



(51) International Patent Classification:

C12N 15/63 (2006.01) C12N 1/38 (2006.01)
C12N 1/00 (2006.01) C12N 15/79 (2006.01)

(21) International Application Number:

PCT/IB2015/054011

(22) International Filing Date:

28 May 2015 (28.05.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

3757/MUM/2013 29 May 2014 (29.05.2014) IN

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Declarations under Rule 4.17:

- as to the identity of the inventor (Rule 4.17(i))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))

(54) Title: METHOD FOR INCREASING LIPID CONTENT IN MICROORGANISMS AND MODIFIED MICROORGANISMS THEREFROM

(57) Abstract: The present disclosure relates to a method for increasing lipid content in microorganisms. The method comprises de-
creasing the expression of molecules involved in the protein synthesis to decrease protein synthesis and thereby increase lipid syn-
thesis in the microorganisms. The present disclosure also provides a modified microorganism having increased lipid content.



METHOD FOR INCREASING LIPID CONTENT IN MICROORGANISMS AND MODIFIED MICROORGANISMS THEREFROM

This application is a Divisional Patent Application to the Indian Patent Application No. 3757/MUM/2013 filed on 29th November, 2013, (post. dt. to 29th May, 2014) the entire contents of which are specifically incorporated herein by reference.

FIELD

The present disclosure relates to a method for increasing the lipid content in microorganisms and modified microorganisms having increased lipid content therefrom.

BACKGROUND

Biofuels produced from living organisms such as plants and algae are alternative fuels to those derived from petroleum such as diesel and petrol. Algae are the preferred choice for producing biofuels as they are able to efficiently convert sunlight and carbon dioxide to biomass and synthesize lipids, and can grow in harsh environmental conditions. Many strains of algae produce triglycerides, which can be converted to biofuels.

Methods for increasing lipid content in microorganisms include nutrient-limitation, modulating enzyme activity, growing the microorganisms in a stress inducing environment and genetic engineering techniques. Though, these methods lead to an increase in lipid content, there is an overall decrease in the growth and cell-mass produced, which is undesirable.

It is known that a major amount of fixed carbon from photosynthesis is channeled to the production of proteins. Hence, decreased protein synthesis would lead to the utilization of this fixed carbon in making lipids, which can be used for the production of biofuels from the microorganisms, specifically from algae/cyanobacteria. The protein synthesis can be down-regulated and the carbon flux is redirected to the production of lipids.

Therefore, the inventors of the present disclosure envisage a method for decreasing the levels of protein synthesis in microorganisms whereby increased lipid content is obtained.

The disclosure also envisages modified strains of microorganisms such as algae and/or cyanobacteria having increased lipid content.

OBJECTS

Some of the objects of the present disclosure which at least one embodiment is adapted to provide, are described herein below:

It is an object of the present disclosure to provide a method for decreasing (down regulating) the expression of tRNA molecules involved in the protein synthesis to decrease protein synthesis and thereby increase lipid content in microorganisms.

It is another object of the present disclosure to provide a method for decreasing (down regulating) the rate of initiation process involved in the protein synthesis in microorganisms to decrease the levels of protein synthesis and thereby increase the lipid content in microorganisms.

It is still another object of the present disclosure to provide a method for decreasing the levels of protein synthesis in microorganisms to obtain increased lipid content leading to an increased overall productivity of biofuels.

It is yet another object of the present disclosure to provide modified strains of microorganisms having increased lipid content.

Other objects and advantages of the present disclosure will be more apparent from the following description when read in conjunction with the accompanying drawings, which is not intended to limit the scope of the present disclosure.

SUMMARY

The present disclosure in one aspect provides a method for increasing the lipid content in microorganisms. The method is characterized by the following steps: obtaining a

knock-out gene construct for knocking out initiator tRNA gene; cloning the knock-out gene construct in a vector; introducing the vector containing the knock-out gene construct into a microorganism; and growing the microorganism on a medium containing a selective agent under conducive conditions and obtaining the microorganism with increased lipid content.

In another aspect of the present disclosure there is provided a modified strain of *Synechococcus elongatus* PCC 7942 having increased lipid content and having CCAP Accession Number 1479/17.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

The method of the present disclosure will now be described with the help of the accompanying drawings, in which:

Figure 1 illustrates a schematic process for increasing the lipid content in microorganisms by decreasing (down regulating) the protein synthesis in microorganisms;

Figure 2 illustrates the tRNA-Met2_deletion vector containing the knock-out gene construct prepared by deleting the initiator tRNA met2;

Figure3 illustrates the PCR confirmation of the transformants prepared in accordance with the present disclosure;

Figure 4A illustrates the graphical representation of fluorescence observed for *Chlamydomonas reinhardtii* CW-15 cells (Day 4) in Tris Acetate Phosphate medium grown with and without cycloheximide using Nile Red Assay;

Figure 4B illustrates Nile Red stained *Chlamydomonas reinhardtii* CW-15 cells grown in the absence of cycloheximide;

Figure 4C illustrates Nile Red stained *Chlamydomonas reinhardtii* CW-15 cells grown in the presence of cycloheximide;

Figure 5 illustrates the spot inoculation of *Synechococcus elongatus* PCC 7942 (with the tRNA Met2 gene knocked out) on a medium containing a selective agent; and

Figure 6 depicts a graph illustrating the increase in lipid content in the modified strains of *Synechococcus elongatus* PCC 7942 as compared to the wild type.

DETAILED DESCRIPTION

A major amount of fixed carbon from photosynthesis is channeled to the production of proteins. Hence, decreased protein synthesis would lead to utilization of this fixed carbon in making lipids, which can be used for the production of biofuels from the microorganisms, specifically from algae/cyanobacteria. The protein synthesis can be down-regulated and the carbon flux is redirected to the production of lipids.

It is observed that the initiator tRNA molecules or initiation factor proteins/ribosomal RNA present in microorganisms are responsible for initiation of protein synthesis. It is possible to decrease the amounts of cellular initiator tRNA molecules by genetic modification of the microorganisms so that the protein synthesis would be decreased.

Therefore, decreasing the availability of proteins by decreasing the rate of initiation of protein synthesis in microorganisms would lead to increased lipid content in the microorganisms. This would lead to increased biofuel production from these microorganisms.

Therefore, in accordance with the present disclosure there is envisaged a method for decreasing (down regulating) protein synthesis in microorganisms, specifically in algae and cyanobacteria (as depicted in **Figure 1**). The decrease in protein synthesis in the transformants would lead to utilization of the fixed carbon for the synthesis of lipid.

In an aspect of the present disclosure, there is provided a method for decreasing (down regulating) protein synthesis and thereby increasing the lipid content in microorganisms.

Chemical inhibition of protein synthesis is carried out using cycloheximide. *Chlamydomonas reinhardtii* CW-15 (*Chlamydomonas* Resource Center, USA), is grown in the presence of varying amounts of cycloheximide (50 μ M to 500 μ M) to find out the effect of inhibition of protein synthesis on lipid content. It is found that the chemical inhibition of protein synthesis results in an increase in lipid content (**Figure 4A**). Highest lipid content is observed when the *Chlamydomonas reinhardtii* CW-15 cells are grown in the presence of 50 μ M of cycloheximide. When higher amounts of cycloheximide (more than 200 μ M) are used, a decrease in the lipid content is observed as illustrated in **Figure 4A**. **Figures 4B** and **4C** depict the Nile Red stained cells of *Chlamydomonas reinhardtii* CW-15 cells grown without the protein inhibitor and in the presence of the protein inhibitor, respectively. As seen from the **Figure 4C**, an increase in the fluorescence is observed when the cells are grown in the presence of the protein inhibitor (cycloheximide), indicating an increase in the lipid content of the *Chlamydomonas reinhardtii* CW-15 cells as compared to the *Chlamydomonas reinhardtii* CW-15 cells grown in the absence of the protein inhibitor.

Transfer RNA Met1 and tRNA Met2 are two initiator tRNA genes present in cyanobacteria and both are involved in the regulation of protein synthesis. In accordance with the present disclosure, tRNA Met2 gene is knocked out. A knock-out gene construct in accordance with the present disclosure is prepared by taking out 1 kb flanking sequences of the initiator tRNA gene and cloning in a vector containing a selective marker. In an embodiment of the present disclosure the selective marker is an antibiotic selected from the group including but not limited to kanamycin, ampicillin and chloramphenicol.

In accordance with another aspect of the present disclosure, there are provided modified strains of microorganism, specifically modified strains of algae and/or cyanobacteria having increased lipid content, particularly, a modified strain in accordance with the present invention can be *Synechococcus elongatus* PCC 7942 deposited in the Culture Collection of Algae and Protozoa (CCAP), SAMS Limited,

Scottish Marine Institute, Dunbeg, Oban, Argyll, PA37 1QA, UK and having CCAP Accession Number 1479/17.

The present disclosure is further described in light of the following laboratory experiments which are set forth for illustration purposes only and not to be construed for limiting the scope of the disclosure.

Experiment 1: Transformation of *Synechococcus elongatus* PCC 7942

One kilo base flanking sequences of the initiator tRNA (tRNA Met2) was taken out and then cloned into a vector containing an antibiotic (chloramphenicol) selection marker. The vector used in the present disclosure is tRNA-Met2_deletion. This vector was then used to knock-out the methionine tRNA (tRNA Met2) from *Synechococcus elongatus* PCC 7942.

Synechococcus elongatus PCC 7942 (Institut Pasteur, France) was inoculated into 50 ml of BG-11 Basal Solution (HiMedia) and allowed to grow overnight at 30 °C till an OD₇₃₀ of 1 (approximately 10⁸ cells/ml) was obtained. The culture was centrifuged at 4000 rpm and the pellet obtained was collected for further experiments.

The pellet was washed thrice with 10 ml of ice cold 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer having pH 7.5. The pellet was soaked in the HEPES buffer for 5 minutes during each washing step.

The supernatant was removed and the pellet was suspended in 500 µl of HEPES buffer containing 6 % dimethyl sulfoxide (DMSO) and 10 % glycerol and mixed properly by vortexing to obtain a cell mixture.

Hundred microliters of the above cell mixture was transferred into a pre-chilled Electroporation Cuvette. Five micro grams of linear DNA (dissolved in water) was added to the Electroporation Cuvette. One cuvette not having DNA was used as the control. The outside of the cuvette was completely dried and then placed in a Gene Pulser for electroporation. The electroporation parameters used in the present disclosure are given below:

- i) Field strength 1800 V/cm

- ii) Capacitance 25 μ F
- iii) Resistance 200 Ω
- iv) Exponential decay wave pulse

After completion of electroporation, the cuvettes were immediately incubated in ice for 5 minutes. The cells were then suspended in 1 ml of BG-11 Basal Solution and then inoculated in an Erlenmeyer flask containing 20 ml of BG-11 Basal Solution. The cells were incubated for 5 days at 30 °C under white light.

After 5 days the cells were collected by centrifugation. The pellet obtained was suspended in 200 μ l of BG-11 Basal Solution. The culture was inoculated on BG-11 Basal Solution containing 1 % agar and chloramphenicol. Colonies were observed on the plates after 20 days (**Figure-5A**). Single colony was further propagated in liquid and solid BG-11 medium containing chloramphenicol for 8 generations for homoplasmy.

The transformation was confirmed by PCR using Chloramphenicol Acetyltransferase (CAT) gene primers as illustrated in **Figure 3**. The DNA was isolated using Sigma plant DNA extraction kit and the genomic DNA was used as the template.

From left to right: Lane-1 depicts the wild type, Lane-6 depicts the positive control containing pMet2 KO plasmid DNA, Lane-7 depicts the negative control without any DNA and Lane-8 depicts the 1 kb plus ladder marker (Thermo Scientific). Lane-2, Lane-3, Lane-4 and Lane-5 are the different transformants: Clone-1, Clone-3, Clone-4 and Clone-19, respectively prepared in accordance with the present disclosure. The vector tRNA-Met2_deletion has a size of 4775 bp. The arrow pointing to the band on Lane-6 (positive control), corresponds to 660 bp of the CAT gene. The wild type genomic DNA does not have a CAT gene and hence, no PCR product is observed after PCR and hence, there is no band corresponding to the wild type (Lane-1) as illustrated in **Figure-3**. In the transformants, the tRNA Met2 gene was knocked-out and replaced with the CAT gene (660 bp). The arrows pointing to the bands in Lane-5 (Clone-19), Lane-4 (Clone-4) and Lane-3 (Clone-3) correspond to the CAT gene,

confirming the transformation. The second band on Lane-2 is a non-specific band formed due to the PCR condition.

Experiment 2: Comparison of the lipid produced by the wild type and the transformants

Ten milligram of dry mass of the wild type and the transformants were used for performing GC analysis of Octadecanoic acid, methyl ester (C18:0) produced. The amount (in %) of C18:0 produced by the wild type and the transformants (Clone-3 and Clone-19) are given in **Figure-6** and **Table-1**.

Octadecanoic acid, methyl ester (C18:0) produced by the wild type and two transformants (Clone-3 and Clone-19) were compared as illustrated in **Figure-6**. A 31 % increase in the amount of lipid (C18:0) produced was observed for Clone-19 as compared to the wild type.

Table-1

10 mg of dry mass	% C18:0
WT	11.83
Clone-3	14.47
Clone-19	15.54

It is clear from **Figure-6** and **Table-1** that the transformants (clone-3 and clone-19) show an increase in the lipid content as compared to the wild type, indicating that the down regulation of protein synthesis results in an overall increase of lipid content in the transformants.

TECHNICAL ADVANCEMENTS

The technical advancements offered by the present disclosure are as follows:

-The present disclosure provides a method for decreasing (down regulating) the different molecules involved in protein synthesis to decrease the protein synthesis and hence, increase the lipid content of the microorganism.

-The present disclosure provides a method for increasing the lipid content of microorganisms to obtain increased production of biofuels.

-The present disclosure provides modified microorganisms with increased lipid content as compared to the wild type.

The embodiments as described herein above, and various features and advantageous details thereof are explained with reference to the non-limiting embodiments in the description. Descriptions of well-known aspects, components and molecular biology techniques are omitted so as to not unnecessarily obscure the embodiments herein.

The foregoing description of specific embodiments will so fully reveal the general nature of the embodiments herein, that others can, by applying current knowledge, readily modify and/or adapt for various applications of such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. Therefore, while the embodiments herein have been described in terms of preferred embodiments, those skilled in the art will recognize that the embodiments herein can be practiced with modification within the spirit and scope of the embodiments as described herein. Further, it is to be distinctly understood that the foregoing descriptive matter is to be interpreted merely as illustrative of the disclosure and not as a limitation.

Having described and illustrated the principles of the present disclosure with reference to the described embodiments, it will be recognized that the described embodiments can be modified in arrangement and detail without departing from the scope of such principles.

While considerable emphasis has been placed herein on the particular features of this disclosure, it will be appreciated that various modifications can be made, and that many changes can be made in the preferred embodiment without departing from the

principles of the disclosure. These and other modifications in the nature of the disclosure or the preferred embodiments will be apparent to those skilled in the art from the disclosure herein, whereby it is to be distinctly understood that the foregoing descriptive matter is to be interpreted merely as illustrative of the disclosure and not as a limitation.

CLAIMS:

1. A method for increasing the lipid content in a microorganism, said method characterized by the following steps:
 - a. obtaining a knock-out gene construct for knocking out initiator tRNA Met2 gene;
 - b. cloning said knock-out gene construct in a vector;
 - c. introducing said vector containing the knock-out gene construct into a microorganism; and
 - d. growing said microorganism on a medium containing a selective agent under conducive conditions and obtaining the microorganism with increased lipid content.
2. The method as claimed in claim 1, wherein said microorganism is a photosynthetic microorganism.
3. The method as claimed in claim 1, wherein said microorganism is an alga selected from the group comprising *Synechococcus* and *Chlamydomonas*.
4. The method as claimed in claim 1, wherein said knock-out gene construct is prepared by knocking out 1 kb flanking sequences of initiator tRNA gene.
5. The method as claimed in claim 1, wherein said vector is tRNA-Met2_deletion.
6. The method as claimed in claim 1, wherein said selective agent is at least one antibiotic selected from the group consisting of kanamycin, ampicillin and chloramphenicol.
7. A method for manufacturing a modified microorganism having increased lipid content, said method characterized by the following steps:

- a. obtaining a knock-out gene construct for knocking out initiator tRNA gene;
 - b. cloning said knock-out gene construct in a vector;
 - c. introducing said vector containing the knock-out gene construct into a microorganism; and
 - d. growing said microorganism on a medium containing a selective agent under conducive conditions to obtain the microorganism having increased lipid content,
- wherein, the lipid content synthesized/produced by the modified microorganism is increased relative to the unmodified microorganism.
8. A modified microorganism manufactured by the method as claimed in claim 7.
 9. A modified strain of *Synechococcus elongatus* PCC 7942 having increased lipid content and having CCAP Accession Number 1479/17.

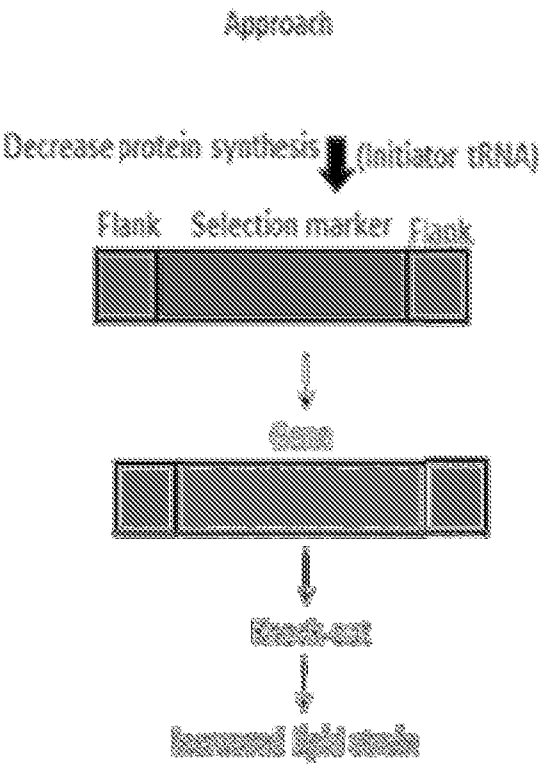


FIGURE 1

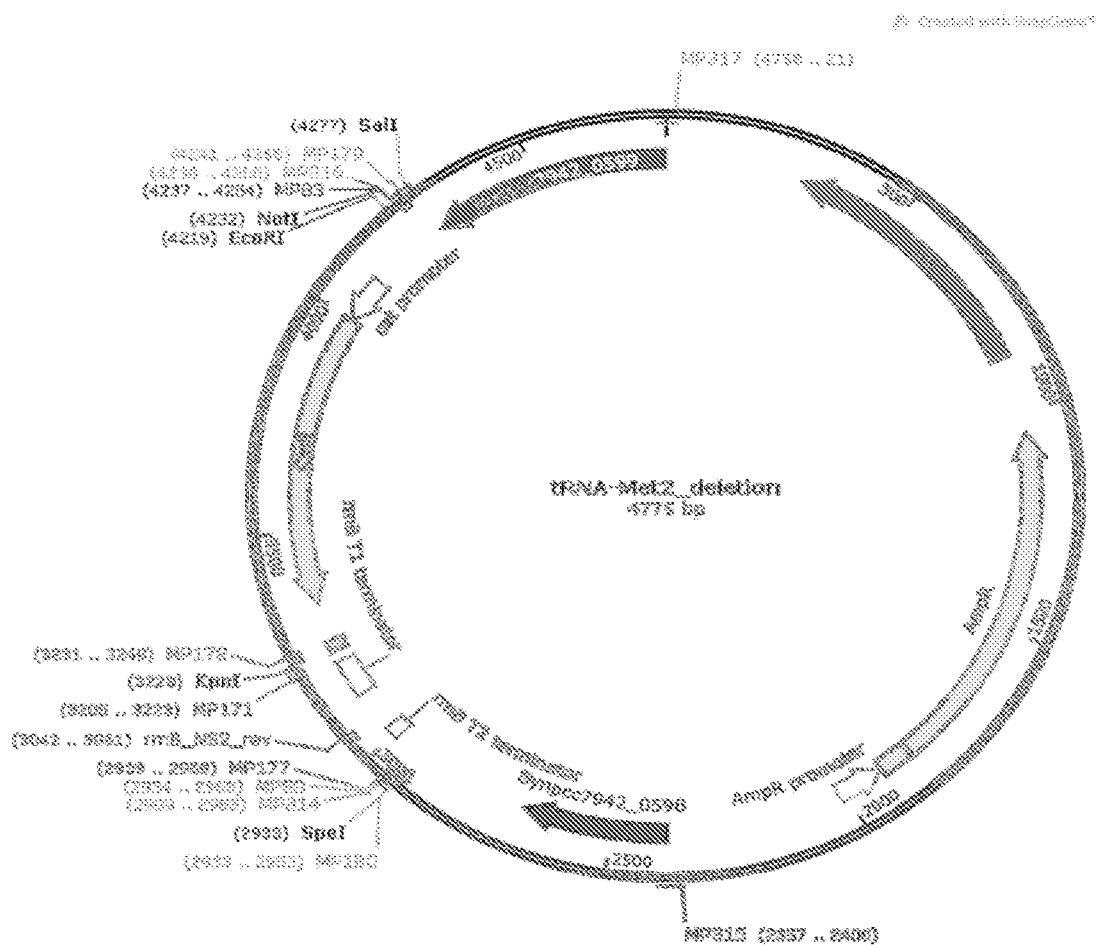


FIGURE 2

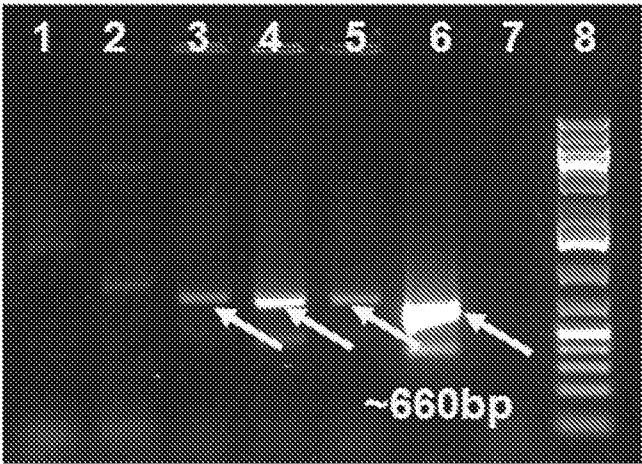


FIGURE 3

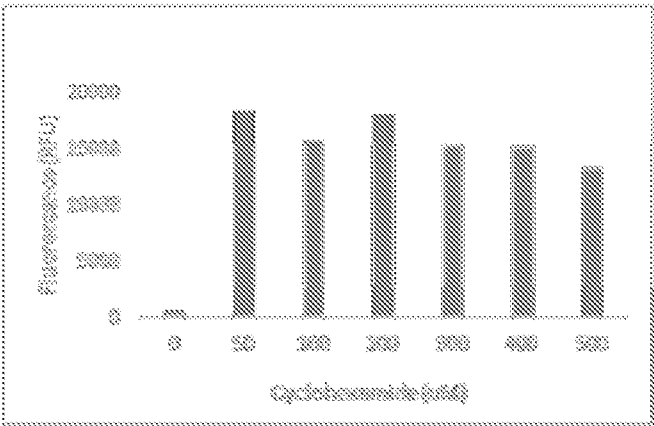


FIGURE 4A

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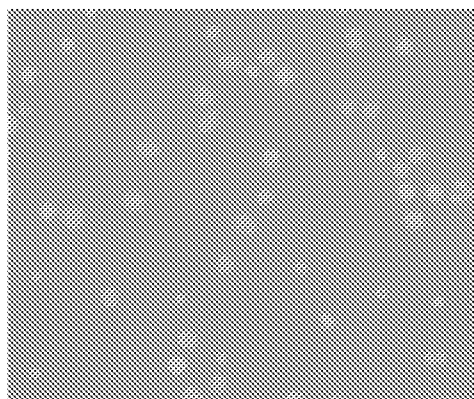


FIGURE 4B

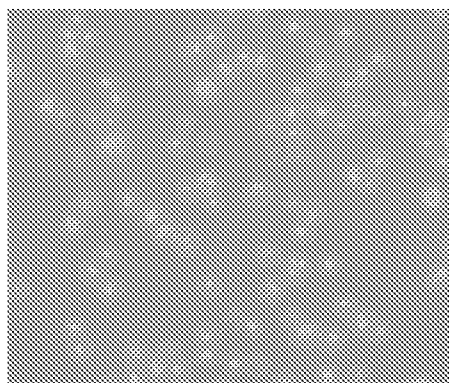


FIGURE 4C

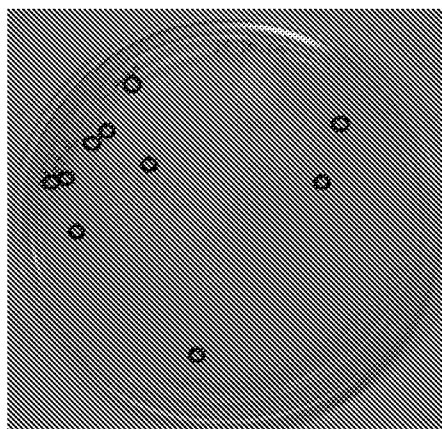


FIGURE 5

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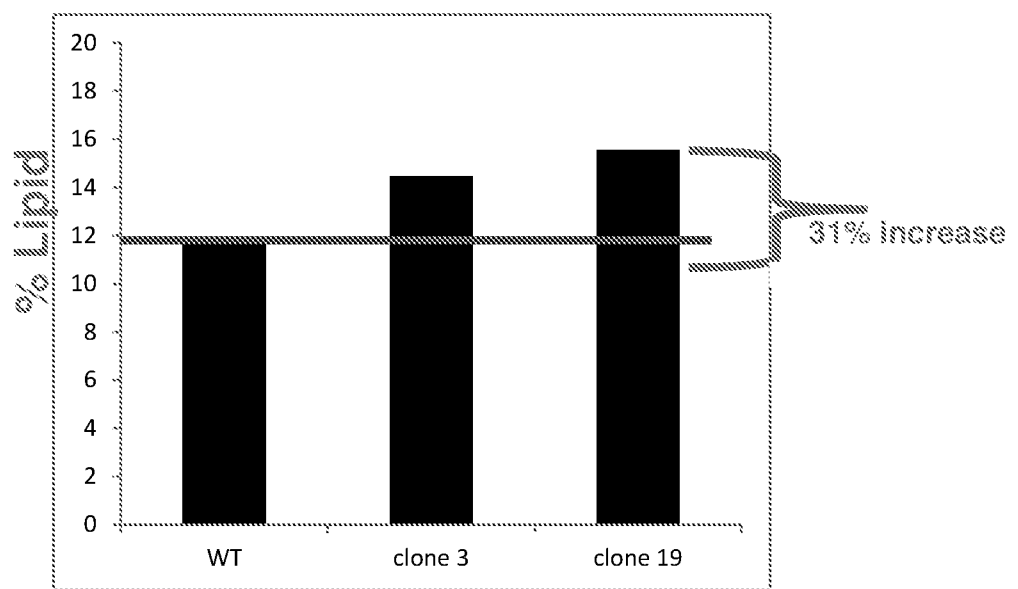


FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2015/054011

A. CLASSIFICATION OF SUBJECT MATTER C12N15/63, C12N1/00, C12N1/38, C12N15/79 Version=2015.01 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) IPO INTERNAL DATABASE, PATSEER DATABASE, PUBMED		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/087963 A1, (TARGETED GROWTH, INC.), 28-06-2012. Claims; page 69, last para; page 70, 1-4 lines, -----	1-9
Y	KAPOOR SUMAN et al, "Crucial contribution of the multiple copies of the initiator tRNA genes in the fidelity of tRNA ^{fMet} selection on the ribosomal P-site in Escherichia coli," Nucleic Acids Research, 2011, Vol. 39, No. 1, Pages 202-212. page 202, right column -----	1-9
A	ANNE M. RUFFING et al, "Physiological Effects of Free Fatty Acid Production in Genetically Engineered Synechococcus elongatus PCC 7942," Biotechnol Bioeng., 2012 September, 109(9), Pages 2190-2199. whole document -----	1-9
A	ANNE M. RUFFING, "Improved free fatty acid production in cyanobacteria with Synechococcus sp. PCC 7002 as host," Frontiers in Bioengineering and Biotechnology, 26 May 2014, Vol. 2, article 17, Page 1-10. whole document	1-9
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
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Date of the actual completion of the international search 14-08-2015		Date of mailing of the international search report 14-08-2015
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2015/054011

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	----- BRIAN SGAARD LAURSEN et al, "Initiation of Protein Synthesis in Bacteria," MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Mar. 2005, Vol. 69, No. 1, Pages 101-123. whole document	1-9

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB2015/054011

Citation	Pub.Date	Family	Pub.Date
WO 2012087963 A1	28-06-2012	EP 2655605 A1	30-10-2013
		US 2014004580 A1	02-01-2014
		AU 2011349444 A1	11-07-2013