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— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
3 November 2016

(54) Title: CELL-FREE PRODUCTION OF BUTANOL

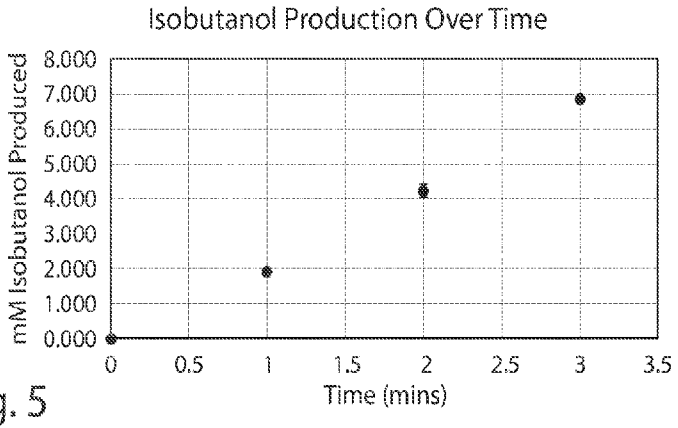

Fig. 5

(57) Abstract: Provided herein, in some aspects, are methods and compositions for producing large- scale quantities of butanol, including normal butanol (*n*-butanol), isobutanol, and 2-butanol using a cell-free system.

WO 2016/149631 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/23173

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 7/16, 7/06, 7/04; C12N 1/00, 1/21, 1/19 (2016.01)
CPC - C12P 7/06; C12N 1/00
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)
IPC(8) Classifications: C12P 7/16, 7/06, 7/04; C12N 1/00, 1/21, 1/19 (2016.01)
CPC Classifications: C12P 7/06; C12N 1/00

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)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data), Google/Google Scholar, EBSCO, PubMed
n-butanol, n-butyl alcohol, normal butanol, C4H9OH, biofuel, Cell, lysate, glucokinase, phosphoglucose isomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, ph

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 2011/0312052 A1 (KOLTERMANN, A et al.) 22 December 2011; [0052], [0061], [0063], [0048], [0119], [0072], [0076], [0095], [0100], [0101], [0059] Tables 1, 6, 7	1-4, 34, 69-70 --- 5/1-4, 6/5/1-4, 71/69-70, 72/71/69-70, 73/72/71/69-70
Y	US 2010/0120105 A1 (ANTHONY, LC et al.) 13 May 2010; [0011], [0051], [0079]	5/1-4, 6/5/1-4
Y	WO 2011/140516 A2 (GREENLIGHT BIOSCIENCES, INC., et al.) 10 November 2011; [0032], [0007], [0097], [0088]	71/69-70, 72/71/69-70, 73/72/71/69-70

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* 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
17 August 2016 (17.08.2016)	16 SEP 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas 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/US16/23173

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.: 7-33, 41-67, 75-81, 88-114
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. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-6, 34 and 69-74 are directed toward methods for producing n-butanol and pyruvate.

Group II, Claims 35-40 and 68 are directed toward methods of producing isobutanol.

Group III, Claims 82-87 and 115 are directed toward methods of producing 2-butanol.

-***-Continued Within the Next Supplemental Box-***-

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.:
Claims 1-6, 34 and 69-74

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
Information on patent family members

International application No.
PCT/US16/23173

-***-Continued from Box No. III: Observations where unity of invention is lacking-***-

The inventions listed as Groups I-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: the special technical features of Group I include pyruvate dehydrogenase complex, not present in either of Groups II or III; the special technical features of Group II include ketol-acid reductoisomerase, not present in either of Groups I or III; the special technical features of Group III include acetolactate decarboxylase, not present in either of Groups I or II.

Groups I-III share the technical features including: a method of producing a cell lysate for producing a butanol, the method comprising: (a) culturing engineered cells that express glucokinase, phosphoglucose isomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; and (b) lysing engineered cells cultured in step (a), thereby producing a cell lysate that comprises enzymes of the glycolytic pathway for producing a butanol; a method of producing a butanol, the method comprising: (a) culturing multiple populations of engineered cells, wherein each population of cells express enzymes of a butanol biosynthetic pathway selected *from the group consisting of: glucokinase, phosphoglucose isomerase, phosphofructokinase, fructose bisphosphate aldolase, triose* phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; (b) lysing populations of engineered cells cultured in step (a), thereby producing multiple cell lysates, wherein each cell lysate comprises enzymes of the butanol biosynthetic pathway; (c) combining in a single reaction mixture (i) glucose, (ii) at least a portion of each of the cell lysates and, optionally, (iii) a purified enzyme of the butanol biosynthetic pathway, wherein the single reaction mixture includes the following enzymes of the butanol biosynthetic pathway: glucokinase, phosphoglucose isomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; and (d) incubating the single reaction mixture under conditions that result in production of a butanol. Groups I and II share the technical features including: alcohol dehydrogenase. Groups II and III share the technical features including: acetolactate synthase.

However, these shared technical features are previously disclosed by US 2011/0312052 A1 to Koltermann et al. (hereinafter *'Koltermann') in view of US 2010/0120105 A1 to Anthony et al. (hereinafter 'Anthony').*

Koltermann discloses a method of producing a cell lysate (a method for producing a cell free lysate (a cell lysate); paragraphs [0017], [0061]) for producing a butanol (for producing butanol; paragraph [0016]), the method comprising: (a) culturing engineered cells that express (the method comprising: (a) culturing engineered cells that express; paragraph [0057]) hexokinase, phosphohexoisomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase (hexokinase, phosphohexoisomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; Table 1); and (b) lysing engineered cells cultured in step (a) (lysing engineered cells cultured in step (a); paragraph [0061]), thereby producing a cell lysate that comprises enzymes of the glycolytic pathway for producing a butanol (thereby producing a cell lysate that comprises enzymes of the glycolytic pathway for producing a butanol; paragraphs [0016], [0017], [0061]); a method of producing a butanol (a method of producing a butanol; paragraph [0016]), *the method comprising: (a) culturing multiple populations of engineered cells (culturing engineered host cells (the method* comprising: (a) culturing multiple populations of engineered cells); paragraph [0057]), wherein each population of cells express enzymes of a butanol biosynthetic pathway (wherein each population of cells express enzymes of a butanol biosynthetic pathway; paragraphs [0016], [0017], [0057]), including phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase (*including phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate* dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; Table 1); (b) lysing populations of engineered cells cultured in step (a) (lysing populations of engineered cells cultured in step (a); paragraph [0061]), thereby producing multiple cell lysates, wherein each cell lysate comprises enzymes of the butanol biosynthetic pathway (thereby producing (multiple) cell lysates, wherein each cell lysate comprises enzymes of the butanol biosynthetic pathway); paragraphs [0057], [0061]); (c) combining in a single reaction mixture (combining in a cell free system in a single phase (a single reaction mixture); paragraphs [0017]; [0068]) (i) glucose (glucose; paragraph [0015]), (ii) at least a portion of each of the cell lysates (at least a portion of each of the cell lysates; paragraphs [0061], [0067]), *wherein the single reaction mixture includes the following enzymes of the butanol biosynthetic pathway:* hexokinase, phosphohexoisomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase (wherein the single reaction mixture includes the following enzymes of the butanol biosynthetic pathway: hexokinase, phosphohexoisomerase, phosphofructokinase, fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase; Table 1); and (d) incubating the single reaction mixture under conditions that result in production of a butanol ((d) incubating the single reaction mixture under conditions that result in production of a butanol; paragraphs [0016], [0017], [0061], Table 1); alcohol dehydrogenase (alcohol dehydrogenase; Table 7); and acetolactate synthase (acteolactate synthase; paragraph [0133]).

Koltermann does not disclose glucokinase and phosphoglucose isomerase.

Anthony discloses a microbial host cell for the production of a butanol (a microbial host cell for the production of a butanol; abstract), wherein the cell expresses glucokinase (wherein the cell expresses at least one enzyme of the PPP, including glucokinase (wherein the cell expresses glucokinase); paragraphs [0011], [0053]) and phosphoglucose isomerase (and phosphoglucose isomerase; paragraphs [0053], [0073]).

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from the previous Supplemental Box-***-

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Koltermann to have selected particular isoenzyme forms, such as glucokinase and phosphoglucose isomerase, as disclosed by Anthony, for the glycolytic pathway expressed in cell lysates for the formation of butanol, as disclosed by Koltermann, in order to utilize particular enzymes that possessed the same functional activity for the pathway, while having more desirable characteristics, such as specific activity, temperature optimum, and functional lifetime, in order to enhance the production of a butanol from the lysate.

Since none of the special technical features of the Groups I-III inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Koltermann and Anthony references, unity of invention is lacking.