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2 April 2015

(54) Title: ENGINEERED ORGANISMS FOR PRODUCTION OF NOVEL LIPIDS

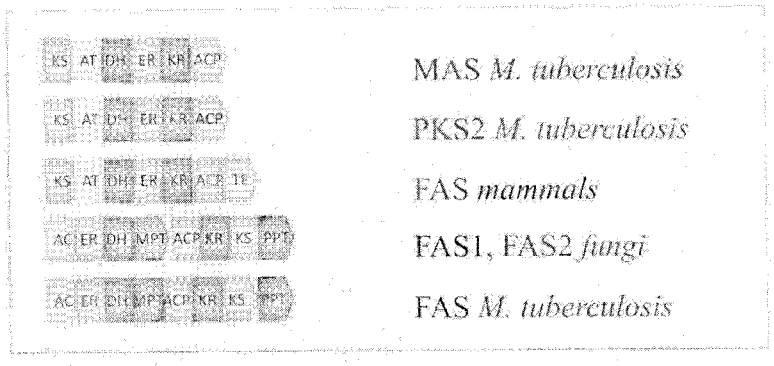

FIGURE 1

(57) Abstract: The present disclosure provides engineered microorganisms, engineered biosynthetic pathways, methods of producing lipid compounds using genetically engineered microorganisms, and the products synthesized by those organisms. In particular, the disclosure provides genetically engineered microorganisms for the production of multi-methyl branched fatty acids (MMBFAs) and MMBFA esters (wax esters) derived from these fatty acids. In addition, the disclosure provides methods for producing acylglycerols with one of more of their acyl substituents being an MMBFA, and methods for producing alcohols derived from MMBFAs (fatty alcohols).

WO 2014/144461 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/28883

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 7/64, C12N 1/00, C12N 1/20 (2014.01)
CPC - C12P 7/6472, C12P 7/6427, C12P 7/6463, C12P 7/6454, C12N 1/20, C12N 1/00, A61K 38/00
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)
CPC- C12P 7/6472, C12P 7/6427, C12P 7/6463, C12P 7/6454, C12N 1/20, C12N 1/00, A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC- C02F 3/34, C12N 1/04, A23D 9/00
USPC- 435/134, 435/243, 435/252.33, 554/1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: MAS, Mycobacterium tuberculosis, fad28, fatty-acid-CoA ligase FadD28, alcohol dehydrogenase, papA5, acyltransferase, PKS-associated protein PapA5, Mycocerosic acid synthase, MMAT domain, long chain fatty acid AMP ligase... GenCore 6.4.1:SEQ ID NO:1, 3, 72

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2011/0166370 A1 (Saunders, et al.) 07 July 2011 (07.07.2011) para [0039], [0041], [0149], [0187]-[0199], [0214], [0221], [0243]-[0251], [0257], SEQ ID NO: 18, 19	1-6 and 84 ---------- 11-13
Y	WO 2001/002555 A1 (Gicquel, et al.) 21 June 2001 (21.06.2001) SEQ ID NO: 60; pg 26, ln 15-32	11-13

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"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
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 October 2014 (08.10.2014)	29 JAN 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/28883

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.: 7, 9, 10, 14-52, 61-83, 85-93, 98-101
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:

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.

•• See Supplemental Sheet to continue ••

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.:
1-6, 11-13, and 84, restricted to SEQ ID NO: 1, 3, 72

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.

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

Group I+: claims 1-6, 8, 11-13, 84, drawn to a multimethyl branched fatty acid (MMBFA) synthesis pathway in which the exogenous or overexpressed polyketide synthase MAS catalyzes the iterative conversion of methylmalonyl-CoA to MMBFA (claim 84) and an engineered microorganism comprising
(i) an exogenous or overexpressed polyketide synthase or active fragment thereof, and
(ii) an exogenous or overexpressed long chain fatty acid AMP ligase,
wherein said recombinant microorganism comprises a MMBFA synthesis pathway in which the exogenous or overexpressed polyketide synthase MAS catalyzes the iterative conversion of methylmalonyl-CoA to MMBFA (claims 1-6, 8, 11-13).
Group I+ will be searched to the extent that it reads on the PKS having an amino acid sequence SEQ ID NO: 1 and 3, the long chain fatty acid AMP ligase comprising SEQ ID NO: 72, i.e. FadD28, without fee. It is believed that claims 1-6, 11-13, and 84 read on this first named invention. Applicants must 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.
An exemplary election would read on PKS4 of SEQ ID NO:26, FadD21 of SEQ ID NO:74, i.e. claims 1, 2, 5, 6, 8, 13. 84.

Group II: claims 53-60, drawn to an engineered microorganism comprising:
(i) a MAS polyketide synthase from Mycobacterium tuberculosis;
(ii) a long chain fatty acid AMP ligase from Mycobacterium tuberculosis;
(iii) a TESA' thioesterase from Escherichia coli (SEQ ID N0:89); and,
(iv) a propionyl-CoA carboxylase (PCC) complex from Streptomyces coelicolor,
wherein the MAS polyketide synthase catalyzes the iterative conversion of methyl-malonyl-CoA to MMBFA.

Group III: claims 94-97, drawn to an MMBFA and an ester thereof.

The inventions listed as Groups I+ through III 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:

Special Technical Features
The special technical feature of each invention of Group I+ is a polyketide synthase of a specific amino acid sequence and a long chain fatty acid AMP ligase of a specific amino acid sequence.
As to the claimed polyketide synthases, a PKS of SEQ ID NO: 1 was known in the art at the time of the invention, as evidenced by US 2011/166370 A1 to SAUNDERS, et al. (hereinafter "Saunders") (SEQ ID NO:18, 100% identity, and no significant structural similarities can readily be ascertained among the claimed polyketide synthases.
As to the claimed long chain fatty acid AMP ligases, a long chain fatty acid AMP ligase of SEQ ID NO: 72, i.e., FadD28, was known in the art at the time of the invention, as evidenced by WO 2001/002555 A1 to GICQUEL, et al. (hereinafter "Gicquel") (SEQ ID NO:60, 100% identity), and no significant structural similarities can readily be ascertained among the claimed long chain fatty acid AMP ligases.

The inventions of Groups I+ and II do not include the shared or common technical feature of an MMBFA and an ester thereof comprising (i) a fatty acid chain length of about 10 to about 40 carbons;(ii) at least 4 methyl branches located at position 2, 4, 6, and 8 of the fatty acid chain; and, (iii) zero or one unsaturation in the fatty acid chain, as required by Group III.

In addition, MMBFAs and their esters do not represent a contribution over prior art as being anticipated by an article titled "Synthesis of diacylated trehalose sulfates: candidates for a tuberculosis vaccine" by Guiard, et al. (Angewandte Chemie, International Edition 2008, 47(50):9734-9738) (hereinafter "Guiard") (pg 9735, Scheme 2, compound F, disclosing an MMBFA, and pg 9736, Table 1col 1, compounds 15 and 20, disclosing an ester of an MMBFA).

The inventions of Group III do not include the shared or common technical feature of an engineered microorganism, as required by Groups I+ and II.

The inventions of Group I+ do not include the shared or common technical feature of an engineered microorganism comprising: (i) a MAS polyketide synthase from Mycobacterium tuberculosis; (ii) a long chain fatty acid AMP ligase from Mycobacterium tuberculosis; (iii) a TESA' thioesterase from Escherichia coli (SEQ ID N0:89); and, (iv) a propionyl-CoA carboxylase (PCC) complex from Streptomyces coelicolor, as required by Group II.
The inventions of Group II do not include the shared or common technical feature of an engineered microorganism comprising an exogenous or overexpressed (i) polyketide synthase and (ii) long chain fatty acid AMP ligase, as required by Group I+.

Common Technical Features
The inventions of Group I+ share the technical feature of an engineered microorganism comprising (i) an exogenous or overexpressed polyketide synthase or active fragment thereof, and (ii) an exogenous or overexpressed long chain fatty acid AMP ligase, wherein said recombinant microorganism comprises a MBFA synthesis pathway in which the exogenous or overexpressed polyketide synthase MAS catalyzes the iterative conversion of methylmalonyl-CoA to MMBFA. However, this shared technical feature does not represent a contribution over prior art as being obvious over Saunders, as above.

********** See the Following Supplemental Sheet to continue **********

********** Supplemental Sheet **********

In Continuation of the Preceding Supplemental Sheet and the Preceding Supplemental Sheet:

Saunders discloses an engineered microorganism (para [0243]-[0251], Production of Host Cells Producing Branched-Chain Fatty Acid, para [0247], "genomic DNAs were screened by PCR for the presence of tetR, lacI.sup.q and fadD88. One such transductant was named K27-Z1 and used in further studies"; para [0257], "strain was engineered to produce branched fatty acids [BL21 Star (DE3) (pTrcHisA Ec sbm So ce epi pZA31 mmat)]"), comprising
(i) an exogenous polyketide synthase or active fragment thereof (para [0221], Example 6, "Creation of an Expression Vector Comprising the Coding Sequence of the MMAT (Methylmalonyl-CoA Acyl Transferase) Domain from Mycobacterium Mycocerosic Acid Synthase (MAS). Mycobacterium MAS is a multifunctional protein that catalyzes the synthesis of mycocerosic acid and that contains a domain with MMAT activity. The MMAT domain (amino acids 508-890) (SEQ ID NO: 18) of MAS from Mycobacterium bovis BCG (YP.sub.--979046) (SEQ ID NO: 19) was codon optimized for E. coli expression"), and
(ii) an exogenous or overexpressed long chain fatty acid AMP ligase (para [0039], [0041], "... the polypeptide is encoded by fabD"; para [0247], fadD88, fatty-acid-CoA ligase; para [0195], "conversion of methylmalonyl-CoA to methylmalonyl-ACP is increased in the cell by engineering the cell to produce an acyl transferase (such as the acyl transferase encoded by fabD in E. coli) to catalyze the formation of methylmalonyl-ACP from methylmalonyl-CoA. Put another way, in one aspect, the cell further comprises an exogenous or overexpressed polynucleotide comprising a nucleic acid sequence encoding an acyl transferase"),
wherein said recombinant microorganism comprises a MMBFA synthesis pathway in which the exogenous or overexpressed polyketide synthase MAS (para [0149], "FIG. 19 is a protein sequence for the Mycobacterium bovis BCG MAS... (SEQ ID NO: 19)") catalyzes the iterative conversion of methylmalonyl-CoA to a methyl branched fatty acid (para [0198], "the cell is modified to produce (or increase the production of) branched acyl-CoA, which is a substrate for elongase in the production of long chain fatty acid.... the cell comprises exogenous or overexpressed polynucleotide(s) comprising a nucleic acid sequence encoding an elongase to increase the length of the carbon backbone... When a methyl-malonyl CoA is used as an extension unit by the enzyme complex, additional methyl branches are introduced at even carbon positions"; para [0214], " ... a branched-chain fatty acid comprising between 10-18 carbons in the carbon backbone, such as fatty acids comprising between 10 and 16 carbons (e.g., fatty acids comprising 10, 11, 12, 13, 14, 15, or 16 carbons), with branching on one or more even numbered carbons (e.g., C2, C4, C6, C8, C10, C12, C14, and/or C16). A composition comprising longer-chain fatty acid also is provided, such as a composition comprising between 19 and 22 carbons in the longest carbon chain").

Saunders does not specifically disclose a specific embodiment of an engineered microorganism producing a multimethyl branched fatty acid. However, Saunders provides a specific guidance as to how to achieve such an microorganism (para [0250], "Production of methylmalonyl-CoA in host cells expressing exogenous propionyl-CoA carboxylase also was studied and is illustrated in FIG. 25. BW25113 (control) and BW25113 containing pZA31-accA1-pccB (labeled as Pcc in the figure) were cultured in LB... Host cells comprising a polynucleotide encoding an exogenous propionyl-CoA carboxylase produced over about 15 ng methylmalonyl-CoA per ml of culture. [0251] When Ec 'tesA was present, less longer-chain (fifteen and seventeen carbons) and more mid-chain (thirteen carbons) branched fatty acids were produced by the host cell, indicating that production of thioesterase increases the proportion of medium chain-length branched fatty acids produced..."; see also para [0187]-[0199] for more guidance as to how to manipulate a host cell genome such as to optimize production of a MMBFA), thereby enabling one of ordinary of ordinary skill in the art to achieve, in the course of routine experimentation and with a reasonable expectation of success an engineered microorganism producing a multimethyl branched fatty acid. As said technical feature would have been obvious to one of ordinary skill in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the inventions.

Groups I+ through III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note re item 4:
Claims 7, 9, 10, 14-52, 61-83, 85-93, 98-101 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.