of the present invention, a method for producing oil, characterized in that the weight ratio of zeaxanthin to lutein is high compared to the oil produced from wild-type microalgae, is provided.

According to one aspect of the present invention, a method for producing an oil comprising astaxanthin is provided.

According to one aspect of the present invention, a method for producing an oil comprising squalene is provided.

According to one aspect of the present invention, a method for producing a functional oil, characterized in that the weight ratio of omega-6 to omega-3 is low compared to the oil produced from wild-type microalgae, is provided. Preferably, the ratio of the oil ranges between 1:1 to 5:1 compared to the oil produced from the wild type microalgae which is 7:1.

According to one aspect of the present invention, a method for producing a functional oil, characterized in that the omega-3 fatty acids increased 3-5-fold and the overall PUFA increased 2-3-fold compared to the wild-type strain.

According to one aspect of the present invention, the microalgal mutant may be a gene knockout or knock-in microalgal mutant by homologous recombination. Preferably, the microalgae may be *Auxenochlorella* genus, more preferably, Auxenochlorella protothecoides PB5.

According to one aspect of the present invention, the microalgae mutant is to provide a method for producing functional oil, characterized in that one or more of the alleles LCYE-1 and LCYE-2 of the Lycopene Cyclase Epsilon gene are knocked out.

According to one aspect of the present invention, the microalgal mutant strain may be to provide a method for producing a functional oil, characterized in that one or more of the alleles SQE-1 and SQE-2 of the Squalene Epoxidase gene are knocked out.

According to one aspect of the present invention, the microalgal mutant may be to provide a method for producing a functional oil, characterized in that the native FAD3 promoter is replaced with a Stearoyl-ACP Desaturase (SAD2) promoter, or the native FAD3 promoter is replaced with an acyl-ACP thioesterase (FATA) promoter.

According to one aspect of the present invention, it may be to provide the use of microalgae as a platform to produce functional oils comprising fatty acids, carotenoids, and/or terpenoids of altered profile by modification of the isoprenoid pathway.

According to an aspect of the present invention, it may be to provide a mutant microalga for the production of oil containing fatty acids, carotenoids, and/or terpenoids of an altered profile.

According to an aspect of the present invention, it may be to provide an oleaginous microalgae mutant that produce a functional oil, the microalgae comprising an ablation of one or more alleles of an endogenous polynucleotide or comprising an exogenous gene.

According to one aspect of the present invention, the microalgal mutant is a microalgal mutant knocked out or knocked-in by homologous recombination, preferably, at least one of the alleles LCYE-1 and LCYE-2 of lycopene cyclase epsilon characterized by knocking out, or knocking out at least one of alleles SQE-1 and SQE-2 of squalene epoxidase, or replacing the native FAD3 promoter with a Stearoyl-ACP Desaturase (SAD2) promoter or a mutant strain in which the native FAD3 promoter is replaced with the promoter of the *A. protothecoides* FATA gene encoding acyl-ACP thioesterase.

Hereinafter, the present invention will be explained in detail.

As an aspect for achieving the object of the present invention, the present invention provides an Auxenochlorella protothecoides mutant for producing a fatty acid, a carotenoid, or terpenoid having a modified profile.

As an embodiment, the Auxenochlorella protothecoides mutant may be a mutant of *A. protothecoides* PB5.

The Auxenochlorella protothecoides mutant of the present invention may be prepared using general mutation treatment methods.

In the present invention, "mutation" refers to a change in a nucleotide sequence due to the insertion, deletion, or substitution of a base into the original nucleotide sequence.

As a means of mutation, the number of inserted bases may be different depending on the mutation and thus is not limited thereto. "Deletion mutation" means a mutation in which a base is removed from the original nucleotide sequence, and "substitution mutation" means that an original nucleotide is changed to another base without changing the number in the original nucleotide sequence.

In a specific embodiment of the present invention, pPB0014 has a transforming DNA with the nucleotide sequence of SEQ ID NO: 1 which is a DNA construct in which LCYE-1 encoding one allele of Lycopene Cyclase Epsilon in Auxenochlorella protothecoides PB5 is prepared in order to change the ratio of lutein derived from alpha-carotene and zeaxanthin derived from beta-carotene compared to wild type. Then, strain 14-2 was prepared by introducing pPB0014. Afterward, in order to generate a double knockout strain completely lacking lutein, a carotenoid derived from alpha-carotene, pPB0038, a DNA construct having a transforming DNA with the nucleotide sequence of SEQ ID NO: 2, in which LCYE-2 encoding the second allele of Lycopene Cyclase Epsilon was prepared. The mutant strain 38-25 is prepared by transforming the strain 14-2 with said pPB0038.

It was confirmed that the above strains increased the ratio of zeaxanthin to lutein compared to the wild type.

In a further embodiment, to prepare a keto-carotenoid, an expression module for *C. reinhardtii* beta-carotene ketolase1 (CrBKT1) SEQ ID NO: 4, was introduced into the pPB0038 backbone to produce construct pPB0123, which targeted the LCYE-2 single allele. Alternatively, strains in which LCYE alleles were disrupted were prepared. In addition, when a single LCYE allele is knocked out, 4-keto-lutein and astaxanthin are produced, while when both LCYE alleles are knocked out strains almost exclusively produced astaxanthin.

In a specific embodiment of the present invention, to obtain squalene, ApSQE-2 encoding the Squalene Epoxidase allele 2 in Auxenochlorella protothecoides PB5 is knocked out. DNA construct having a transforming DNA with the nucleotide sequence of SEQ ID NO: 7 pPB0065 was prepared and introduced into wild-type Auxenochlorella protothecoides PB5 to prepare strain 65-4. Then, in order to generate a double knockout strain, pPB0077, a DNA construct having nucleotide sequence SEQ ID NO: 8 was constructed and transformed into the strain 65-4 in which SQE-1 encoding Squalene Epoxidase allele 1 was knocked out. The prepared mutant strain was named strain 77B-21.

It was confirmed that the above strains can accumulate squalene while the wild type cannot.

In a specific embodiment of the present invention, the native FAD3 promoter in *A. protothecoides* PB5 is replaced with a promoter of a gene that is strongly up-regulated during lipid production, thereby activating the endogenous FAD3 gene encoding Fatty acid Desaturase 3 to reduce the ratio of omega-6 to omega-3 compared to the wild type. Specifically, construct pPB0039 having a transforming DNA with the nucleotide sequence of SEQ ID NO: 13 was constructed in which the ApSAD2 promoter was inserted upstream of the FAD3-1 coding sequence. Alternatively, construct pPB0041 was constructed, in which the native FAD3 promoter was replaced with the promoter of the FATA gene encoding acyl-ACP thioesterase SEQ ID NO: 14. Plasmid construct pPB0039 and pPB0041 were introduced into wild-type *A. protothecoides* PB5 to obtain strains 39-1 and 39-9 strains 41-1 and 41-3, respectively. It was confirmed that the strains increased the ratio of omega 3 to omega 6 compared to the wild type.

In a specific embodiment of the present invention, transfer of C18:1 between DAG and phospholipids was improved by transforming a construct, pPB0118 SEQ ID NO:15, encoding *Arabidopsis* phosphatidylchloline:diacylglycerol choline phosphotransferase (PDCT) into strain 41-3. As a result, C18:2 accumulation increased by 2.5-fold in strains 118B-8 and 118B-20, indicating that expression of AtPDCT during lipid production caused significant enhancement of FAD2 activity, and the incorporation of C18:2 into TAG was favored over desaturation by FAD3.

In a specific embodiment of the present invention, the accumulation of fatty acids C18:3 (ALA) was increased by the introduction of a construct, pPB0142 SEQ ID: NO:16, encoding heterologous (*Linum usitatissimum*) FAD3A into strain 118B-8. As a result, ALA accumulation increased about 3% in strain 142B-11.

Auxenochlorella protothecoides PB5 is a superior system for generating engineered microalgae strains due to its ease of transformation and facile homologous recombination that does not require riboprotein-mediated gene editing. PB5 has a higher intrinsic capacity than non-photosynthetic heterotrophic platforms for production of carotenoids and other terpenoids due to the high flux through these biosynthetic pathways during photosynthetic growth.

The Mutants of the present invention are more industrially useful in that they may provide oils having fatty acids, carotenoids, and squalene content of a profile different from that produced in wild-type Auxenochlorella protothecoides.

In some cases, the percent (w/w) of zeaxanthin in the carotenoids produced by using the microalgal mutants of the present invention is 2-3-fold higher compared to the wild-type microalgae and the zeaxanthin is present as a major carotenoid. In a specific embodiment, the percent of zeaxanthin produced ranges between 40 to 90% (w/w) of the total identified carotenoids.

In some cases, the oil produced may contain keto carotenoids, such as the mixture of keto lutein, and astaxanthin, and the astaxanthin is present as a major carotenoid. In a specific embodiment, the percent of keto carotenoids produced ranges between 20-90% (w/w) of the total identified carotenoids.

In some cases, the oil produced may contain squalene. In a specific embodiment, the amount of squalene in the oil produced ranges from 300 to 1300 ppm.

In some cases, the weight ratio of omega-6 to omega-3 in the oil produced by using the microalgal mutants of the present invention is low compared to the oil produced from wild-type microalgae which are 7:1. In a specific embodiment the percent ratio of omega-6 to omega-3 in the oil produced ranges from 1:1 to 5:1.

The mutant of the present invention may grow appropriately in a growth environment (light conditions, temperature conditions, medium, etc.) capable of culturing conventional Auxenochlorella protothecoides.

The mutant of the present invention may be cultured according to the culture conditions of general Auxenochlorella protothecoides, and specifically, a culture medium capable of culturing algae under weak light conditions may be used. To culture a specific microorganism, it may include a nutrient material required for a culture target, that is, a microorganism to be cultured, and may be mixed by adding material for a special purpose. The medium includes an all-natural medium, synthetic medium, or selective medium. The Auxenochlorella protothecoides mutant may be cultured according to a conventional culture method.

The pH of the culture medium is not particularly limited if the Auxenochlorella protothecoides may survive and grow, for example, it is viable at pH 5 or higher, specifically at pH 6 to 8.

The Auxenochlorella protothecoides mutant of the present invention may produce modified profiles of fatty acids, carotenoids, and terpenoids in cells, so that oils extracted from mutants of the present invention may be effectively used as raw materials for pharmaceuticals, cosmetics, food, feed, etc.

In this aspect, the present invention provides a composition comprising the oil derived from the Auxenochlorella protothecoides mutant. The composition may be a cosmetic composition, a food composition, a composition for a food additive, a feed composition, a composition for a feed additive, a pharmaceutical composition, a raw material composition for food, a raw material composition for feed, a raw material composition for pharmaceutics or a raw material composition for cosmetics.

The composition may be used as a raw material for food, feed or pharmaceutics, and may be used as a formulation for oral administration or parenteral administration. For example, it may be used as a formulation for oral, transdermal or injection administration. Accordingly, the composition of the present invention may be a composition for oral administration in that the composition may be orally supplied to be included in food, medicine, or feed.

In the case of compositions for oral administration may be formulated as powders, granules, tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc. by using methods known in the art. For example, oral preparations may be obtained by mixing the active ingredient with excipients, grinding the mixture, adding suitable additives, and processing it into a granule mixture to obtain tablets or sugar tablets. Examples of suitable excipients include sugars, including lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol and maltitol, and starches, including corn starch, wheat starch, rice starch and potato starch, cellulose, including methylcellulose, sodium carboxymethylcellulose, and hydroxypropylmethyl-cellulose, and the like, fillers such as gelatin, polyvinylpyrrolidone, and the like may be included. In addition, cross-linked polyvinylpyrrolidone, agar, alginic acid, or sodium alginate may be added as a disintegrant if necessary.

The composition may be used for human and animal health promotion. Specifically, the mutant of the present invention has oil production ability with enhanced antioxidant pigment content, so it is not easily oxidized, and functionally, it is possible to provide an oil superior to conventional microalgae-derived vegetable oil in antioxidant activity, and it can be effectively used as a raw material for health functional food, feed, or medicine.

In addition, since the composition may be added to food or feed to achieve a special purpose use, in this respect it may be a food composition, a composition for food additives, a feed composition or a composition for feed additives. When the composition is used in feed or food, it is possible to maintain or enhance body health by pigments and lipids including zeaxanthin produced by the mutant and accumulated in cells.

In the present invention, "additive" is included as long as it is a material added to food or feed other than the main raw material, and specifically, it may be an effective active material having functionality in food or feed.

In the present invention, the composition for feed may be prepared in the form of fermented feed, compounded feed, pellet form, and silage. The fermented feed may include a functional oil derived from the mutant of the present invention, and additionally include various microorganisms or enzymes.

The composition is mixed with a carrier commonly used in the food or pharmaceutical field, such as tablets, troches, capsules, elixirs, syrups, powders. It can be prepared and administered in the form of powder, suspension, or granules. As the carrier, binders, lubricants, disintegrants, excipients, solubilizers, dispersants, stabilizers, suspending agents, and the like may be used. The administration method may be an oral, or parenteral method, but preferably oral administration. In addition, the dosage may be appropriately selected according to the absorption of the active ingredient in the body, the inactivation rate and excretion rate, the age, sex, condition of the subject, and the like. The pH of the composition can be easily changed according to the manufacturing conditions of the drug, food, cosmetics, etc. in which the composition is used.

The composition may include 0.001 to 99.99% by weight, preferably 0.1 to 99% by weight of any one selected from the group consisting of the microalgal mutants of the present invention, the culture of the mutants, the dried product of the mutant, or the culture thereof, and the extract of the mutant or the culture thereof, and the functional oil derived from the mutant, based on the total weight of the composition, and the method of using the composition and the content of the active ingredient may be appropriately adjusted according to the purpose of use.

The mutant may be included in the composition in its own or dried form, and the culture of the mutant may be included in the composition in a concentrated or dried form. In addition, the dried product refers to the dried form of the mutant or its culture and may be in the form of a powder prepared by freeze-drying or the like.

In addition, the extract means that obtained by extraction from the mutant of the present invention, its culture medium or its dried product, an extract using a solvent, etc. Thus, the mutant of the present invention includes those obtained by crushing the mutant of the present invention. Specifically, the oil with the modified profile accumulated in the cells of the mutant of the present invention may be extracted and separated by a physical or chemical method.

In addition, the method for producing oil with the modified profile according to the present invention may include culturing the mutant of the present invention. In addition, the production method may further include; after the culturing step, isolating the mutant of the present invention from the culture.

The culture may be performed in a medium of pH 5.0 to 8.0 conditions. In addition, it may be carried out under a weak light condition, specifically, a light intensity condition in the range of 0.1-1, 1-3, or 3-5 μmol photons/m^2 s.

The production method may further include, in addition to the culturing step, a concentration step to increase the content of algae after culturing, and a drying step of drying by further reducing the moisture of the algae that has undergone the concentration step. However, the concentration step or the drying step is not necessarily required, and in general, the concentration and drying method commonly used in the field to which the present invention pertains, and it can be carried out using a machine.

The production method may further include the step of purifying the material isolated from the culture, which may be performed by a conventional purification method in the art to which the present invention pertains.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Hereinafter, the present invention will be explained in detail through Examples and Experimental Examples, but these Examples and Experimental Examples are presented only as of the illustration of the present invention, and the scope of the present invention is not limited thereby.

The oleaginous Trebouxiophyte alga, Auxenochlorella protothecoides, stores copious amounts of triacylglyceride oil under conditions where the nutritional carbon supply is in excess, but cell division is inhibited due to the limitation of other essential nutrients.

Heterotrophically grown *Auxenochlorella* strains also degrade chlorophyll and down-regulate photosynthesis but maintain significant levels of the yellow carotenoids lutein and zeaxanthin. Bulk biosynthesis of fatty acids with carbon chain lengths up to C18 occurs in the plastids; fatty acids are then exported to the endoplasmic reticulum where incorporation into triacylglycerides (TAGs) occurs. Lipids are stored in large cytoplasmic organelles called lipid bodies until environmental conditions change to favor growth, whereupon they are rapidly mobilized to provide energy and carbon molecules for anabolic metabolism. Wild-type *A. protothecoides* storage lipid is comprised mainly of oleic (~68%), palmitic (~12%), and linoleic (~13%) acids, with minor amounts of stearic, myristic, α-linolenic, and palmitoleic acids. This fatty acid profile results from the relative activities and substrate affinities of the enzymes of the endogenous fatty acid biosynthetic pathway. *A. protothecoides* is amenable to manipulation of fatty acid and lipid biosynthesis using molecular genetic tools, enabling the production of oils with fatty acid profiles that are very different from the wild-type composition. Similarly, the carotenoid and phytosterol profile of the lipid fraction can be altered by genetic engineering of terpenoid biosynthesis pathways.

We have demonstrated efficient transformation and facile nuclear gene targeting via homologous recombination in *A. protothecoides*, PB5. In the following examples, we leverage our ability to perform gene knockouts and knock-ins to produce algal oils with modified fatty acid, carotenoid, and terpenoid profiles.

Wild type *A. protothecoides* PB5 was obtained from the University of Texas Culture Collection of Algae (UTEX catalog number 250), and it is available to the public to purchase via webpage www.utex.org.

Example 1. Production of Strains with Altered Carotenoid Profiles

Lutein is the predominant carotenoid that accumulates in heterotrophic *A. protothecoides* cells. To alter the ratio of lutein (derived from alpha-carotene) to zeaxanthin (derived from beta-carotene) we made a DNA construct to disrupt LCYE-1, encoding one allele of lycopene cyclase epsilon in *A. protothecoides* PB5. In general, flanking regions, promoters, and terminator sequences were PCR amplified from PB5 genomic DNA, and codon-optimized synthetic genes were amplified from plasmid DNA. Herculase II Fusion Enzyme (Agilent, USA) was used for PCR amplification. 50 ml amplification reactions contained 100-500 ng genomic DNA template, 250 micromolar dNTPs, 0.25 micromolar primers, and 0.5 ml Herculase II fusion DNA polymerase. Initial denaturation was at 95° C. for 2 minutes or at 98° C. for 4 minutes for highly GC-enriched templates. PCR products were amplified using 30-35 cycles of 95° C. or 98° C., 20 seconds, 55-65° C. annealing for 20 seconds, and extension at 72° C. for 30 seconds per kb. The sequence of the transforming DNA from the LCYE-1 disruption construct pPB0014 is shown below in FIG. **2**.

Relevant restriction sites used to generate linear DNA and for cloning are indicated in lowercase, bold, and are from 5'-3' HindIII, KpnI, SacI, and HindIII. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *A. protothecoides* PB5 that enable targeted integration of the transforming DNA via homologous recombination at the LCYE-1 locus. Proceeding in the 5' to 3' direction, the *Chlamydomonas reinhardtii* TUB2 promoter (CrTUB2') driving the expression of the *Saccharomyces cerevisiae* SUC2 gene (ScSUC2, codon-optimized for expression in *A. protothecoides* and encoding sucrose invertase, thereby enabling the strain to utilize exogenous sucrose) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for ScSUC2 are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The terminator region of the *A. protothecoides* enolase gene (ApPGH) gene is indicated by small capitals.

Construct pPB0014 was introduced into *A. protothecoides* PB5 using a modified lithium acetate transformation procedure. Briefly, 50 mL of seed growth medium containing 2% glucose in 250 mL Erlenmeyer flask was inoculated with a loop of cells from a plate. Cultures were grown for 2 days at room temperature on an orbital shaker at 140 rpm, to an OD 750 nm 2-4. 50 ml of cell culture was harvested in a Falcon tube and centrifuged for 5 minutes at 3750 rpm. The cell pellet was washed once with 5 ml wash solution (0.1 M Lithium acetate (LiAc) and 1×TE (10 mM Tris, 0.1 mM EDTA) buffer and centrifuged to discard the supernatant. The cells were resuspended in 500 μl wash solution, transferred to a sterile Eppendorf tube, and incubated for 1 hour on a rotary shaker at room temperature at 150 rpm. For each transformation, 150 μl of cell suspension was aliquoted into a 1.5 ml Eppendorf tube. 5-20 μg of linearized DNA was added to the cell suspension, then incubated for 30 minutes at room temperature at 150 rpm. 750 μl of PEG solution (0.1 M LiAc, 1×TE, 40% PEG-4000) was added to the Eppendorf tube, and transformations were incubated overnight on the shaker at room temperature at 150 rpm. Cells were harvested by centrifugation at 5000 rpm for 30 seconds and resuspended in 250 μl of 1 M sorbitol. About 180 ul of the transformation was spread on growth media plates with 1.5% agar with selection, using glass beads, and incubated at room temperature for 1 to 2 weeks. Single colonies were observed on the agar plate after 5-6 days of plating.

Primary transformants were selected for heterotrophic growth on media with sucrose as the sole carbon source. Colonies were clonally purified, and integration of pPB0014 at the LCYE-1 locus was verified for 11 strains by PCR amplification of the regions flanking the 5' and 3' ends of the integration site. Strain 14-2 was selected as the parent strain for subsequent modifications. Next, we disrupted LCYE-2, encoding the second allele of lycopene cyclase epsilon, with the goal of generating double knockout strains completely lacking in alpha-carotene-derived carotenoids. The sequence of the transforming DNA from the LCYE-2 disruption construct pPB0038 is shown below in FIG. **3**. Relevant restriction sites used to generate linear DNA and for cloning are indicated in lowercase, bold, and are from 5'-3' HindIII, KpnI, SacI, and HindIII. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *A. protothecoides* that enable targeted integration of the transforming DNA via homologous recombination at the

LCYE-2 locus. Proceeding in the 5' to 3' direction, the *A. protothecoides* HUP1 promoter (ApHUP1) driving the expression of the *Arabidopsis thaliana* THIC gene (AtTHIC, codon-optimized for expression in *A. protothecoides* and encoding 4-amino-5-hydroxymethyl-2-methylpyrimidine synthase activity, thereby permitting the strain to grow in the absence of exogenous thiamine) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for AtTHIC are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The terminator region of the *A. protothecoides* heat shock protein 90 (ApHSP90) gene is indicated by small capitals.

Construct pPB0038 was transformed into LCYE-1 disruption strain 14-2. Primary transformants were selected for heterotrophic growth on media without thiamine, and with sucrose as the sole carbon source. Colonies were clonally purified, and double knockout of both LCYE-1 and LCYE-2 was verified in strain 38-25 by PCR amplification of the regions flanking the 5' and 3' ends of the pPB0014 and pPB0038 integrations. Spectrophotometric, and HPLC analysis of carotenoid pigments from wild-type *A. protothecoides* PB5, 14-2, and 38-25 is shown in FIGS. **4**A and B. Pigments for spectrophotometric analysis were extracted by mechanical disruption of cell pellets mixed with 0.5 mm glass beads (BioSpec Products, Inc.) suspended in 80:20 acetone:methanol, using a Mini-Beadbeater-16 (BioSpec Products, Inc.). Absorption spectra were acquired using a Thermo Scientific GeneSys 10 uv spectrophotometer. Lutein is the predominant pigment extracted from the wild-type strain. Reduction of lycopene cyclase epsilon activity by disrupting the LCYE-1 allele in 14-2 increases the ratio of zeaxanthin to lutein. Lutein accumulation is abolished in the 38-25 double knockout strain, and zeaxanthin is the major carotenoid.

Cell cultivation—Cells were grown in a growth medium composed of the following chemicals in 1 L deionized water. 0.025 g NaCl, 0.25 g $NaNO_3$, 0.074 g $MgSO_4.7H_2O$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g K_2HPO_4, 0.176 g KH_2PO_4, 2.38 g HEPES, 3 g Yeast extract, 5 ml stock vitamin solutions, and 20 g glucose. pH was adjusted to 6.8 with 20% NaOH. Stock vitamin solution contained the following in 200 ml HEPES solution (50 mM HEPES, pH7.8); 0.005 g Biotin, 0.44 g Thiamine HCl, 0.027 g B12, and 0.619 g D-Pantothenic acid hemicalcium. The stock vitamin solution was filtered, sterilized, and stored at 4° C. refrigerator.

Seed flask cultivation—in 250 ml Erlenmeyer flask containing 95 ml growth medium and 5 ml 40% stock glucose solution, loopful of cells were inoculated from the agar plate and grown for 2 days on an orbital shaker at 115 rpm at 28° C. under 0.1-1 μmol photons/m^2 s low intensity LED light.

Main flask cultivation—in 1 L Erlenmeyer flask containing 332.5 ml growth medium and 17.5 ml 40% stock glucose solution, inoculate cells from seed flask so that initial optical density (OD) at 750 nm wavelength was 0.5-0.6. Incubate the flask on the orbital shaker under 0.1-1 μmol photons/m^2 s low intensity LED light at 115 rpm at 28° C. for 4-5 days until all glucose is exhausted. Harvest the cells via centrifugation and freeze dry the cell pellet. Store the freeze-dried cell pellet at −20° C. freezer.

The lipid extraction method and HPLC parameters are described below.

About 3-5 g lyophilized PB5 and modified strain cell powders were finely ground in a mortar using a pestle. 30 ml of ether was added to the milled microalga powders then vortexed for about 30 seconds to extract oil and carotenoids. 30 ml of additional ether was used if necessary to complete the extraction of all the oil and carotenoids. The remaining ether was evaporated using an evaporator at room temperature. The extracted oil was dissolved and diluted at a 1:10 ratio with a mixed Hexane:Ethyl Acetate (70:30, v/v) solvent. 10 ul of the diluted solution was injected into an HPLC (Agilent 1260 Infinity II, Agilent, USA) and separation of analytes was conducted on a Luna silica 100 A column (25 cm×4.6 mm; 5 μm, Phenomenex, USA). The mobile phase consisted of hexane:ethyl acetate (70:30, v/v) at a flow rate of 1.5 ml/min. The column temperature was set at 30° C. and lutein, zeaxanthin, and standards were detected at 446 nm. The lutein standard eluted at a retention time of 12.6 minutes and the zeaxanthin standard eluted at a retention time of 13.5 minutes. Table 1 summarized the results of carotenoid analysis of each genetically modified strain and wild-type strain PB5 (control).

TABLE 1

Carotenoids identified in extracted oil from PB5 and modified strains via HPLC.

Strains	Carotenoids	Retention time	Percent (%)
PB5 (Control)	Lutein	12.689	67.0392
	Zeaxanthin	13.548	27.9272
14-2	Lutein	12.685	50.1228
	Zeaxanthin	13.55	45.4815
38-25	Zeaxanthin	13.554	87.8917

Next, we sought to express beta-carotene ketolase activity in *A. protothecoides* cells, allowing them to make ketocarotenoids with high value as antioxidants. To achieve this, we introduced an expression module for the *C. reinhardtii* beta-carotene ketolase gene 1 (CrBKT1) into the pPB0038 backbone. The sequence of the expression module cloned into the KpnI site of pPB0038 to generate construct pPB0120 is shown below in FIG. **5**. KpnI and SpeI sites flanking the expression module and used for cloning are indicated in lowercase, bold. Proceeding in the 5' to 3' direction, the *A. protothecoides* SAD2 promoter (ApSAD2) driving the expression of the CrBKT1 gene is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for CrBKT1 are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The sequence encoding the predicted plastid transit peptide is underlined. The terminator region of *A. protothecoides* SAD2 is indicated by small capitals.

We considered that the heterologous CrBKT1 protein might not be imported efficiently into *Auxenochlorella* plastids, so we also made a version of the CrBKT1 expression module that replaced the sequence encoding the native plastid transit peptide with the corresponding sequence from the endogenous *Auxenochlorella* SAD2 gene. The sequence of the chimeric ApSAD2tp_CrBKT1 coding sequence in construct pPB0123 is shown below in FIG. **6**. pPB0123 was otherwise identical to pPB0120 and targeted the AtTHIC transformation marker and the CrBKT1 expression module to the LCYE-2 locus. The sequences of the native CrBKT1 protein and the ApSAD2tp_CrBKT1 chimeric proteins are shown in FIGS. **7**A and **7**B, respectively.

Constructs pPB0120 and pPB0123 were both transformed into wild-type *A. protothecoides* PB5 to make LCYE-2 single allele knockouts expressing CrBKT1, and into LCYE-1 disruption strain 14-2 to make LCYE double knockouts expressing CrBKT1. Primary transformants made in the wild-type *A. protothecoides* background were selected for heterotrophic growth on glucose-containing media without thiamine, while transformants generated in

the 14-2 parent strain were selected on media without thiamine, supplemented with sucrose. Primary transformants were clonally purified and shake flask cultures of representative strains are shown in FIG. **8**A. Strains transformed with pPB0123, expressing chimeric ApSAD2tp_CrBKT1 produced more red-colored ketocarotenoids than pPB0120 transformants that expressed native CrBKT1, suggesting that the chimeric protein was imported and processed more efficiently than the native protein. HPLC analysis, shown in FIG. **8**B, showed that the predominant ketocarotenoids in LCYE-2 single allele knockouts (A strains, wild-type *A. protothecoides* parent) were 4-keto-lutein and astaxanthin, derived from lutein and zeaxanthin, respectively. The strains that expressed CrBKT1 with both LCYE alleles disrupted (B strains) made astaxanthin almost exclusively.

The lipid extraction method and HPLC parameters for keto-carotenoids analysis—Ether extraction of oil and carotenoids was performed as described above. The extracted oil was dissolved and diluted at a 1:100 ratio with a solvent mixture of Hexane:Acetone (82:18, v/v). 20 ul of the diluted solution was injected into an HPLC (Agilent 1260 Infinity II, Agilent, USA) and separation of analytes was conducted on a Luna silica column (15 cm×4.6 mm; 3 μm, Phenomenex, USA). The mobile phase consisted of Hexane:Acetone (82:18, v/v) ran at a flow rate of 1.2 ml/min. The column temperature was not set and astaxanthin and standards were detected at 474 nm. The astaxanthin standard was eluted at a retention time of 7.6 minutes. Table 2 summarized HPLC analysis results of genetically modified strains producing different carotenoids.

TABLE 2

Carotenoids identified in extracted oil from modified strains via HPLC.

Strains	Carotenoids	Retention time	Percent (%)
120A-2	Astaxanthin	7.638	29.8938
	Keto Lutein	9.258	39.4228
	Lutein	12.682	11.5903
123A-5	Astaxanthin	7.634	30.0058
	Keto Lutein	9.254	39.9874
	Lutein	12.685	7.5039
120B-2	Astaxanthin	7.631	71.5094
	Zeaxanthin	13.571	3.3719
123B-24	Astaxanthin	7.632	77.876
	Zeaxanthin	13.582	2.5516

Example 2. Production of Strains with Accumulating Squalene

Squalene is used extensively in cosmetics as an emollient and moisturizer, while its antioxidant properties are exploited in sunscreens and anti-aging products. There are numerous pharmacological applications, including use in formulations as a chemoprotective agent, an anti-bacterial and anti-fungal agent, an adjuvant for vaccines, and a drug carrier. In vivo studies have demonstrated its value as a food supplement with benefits for weight and cholesterol control. Squalene is also a valuable precursor for the chemical synthesis of steroids and other bioactive molecules.

We have enhanced the value of *A. protothecoides*, PB5, storage lipid by engineering strains to accumulate squalene during the lipid production phase by targeted disruption of the squalene epoxidase (SQE) gene. Squalene epoxidase catalyzes the squalene cyclization step in phytosterol biosynthesis. Knockout of the equivalent ERG1 gene in *S. cerevisiae* led to squalene accumulation. Similar metabolic engineering strategies have been applied in cyanobacteria and purple non-sulfur bacteria, and elevated squalene levels were observed in *C. reinhardtii* strains where RNAi was used to knock-down SQE gene expression.

In this example, we describe genetically engineered *A. protothecoides*, PB5 strains in which we have disrupted both alleles of SQE. These modifications block downstream phytosterol biosynthesis and cause the accumulation of squalene in cellular lipids. The sequence of the transforming construct pPB0065, targeting disruption of ApSQE-2 is provided in FIG. **9**. Restriction sites are indicated with bold, lowercase text. HindIII sites delimit the 5' and 3' ends of the transforming DNA. EcoRI and SacI sites flank the selection cassette. Underlined sequences represent SQE-2 genomic DNA targeting integration at the SQE-2 locus via homologous recombination. Proceeding from 5' to 3', the selection cassette contains the ApHUP1 promoter (lowercase, boxed text), driving the expression of codon-optimized AtTHIC. The initiator ATG and terminator TGA for AtTHIC are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApHSP90 terminator region is indicated by small capitals.

SQE-2 knockout strains were generated by transformation of pPB0065 into *A. protothecoides* PB5. Primary transformants were selected on glucose-containing growth media without thiamine. Colonies were clonally purified, and targeted disruption of SQE-2 was verified by PCR amplification and sequencing of the regions flanking the integration site. Transformant 65-4 was selected as the parent strain for subsequent transformation with construct pPB0077, targeting ApSQE-1. The sequence of the transforming DNA from pPB0077 is shown below in FIG. **10**. Restriction sites are indicated with bold, lowercase text. HindIII sites delimit the 5' and 3' ends of the transforming DNA. EcoRI and SacI sites flank the selection cassette. Underlined sequences represent ApSQE-1 genomic DNA targeting integration at the ApSQE-1 allele via homologous recombination. Proceeding from 5' to 3', the selection cassette contains the CrTUB2 promoter (lowercase, boxed text), driving the expression of codon-optimized ScSUC2. The initiator ATG and terminator TGA for ScSUC2 are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApPGH terminator region is indicated by small capitals.

Primary transformants from the introduction of pPB0077 into strain 65-4 were selected on growth media with sucrose and without thiamine. Colonies were clonally purified and targeted disruption of both ApSQE-1 and ApSQE-2 was verified by PCR amplification and sequencing of the regions flanking the integration site.

A representative strain, 77B-21, was grown in lipid production media and the squalene content of crude lipid extracts was measured by HPLC (FIG. **11**).

Cell cultivation conditions—Cells were grown in a growth medium containing the followings in 1 L deionized water: 4.2 g K_2HPO_4, 3.57 g $NaH_2PO_4{\cdot}H_2O$, 0.24 g $MgSO_4{\cdot}7H_2O$, 0.025 g $CaCl_2{\cdot}2H_2O$, 0.25 g citric acid, 2 micromolar thiamine-HCl, and 10 mL of trace metal solution. 1 L of trace metal solution contained 2.75 g citric acid, 0.011 g $CuSO_4{\cdot}5H_2O$, 0.081 g $CoCl_2{\cdot}6H_2O$, 0.33 g H_3BO_3, 1.4 g $ZnSO_4{\cdot}7H_2O$, 0.9485 g $MnCl_2{\cdot}4H_2O$, 0.039 g $Na_2MoO_4{\cdot}2H_2O$, 0.11 g $FeSO_4{\cdot}7H_2O$, 0.0144 g $NiSO_4{\cdot}6H_2O$. Pre-seed and seed medium contained 0.991 g/L of $(NH_4)_2SO_4$, and lipid production media contained 0.248 g of $(NH_4)_2SO_4$. The pre-seed medium was supple-

mented with 5 g/L glucose; seed medium contained 20 g/L glucose and lipid production contained 40 g/L glucose.

The lipid extraction method and HPLC parameters for squalene analysis—About 3 g of lyophilized PB5 and modified strain cell powders were finely ground in a mortar using a pestle. 30 ml of ether was added to the ground PB5 powder and the resulting suspension was vortexed for about 30 seconds to extract oil. An additional 30 ml of ether was used to complete oil extraction. The remaining ether was evaporated at room temperature using an evaporator. The extracted oil was dissolved and diluted at a 1:10 ratio with a solvent mixture solvent of Acetonitrile:Methanol (8:2, v/v), which was also used as the mobile phase. 30 ul of the diluted solution was injected into an HPLC (Agilent 1260 Infinity II) and separation of analytes was conducted on a C18 reverse column (15 cm×4.6 mm; Shiseido, Japan). The mobile phase's flow rate was 1.5 ml/min. The column temperature was set at 35° C. and squalene and the standard was detected at 195 nm. Squalene standard eluted at a retention time of 18.090.

Squalene accumulated to 1222 ppm in oil from strain 77B-21, a level that is on par with the squalene content of olive oil, a common commercial source.

The value of *A. protothecoides* oil would be enhanced by further increases in squalene levels. This may be achieved through overexpression of key enzymes in the isoprenoid biosynthesis pathway to increase flux. The amino acid sequences of *A. protothecoides* 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), farnesyl diphosphate synthase (FDPS), and squalene synthase are provided in FIGS. **12-15**. Overexpression of the native *A. protothecoides* enzymes, or of heterologous enzymes from plants or other algae, in the SQE double knockout background may improve squalene accumulation.

Example 3. Production of Strains with Increased Levels of Omega-3 and Omega-6 Fatty Acids

The fatty acid composition of *A. protothecoides* storage lipid is typical for Trebouxiophyte algae, consisting mainly of oleic (~68%), palmitic (~12%), and linoleic (~13%) acids, with minor amounts of stearic, myristic, α-linolenic, and palmitoleic acids. We sought to improve the nutritional quality of *A. protothecoides* triacylglyceride oils by increasing the polyunsaturated fatty acid (PUFA) content. First, we activated the endogenous FAD3 gene, encoding fatty acid desaturase 3, by swapping the native FAD3 promoter with promoters from genes that are strongly upregulated during lipid production. FAD3 introduces a double bond at the Δ15 position of linoleic acid (C18:2) to make α-linolenic acid. The sequence of the transforming construct pPB0039, targeting insertion of the ApSAD2 promoter upstream of the FAD3-1 coding sequence is provided in FIG. **16**. Restriction sites are indicated with bold, lowercase text. HindIII sites delimit the 5' and 3' ends of the transforming DNA. KpnI and SpeI sites flank the selection cassette. Underlined sequences represent FAD3-1 genomic DNA targeting integration at the FAD3-1 locus via homologous recombination. Proceeding from 5' to 3', the selection cassette contains the ApHUP1 promoter (lowercase, boxed text), driving the expression of codon-optimized AtTHIC. The initiator ATG and terminator TGA for AtTHIC are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApHSP90 terminator region is indicated by small capitals. Lowercase, boxed text delineates the ApSAD2 promoter.

We also made a version of the FAD3 integration construct expression module that replaced the native FAD3 promoter with the promoter from the *A. protothecoides* FATA gene, encoding the acyl-ACP thioesterase. Construct pPB0041 was identical to pPB0039 except that ApFATA promoter shown below in FIG. **17** in lowercase, boxed text, was used to drive the expression of FAD3.

pPB0039 and pPB0041 were transformed into *A. protothecoides* PB5 and primary transformants were selected on glucose-containing growth media without thiamine. Colonies were clonally purified and insertion of the constructs at the FAD3 locus was verified by PCR amplification and sequencing of the regions flanking the integration site.

Cell cultivation conditions—Cells were grown in a growth medium composed of the following chemicals in 1 L of deionized water: 4.2 g K_2HPO_4, 3.57 g $NaH_2PO_4 \cdot H_2O$, 0.24 g $MgSO_4 \cdot 7H_2O$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.25 g citric acid, 2 micromolar thiamine-HCl, and 10 mL of trace metal solution. 1 L of trace metal solution contained 2.75 g citric acid, 0.011 g $CuSO_4 \cdot 5H_2O$, 0.081 g $CoCl_2 \cdot 6H_2O$, 0.33 g H_3BO_3, 1.4 g $ZnSO_4 \cdot 7H_2O$, 0.9485 g $MnCl_2 \cdot 4H_2O$, 0.039 g $Na_2MoO_4 \cdot 2H_2O$, 0.11 g $FeSO_4 \cdot 7H_2O$, 0.0144 g $NiSO_4 \cdot 6H_2O$. Pre-seed and seed medium contained 0.991 g/L of $(NH_4)_2SO_4$, and lipid production media contained 0.248 g of $(NH_4)_2SO_4$. The pre-seed medium was supplemented with 5 g/L glucose; seed medium contained 20 g/L glucose and lipid production contained 40 g/L glucose.

Freeze-dried microalgal samples were sent to Microbial ID Inc., (Delaware, USA) for fatty acid methyl ester analysis (FAME). Microbial ID Inc. utilized standardized gas chromatographic analysis of fatty acid methyl esters method developed by MIDI Inc., (Delaware, USA) and the reference could be found in their technical note #101, published and revised in 2006. The fatty acid profiles of lipids from shake flask assays of representative strains are shown in Table 3. Strains driving expression of one allele of FAD3 with the ApSAD2 promoter (39-1 and 39-9 strains) or the ApFATA promoter (41-1 and 41-3 strains) showed a slight increase in α-linolenic acid (ALA, omega-3) from 2% in the wild-type (PB5) to about 3%. The increase in ALA came at the expense of linoleic acid (omega-6), improving the omega-6:omega-3 ratio from 7 to about 4 (lower is better).

TABLE 3

Fatty acid profiles as a percentage of total fatty acids for *A. protothecoides* strains transformed with pPB0039 or pPB0041.

strain	PB5	39-1	39-9	41-1	41-3
C14:0 myristic	1.7	1.6	1.7	1.6	1.7
C16:0 palmitic	12.7	13.0	12.6	12.0	12.6
C16:1n-7 palmitoleic	0.3	0.3	0.3	0.3	0.3
C18:0 stearic	2.5	2.5	2.6	2.4	2.3
C18:1n-9 oleic	66.3	68.9	67.8	68.5	67.5
C18:2n-6 linoleic LA	14.2	10.5	11.1	11.7	11.9
C18:3n-3 α-linolenic	2.0	2.9	3.3	3.0	3.1
omega-6:omega-3	6.9	3.6	3.4	4.0	3.9
% PUFA	16.2	13.4	14.4	14.6	15.0

The Δ9 double bond in C18:1 is introduced by the stearoyl-ACP desaturases (SADs) in the plastid. Formation of the Δ12 and Δ15 double bonds, catalyzed by FAD2 and FAD3, respectively, occurs in the endoplasmic reticulum, and these enzymes use membrane lipids as their substrate. The relatively low abundance of C18:2 and C18:3 α in wild-type *A. protothecoides* storage lipid results from the competition between the acyltransferases of the Kennedy pathway for the formation of TAG, and the enzymes of the

Lands cycle, which control the exchange of fatty acids between diacylglycerol (DAG) and membrane phospholipids. We considered that increasing the transfer of C18:1 between DAG and phospholipids might improve PUFA production, so we introduced a construct to overexpress *Arabidopsis* PDCT, encoding phosphatidylchloline:diacylglycerol choline phosphotransferase into strain 41-3. The sequence of the transforming construct pPB0118, targeting AtPDCT and ScSUC2 expression to the THI4 locus is provided in FIG. **18**. Restriction sites are indicated with bold, lowercase text. HindIII sites delimit the 5' and 3' ends of the transforming DNA. KpnI, SpeI, and XhoI sites separate the AtPDCT expression module and the ScSUC2 expression cassette. Underlined sequences represent THI4 genomic DNA targeting integration at the THI4 locus via homologous recombination. Proceeding from 5' to 3', the selection cassette contains the ApSAD2 promoter (lowercase, boxed text), driving the expression of codon-optimized AtPDCT. The initiator ATG and terminator TGA for AtPDCT are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApSAD2 terminator region is indicated by small capitals. Lowercase, boxed text delineates the CrTUB2 promoter, driving the expression of codon-optimized ScSUC2. The initiator ATG and terminator TGA for ScSUC2 are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApPGH terminator region is indicated by small capitals.

pPB0118 was transformed into strain 41-3, and primary transformants were selected on sucrose-containing growth media without thiamine. Colonies were clonally purified and insertion of the constructs at the THI4 locus was verified by PCR amplification and sequencing of the regions flanking the integration site. Freeze-dried microalgal cell samples were sent to Microbial ID inc. (Delaware, USA) for fatty acid methyl ester analysis and the profiles of lipids from shake flask assays of representative strains containing the pPB0118 construct are shown in Table 4. C18:2 accumulation increased by 2.5-fold in strains 118B-8 and 118B-20, indicating that expression of AtPDCT during lipid production caused significant enhancement of FAD2 activity. ALA levels only increased by about 1% compared to the 41-3 parent strain, suggesting that incorporation of C18:2 into TAG was favored over desaturation by FAD3. The omega-6:omega-3 ratio increased from 3.8 in 41-3 to above 8, due to the increase in C18:2 without a concomitant increase in ALA.

TABLE 4

Fatty acid profiles as a percentage of total fatty acids for strain 41-3 transformed with pPB0118.

strain	PB5	41-3	118B-8	118B-20
C14:0 myristic	1.7	1.8	2.1	2.1
C16:0 palmitic	12.3	12.5	12.4	13.3
C16:1n-7 palmitoleic	0.3	0.3	0.3	0.4
C18:0 stearic	2.4	2.3	1.8	2.2
C18:1n-9 oleic	67.9	67.5	45.4	46.4
C18:2n-6 linoleic LA	13.1	12.0	33.3	30.8
C18:3n-3 α-linolenic	1.8	3.1	4.0	3.9
omega-6:omega-3	7.2	3.8	8.3	8.0
% PUFA	15.0	15.1	37.3	34.7

Upregulation of the endogenous FAD3 gene and overexpression of AtPDCT only resulted in small improvements in ALA accumulation, so we tested whether ALA biosynthesis could be further improved by the expression of heterologous FAD3. We chose to test FAD3A from *Linum usitatissimum* (flax), which has been shown previously to have desaturase activity in *Prototheca moriformis*. The sequence of the transforming construct pPB0142, targeting LuFAD3A and neoR expression to the THI4 locus is provided in FIG. **19**. Restriction sites are indicated with bold, lowercase text. HindIII sites delimit the 5' and 3' ends of the transforming DNA. KpnI, SpeI, and BamHI sites separate the LuFAD3A expression module and the neoR expression cassette. Underlined sequences represent THI4 genomic DNA targeting integration at the THI4 locus via homologous recombination. Proceeding from 5' to 3', the selection cassette contains the ApSAD2 promoter (lowercase, boxed text), driving the expression of codon-optimized LuFAD3A. The initiator ATG and terminator TGA for LuFAD3A are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApFBA1 terminator region is indicated by small capitals. Lowercase, boxed text delineates the ApPGK1 promoter, driving the expression of codon-optimized neoR, which confers resistance to the antibiotic G418. The initiator ATG and terminator TGA for neoR are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The ApPGK1 terminator region is indicated by small capitals.

pPB0142 was transformed into strain 118B-8, and primary transformants were selected on G418-containing growth media supplemented with sucrose and without thiamine. Colonies were clonally purified and insertion of the constructs at the THI4 locus was verified by PCR amplification and sequencing of the regions flanking the integration site. We also verified that strains containing both constructs pPB0118 and pPB0142 disrupted both alleles of the THI4 locus, rendering them unable to grow without supplementation with hydroxymethyl thiazole (FIG. **20**).

As shown in FIG. **20**, Thiamine prototrophy is observed in strain 118B-8, expressing AtTHIC, targeted to one allele of THI4. Disruption of the second THI4 allele by pPB0142 renders the transformants thiazole auxotrophs.

Freeze-dried microalgal cell pellets were sent to Microbial ID (Delaware, USA) for fatty acid methyl ester analysis (FAME) and the profiles of lipids from shake flask assays of representative strains containing the pPB0142 construct are shown in Table 5. ALA accumulation increased from 5.4% in the 118B-8 parent to up to 8.7% in strain 142B-11, indicating that expression of LuFAD3A during lipid production enhanced desaturation of C18:2. The omega-6:omega-3 ratio was reduced to 4, similar to the original ratio observed in the parent strain 41-3 (see Table 4), but with a 5-fold increase in omega-3 fatty acids and a 3-fold improvement in overall PUFA compared to the wild-type strain.

TABLE 5

Fatty acid profiles as a percentage of total fatty acids for strain 118B-8 transformed with pPB0142.

strain	PB5	118B-8	142B-8	142B-11
C14:0 myristic	2.0	2.5	2.3	2.2
C16:0 palmitic	12.0	11.8	12.5	12.0
C16:1n-7 palmitoleic	0.4	0.3	0.4	0.4
C18:0 stearic	2.5	1.8	1.9	1.8
C18:1n-9 oleic	67.9	44.1	39.9	39.2
C18:2n-6 linoleic LA	13.5	34.0	34.4	34.6
C18:3n-3 α-linolenic	1.8	5.4	8.2	8.7
omega-6:omega-3	7.6	6.3	4.2	4.0
% PUFA	15.3	39.4	42.6	43.3

We aim to produce a nutritionally superior oil from *A. protothecoides* PB5, with very low saturates and a 1:1 ratio of omega-6:omega-3 fatty acids. Optimization of C18:2 and C18:3 α levels may be achieved by modulating the expression of AtPDCT and LuFAD3A. The main saturated fatty acids in wild-type *A. protothecoides* oil are C14:0, C16:0, and C18:0 (see Tables 3, 4 & 5). Levels of C14:0 and C16:0 can be reduced by over-expressing the native KASII gene, encoding beta-ketoacyl-ACP synthase II, which extends C16:0 to C18:0. The coding sequence of the *Auxenochlorella* KASII gene, optimized for translation, is shown in FIG. **21** and the corresponding amino acid sequence is detailed in FIG. **22**.

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ttcaagggcc tggaggaccc cgaggagtac ctccgcatgg gcttcgaggt gtccgcgtcc 2460

tccttcttcc tggaccgcgg gaacagcaag gtgaagttcg tgaaggagaa cccctacttc 2520

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accaaccgca tgagcgtgaa caaccagccc ttcaagagcg agaacgacct gtcctactac 2580

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acgggggtgg acaacctgtt ctacatcgac aagttccagg tgcgcgaggt caagtgattg 2760

attggaactc acaaagcggc ccacggcttc gaacgtcccg tgtcaattgc gcggggtgtg 2820

ccagagtttc tgcgccaccg atgctcaccc taggggggga tgcccttga cattcatgtg 2880

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tggtggaggc ctgccaggca aacggtgtca ccttccagcc gggggaggta gtggatgtgg 3420

gcgtgaagaa cggcacagcc tcggtcacct gccaagatgg ctccgtcctg actgcgaggt 3480

gggcgctgtg catggcattt gttggcacga gtctgcatct ctgaagctgc tgggtagcgt 3540

cagagcagtg gagtcaacag cacacagctc tggcggtgct cagggaacat acatcgcact 3600

gtttcctgga gttgctggcc ctctgtgggg caaccaggac cccccgacgc atgcatgccc 3660

cctcgcacat cccgcacagg ctggtgaccc tggcctccgg cgcggcggcg gggcgcttcc 3720

tcaagtacga gagaagctt 3739

<210> SEQ ID NO 2
<211> LENGTH: 4611
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA
from pPB0038

<400> SEQUENCE: 2

aagcttcaag tgcgtgcgtt acagtgttac caacaacagt ccaacctaac cctttcggtc 60

attctgtcct ttggcaaggg ctcagaatga agtgtgcctg cacatcgagc tagtgctgtg 120

agcgaagaca aggaagtccc cactcaccca cgtggccaga ttttatcttt tttcagattg 180

caagggccac gcccagcgaa ccccgcgatg gggccgagcc atgcccgaca tctcgacatc 240

ttcatatgac aaggcgcttc aaagtgcaat ttatgtgcat ggcatcgatt aggagagtgg 300

ttgaacacca gcccatcttc caccggggaa ggaccgtcga aatgcctctg cagacggcca 360

ccgtctgatc gctgcctgtc ccgaggtgac ggcgatgtcg tccttatccc aaacaatcgt 420

tcgaagacct ttcttttgtt cgctcaaccc accgaggaga ccgtctggat tccatgccgc 480

tgtgacgcct agccccctga gaccctccaa gtgggcggtc ccctccctag cccccagcct 540

ctctgacgtg gcagatgcct ccgcggaagc aaatcaggat cgcagggagg gctcctacga 600

gcagcccctg gtccaacgcc aggtgcctag ggggaaagga gggcaggggg ccttgaggcg 660

agcctggccc aggcagggct tccatggtca gtcgtggcag tgccatgaca gccgaagccc 720

accgcgacac cgtgggtgca gcatgcgtgg acggaaacat tggcaatgcc ttgccccatt 780

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acaatgcctg tcccgtccat gcatctcaac agcctcatgc aaggtttgca caagcaagac 960

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caccgcgatt gtctcacgcg catttcggag aaggtttgca gaacactcca ggacatgaaa 1140

tgccttgtca cgtatgaacc atctcccacg gccttgaaaa gatcgctcga cttccattct 1200

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cccggcgctc ctgacgggga ttaaccccca cgactgtatc catcgacact cgtctcgggg 1500

gaataaaagc ggcgacccag ctccagaggc gcaatccttc tcacaatctg tttaactttc 1560

aacaaagtat aagtcaattc aacttgacac aatggccgcg tccgtccact gcaccctgat 1620

gtccgtggtc tgcaacaaca agaaccactc cgcccgcccc aagctgccca actcctccct 1680

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gcgcaagcac accatcgacc cctcctcccc cgacttccag cccatcccct ccttcgagga 1860

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cgacacgtcc ggcccccaga acgtcaacgc ccacatcggc ctggcgaagc tgcgcaagga 2040

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caactccgcc gtggcctcct ccatcgagga ggaggtctac aaggtgcagt gggccaccat 2340

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ccgttggcat aggcagaagc tgcggcagca cctggtggag gcctgccagg caaacggtgt 4260

caccttccag ccgggggagg tagtggatgt gggcgtgaag aacggcacag cctcggtcac 4320

ctgccgagac ggctccgtcc tgactgcgag gtgggcgctg tgcatggcac ttgttggcac 4380

gagtctgcat ccctgaagct gctgggtagc gtcagagcag tggcgtcaac agcacacagc 4440

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cctggcctcc ggcgcggcgg cggggcgctt cctcaagtac gagagaagct t 4611

<210> SEQ ID NO 3
<211> LENGTH: 2081
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the CrBKT1 expression module from pPB0120

<400> SEQUENCE: 3

ggtacccttg cagtgcccca aaaactggct accacctaac aattctcacg cagttttatc 60

ctctgcactt tgatgtcagc tttttgattc gtctgcgtac attacagcgt tgagtggcca 120

gcaggaagga gaccgcggtc cgagacgagt ctgagggcgc gctctcgcaa cttggattcc 180

ggatttctta ccctgcatcg acctcggcct ggagtcgatc agaaattgtc attgccagat 240

tgcctggcga ggacgggtga tatactcaag gcgttgcatc gcccacaaaa cacacactta 300

tctgcaaggg agttactgca tcaggctctg ctcaacagct cgtgacatcg atcgttcagc 360

tccccagcag gtgcgtgtcc gcatggagca cccctcccga gacacctgcg ttgggtgtcg 420

gaggagctca catgccaggg aggtgcccac attgcaccac gcgaccgcga aataggcaga 480

cttcgggcat cctgtcatcg catgtccgct ggccgggaat catggcctcc ccaccaggcg 540

tcacgcgctg cccacctccc tccccttgct gcgcagggca ccgcgttcct gtggagagcc 600

-continued

gaccacatgg gccccggcat ccagcccacc tccgcccgcc cctgctcccg caccaagcac 660

tcccgcttcg ccctgctggc cgccgccctg accgcccgcc gcgtgaagca gttcaccaag 720

cagttccgct cccgccgcat ggccgaggac atcctgaagc tgtggcagcg ccagtaccac 780

ctgccccgcg aggactccga caagcgcacc ctgcgcgagc gcgtgcacct gtaccgcccc 840

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aacgacttcc tgggccagct cgcgatctcc ctgtacgcct ggttcgacta ctccgtgctg 1140

caccgcaagc actgggagca ccacaaccac accggcgagc cccgcgtgga ccccgacttc 1200

caccgcggca accccaacct ggccgtgtgg ttcgcccagt tcatggtgtc ctacatgacc 1260

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tgacgggcat ggccgttccg cgagggcgcc aactgcggcg gcctgcccgc tggctcgtgc 1800

ccccctaccc ccaccattgc ctggagcgtt tccatcccca aatcacattc catccaagtt 1860

gtatcactat gcccctttgg ctctatacac tcacggcctg aggtcccttc tcggccgtgg 1920

cggcacacgc ccaacccccc accatactct ttccatacac tgcaatgctt cgagcctgcc 1980

tgccacctgc tctgcttgtc tcccctccct tcccttgagg ttttccaatg cagtaagaga 2040

agtcgacgtg catggacaga tgattgagag atgagactag t 2081

<210> SEQ ID NO 4
<211> LENGTH: 981
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the ApSAD2tp_CrBKT1 expression module from pPB0123

<400> SEQUENCE: 4

atggccaccg catccacttt ctcggcgttc aatgcccgct gcggcgacct gcgtcgctcg 60

gcgggctccg ggccccggcg cccagcgagg cccctccccg tgcgcgctgc catccgctcc 120

cgccgcatgg ccgaggacat cctgaagctg tggcagcgcc agtaccacct gccccgcgag 180

gactccgaca agcgcaccct gcgcgagcgc gtgcacctgt accgcccccc ccgctccgac 240

ctgggcggca tcgccgtggc cgtgaccgtg atcgccctgt gggccaccct gttcgtgtac 300

ggcctgtggt tcgtgaagct gccctgggcc ctgaaggtgg gcgagaccgc cacctcctgg 360

gccaccatcg ccgccgtgtt cttctccctg gagttcctgt acaccggcct gttcatcacc 420

acccacgacg ccatgcacgg caccatcgcc ctgcgcaacc gccgcctgaa cgacttcctg 480

ggccagctcg cgatctccct gtacgcctgg ttcgactact ccgtgctgca ccgcaagcac 540

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tgggagcacc acaaccacac cggcgagccc cgcgtggacc ccgacttcca ccgcggcaac    600

cccaacctgg ccgtgtggtt cgcccagttc atggtgtcct acatgaccct gtcccagttc    660

ctgaagatcg ccgtgtggtc caacctgctg ctgctggccg gcgccccccc ggccaaccag    720

ctgctgttca tgaccgccgc ccccatcctg tccgccttcc gcctgttcta ctacggcacc    780

tacgtgcccc accaccccga gaagggccac accggcgcca tgccctggca ggtgtcccgc    840

acctcctccg cctcccgcct gcagtccttc ctgacctgct accacttcga cctccactgg    900

gagcaccacc gctggcccta cgccccctgg tgggagctgc ccaagtgccg ccagatcgcc    960

cgcggcgccg ccctggcctg a                                              981


<210> SEQ ID NO 5
<211> LENGTH: 328
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of native CrBKT1

<400> SEQUENCE: 5

Met Gly Pro Gly Ile Gln Pro Thr Ser Ala Arg Pro Cys Ser Arg Thr
1               5                   10                  15

Lys His Ser Arg Phe Ala Leu Leu Ala Ala Ala Leu Thr Ala Arg Arg
            20                  25                  30

Val Lys Gln Phe Thr Lys Gln Phe Arg Ser Arg Arg Met Ala Glu Asp
        35                  40                  45

Ile Leu Lys Leu Trp Gln Arg Gln Tyr His Leu Pro Arg Glu Asp Ser
    50                  55                  60

Asp Lys Arg Thr Leu Arg Glu Arg Val His Leu Tyr Arg Pro Pro Arg
65                  70                  75                  80

Ser Asp Leu Gly Gly Ile Ala Val Ala Val Thr Val Ile Ala Leu Trp
                85                  90                  95

Ala Thr Leu Phe Val Tyr Gly Leu Trp Phe Val Lys Leu Pro Trp Ala
            100                 105                 110

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

Phe Phe Ser Leu Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His
    130                 135                 140

Asp Ala Met His Gly Thr Ile Ala Leu Arg Asn Arg Arg Leu Asn Asp
145                 150                 155                 160

Phe Leu Gly Gln Leu Ala Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser
                165                 170                 175

Val Leu His Arg Lys His Trp Glu His His Asn His Thr Gly Glu Pro
            180                 185                 190

Arg Val Asp Pro Asp Phe His Arg Gly Asn Pro Asn Leu Ala Val Trp
        195                 200                 205

Phe Ala Gln Phe Met Val Ser Tyr Met Thr Leu Ser Gln Phe Leu Lys
    210                 215                 220

Ile Ala Val Trp Ser Asn Leu Leu Leu Leu Ala Gly Ala Pro Leu Ala
225                 230                 235                 240

Asn Gln Leu Leu Phe Met Thr Ala Ala Pro Ile Leu Ser Ala Phe Arg
                245                 250                 255

Leu Phe Tyr Tyr Gly Thr Tyr Val Pro His His Pro Glu Lys Gly His
            260                 265                 270

Thr Gly Ala Met Pro Trp Gln Val Ser Arg Thr Ser Ser Ala Ser Arg
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        275                 280                 285

Leu Gln Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His
    290                 295                 300

His Arg Trp Pro Tyr Ala Pro Trp Trp Glu Leu Pro Lys Cys Arg Gln
305                 310                 315                 320

Ile Ala Arg Gly Ala Ala Leu Ala
                325


<210> SEQ ID NO 6
<211> LENGTH: 326
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of chimeric ApSAD2tp_CrBKT1

<400> SEQUENCE: 6

Met Ala Thr Ala Ser Thr Phe Ser Ala Phe Asn Ala Arg Cys Gly Asp
1               5                   10                  15

Leu Arg Arg Ser Ala Gly Ser Gly Pro Arg Arg Pro Ala Arg Pro Leu
            20                  25                  30

Pro Val Arg Ala Ala Ile Arg Ser Arg Arg Met Ala Glu Asp Ile Leu
        35                  40                  45

Lys Leu Trp Gln Arg Gln Tyr His Leu Pro Arg Glu Asp Ser Asp Lys
    50                  55                  60

Arg Thr Leu Arg Glu Arg Val His Leu Tyr Arg Pro Pro Arg Ser Asp
65                  70                  75                  80

Leu Gly Gly Ile Ala Val Ala Val Thr Val Ile Ala Leu Trp Ala Thr
                85                  90                  95

Leu Phe Val Tyr Gly Leu Trp Phe Val Lys Leu Pro Trp Ala Leu Lys
            100                 105                 110

Val Gly Glu Thr Ala Thr Ser Trp Ala Thr Ile Ala Ala Val Phe Phe
        115                 120                 125

Ser Leu Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala
    130                 135                 140

Met His Gly Thr Ile Ala Leu Arg Asn Arg Arg Leu Asn Asp Phe Leu
145                 150                 155                 160

Gly Gln Leu Ala Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Val Leu
                165                 170                 175

His Arg Lys His Trp Glu His His Asn His Thr Gly Glu Pro Arg Val
            180                 185                 190

Asp Pro Asp Phe His Arg Gly Asn Pro Asn Leu Ala Val Trp Phe Ala
        195                 200                 205

Gln Phe Met Val Ser Tyr Met Thr Leu Ser Gln Phe Leu Lys Ile Ala
    210                 215                 220

Val Trp Ser Asn Leu Leu Leu Leu Ala Gly Ala Pro Leu Ala Asn Gln
225                 230                 235                 240

Leu Leu Phe Met Thr Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe
                245                 250                 255

Tyr Tyr Gly Thr Tyr Val Pro His His Pro Glu Lys Gly His Thr Gly
            260                 265                 270

Ala Met Pro Trp Gln Val Ser Arg Thr Ser Ser Ala Ser Arg Leu Gln
        275                 280                 285

Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His Arg
    290                 295                 300

Trp Pro Tyr Ala Pro Trp Trp Glu Leu Pro Lys Cys Arg Gln Ile Ala
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305 310 315 320

Arg Gly Ala Ala Leu Ala
325

<210> SEQ ID NO 7
<211> LENGTH: 4637
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA from pPB0065

<400> SEQUENCE: 7

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ggatgggagg gaacccagct gggagccgga ggagcatgtg accccggacc tgatcgagct 120

atatgccgcc agggacgctc gagcacggga tgtcaaggcc gcggctgaca aggctggcaa 180

gggtaggaca cctgcgttga ggcaaacgct ggagacggcc ggtctcagca tgtgatcctg 240

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ataccggcac gccagatcat ctgtacagga gtctgtgcag gagtgaaaag gtaagagcca 360

gagccatgac cggcgaccat ccatgtcccg tcactcggat gcactggctg acatcggcgg 420

caggtcatcc aacggtcctg aatgatcgag aggaagctgc ccgatttcaa aacgcccccc 480

cacgtcgccc tccatggccg cacagcatgc tcagcacagg ttgctgcgtg tcctcacaca 540

aactgctcct ttaaagcgat caactttcca gggcatgggg cactcgtact gacaatcacc 600

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ggcgaggcgt gccgcggggg tgaattcaga gtcaaatctg cgcctgcccc ggcgctcctg 1440

acggggatta acccccacga ctgtatccat cgacactcgt ctcgggggaa taaaagcggc 1500

gacccagctc cagaggcgca atccttctca caatctgttt aactttcaac aaagtataag 1560

tcaattcaac ttgacacaat ggccgcgtcc gtccactgca ccctgatgtc cgtggtctgc 1620

aacaacaaga accactccgc ccgccccaag ctgcccaact cctccctgct gcccggcttc 1680

gacgtggtgg tccaggccgc ggccacccgc ttcaagaagg agacgacgac cacccgcgcc 1740

acgctgacgt tcgacccccc cacgaccaac tccgagcgcg ccaagcagcg caagcacacc 1800

atcgaccct cctcccccga cttccagccc atcccctcct tcgaggagtg cttccccaag 1860

tccacgaagg agcacaagga ggtggtgcac gaggagtccg gccacgtcct gaaggtgccc 1920

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ttccgccgcg tgcacctgtc cggcggcgag cccgccttcg acaactacga cacgtccggc   1980
ccccagaacg tcaacgccca catcggcctg gcgaagctgc gcaaggagtg gatcgaccgc   2040
cgcgagaagc tgggcacgcc ccgctacacg cagatgtact acgcgaagca gggcatcatc   2100
acggaggaga tgctgtactg cgcgacgcgc gagaagctgg accccgagtt cgtccgctcc   2160
gaggtcgcgc ggggccgcgc catcatcccc tccaacaaga agcacctgga gctggagccc   2220
atgatcgtgg gccgcaagtt cctggtgaag gtgaacgcga acatcggcaa ctccgccgtg   2280
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ggcgtggact acttcacgat ccacgcgggc gtgctgctgc gctacatccc cctgaccgcc   2580
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gacacggccc agttcgccga gctgctgacc cagggcgagc tgacgcgccg cgcgtgggag   2820
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acgaccgaca tcgcgcccgg ctacgaccac atcacctccg ccatcggcgc ggccaacatc   3000
ggcgccctgg gcaccgccct gctgtgctac gtgacgccca aggagcacct gggcctgccc   3060
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gagttccgct ggatggacca gttcgcgctg tccctggacc ccatgacggc gatgtccttc   3240
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tacggctccg ccgaggaggc catccgccag ggcatggacg ccatgtccga ggagttcaac   3420
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cccgagtcct acgtcaaggc cgcgcagaag tgagtcctgg cgaccctgct cccctgaccc   3540
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agtcgcgttg gcgaggggggt ggtcacgatg accacatctg cgggataatt gaatcctcag   3780
gggaaaatac cagtctctgc ttccaggtgc tccggagctc cccaggcgag tcaatcagtt   3840
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gcgtggccgg cgcggcgctg gcgcatcagc agggcttgga cggccgacgc gtgctgctcc   4080
tcgagcggga tctggcccag cccgaccgca tcgtgggcga gctgctgcag cctggcggcg   4140
tgctggccct ggagcgcctg ggcctgggcg gcgccgtgga cggcatcgac gcgcagcccg   4200
tggtcgggta ctgcatgttc aagggcgggc gcgaggcgtg catcgcctac cccacccccg   4260
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ccgagctggg gggtccagcg gctgcggctg cggcatgcag gggccccact ggaagcgcca 4320

gcgccgcgcc cgccggcgac gcccccgtca cgggcttctc cttccacaac gggcgattcg 4380

tgcagcggct gcgcgccgcg gcggcggctg cgcccggggt cacgctgcgt cgcggcacgg 4440

tgcgcgcgct ggtggatgac gccggcgcgg actgggagga ggggcgcgtg gtgacgggcg 4500

tgcggtaccg cgcgggcgac ggcggcgagc gcgtggcact gggccacctc accgtggtct 4560

gcgacggcat gtactcggcc ctgcggtcca agctggcggt gcccgacctg cgcacgccct 4620

cccacttcat caagctt 4637

<210> SEQ ID NO 8
<211> LENGTH: 3767
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA from pPB0077

<400> SEQUENCE: 8

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ggatgggagg gaacccagct gggagccaga ggagcatgtg accccggacc tgatcaagct 120

atatgccgcc agggacgctc gagcacggga tgtcaaggcc gcggctgaca aggctggcaa 180

gggcaggaca cctgcgccga ggcaaacgct ggagacggcc ggtctcagca tgtgatcctg 240

tacttgctgt gacaaggtgg acaatgcagt acgagttgta ccaggctaag tatccctagt 300

ataccggcac gccagatcat ctgtacagga gtctgtgcag gagtaaaaag gcaacagcca 360

gagccatgac cggcgactat ccacatcccg tcactccgat gcactggctg acatcggcgg 420

caggtcgtcc aacggtcctg aatgatcgag aggaagctgc ccgatttcac ccccccccc 480

cacgtcgccc tccatggccg cacagcatgc tcagcacagg ttgctgcgtg tcctcacaca 540

aactgctcct ttaaagcgat caactttcca gggcatgggg cactcgtact gacaatcacc 600

cacattcgta tacctttgac gtcatttttt tttcgcccca acgcggttgc catcccgagt 660

tgtacctccg cggctaccat acccctgtct tctggccctc accgtcgctc gcaggcggga 720

tccagcgcag ccagtctgaa tacttttaca caacatagta cgtaacgcgc attaggcccc 780

caataccagc agctggctcc agcatgggca agggtgcgcg gcaggaggaa ttcctttctt 840

gcgctatgac acttccagca aaaggtaggg cgggctgcga gacggcttcc cggcgctgca 900

tgcaacaccg atgatgcttc gacccccccga agctccttcg gggctgcatg ggcgctccga 960

tgccgctcca gggcgagcgc tgtttaaata gccaggcccc cgattgcaaa gacattatag 1020

cgagctacca aagccatatt caaacaccta gatcactacc acttctacac aggccactcg 1080

agcttgtgat cgcactccgc taaggggggcg cctcttcctc ttcgtttcag tcacaacccg 1140

caaacatgct gctgcaggcc ttcctgttcc tgctggccgg cttcgccgcc aagatcagcg 1200

cctccatgac gaacgagacg tccgaccgcc ccctggtgca cttcaccccc aacaagggct 1260

ggatgaacga ccccaacggc ctgtggtacg acgagaagga cgccaagtgg cacctgtact 1320

tccagtacaa cccgaacgac accgtctggg ggacgccctt gttctggggc cacgccacgt 1380

ccgacgacct gaccaactgg gaggaccagc ccatcgccat cgccccgaag cgcaacgact 1440

ccggcgcctt ctccggctcc atggtggtgg actacaacaa cacctccggc ttcttcaacg 1500

acaccatcga cccgcgccag cgctgcgtgg ccatctggac ctacaacacc ccggagtccg 1560

aggagcagta catctcctac agcctggacg gcggctacac cttcaccgag taccagaaga 1620

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accccgtgct ggccgccaac tccacccagt tccgcgaccc gaaggtcttc tggtacgagc   1680

cctcccagaa gtggatcatg accgcggcca agtcccagga ctacaagatc gagatctact   1740

cctccgacga cctgaagtcc tggaagctgg agtccgcgtt cgccaacgag ggcttcctcg   1800

gctaccagta cgagtgcccc ggcctgatcg aggtccccac cgagcaggac cccagcaagt   1860

cctactgggt gatgttcatc tccatcaacc ccggcgcccc ggccggcggc tccttcaacc   1920

agtacttcgt cggcagcttc aacggcaccc acttcgaggc cttcgacaac cagtcccgcg   1980

tggtggactt cggcaaggac tactacgccc tgcagacctt cttcaacacc gacccgacct   2040

acgggagcgc cctgggcatc gcgtgggcct ccaactggga gtactccgcc ttcgtgccca   2100

ccaacccctg gcgctcctcc atgtccctcg tgcgcaagtt ctccctcaac accgagtacc   2160

aggccaaccc ggagacggag ctgatcaacc tgaaggccga gccgatcctg aacatcagca   2220

acgccggccc ctggagccgg ttcgccacca acaccacgtt gacgaaggcc aacagctaca   2280

acgtcgacct gtccaacagc accggcaccc tggagttcga gctggtgtac gccgtcaaca   2340

ccacccagac gatctccaag tccgtgttcg cggacctctc cctctggttc aagggcctgg   2400

aggaccccga ggagtacctc cgcatgggct tcgaggtgtc cgcgtcctcc ttcttcctgg   2460

accgcgggaa cagcaaggtg aagttcgtga aggagaaccc ctacttcacc aaccgcatga   2520

gcgtgaacaa ccagcccttc aagagcgaga acgacctgtc ctactacaag gtgtacggct   2580

tgctggacca gaacatcctg gagctgtact tcaacgacgg cgacgtcgtg tccaccaaca   2640

cctacttcat gaccaccggg aacgccctgg gctccgtgaa catgacgacg ggggtggaca   2700

acctgttcta catcgacaag ttccaggtgc gcgaggtcaa gtgattgatt ggaactcaca   2760

aagcggccca cggcttcgaa cgtcccgtgt caattgcgcg gggtgtgcca gagtttctgc   2820

gccaccgatg ctcaccctag ggggggatgc cctttgacat tcatgtgtgc ctgcatgcac   2880

gtttgtatca gtctcaccac accttgaaga tttttgggag gggggggaa gtcggaatgg   2940

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gcgccgtgga cggcatcgac gcgcagcccg tggtcgggta ctgcatgttc aagggcgggc   3360

gcgaggcgtg catcgcctac cccacccccg ccgagctggg gggtccagcg gctgcggctg   3420

cggcatgcag gggccccact ggaagcgcca gcgccgcgcc cgccggcgac gcccccgtca   3480

cgggcttctc cttccacaac gggcgattcg tgcagcggct gcgcgccgcg gcggcggctg   3540

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actgggagga ggggcgcgtg gtgacgggcg tgcggtaccg cgcgggcgac ggcggcgagc   3660

gcgtggcact gggccacctc accgtggtct gcgacggcat gtactcggcc ctgcggtcca   3720

agctggcggt gcccgacctg cgcacgccct cccacttcat caagctt                  3767
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<210> SEQ ID NO 9
<211> LENGTH: 730
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of A. protothecoides

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1-deoxy-D-xylulose 5-phosphate synthase (DXS)

<400> SEQUENCE: 9

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Met Gln Phe Ser Leu Ala Gly Met Asn Thr Arg Ala Leu Gln Thr Gly
1               5                  10                  15

Ala Arg Pro Ser Leu Pro Ala Ala Arg Pro Ser Arg Arg Val Arg Pro
            20                  25                  30

Ala Arg Arg Ser Ala Pro Cys Pro Val Ala Arg Thr Met Gly Gly Gly
        35                  40                  45

Glu Glu Gln Pro Ser Ser Ala Glu Gly Val Ala Trp Asp Lys Ile Ser
    50                  55                  60

Thr Asp Glu Leu Ala Asp Trp Ala Gly Ala Gly Pro Pro Thr Pro Leu
65                  70                  75                  80

Leu Asp Thr Val Ala Phe Pro Val His Ile Lys Asn Phe Asn Ser Arg
                85                  90                  95

Gln Leu Gln Gln Leu Cys Lys Glu Leu Arg Ala Asp Leu Ile His Thr
            100                 105                 110

Val Ala Lys Thr Gly Gly His Leu Gly Ser Ser Leu Gly Val Val Glu
        115                 120                 125

Leu Thr Val Ala Leu His His Val Phe Asn Thr Pro Glu Asp Arg Ile
    130                 135                 140

Val Trp Asp Val Gly His Gln Ala Tyr Ile His Lys Met Leu Thr Gly
145                 150                 155                 160

Arg Arg Ala Arg Met His Thr Ile Arg Gln Gln Gly Gly Leu Ser Gly
                165                 170                 175

Phe Thr Arg Arg Ala Glu Ser Val Tyr Asp Pro Phe Gly Ala Gly His
            180                 185                 190

Ser Ser Thr Ser Val Ser Ala Ala Leu Gly Met Ala Val Gly Arg Asp
        195                 200                 205

Arg Lys Gly Arg Ala Asn Asn Cys Ile Ala Val Ile Gly Asp Gly Ala
    210                 215                 220

Ile Thr Gly Gly Met Ala Tyr Glu Ala Met Asn His Ala Gly Phe Leu
225                 230                 235                 240

Asp Thr Asn Met Ile Val Ile Leu Asn Asp Asn Gln Gln Val Ser Leu
                245                 250                 255

Pro Thr Gln Tyr Asn Gly Lys Asn Gln Glu Pro Val Gly Ala Leu Ser
            260                 265                 270

Ser Ala Leu Ala Arg Leu Gln Ala Asn Arg Gln Leu Arg Glu Leu Arg
        275                 280                 285

Glu Ile Ala Lys Gly Val Thr Lys Gln Leu Pro Asp Val Ile Gln Asn
    290                 295                 300

Ala Thr Ala Lys Ile Asp Glu Tyr Ala Arg Gly Met Ile Ser Gly Thr
305                 310                 315                 320

Gly Ser Thr Leu Phe Glu Glu Leu Gly Phe Tyr Tyr Ile Gly Pro Val
                325                 330                 335

Asp Gly His Asn Met Gln Asp Leu Val Asp Val Leu Ser Glu Ile Lys
            340                 345                 350

Ala Thr Glu Thr Val Gly Pro Val Leu Leu His Val Val Thr Gln Lys
        355                 360                 365

Gly Arg Gly Tyr Thr Pro Ala Glu Thr Ala Ser Asp Arg Met His Gly
    370                 375                 380

Val Val Gln Tyr Asp Thr Leu Thr Gly Lys Gln Lys Lys Gly Ser Gly
385                 390                 395                 400
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Gly Pro Gln Ser Tyr Thr Asn Tyr Phe Ala Asp Ala Leu Val Ala Glu
                405                 410                 415

Ala Lys Arg Asp Ala Arg Val Leu Gly Ile His Ala Ala Met Gly Gly
            420                 425                 430

Gly Thr Gly Met Asn Arg Phe Glu Ala Ala Phe Pro Asp Arg Val Phe
        435                 440                 445

Asp Thr Gly Ile Ala Glu Gln His Ala Val Thr Phe Ala Ala Gly Leu
    450                 455                 460

Ala Thr Glu Gly Leu Val Pro Phe Val Ala Ile Tyr Ser Thr Phe Leu
465                 470                 475                 480

Gln Arg Gly Tyr Asp Gln Ile Val His Asp Val Ser Leu Gln Ser Leu
                485                 490                 495

Pro Val Arg Phe Ala Leu Asp Arg Ala Gly Asn Val Gly Ala Asp Gly
            500                 505                 510

Ala Thr His Ala Gly Ala Phe Asp Val Thr Tyr Leu Ala Cys Leu Pro
        515                 520                 525

Asn Met Val Val Met Ala Pro Ser Asn Glu Ala Glu Leu Val His Ala
    530                 535                 540

Val Ala Thr Ala Ala Ala Ile Asp Asp Arg Pro Ser Ala Phe Arg Phe
545                 550                 555                 560

Pro Arg Gly Asn Gly Leu Gly Val Asp Leu Ala Ala Ala Gly Val Thr
                565                 570                 575

Asp Asp Leu Lys Gly Gln Pro Met Glu Val Gly Arg Gly Val Val Arg
            580                 585                 590

Arg Gly Gly Ala Asp Val Ala Leu Leu Gly Tyr Gly Thr Cys Val Asn
        595                 600                 605

Ala Cys Leu Ala Ala Ala Asp Leu Leu Ala Ala Gln Gly Val Ser Ala
    610                 615                 620

Thr Val Val Asp Ala Arg Phe Cys Lys Pro Leu Asp Thr Ala Leu Val
625                 630                 635                 640

Arg Arg Met Ala Ala Glu His Pro Val Met Ile Thr Val Glu Glu Gly
                645                 650                 655

Ser Ile Gly Gly Phe Ala Ala His Val Met Gln Phe Leu Ala Leu Glu
            660                 665                 670

Gly Leu Leu Asp Gly Lys Leu Lys Phe Arg Pro Met Thr Leu Pro Asp
        675                 680                 685

Arg Tyr Ile Glu His Gly Thr Gln Ala Glu Gln Met Ala Glu Ala Gly
    690                 695                 700

Leu Thr Ala Ser His Ile Ala Gly Thr Ala Leu Ser Val Met Gly Val
705                 710                 715                 720

Lys Arg Asp Ala Pro Ser Ile Phe Ser Thr
                725                 730


<210> SEQ ID NO 10
<211> LENGTH: 453
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of A. protothecoides
      1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)

<400> SEQUENCE: 10

Met Arg Cys Ser Ala Gln Leu Asn Thr Arg Gly Pro Thr Leu Pro Asn
1               5                   10                  15

Ser Ala Arg Pro Arg Thr Cys Arg Val Val Ser Ala Ser Ala Ala Pro
            20                  25                  30
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Val Pro Ser Ala Trp Pro Gly Arg Val Val Leu Pro Glu Lys Ser Ala
        35                  40                  45

Ser Arg Thr Gly Pro Lys Lys Phe Ser Leu Leu Gly Ser Thr Gly Ser
    50                  55                  60

Ile Gly Thr Gln Thr Leu Asp Ile Val Ala Glu His Pro Asp Arg Phe
65                  70                  75                  80

Gln Val Val Ser Leu Ala Ala Gly Gly Asn Val Ala Leu Leu Ala Glu
                85                  90                  95

Gln Ile Ala Arg Phe Ser Pro Ser Leu Val Ser Val Arg Asp Ser Gly
            100                 105                 110

Gly Ala Arg Ala Leu Glu Ala Ala Leu Asp Ala Ala Gly Val Asp Arg
        115                 120                 125

Arg Pro Glu Ile Gln Ile Gly Ala Ala Gly Ile Asp Ala Val Ala Ala
    130                 135                 140

His Pro Glu Ala Asp Ala Cys Val Thr Gly Ile Val Gly Cys Ala Gly
145                 150                 155                 160

Leu Arg Pro Thr Met Ala Ala Ile Glu Ala Gly Lys Asp Ile Cys Leu
                165                 170                 175

Ala Asn Lys Glu Thr Leu Ile Ala Gly Gly Pro Thr Val Leu Pro Ala
            180                 185                 190

Ala Ala Lys His Gly Val Ser Ile Leu Pro Ala Asp Ser Glu His Ser
        195                 200                 205

Ala Ile Phe Gln Cys Leu Gln Gly Leu Pro Glu Gly Gly Leu Arg Arg
    210                 215                 220

Ile Ile Leu Thr Ala Ser Gly Gly Ala Phe Arg Asp Leu Pro Val Ser
225                 230                 235                 240

Glu Leu Pro Lys Val Thr Val Ala Asp Ala Leu Lys His Pro Asn Trp
                245                 250                 255

Ala Met Gly Lys Lys Ile Thr Ile Asp Ser Ala Thr Leu Met Asn Lys
            260                 265                 270

Gly Leu Glu Val Ile Glu Ala His Tyr Leu Phe Gly Ala Ser Tyr Asp
        275                 280                 285

Asn Ile Asp Ile Val Ile His Pro Gln Ser Ile Val His Ser Met Ile
    290                 295                 300

Glu Thr Gln Asp Ser Ser Val Leu Ala Gln Leu Gly Trp Pro Asp Met
305                 310                 315                 320

Arg Leu Pro Ile Leu Tyr Thr Met Ser Trp Pro Glu Arg Val Pro Cys
                325                 330                 335

Ser Glu Val Thr Trp Pro Arg Leu Asp Phe Val Lys Ala Gly Asn Leu
            340                 345                 350

Thr Phe Arg Gln Pro Asp His Ala Lys Tyr Pro Ala Met Glu Leu Ala
        355                 360                 365

Tyr Ser Ala Gly Arg Ala Gly Gly Thr Met Thr Gly Val Met Ser Ala
    370                 375                 380

Ala Asn Glu Ala Ala Val Glu Leu Phe Leu Glu Glu Ala Ile Gly Tyr
385                 390                 395                 400

Leu Asp Ile Val Pro Val Val Glu Ala Ala Cys Glu Ala His Arg Val
                405                 410                 415

Glu Leu Val Glu Arg Pro Ser Leu Glu Glu Ile Val His Tyr Asp Gln
            420                 425                 430

Trp Ala Arg Arg His Val Arg Glu Ser Val Ala Lys Arg Ala Pro Ala
        435                 440                 445
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Ala Val Pro Ala Leu
450

<210> SEQ ID NO 11
<211> LENGTH: 359
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of A. protothecoides farnesyl diphosphate synthase (FDPS)

<400> SEQUENCE: 11

Met Ala Ala Val Val Glu Ala Gly His Ala Ala Ser Lys Gln Lys Thr
1 5 10 15

Glu Ala His Gln Thr Lys Gln Glu Phe Leu Ala Val Phe Glu Lys Leu
20 25 30

Arg Asp Glu Leu Leu Glu Asp Ser Ile Leu Ala Gly Gln Pro Glu Ser
35 40 45

Ser Lys Asp Trp Leu Arg Thr Met Leu Asp Tyr Asn Val Pro His Gly
50 55 60

Lys Leu Asn Arg Gly Met Ala Val Leu Asp Val Leu Leu Ala Ala Arg
65 70 75 80

Gly Gly Asp Val Thr Glu Lys Glu Arg Glu Ala Ala Asn Val Leu Gly
85 90 95

Trp Cys Ile Glu Leu Leu Gln Ala Tyr Phe Leu Val Ala Asp Asp Ile
100 105 110

Met Asp Ser Ser Leu Thr Arg Arg Gly Gln Pro Cys Trp Tyr Arg Gln
115 120 125

Pro His Val Gly Met Val Ala Ile Asn Asp Gly Ile Ile Leu Glu Ser
130 135 140

Cys Ile Tyr Arg Leu Leu Lys Leu His Phe Arg Ala His Pro Ala Tyr
145 150 155 160

Val His Leu Leu Glu Leu Phe His Asp Thr Thr His Arg Thr Ala His
165 170 175

Gly Gln Leu Leu Asp Thr Thr Thr Ala Pro Pro Gly Gly Val Asp Leu
180 185 190

Thr Arg Tyr Thr Glu Gly Thr Tyr Leu Arg Ile Val Thr Tyr Lys Thr
195 200 205

Ala Phe Tyr Thr Ile Tyr Leu Pro Val Ala Cys Gly Leu Ala Leu Ala
210 215 220

Gly Val Thr Asp Glu Ala Ser Leu Ala Leu Ala Glu Asp Leu Ser Val
225 230 235 240

Arg Met Gly Arg Tyr Phe Gln Ile Gln Asp Asp Val Leu Asp Ala Phe
245 250 255

Gly Glu Pro Glu Val Ile Gly Lys Val Gly Thr Asp Ile Gln Asp Ser
260 265 270

Lys Cys Ser Trp Leu Val Val Arg Ala Leu Ala Val Ala Ser Ala Glu
275 280 285

Gln Arg Glu Ala Ile Lys Ala Asn Tyr Gly Arg Asp Asp Ala Glu Ala
290 295 300

Val Glu Ala Val Lys Ala Val Tyr Arg Glu Leu Asp Leu Pro Ala Ala
305 310 315 320

Phe Ala Ala Tyr Glu Gln Glu Ser Tyr Asp Gly Leu Val Gln Ala Ile
325 330 335

Glu Gly Gln Asp Lys Phe Pro Pro Ala Val Phe Met Gly Ile Leu Ala
340 345 350

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Lys Ile Tyr Lys Arg Thr Lys
355

<210> SEQ ID NO 12
<211> LENGTH: 458
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of A. protothecoides squalene synthase (SQS)

<400> SEQUENCE: 12

Met Gly Lys Leu Gly Glu Leu Leu Ser His Pro Asp Glu Ile Ile Pro
1 5 10 15

Met Ala Ala Met Tyr Leu Ala Ala Arg Arg Ala Ala Val Leu Pro His
20 25 30

Asp Pro Asp Leu Ala Phe Cys Tyr Ser Met Leu Asn Lys Val Ser Arg
35 40 45

Ser Phe Ala Ile Val Ile Gln Gln Leu Pro Glu Gln Leu Arg Asp Ala
50 55 60

Val Cys Val Phe Tyr Leu Val Leu Arg Ala Leu Asp Thr Val Glu Asp
65 70 75 80

Asp Met Ala Ile Asp Gln Ala Glu Lys Val Pro Ile Leu Leu Ser Phe
85 90 95

His Glu Lys Thr Tyr Glu Lys Asp Trp Ser Met Lys Cys Gly His Gly
100 105 110

His Tyr Val Glu Leu Met Glu Gln Tyr Pro Val Val Cys Ala Ala Phe
115 120 125

Gln Gly Leu Glu Pro Gln Tyr Gln Glu Val Ile Thr Asp Ile Cys Arg
130 135 140

Arg Met Gly Ala Gly Met Ala Glu Phe Ile Val Lys Glu Val Glu Thr
145 150 155 160

Val Lys Asp Tyr Asp Leu Tyr Cys His Tyr Val Ala Gly Leu Val Gly
165 170 175

Val Gly Leu Ser Asn Leu Phe Ala Gly Ser Gly Leu Glu Ser Glu Asp
180 185 190

Phe Ala Ser Leu His Glu Leu Ser Asn Gly Met Gly Leu Phe Leu Gln
195 200 205

Lys Thr Asn Ile Ile Arg Asp Tyr Leu Glu Asp Ile Met Glu Glu Pro
210 215 220

Ala Pro Arg Met Phe Trp Pro Lys Glu Ile Trp Gly Lys His Gly Asp
225 230 235 240

Ser Leu Glu Asp Phe Lys Asp Pro Glu Asn Ala Glu Ala Ala Val Ala
245 250 255

Cys Leu Asn Asp Met Ile Ala Asp Ala Leu Arg His Val Asp Ala Ser
260 265 270

Leu Asp Tyr Met Gln Arg Leu Arg Asn Arg Pro Ile Phe Arg Phe Cys
275 280 285

Ala Val Pro Gln Ile Met Ala Ile Gly Thr Leu Ala Ala Cys Phe Asp
290 295 300

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

Ala Lys Ile Met His Asp Val Glu Asp Tyr Ala Asp Leu Leu Ala Tyr
325 330 335

Phe Arg Ala Phe Gly Gln Ala Leu Ala Ala Lys Ala Arg Ala Ala Arg

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            340                 345                 350

Gly Lys Gly Ala Glu Ser Val Gly Arg Ala Ala Glu Arg Val Val Ala
        355                 360                 365

Gly Cys Ser Ala Ala Leu Ala Asp Leu Ser Arg Ala Glu Asn Ala Arg
    370                 375                 380

Met Ala Ala Ala Ala Arg Arg Pro Leu Ser Leu Pro Ala Arg Ala Leu
385                 390                 395                 400

Leu Leu Val Ala Ala Leu Leu Tyr Leu Phe Leu Ala Trp Arg Ala Glu
                405                 410                 415

Gly Val Arg Arg Trp Leu Gly Val Asp Ser Pro Pro Ala Ala His Lys
            420                 425                 430

Leu Asp Tyr Tyr Asn Gln Ile Val Ala Ser Met Phe Leu Gly Tyr Ser
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Leu Phe Ala Val Gly Thr Gly Arg Arg Pro
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<210> SEQ ID NO 13
<211> LENGTH: 5161
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA
      from pPB0039

<400> SEQUENCE: 13

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aggcaaggac gaccatgccg ggagaaaccc aaggtgacga agtgacattg tgctcgatca    180

ctccatgcac tgcctcactc gcccatgtac cttggtcatg tactccccag tctgcatctt    240

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gatgtaaaag gcagggacac ccacccccag ggccactccc agtagagccc cccctacagc    360

aaccagggtg tccggatcgc taaagtcgat gttgatcgcg cggcacggtg gcattatcgg    420

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tagatggtgc aaaaccctac gactcaagaa ggtgccaccg actcaggcat tgggcacggc   1200

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gagcagcaca tgatgcatcg aggtggcgcc gttgcaaagg agttgcgcat gggtcgaagc   1320

agggagaagg aaacggcgag gcgtgccgcg ggggtgaatt cagagtcaaa tctgcgcctg   1380
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caatcaacac cccaggcaca ttaagagtgc acagcatgac gcagcatagg gtttgtgtcg 3660

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<210> SEQ ID NO 14
<211> LENGTH: 774
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the ApFATA promoter in pPB0041

<400> SEQUENCE: 14

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gctgcagtgc aagtgtacat ataggtcaat cttatgaccc ggcactacca atgatgatca    180

acaccgagcg gccctctgtg ttgtgcttgc ctctttacct tcactgcgta ctgctgcagg    240

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tccagtacga ggtcctggct gaccatgatg gtaggtgaag ttgggccctg ggaggagcgc    360

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ctccccctttg aagtcggctc tcagtttgcg ttgtttcgaa atcgagccac aatcgaatat    480

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<210> SEQ ID NO 15
<211> LENGTH: 5651
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA from pPB0118

<400> SEQUENCE: 15

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atgctgtgtc gacttaagtt accgagctgg ctgacagaga caatcttcag gtcaaatgtt 300

ggcaccaatg atcgcgacga tcgttcaggg gttatcaagt cagatctgaa cgaaaaccag 360

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agaattgcaa tgtgccatat tccagatatc ccagccggcc cttctataaa ccacctgcgg 540

gctcagatac ctacgaagag gctcagataa ctcaaggacg tgcattcgaa ttatccctgc 600

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<210> SEQ ID NO 16
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of the transforming DNA
from pPB0142

<400> SEQUENCE: 16

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agaattgcaa tgtgccatat tccagatatc ccagccggcc cttctataaa ccacctgcgg 540

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Val Thr Gly Gln Gly Val Val Thr Ser Leu Gly Gln Ser Thr Gln Gln
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Phe Tyr Asp Gln Leu Leu Ala Gly Ala Ser Gly Ile Thr His Ile Glu
65                  70                  75                  80

Gly Phe Asp Thr Ser Asp Tyr Ser Thr Lys Ile Ala Gly Glu Val Lys
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Ser Val Asp Ala Ala Pro Tyr Val Ala Arg Lys Trp Val Lys Arg Met
            100                 105                 110

Asp Glu Val Met Lys Phe Met Phe Val Ala Gly Lys Gln Ala Leu Glu
        115                 120                 125

Asp Ala Gly Leu Pro Phe Glu Gly Pro Gly Leu Glu Asp Leu Asp Arg
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What is claimed is:

1. A method for producing oils with modified profiles of omega-3 fatty acids, omega-6 fatty acids, lutein, zeaxanthin, astaxanthin, 4-keto-lutein, or squalene, comprising: growing a microalgal mutant, wherein the microalgal mutant is made by knock-out, knock-in or homologous recombination, wherein the microalgal mutant is a mutant of Auxenochlorella protothecoides, wherein the microalgal mutant comprises a knock-out of at least one allele of lycopene cyclase epsilon LCYE-1, lycopene cyclase epsilon LYCE-2, squalene epoxidase SQE-1, or squalene epoxidase SQE-2, or a replacement of a native FAD3 promoter by a FATA gene promoter from Auxenochorella protothecoides or a stearoyl-ACP desaturase (SAD2) promoter from Auxenochorella protothecoides, and

isolating the oil from the microalgal mutant.

2. The method of claim **1**, wherein the zeaxanthin has a percent (w/w) of zeaxanthin is 2-3-fold higher compared to the wild-type microalgae and the zeaxanthin is present as a major carotenoid.

3. The method of claim **2**, wherein the percent (w/w) of zeaxanthin produced ranges between 40 to 90% of a total identified carotenoids.

4. The method of claim **1**, wherein the oil contains a mixture of 4-keto lutein and astaxanthin, and wherein the astaxanthin is present as a major carotenoid.

5. The method of claim **4**, wherein the percent (w/w) of keto carotenoids produced ranges between 20-90% of a total identified carotenoids.

6. The method of claim **1**, wherein the oil contains squalene.

7. The method of claim **1**, wherein the omega-6 fatty acids and the omega-3 fatty acids have a weight ratio of omega-6 fatty acids to omega-3 fatty acids in the oil that is low compared to the oil produced from wild type microalgae.

8. The method of claim **7**, wherein the weight ratio of omega-6 to omega-3 in the oil ranges from 1:1 to 5:1 compared to the oil produced from wild type microalgae which is 7:1.

9. The method of claim **1**, wherein the omega-3 fatty acids increased 3-5-fold and the overall PUFA polyunsaturated fatty acids increased 2-3-fold compared to the wild-type strain.

10. The method of claim **1**, wherein the microalgal mutant is characterized in that one or more of the alleles of the lycopene cyclase epsilon LCYE-1 gene, lycopene cyclase epsilon LYCE-2 gene are knocked out.

11. The method of claim **1**, wherein the microalgal mutant is characterized in that one or more of the alleles of the squalene epoxidase SQE-1 gene, or squalene epoxidase SQE-2 gene are knocked out.

12. The method of claim **1**, wherein the microalgal mutant is characterized in that the native FAD3 promoter is replaced with a stearoyl-ACP desaturase (SAD2) promoter or a promoter from the Auxenochorella protothecoides FATA gene encoding acyl-ACP thioesterase.

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