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(54) Title: METHODS AND ORGANISMS WITH INCREASED CARBON FLUX EFFICIENCIES

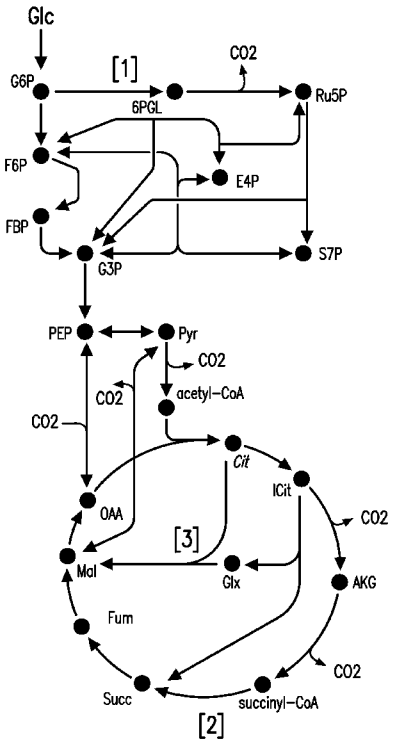

FIG. 1

(57) Abstract: The invention is directed to a non-naturally occurring microbial organism comprising a first attenuation of a succinyl-CoA synthetase or transferase and at least a second attenuation of a succinyl-CoA converting enzyme or a gene encoding a succinate producing enzyme within a multi-step pathway having a net conversion of succinyl-CoA to succinate.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/72178

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/21, C12P 7/52, C12P 1/04, C12P 7/16 (2015.01)
CPC - C12N 1/20, C12R 1/19, C12P 7/52, C12P 1/04, C12P 7/16
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) - C12N 1/21, C12P 7/52, C12P 1/04, C12P 7/16 (2015.01)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12R 1/185, C12N 15/00, C12N 15/09
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, PubMed Central
Search terms: cydA, cydB, attenuate, pntAB, non-naturally occurring, microbe, Escherichia coli, TCA, cycle, 4-hydroxybutyrate, 1,4-butanediol, 1,3-butanediol, polyhydroxylbutanoate, butadiene, adipate, 6-aminocaproate, caprolactam, methacrylic acid, isopropanol,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIM et al., Large-Scale Bi-Level Strain Design Approaches and Mixed-Integer Programming Solution Techniques. PLoS One. 2011, Vol 6, No 9, page e24162. Especially p 7, col 1 -- col 2, para 1, para 2; p 8, Table 2	1-7
Y	HANKE et al., Combined Fluxomics and Transcriptomics Analysis of Glucose Catabolism via a Partially Cyclic Pentose Phosphate Pathway in Gluconobacter oxydans 621H. Appl Environ Microbiol. April 2013, Vol 79, No 7, pages 2336-2348. Especially p 2342, col 2, para 2	1-7
Y	US 2012/0122171 A1 (BURK et al.) 17 May 2012 (17.05.2012) abstract; para [0026], [0066]	3
A	GABRIEL et al., Regulation of the Bacillus subtilis yciC gene and insights into the DNA-binding specificity of the zinc-sensing metalloregulator Zur. J Bacteriol. May 2008, Vol 190, No 10, pages 3482-3488. Entire document	1-7
A	PRZYBYLA-ZAWISLAK et al., Genes of succinyl-CoA ligase from Saccharomyces cerevisiae. Eur J Biochem, 01 December 1998, Vol 258, No 2, pp 736-43. Entire document	1-7
Y, P	MCMAHON et al., Functional Screening and In Vitro Analysis Reveal Thioesterases with Enhanced Substrate Specificity Profiles That Improve Short-Chain Fatty Acid Production in Escherichia coli. Appl Environ Microbiol. February 2014, Vol 80, No 3, pages 1042-1050. Entire document	1-7

☐ 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
01 July 2015 (01.07.2015)	23 JUL 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. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/72178

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.: 8-9, 28-35, 49-55, 71-75
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:

--------Please see continuation in extra sheet--------

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.:
1-7, limited to an attenuation of cydA or cydB and a genetic alteration that increases expression of a protein encoded by pntAB

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/US 14/72178

Continuation of:
Box No III Observations where unity of invention is lacking

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 must be paid.

Group I+: Claims 1-7, 10-27, and 36-40, drawn to a non-naturally occurring microbial organism comprising a genetic alteration in a combination of genes selected from the following genes: cydA, cydB, pntAB, pykF, sucCD, yciA, ackA, pta, cyoB, pykA, arcA, err, clpA, menC. The non-naturally occurring microbial organism will be searched to the extent that the genetic alterations encompasses the first named combination of genetic alterations, namely a genetic alteration comprising attenuation of cydA or cydB and a genetic alteration that increases expression of a protein encoded by pntAB. It is believed that claims 1-7 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a non-naturally occurring microbial organism comprising attenuation of cydA or cydB and a genetic alteration that increases expression of a protein encoded by pntAB. [Note that claim 10 is not included in the first named invention because cydA, cydA and pntAB are not succinyl-CoA synthetase or transferase as required in claim 10]. Additional combination of genetic alterations will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected combination of genetic alterations. Applicants must further indicate, if applicable, the claims which encompass 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. An exemplary election would be a non-naturally occurring microbial organism comprising an attenuation of cydA or cydB, and an attenuation of the protein encoded by pykF, i.e. claims 1-7.

Group II: Claims 41-48 and 56-60, drawn to a non-naturally occurring microbial organism comprising a gene disruption of a gene encoding YciA CoA hydrolase and a metabolically engineered pathway for producing a bioderived compound from a TCA cycle intermediate

Group III: Claims 61-70, drawn to a non-naturally occurring microbial organism comprising a genetic alteration that increases expression of a NADH dehyrogenase Ndh-I (nuo), cytochrome bo oxidase (cyoABCDE) or both and further comprising a gene disruption of one or more endogenous nucleic acids encoding a menaquinol biosynthetic enzyme or one or more nucleic acids encoding a dimethylmenaquinol biosynthetic enzyme

The inventions listed as Groups I+, II, and 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 technical feature of each of the inventions listed as Group I+ is the specific combination of genetic alterations recited therein. Each invention requires a combination of genetic alterations not required by the other inventions, and not required by Group II and III.

Group II requires a metabolically engineered pathway for producing a bioderived compound from a TCA cycle intermediate, not required by Groups I+ and III.

Group III requires a genetic alteration that increases expression of a NADH dehyrogenase Ndh-I (nuo), cytochrome bo oxidase (cyoABCDE) or both and further comprising a gene disruption of one or more endogenous nucleic acids encoding a menaquinol biosynthetic enzyme or one or more nucleic acids encoding a dimethylmenaquinol biosynthetic enzyme, not required by Group I+ and II.

Common Technical Features

The feature shared by Groups I+, II, and III is a non-naturally occurring microbial organism having genetic alterations in a metabolic pathway.

The feature shared by Group I+ is the non-naturally occurring microbial organism of claim 1, such as a microbial organism having a genetic alteration comprising attenuation of cydA or cydB and a genetic alteration that increases expression of a protein encoded by pntAB

However, these shared technical features do not represent a contribution over prior art, because the shared technical features were obvious over the article entitled "Large-Scale Bi-Level Strain Design Approaches and Mixed-Integer Programming Solution Techniques" by Kim et al. (PLoS One. 2011, Vol 6, No 9, pp e24162) (hereinafter 'Kim') in view of the article entitled "Combined Fluxomics and Transcriptomics Analysis of Glucose Catabolism via a Partially Cyclic Pentose Phosphate Pathway in Gluconobacter oxydans 621H" by Hanke et al. (Appl Environ Microbiol. April 2013, Vol 79, No 7, pp 2336?2348) (hereinafter 'Hanke').

Kim discloses non-naturally occurring microbial organism that are engineered to have genetic alterations in a metabolic pathway (abstract).

---please see continuaiton on next extra sheet---

Continuation of:
Box No III Observations where unity of invention is lacking

Kim further discloses a non-naturally occurring microbe engineered to increase succinate production (p 7, col 1, para 2), wherein the microbe has an attenuation of cydA (p 7, col 1, para 2 "we were able to identify non-native reactions which can significantly improve the amount of succinate produced when the E. coli mutant strains achieve their maximum growth rate (Table 2)"; p 8, Table 2, showing cydA is a deleted gene).

Kim does not teach that the microbe has a genetic alteration that increases expression of a protein encoded by pntAB. However, Kim does disclose that the engineered strains having increased succinate production had the common characteristic of the use of NADP(H) instead of NAD(H) cofactors which increased fluxes in the TCA cycle thereby improving succinate production (p 7, col 1, para 2 – col 2, para 1 "A common characteristic of the identified non-native reaction additions was the use of NADP(H) instead of NAD(H) cofactors (Figure 6A). ... This reduces the flux through glutamate dehydrogenase, which uses NADPH to convert ammonia and 2-oxoglutarate into glutamate. This additional NADPH (from reduced glutamate dehydrogenase flux) lowered fluxes in the pentose phosphate pathway and increased fluxes in the TCA cycle thereby improving succinate production (Figure 6B)"). Further, Hanke discloses pntAB as a transhydrogenase that is strongly upregulated and which couples reduction of NADP+ by NADH to the import of protons across the membrane (p 2342, col 2, para 2 "The genes encoding pyridine nucleotide transhydrogenase (pntA1A2B, GOX0310 to GOX0312, mRNA ratios of 4.4 to 6.0) belonged to the most strongly upregulated genes at sample point II. Transhydrogenase PntAB is located in the cytoplasmic membrane of many bacteria and couples the reduction of NADP+ by NADH to the import of protons across the membrane."). Given that Kim teaches improvements in succinate production from use of NADP(H), and Hanke teaches highly upregulated pntAB as being responsible for reduction of NADP+ by NADH, one of ordianry skill in the art would have found it obvious to combine an attenuation of cydA with an increase in pntAB.

As the technical features were known in the art at the time of the invention, they cannot be considered a special technical feature that would otherwise unify the groups.

The feature shared by Groups I+ and II is a gene disruption of a gene encoding YciA CoA hydrolase.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by the article entitled
"Regulation of the Bacillus subtilis yciC gene and insights into the DNA-binding specificity of the zinc-sensing metalloregulator Zur" by Gabriel et al. (hereinafter 'Gabriel') (J Bacteriol. May 2008, Vol 190, No 10, pp 3482-8). Gabriel discloses
a gene disruption of a gene encoding YciA CoA hydrolase (abstract "Using a zur merodiploid strain, we obtained two cis-acting mutations that contained large deletions in the yciC regulatory region").As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

Another feature shared by Group I+ is [claim 10] a non-naturally occurring microbial organism comprising a first attenuation of a succinyl -CoA synthetase or transferase and at least a second attenuation of a succinyl-CoA converting enzyme or a gene encoding a succinate producing enzyme within a multi-step pathway having a net conversion of succinyl-CoA to succinate.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by the article entitled "Genes of succinyl-CoA ligase from Saccharomyces cerevisiae" by Przybyla-Zawislak et al. (Eur J Biochem, 1 December 1998, Vol 258, No 2, pp 736-43) (hereinafter 'Przybyla-Zawislak'). Przybyla-Zawislak discloses a succinyl-CoA synthetase and a second attenuation of a succinyl-CoA converting enzyme (abstract "Succinyl-CoA ligase (succinyl-CoA synthetase) catalyzes the nucleotide-dependent conversion of succinyl-CoA to succinate. This enzyme functions in the tricarboxylic acid (TCA) cycle ... Two genes, LSC1 (YOR142W) and LSC2 (YGR244C), with high similarity to succinyl-CoA ligase subunits from other species were isolated from Saccharomyces cerevisiae. ... The LSC genes were deleted singly and in combination"; Note: the instant specificaitons at para [0021] defines "succinyl-CoA converting enzyme" to include succinyl-CoA synthetases). As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

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

NOTES: Claims 8-9, 28-35, 49-55, 71-75 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).