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[Continued on next page]

(54) Title: RECOMBINANT SYNTHESIS OF ALKANES

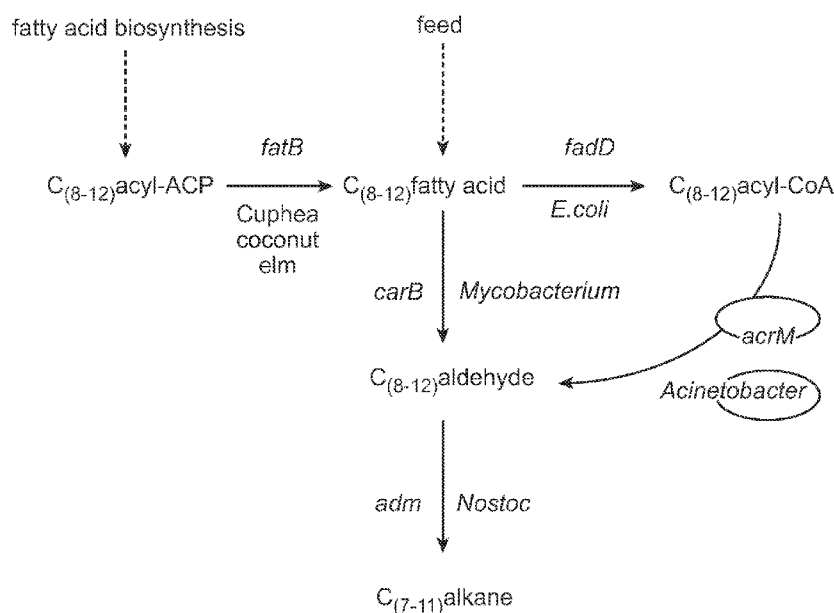


FIG. 6

(57) Abstract: The present disclosure identifies methods and compositions for modifying photoautotrophic organisms as hosts, such that the organisms efficiently produce alkanes, and in particular the use of such organisms for the commercial production of alkanes and related molecules. Other materials, methods, and compositions are also described. Many existing photoautotrophic organisms (i.e., plants, algae, and photosynthetic bacteria) are poorly suited for industrial bioprocessing and have therefore not demonstrated commercial viability. Recombinant photosynthetic microorganisms have been engineered to produce hydrocarbons and alcohols in amounts that exceed the levels produced naturally by the organism.

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9 October 2014

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/013189

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 1/21 (2014.01)

USPC - 435/166

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)

IPC(8) - C07C 37/20; C12N 1/21, 15/09, 15/82; C12P 5/00, 5/02 (2014.01)

USPC - 435/134, 157, 166, 167, 252.3; 568/448, 449; 585/16

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

CPC - C12N 9/0006, 15/52; C12P 5/02, 5/026; Y02E 50/10, 50/13 (2014.02)

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

PatBase, Google Patents, Google Scholar, ProQuest

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0157717 A1 (RUDE et al) 21 June 2012 (21.06.2012) entire document	43, 45, 62, 63, 65-70, 76-80

Y		1-3, 16, 44, 52-54, 60, 64, 81
Y	WO 2012/154329 A1 (GREENFIELD et al) 15 November 2012 (15.11.2012) entire document	1-3, 16
Y	US 2011/0245091 A1 (GOLOVLEV) 06 October 2011 (06.10.2011) entire document	3
Y	US 2012/0021478 A1 (OSTERHOUT et al) 26 January 2012 (26.01.2012) entire document	44, 53, 54, 64
Y	US 2012/0276637 A1 (ZHOU et al) 01 November 2012 (01.11.2012) entire document	52-54, 60
Y	US 2007/0092957 A1 (DONALDSON et al) 26 April 2007 (26.04.2007) entire document	81
A	US 2012/0244589 A1 (ROBERTSON et al) 27 September 2012 (27.09.2012) entire document	1-3, 16, 43-45, 52-54, 60, 62-70, 76-81

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 June 2014

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/013189

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☐

together with the international application in electronic form

☒

subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO:36 was searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/013189

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 17-42, 51, 61
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Extra Sheet(s)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 16, 43-45, 52-54, 60, 62-70, and 76-81 to the extent that they read on an alkane deformylative monooxygenase, a thioesterase, a carboxylic acid reductase, and a phosphopantetheinyl transferase, wherein the alkane deformylative monooxygenase has EC number 4.1.99.5, the thioesterase has EC number 3.1.2.14, the carboxylic acid reductase has EC number 1.2.99.6, and the phosphopantetheinyl transferase has EC number 2.7.8.7.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/013189

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-16, 43-50, 52-60, and 62-81 are drawn to an engineered microorganism, and method and cell culture comprising the same.

The first invention of Group I+ is restricted to a an engineered microorganism, and method and cell culture comprising the same, wherein said engineered microorganism comprises one or more recombinant nucleic acid sequences encoding one or more enzymes having enzyme activities which catalyze the production of alkanes, wherein the enzyme activities are selected to be an alkane deformylative monooxygenase, a thioesterase, a carboxylic acid reductase, and a phosphopanthetheinyl transferase, wherein the alkane deformylative monooxygenase has EC number 4.1.99.5, the thioesterase has EC number 3.1.2.14, the carboxylic acid reductase has EC number 1.2.99.6, and the phosphopanthetheinyl transferase has EC number 2.7.8.7. It is believed that claims 1-3, 16, 43-45, 52-54, 60, 62-70, and 76-81 read on this first named invention and thus these claims will be searched without fee to the extent that they read on an alkane deformylative monooxygenase, a thioesterase, a carboxylic acid reductase, and a phosphopanthetheinyl transferase, wherein the alkane deformylative monooxygenase has EC number 4.1.99.5, the thioesterase has EC number 3.1.2.14, the carboxylic acid reductase has EC number 1.2.99.6, and the phosphopanthetheinyl transferase has EC number 2.7.8.7.

Applicant is invited to elect additional enzyme activities for each engineered microorganism, method, and cell culture to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be an engineered microorganism, and method and cell culture comprising the same, wherein said engineered microorganism comprises one or more recombinant nucleic acid sequences encoding one or more enzymes having enzyme activities which catalyze the production of alkanes, wherein the enzyme activities are selected to be an alkane deformylative monooxygenase, a thioesterase, a carboxylic acid reductase, and a phosphopanthetheinyl transferase, wherein the enzyme having alkane deformylative monooxygenase activity has EC number 4.1.99.5, the enzyme having thioesterase activity has EC number 3.1.2.14, the enzyme having carboxylic acid reductase activity has EC number 1.2.99.6, and the enzyme having phosphopanthetheinyl transferase activity has EC number 2.7.8.7. Additional enzyme activities for each engineered microorganism, method, and cell culture will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element, requiring the selection of alternatives for the enzyme activities "said engineered microorganism comprises one or more recombinant nucleic acid sequences encoding one or more enzymes having enzyme activities which catalyze the production of alkanes, wherein the enzyme activities comprise an alkane deformylative monooxygenase activity and a thioesterase activity, a carboxylic acid reductase activity, and a phosphopanthetheinyl transferase activity; a thioesterase activity, a long-chain fatty acid CoA-ligase activity, and a long-chain acyl-CoA reductase activity; and/or a pyruvate decarboxylase activity and a 2-ketoacid decarboxylase activity".

The Groups I+ share the technical features of an engineered microorganism, wherein said engineered microorganism comprises one or more recombinant nucleic acid sequences encoding one or more enzymes having enzyme activities which catalyze the production of alkanes, wherein the enzyme activities comprise an alkane deformylative monooxygenase activity and a thioesterase activity, a carboxylic acid reductase activity, and a phosphopanthetheinyl transferase activity; a thioesterase activity, a long-chain fatty acid CoA-ligase activity, and a long-chain acyl-CoA reductase activity; and/or a pyruvate decarboxylase activity and a 2-ketoacid decarboxylase activity; a method of producing a short-chain alkane or alkene from an engineered organism, the method comprising: a. expressing a recombinant alkanal deformylative monooxygenase ("ADM") in said engineered microorganism; and b. culturing said engineered microorganism in a culture medium containing a carbon source under conditions effective to produce a short-chain alkane or alkene; an engineered microorganism, wherein said engineered microorganism comprises a recombinant gene encoding an alkanal deformylative monooxygenase ("ADM"), and wherein said engineered microorganism further comprises a recombinant gene encoding an enzyme selected from the group consisting of: pyruvate decarboxylase and 2-ketoacid decarboxylase; a cell culture comprising a recombinant microorganism and a culture medium containing a carbon source, wherein a polypeptide that catalyzes the conversion of an aldehyde to an alkane is overexpressed in said recombinant microorganism and an alkane or alkene is produced in the cell culture when said recombinant microorganism is cultured in the culture medium under conditions effective to express said polypeptide; and a method for producing isobutane or a derivative of isobutane, comprising contacting ADM with an aldehyde in vitro. However, these shared technical features do not represent a contribution over the prior art.

<continued on next sheet>

Specifically, US 2012/0244589 A1 to Robertson et al. discloses an engineered microorganism (engineered cyanobacterium, Para. [0011]), wherein said engineered microorganism comprises one or more recombinant nucleic acid sequences encoding one or more enzymes having enzyme activities which catalyze the production of alkanes (engineered cyanobacterium comprises a recombinant AAR enzyme and a recombinant ADM enzyme; and exposing said engineered cyanobacterium to light and inorganic carbon, wherein said exposure results in the conversion of said inorganic carbon by said engineered cyanobacterium into n-alkanes, Para. [0011]), wherein the enzyme activities comprise an alkane deformylative monooxygenase activity and a thioesterase activity, a carboxylic acid reductase activity, and a phosphopantetheinyl transferase activity; a thioesterase activity, a long-chain fatty acid CoA-ligase activity, and a long-chain acyl-CoA reductase activity; and/or a pyruvate decarboxylase activity and a 2-ketoacid decarboxylase activity (an engineered host cell for producing an n-alkane, wherein said cell comprises one or more recombinant protein activities selected from the group consisting of an acyl-CoA reductase activity, an acyl-ACP reductase activity, an alkanal deformylative monooxygenase activity, and an electron donor activity, Para. [0023]); a method of producing a short-chain alkane or alkene from an engineered organism (a method for producing hydrocarbons ...for producing an n-alkane, Para. [0011]; alkanes such as propane, Para. [0118]), the method comprising: a. expressing a recombinant alkanal deformylative monooxygenase ("ADM") in said engineered microorganism (said engineered cyanobacterium comprises a recombinant AAR enzyme and a recombinant ADM enzyme, Para. [0011]; an engineered host cell for producing an n-alkane, wherein said cell comprises one or more recombinant protein activities selected from the group consisting of an acyl-CoA reductase activity, an acyl-ACP reductase activity, an alkanal deformylative monooxygenase activity, and an electron donor activity, Para. [0023]); and b. culturing said engineered microorganism in a culture medium containing a carbon source under conditions effective to produce a short-chain alkane or alkene (culturing an engineered cyanobacterium in a culture medium ...exposing said engineered cyanobacterium to light and inorganic carbon, wherein said exposure results in the conversion of said inorganic carbon by said engineered cyanobacterium into n-alkanes, Para. [0011]); a cell culture (cell or cell culture, Para. [0222]) comprising a recombinant microorganism and a culture medium containing a carbon source, wherein a polypeptide that catalyzes the conversion of an aldehyde to an alkane is overexpressed in said recombinant microorganism and an alkane or alkene is produced in the cell culture (culturing an engineered cyanobacterium in a culture medium, wherein said engineered cyanobacterium comprises a recombinant AAR enzyme and a recombinant ADM enzyme (ADM catalyzes the conversion of an aldehyde to an alkane); and exposing said engineered cyanobacterium to light and inorganic carbon, wherein said exposure results in the conversion of said inorganic carbon by said engineered cyanobacterium into n-alkanes, Para. [0011]) when said recombinant microorganism is cultured in the culture medium under conditions effective to express said polypeptide (said cyanobacterium does not synthesize alkanes in the absence of the expression of one or both of the recombinant enzymes, Para. [0013]; recombinant AAR and/or ADM enzymes are encoded by genes which are part of an operon, wherein the expression of said genes is controlled by a single promoter, Para. [0016]; the promoter in each case is selected to provide optimal expression of the protein, Para. [0230]); and a method for producing isobutane or a derivative of isobutane (a method for producing hydrocarbons ...for producing an n-alkane, Para. [0011]; butanol, Para. [0118]), comprising contacting ADM with an aldehyde in vitro (culturing an engineered cyanobacterium in a culture medium, wherein said engineered cyanobacterium comprises a recombinant AAR enzyme and a recombinant ADM enzyme (ADM catalyzes the conversion of an aldehyde to an alkane); and exposing said engineered cyanobacterium to light and inorganic carbon, wherein said exposure results in the conversion of said inorganic carbon by said engineered cyanobacterium into n-alkanes, Para. [0011]; fatty aldehyde --> alkane/alkene, of the AAR-ADM pathway, Para. [0192]).

Further, WO 2012/116345 A2 to Zhou et al. discloses an engineered microorganism (genetically modified cyanobacteria such as *Anabaena* spp., Para. [0005]), wherein said engineered microorganism comprises a recombinant gene encoding an alkanal deformylative monooxygenase ("ADM"), and wherein said engineered microorganism further comprises a recombinant gene encoding an enzyme selected from the group consisting of: pyruvate decarboxylase and 2-ketoacid decarboxylase (the *Anabaena* spp. is genetically engineered by expression of at least one recombinant polynucleotide expression construct comprising an enzyme capable of increasing production of a carbon based product of interest, Para. [0005]; polynucleotide encodes pyruvate decarboxylase, Para. [0044]; combined with aldehyde decarboxylase (AD) convert fatty acid to alkanes/alkenes, Para. [0055]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.