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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/25/2012	8273565	GEVO-041/13US 310142-2263	5847

58249 7590 09/05/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Catherine Asleson Dundon, Englewood, CO;
Aristos Aristidou, Highlands Ranch, CO;
Andrew Hawkins, Parker, CO;
Doug Lies, Parker, CO;
Lynne H. Albert, Golden, CO;

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
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58249 7590 08/21/2012

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Certificate of Mailing or Transmission

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(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$0	$0	$870	$0	11/21/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
KIM, ALEXANDER D	1656	435-254200

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 COOLEY LLP
2
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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: GEVO, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): ENGLEWOOD, COLORADO

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☐ Issue Fee
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☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date August 24, 2012

Typed or printed name Angie S. Mah Registration No. 64,902

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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Electronic Acknowledgement Receipt

EFS ID:	13579096
Application Number:	13246693
International Application Number:	
Confirmation Number:	5847
Title of Invention:	METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS
First Named Inventor/Applicant Name:	Catherine Asleson Dundon
Customer Number:	58249
Filer:	Angie S.Y. Mah
Filer Authorized By:	
Attorney Docket Number:	GEVO-041/13US 310142-2263
Receipt Date:	24-AUG-2012
Filing Date:	27-SEP-2011
Time Stamp:	10:50:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	GEVO_041_13US_IssueFeeTransmittal.pdf	100395 c93631188b7328a60cd14729da2acb06e60741d0	no	1

Warnings:	
Information:	

Total Files Size (in bytes):	100395

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IFW

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
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(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

08/27/2012 MBELETE2 00000033 501283 13246693
01 FC:2501 870.00 DA

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$0	$0	$0	$870	11/27/2012

Adjustment date: 08/27/2012 MBELETE2
05/25/2012 INTEFSW 00010040 501283 13246693
01 FC:2501 870.00 CR

EXAMINER	ART UNIT	CLASS-SUBCLASS
KIM, ALEXANDER D	1656	435-254200

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. Use of a Customer Number is required.

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(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

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(A) NAME OF ASSIGNEE: GEVO, INC.
(B) RESIDENCE: (CITY and STATE OR COUNTRY): ENGLEWOOD, COLORADO

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☐ Issue Fee
☐ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)
☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)
☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature Angie S. Mah Date August 24, 2012

Typed or printed name Angie S. Mah Registration No. 64,902

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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NOTICE OF ALLOWANCE AND FEE(S) DUE

58249 7590 08/21/2012

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WASHINGTON, DC 20001

EXAMINER
KIM, ALEXANDER D

ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 08/21/2012

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$0	$0	$870	$0	11/21/2012

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

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If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

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(Signature)

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EXAMINER	ART UNIT	CLASS-SUBCLASS
KIM, ALEXANDER D	1656	435-254200

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1 _____

2 _____

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❑ Issue Fee

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EXAMINER	
KIM, ALEXANDER D	

ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 08/21/2012

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)

(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

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4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *06/15/2012*.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1-19*.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 08/03/2012,06/15/2012
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

DETAILED ACTION

Application Status

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 06/15/2012 has been entered.

Claims 1-19 are pending. In view of approval of the Examiner's amendment shown below, claims 1-19 is allowed.

Information Disclosure Statement

2. The information disclosure statements (IDSs) submitted on 06/15/2012 and 08/03/2012 have been reviewed, and its references have been considered except for those which have been lined through. A copy of Form PTO/SB/08 is attached to the instant Office action.

Examiner's amendment to the Claims

3. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided

by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment shown below was given in a telephone interview with Angie S. Mah on 5/17/2012.

Amend the claim listing filed on 05/08/2012 with the following changes.

Claim 1: A recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to comprise at least one inactivated ~~endogenous gene encoding a~~ monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated monothiol glutaredoxin ~~endogenous gene~~ results from the deletion of one or more nucleotides of ~~said~~an endogenous gene encoding [[a]]said monothiol glutaredoxin, the insertion of one or more nucleotides into an~~said~~ endogenous gene encoding [[a]]said monothiol glutaredoxin, [[and]]or combinations thereof.

Claim 2: Insert ---further--- in between "microorganism" and "comprises"; to recite ---microorganism further comprises---, in 2nd line of claim 2.

Claims 3-7: No change.

Claims 8-9: Delete the term "derived".

Claims 10-12: No change.

Claims 13-14: Delete the term "genes" at the end of claims.

Claims 15-19: No change.

Statement of Reasons for Allowance

4. Claims 1-19 are allowed. The following is an examiner's statement of reasons for allowance:

The instant invention is drawn to a recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to comprise at least one inactivated (i.e., no activity) monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated monothiol glutaredoxin results from the deletion of one or more nucleotides of an endogenous gene encoding said monothiol glutaredoxin, the insertion of one or more nucleotides into an endogenous gene encoding said monothiol glutaredoxin, or combinations thereof. The instant claims are novel over prior art of record and useful for production of various chemical product such as isobutanol, for example.

In view of the examiner's amendment and approval of terminal disclaimers, all outstanding rejections are withdrawn.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance".

Conclusion

5. Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALEXANDER D. KIM whose telephone number is (571)272-5266. The examiner can normally be reached on 9AM-5:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on (571) 272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Alexander D Kim/
Primary Examiner, Art Unit 1656

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Catherine Asleson Dundon *et al.*	Confirmation No.: 5847
Application No.: 13/246,693	Group Art Unit: 1656
Filed: September 27, 2011	Examiner: Alexander D. Kim

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window, **Mail Stop Amendment**
Randolph Building
401 Dulany Street
Alexandria, VA 22314

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97(b)

In accordance with the duty of disclosure set forth in 37 C.F.R. § 1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§ 1.97 and 1.98.

[x] Pursuant to 37 C.F.R. §1.98, copies of documents 8-25 cited in the attached Form PTO/SB/08a are enclosed.

[x] No copies of any U.S. patents or U.S. patent application publications listed on the attached Form PTO/SB/08a are being provided pursuant to 37 C.F.R. § 1.98.

[x] The Examiner's attention is directed to related co-pending United States Patent Application Serial Nos.:

13/246,718, filed September 27, 2011;

13/279,166, filed October 21, 2011; and

13/528,106, filed June 20, 2012.

[x] The following document is in a foreign language:

• 9, WO 2009/103533 A1: An English abstract may be found on the front page of the document.

This Information Disclosure Statement is filed within any one of the following time periods:

[ ] within three months from the filing date of this national application other than a CPA under 37 C.F.R. § 1.53(d);

[ ] within three months from the date of entry of the national stage as set forth in 37 C.F.R. §1.491 in this international application;

[ ] before the mailing date of a first office action on the merits; or

[x] before the mailing of a first office action after the filing of a request for continued examination under 37 C.F.R. § 1.114.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Forms PTO/SB/08a to the undersigned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-1283.

Dated: August 3, 2012

Respectfully submitted,
COOLEY LLP

By: _____
Angie S. Mah, Ph.D.
Reg. No. 64,902

USPTO Customer No. 58249
COOLEY LLP
ATTN: Patent Group
777, 6th Street NW, Suite 1100
Washington, DC 20001
Phone: (202) 842-7800
Fax: (202) 842-7899

PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code2 (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	2006/0263864 A1	11-23-2006	Busby et al.	
	2.	2011/0183393 A1	07-28-2011	Dundon et al.	
	3.	2011/0287500 A1	11-24-2011	Urano et al.	
	4.	2012/0015417 A1	01-19-2012	Dundon et al.	
	5.	2012/0034666 A1	02-09-2012	Hawkins et al.	
	6.	8,071,358 B1	12-06-2011	Dundon et al.	
	7.	8,232,089 B2	07-31-2012	Urano et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	8.	WO 2006/059111 A2	06-08-2006	F2G Ltd. et al.		
	9.	WO 2009/103533 A1	08-27-2009	Butalco GMBH et al.		abstract

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
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Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

NON PATENT LITERATURE DOCUMENTS

Examiner's Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	10.	ADAM et al., "The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria." EMBO Journal (2006), 25(1): 174-183 (2006)	
	11.	CHEN et al., "Inhibition of Fe-S cluster biosynthesis decreases mitochondrial iron export: evidence that Yfh1p affects Fe-S cluster synthesis." PNAS 99(19): 12321-12326 (2002)	
	12.	COOK et al., "Molecular details of the yeast frataxin-Isu1 interaction during mitochondrial Fe-S cluster assembly." Biochemistry 49(40): 8756-8765 (Sept. 2010)	
	13.	International Search Report and Written Opinion mailed February 16, 2012 in the International (PCT) Application No.: PCT/US2011/057299, 8 pages	
	14.	Kassow, A. (1992). Metabolic effects of deleting the region encoding the transit peptide in Saccharomyces cerevisiae ILV5. PhD thesis, University of Copenhagen.	
	15.	LI et al., "Oligomeric yeast frataxin drives assembly of core machinery for mitochondrial iron-sulfur cluster synthesis." Journal of Biological Chemistry 284(33): 21971-21980 (June 2009)	
	16.	LI et al., "Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution." Journal of Biological Chemistry 274(46): 33025-33034 (1999)	
	17.	Lill et al., "Iron-Sulfur Protein Biogenesis in Eukaryotes: Components and Mechanisms," Annu. Rev. Cell Dev. Biol., 2006, Vol. 22, pages 457-486.	
	18.	Lill et al., "Mechanisms of iron-sulfur protein maturation in mitochondria, cytosol and nucleus of eukaryotes," Biochimica et Biophysica Acta (2006), Vol. 1763, pages 652-667.	

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

NON PATENT LITERATURE DOCUMENTS

Examiner's Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	19.	Lill, "Function and biogenesis of iron-sulphur proteins," Nature 460:831-838 (2009)	
	20.	Muehlenhoff et al., "Functional characterization of the eukaryotic cysteine desulfurase Nfs1p from Saccharomyces cerevisiae." Journal of Biological Chemistry 279(35): 36906-36915 (2004)	
	21.	Petition under 37 C.F.R. § 1.181 for a Proper First Office Action for *Inter Partes* Reexamination Control No. 95/001,870, 49 pages (filed July 11, 2012)	
	22.	Petition under 37 C.F.R. § 1.183 for Reconsideration of Portions of Order Granting Reexamination for *Inter Partes* Reexamination Control No. 95/001,870, 51 pages (filed July 11, 2012)	
	23.	Rawat et al., "Key players and their role during mitochondrial iron-sulfur cluster biosynthesis." Chem. Eur. J. 17: 746-753 (Jan. 2011)	
	24.	Third Party Requester Comments Under 37 C.F.R. § 1.947 for *Inter Partes* Reexamination Control No. 95/001,870, 61 pages (filed July 20, 2012)	
	25.	Wiedemann et al., "Essential role of Isd11 in mitochondrial iron-sulfur cluster synthesis on Isu scaffold proteins." EMBO Journal 25(1): 184-195 (2006)	

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

PATENT WITHDRAWAL NOTICE

DATE WITHDRAWN	WITHDRAWAL NUMBER
6/25/2012	20898

The following application has been **WITHDRAWN** from the

7/10/2012 issue.

SERIAL NO.	PATENT NUMBER
13/246,693	8,216,824
DRAWINGS	**CLASS**
000	726/004

TITLE

GENERATING AUTHENTICATION CHALLENGES BASED ON PREFERENCES OF A USER'S CONTACTS

NAME AND ADDRESS

JESSICA STADDON
REDWOOD CITY, CA

REASON FOR WITHDRAWAL

Office of Petitions granted applicant's request to withdraw patent from issue.

APPROVED

/Kimberly Terrell/, Manager

Patent Publication Branch
Office of Data Management

FORM PTO-302 -- (REV. 05-2009)

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	07/10/2012	8216824	GEVO-041/13US 310142-2263	5847

58249 7590 06/21/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Catherine Asleson Dundon, Englewood, CO;
Aristos Aristidou, Highlands Ranch, CO;
Andrew Hawkins, Parker, CO;
Doug Lies, Parker, CO;
Lynne H. Albert, Golden, CO;

PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

This is a Request for Continued Examination (RCE) under 37 CFR 1 .114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

   b. ☒ Enclosed

      i. ☐ Amendment/Reply
      ii. ☐ Affidavit(s)/ Declaration(s)
      iii. ☒ Information Disclosure Statement (references filed under MPEP 724)
      iv. ☒ Other Petition to Withdraw from Issue Under 37 CFR 1.313(c)(2); Petition to Expunge Under 37 CFR 1.59(b)

2. Miscellaneous

   Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a

   a. ☐ period of ___ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)
   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to

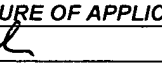
   a. ☒ Deposit Account No. 50-1283 .

      i. ☒ RCE fee required under 37 CFR 1.17(e)
      ii ☐ Extension of time fee (37 CFR 1.136 and 1.17)
      iii ☒ Other Petition fee under 37 CFR 1.17(h); Petition fee under 37 CFR 1.17(g)

   b. ☐ Check in the amount of $ _____ enclosed
   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature		Date	June 15, 2012
Name (Print/Type)	Angie S. Mah	Registration No.	64,902

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature	
Name (Print/Type)	Date

06/18/2012 LEKUBAY1 00000058 501283 13246693
01 FC:2801 465.00 DA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Catherine Asleson Dundon *et al.* Confirmation No.: 5847

Application No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Alexander D. Kim

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

PETITION TO WITHDRAW FROM ISSUE UNDER 37 C.F.R. § 1.313(c)(2)

Sir:

In accordance with 37 C.F.R. § 1.313(c)(2), Applicants respectfully petition the Director of Patents to withdraw the above-referenced application from issue to permit consideration of a Request for Continued Examination ("RCE") under 37 C.F.R. § 1.114. Accordingly, submitted herein with is a RCE and an accompanying Submission, in the form of an Information Disclosure Statement, are concurrently filed with the present petition. Select materials cited in the Information Disclosure Statement are provided in a sealed container as the materials are subject to a protective order and are being submitted herewith for consideration under MPEP § 724.

In accordance with MPEP § 1308, this petition is filed with sufficient time before the patent issue date. The petition fee as set forth in 37 C.F.R. § 1.17(h), the filing fee for the RCE as set forth in 37 C.F.R. § 1.17(e), are enclosed herewith. Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and

1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-1283.

Prompt and favorable consideration of this petition is respectfully requested.

Dated: June 15, 2012

Respectfully submitted,
COOLEY LLP

By: _____
Angie S. Mah, Ph.D.
Reg. No. 64,902

COOLEY LLP
ATTN: Patent Group
777 6th Street NW, Suite 1100
Washington, DC 20001

Tel: (202) 728-7138
Fax: (202) 842-7899

Attorney Docket No: GEVO-041/13US 310142-2263 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Catherine Asleson Dundon *et al.* Confirmation No.: 5847

Application No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Alexander D. Kim

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

TRANSMITTAL OF DOCUMENTS

1. Request for Continued Examination;
2. Petition To Withdraw From Issue Under 37 C.F.R. § 1.313(c)(2);
3. Petition To Expunge Under 37 C.F.R. § 1.59(b);
4. Information Disclosure Statement Under 37 C.F.R. § 1.97(b);
5. PTO/SB/08A (2 pages) citing ten documents;
6. Copies of seven cited (NPL4 – NPL10) documents containing materials subject to a protective order in a sealed container submitted for consideration under M.P.E.P. § 724.

[X] Please charge **$795.00** to Deposit Account No. 50-1283

RCE	$465.00
Petition fee set forth in 37 CFR 1.17(g)	$200.00
Petition fee set forth in 37 CFR 1.17(h)	$130.00

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283.

Respectfully submitted,
COOLEY LLP

Dated: June 15, 2012

By: _____
Angie S. Mah
Reg. No. 64,902

COOLEY LLP
ATTN: Patent Group
777 6th Street NW, Suite 1100
Washington, DC 20001
Tel: (202) 842-8000
Fax: (202) 842-7899

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Catherine Asleson Dundon *et al.* Confirmation No.: 5847

Application No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Alexander D. Kim

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. § 1.97(b)

In accordance with the duty of disclosure set forth in 37 C.F.R. § 1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§ 1.97 and 1.98.

No copies of any U.S. patent application publications listed on the attached Form PTO/SB/08 are being provided pursuant to 37 C.F.R. § 1.98.

Pursuant to 37 C.F.R. § 1.98, copies of documents 4-10 cited in the attached Form PTO/SB/08 are provided. The documents are provided in a sealed container and are being submitted herewith for consideration under MPEP § 724 as the materials are subject to the Protective Order issued July 17, 2011 in the currently pending litigation in the United States District Court for the District of Delaware, docket no. 1:11-cv-00054-SLR, filed January 14, 2011.

The Examiner's attention is directed to currently pending litigation in the United States District Court for the District of Delaware, docket no. 1:11-cv-00054-SLR, in which related U.S. Application No. 12/953,884, issued as U.S. Patent No. 8,017,376, is involved in the litigation. Select filings have been cited herein as documents 4-10, which are provided in a sealed container as the materials are subject to the Protective Order. The Protective Order implemented a Prosecution Bar in which materials designated as "HIGHLY CONFIDENTIAL – ATTORNEY'S EYES ONLY" by the producing party in the litigation are not to be disclosed to

any person who participates or advises in the preparation or prosecution of a patent application for the receiving party, in which the patent application concerns the use of microorganisms for the production of isobutanol, 1-butanol or 2-butanol. The select filings, cited herein as documents 4-10, have the highly confidential sections, as well as non-material sections, redacted. The highly confidential and non-material sections were redacted prior to the receipt of these documents for submission herewith by the person(s) participating or advising in the preparation or prosecution of the instant application.

This Information Disclosure Statement is filed within any one of the following time periods:

[ ] within three months from the filing date of this national application other than a CPA under 37 C.F.R. § 1.53(d);

[ ] within three months from the date of entry of the national stage as set forth in 37 C.F.R. §1.491 in this international application;

[ ] before the mailing date of a first office action on the merits; or

[x] before the mailing of a first office action after the filing of a request for continued examination under 37 C.F.R. § 1.114.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Forms PTO/SB/08a to the undersigned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-1283.

Dated: June 15, 2012

Respectfully submitted,
COOLEY LLP

By: _____
Angie S. Mah, Ph.D.
Reg. No. 64,902

USPTO Customer No. 58249
COOLEY LLP
ATTN: Patent Group
777, 6th Street NW, Suite 1100
Washington, DC 20001
Phone: (202) 842-7800
Fax: (202) 842-7899

PTO/SB/08b (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	DUNDON, Catherine Asleson
Art Unit	1656
Examiner Name	Alexander D. KIM
Attorney Docket Number	GEVO-041/13US 310142-2263

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	2007/0092957 A1	04-26-2007	Donaldson; Gail K. et al.	
	2	2009/0163376 A1	06-25-2009	Li; Yougen et al.	
	3	2011/0076733 A1	03-31-2011	Urano; Jun et al.	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	4	"ANSWER to Amended Complaint ANSWER to Amended Complaint, with Jury Demand, COUNTERCLAIM against Butamax(TM) Advanced Biofuels LLC, E.I. DuPont De Nemours and Co. by Gevo Inc.," Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (filed September 13, 2011)	
	5	"SEALED ANSWER to Answer to Amended Complaint, Counterclaim, COUNTERCLAIM against Gevo Inc. by E.I. DuPont De Nemours and Co., Butamax(TM) Advanced Biofuels LLC," Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (filed November 18, 2011)	
	6	"OBJECTIONS AND RESPONSES TO GEVO, INC.'S SECOND SET OF INTERROGATORIES TO BUTAMAX ADVANCED BIOFUELS LLC (NOS. 8--16)" for Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (dated March 26, 2012)	
	7	"SEALED OPENING BRIEF in Support re SEALED MOTION for Leave to File Amended Answer to Counterclaims, Defenses, and Counter-Counterclaims filed by Butamax(TM) Advanced Biofuels LLC, E.I. DuPont De Nemours and Co.," Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (filed March 30, 2012)	
	8	"ANSWERING BRIEF in Opposition re SEALED MOTION for Leave to File Amended Answer to Counterclaims, Defenses, and Counter-Counterclaims filed by Gevo Inc.," Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (filed April 16, 2012)	
	9	"SEALED REPLY BRIEF re SEALED MOTION for Leave to File Amended Answer to Counterclaims, Defenses, and Counter-Counterclaims REPLY BRIEF IN SUPPORT OF PLAINITFF'S AND COUNTERCLAIM DEFENDANTS' MOTION FOR LEAVE TO AMEND THE PLEADINGS filed by Butamax(TM) Advanced Biofuels LLC, E.I. DuPont De Nemours and Co.," Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (filed April 26, 2012)	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	DUNDON, Catherine Asleson
Art Unit	1656
Examiner Name	Alexander D. KIM
Attorney Docket Number	GEVO-041/13 310142-2263

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	10	"FIRST SUPPLEMENTAL OBJECTIONS AND RESPONSES TO GEVO, INC.'S SECOND SET OF INTERROGATORIES TO BUTAMAX ADVANCED BIOFUELS LLC (NOS. 8–16)" for Butamax™ Advanced Biofuels LLC v. Gevo, Inc. v. E.I. DuPont de Nemours & Co., 1:11-cv-00054-SLR, United States District Court for the District of Delaware (dated May 18, 2012)	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.
[1] Applicant's unique citation designation number (optional). [2] Applicant is to place a check mark here if English language Translation is attached.
This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO:**
Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

58249 7590 05/24/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$0	$0	$870	08/24/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
KIM, ALEXANDER D	1656	435-254200

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 COOLEY LLP
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: GEVO, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): ENGLEWOOD, COLORADO

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☒ Issue Fee

☒ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

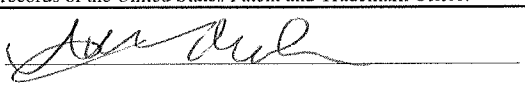
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 50-1283 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature  Date May 25, 2012

Typed or printed name Angie S. Mah Registration No. 64,902

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

58249 7590 05/24/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER
KIM, ALEXANDER D

ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 05/24/2012

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$0	$0	$870	08/24/2012

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

58249 7590 05/24/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

TITLE OF INVENTION: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$0	$0	$870	08/24/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
KIM, ALEXANDER D	1656	435-254200

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

58249 7590 05/24/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER	
KIM, ALEXANDER D	

ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 05/24/2012

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)

(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *05/08/2012*.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1-19*.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 05/08/2012
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

DETAILED ACTION

Application Status

1. In response to the previous Office action, a Final rejection (mailed on 05/01/2012), Applicants filed a response and amendment received on 05/08/2012.

Said amendment amended Claim 1. Claims 1-19 are pending.

Information Disclosure Statement

2. The information disclosure statement (IDS) submitted on 05/11/2012 was filed after the mailing date of the Non-Final rejection on 12/13/2011. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

Terminal disclaimers

3. The three terminal disclaimers filed on 05/08/2012 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of US Patent Application 12/240,718, US Pat. 8,071,358, US Pat. 8,071,376 have been reviewed and is accepted. The terminal disclaimers have been recorded.

Examiner's amendment to the Claims

4. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided

by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment shown below was given in a telephone interview with Angie S. Mah on 5/17/2012.

Amend the claim listing filed on 05/08/2012 with the following changes.

Claim 1: A recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to comprise at least one inactivated ~~endogenous gene encoding a~~ monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated monothiol glutaredoxin ~~endogenous gene~~ results from the deletion of one or more nucleotides of ~~said~~an endogenous gene encoding [[a]]said monothiol glutaredoxin, the insertion of one or more nucleotides into an~~said~~ endogenous gene encoding [[a]]said monothiol glutaredoxin, [[and]]or combinations thereof.

Claim 2: Insert ---further--- in between "microorganism" and "comprises"; to recite ---microorganism further comprises---, in 2nd line of claim 2.

Claims 3-7: No change.

Claims 8-9: Delete the term "derived".

Claims 10-12: No change.

Claims 13-14: Delete the term "genes" at the end of claims.

Claims 15-19: No change.

Statement of Reasons for Allowance

5. Claims 1-19 are allowed. The following is an examiner's statement of reasons for allowance:

The instant invention is drawn to a recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to comprise at least one inactivated (i.e., no activity) monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated monothiol glutaredoxin results from the deletion of one or more nucleotides of an endogenous gene encoding said monothiol glutaredoxin, the insertion of one or more nucleotides into an endogenous gene encoding said monothiol glutaredoxin, or combinations thereof. The instant claims are novel over prior art of record and useful for production of various chemical product such as isobutanol, for example.

In view of the examiner's amendment and approval of terminal disclaimers, all outstanding rejections are withdrawn.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance".

Conclusion

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALEXANDER D. KIM whose telephone number is (571)272-5266. The examiner can normally be reached on 9AM-5:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on (571) 272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Alexander D Kim/
Primary Examiner, Art Unit 1656

OK TO ENTER: /AK/ 05/18/2012

Attorney Docket No. GEVO-041/13US 310142-2263 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re App'n of: Catherine Asleson DUNDON *et al.* Confirmation No.: 5847

Serial No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Kim, Alexander D.

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT UNDER 37 CFR § 1.116

This paper responds to the Office Action dated May 1, 2012. Applicants respectfully request reconsideration of this application in view of the following amendments and remarks.

Amendments to the Claims are reflected on the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 6 of this paper.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

58249 7590 05/17/2012

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777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER	
KIM, ALEXANDER D	
ART UNIT	**PAPER NUMBER**
1656	

MAIL DATE	DELIVERY MODE
05/17/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Examiner-Initiated Interview Summary	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

All participants (applicant, applicant's representative, PTO personnel):

(1) *ALEXANDER KIM*.

(2) *Angie S. Mah*.

(3) *Paul Wickman*.

(4) _____.

Date of Interview: *15 May 2012*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1, 8 and 9*.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The Examiner propsed amendments in claim 1 to includes functional language such as eliminating activity of the endogenous GRX3 or GRX4 since inactivating gene as written in the context of claim still encompasses said gene encoding partially functional GRX3 or GRX4 polypeptide. The Applicants noted that the meaning of "inactivate" is to render inactive so that GRX3 and GRX4 protein have no activity thereof. The Examiner agree with the meaning of inactivate; thus, amending claim 1 to recite "inactivated" in term of GRX3 or GRX4 protein is suggested.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Alexander D Kim/ Primary Examiner, Art Unit 1656	

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	13/246,693	DUNDON ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 08 MAY 2012	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:

THREE TDS FILED AND APPROVED

JAB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re App'n of: Catherine Asleson DUNDON *et al.* Confirmation No.: 5847

Serial No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Kim, Alexander D.

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

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Commissioner for Patents
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Alexandria, VA 22313-1450

AMENDMENT UNDER 37 CFR § 1.116

This paper responds to the Office Action dated May 1, 2012. Applicants respectfully request reconsideration of this application in view of the following amendments and remarks.

Amendments to the Claims are reflected on the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 6 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior listings in the application. Please amend the claims as follows.

1. (Currently Amended) A recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated endogenous gene results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin ~~or regulatory region thereof~~, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin ~~or a regulatory region thereof~~, and combinations thereof.

2. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, said isobutanol producing metabolic pathway comprising the following substrate to product conversions:

(a) pyruvate to acetolactate;

(b) acetolactate to 2,3-dihydroxyisovalerate;

(c) 2,3-dihydroxyisovalerate to α-ketoisovalerate;

(d) α-ketoisovalerate to isobutyraldehyde; and

(e) isobutyraldehyde to isobutanol;

and wherein said DHAD catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate.

3. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of pyruvate to acetolactate is an acetolactate synthase.

4. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of acetolactate to 2,3-dihydroxyisovalerate is a ketol-acid reductoisomerase.

5. (Previously Presented) The recombinant yeast microorganism of claim 4, wherein said ketol-acid reductoisomerase is an NADH-dependent ketol-acid reductoisomerase.

6. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the cytosol.

7. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the mitochondria.

8. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Lactococcus lactis.*

9. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Streptococcus mutans.*

10. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde is a 2-keto acid decarboxylase.

11. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol is an alcohol dehydrogenase.

12. (Previously Presented) The recombinant yeast microorganism of claim 11, wherein said alcohol dehydrogenase is an NADH-dependent alcohol dehydrogenase.

13. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is further engineered to inactivate one or more endogenous pyruvate decarboxylase (PDC) genes.

14. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is further engineered to inactivate one or more endogenous glycerol-3-phosphate dehydrogenase (GPD) genes.

15. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is further engineered to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins as compared to the corresponding yeast microorganism that has not been engineered to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins.

16. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is further engineered to express one or more polynucleotides encoding one or more constitutively active activator of ferrous transport (Aft) proteins.

17. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following genera: *Saccharomyces*, *Kluyveromyces*, *Pachysolen*, *Zygosaccharomyces*, *Debaryomyces*, *Pichia*, *Schizosaccharomyces*, *Candida*, *Issatchenkia*, *Hansenula*, *Yarrowia*, *Tricosporon*, *Rhodotorula,* and *Myxozyma.*

18. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following species: *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces bayanus*, *Saccharomyces paradoxus*, *Saccharomyces castelli*, *Saccharomyces kluyveri*, *Kluyveromyces thermotolerans*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces waltii*,

Pachysolen tannophilis, *Zygosaccharomyces bailli*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Debaromyces carsonii*, *Pichia pastorius*, *Pichia anomala*, *Pichia stipitis*, *Pichia castillae*, *Schizosaccharomyces pombe*, *Candida utilis*, *Candida glabrata*, *Candida tropicalis*, *Candida xestobii*, *Issatchenkia orientalis*, *Issatchenkia occidentalis*, *Issatchenkia scutulata*, *Hansenula anomala*, and *Yarrowia lipolytica*.

19. (Previously Presented) A method of producing isobutanol comprising: (a) providing the recombinant yeast microorganism of claim 2; and (b) cultivating the recombinant yeast microorganism of claim 2 in a culture medium containing a feedstock providing a carbon source, until a recoverable quantity of the isobutanol is produced.

Remarks

Status of Claims and Claim Amendments

In this Amendment, claim 1 has been amended. After entry of this Amendment, claims 1-19 will be pending.

Claim 1 has been amended to remove the recitation of "or regulatory region thereof". Applicants respectfully submit that entry and consideration of this amendment is appropriate pursuant to 37 C.F.R. 1.116, as this amendment only cancels subject matter and therefore narrows issues for appeal. No new matter has been introduced by way of this amendment.

The Office Action dated May 1, 2012 has been carefully reviewed and the following reply is made in response thereto. In view of the following remarks, Applicants respectfully request reconsideration of this application and the timely allowance of the pending claims.

Claim Rejections under 35 USC § 112, First Paragraph, Written Description and Enablement

At pages 3-7 of the Office Action, the Office rejected claims 1-19 as allegedly failing to comply with the written description and enablement requirements. Specifically, the Office alleges that the instant claims encompass genetic mutations in a broad genus of regulatory region(s) which regulate GRX3 and/or GRX4 expression and/or activity. The Office further alleges that the specification and/or the art do not sufficiently describe the structure of any regulatory region to be mutated necessary to inactivate GRX3 and/or GRX4.

Solely to advance prosecution and not in acquiescence to the propriety of this rejection, claim 1 has been amended to remove the recitation "or regulatory region thereof". Accordingly, Applicants submit that claim 1, and claims 2-19 depending therefrom, should no longer be subject to the Office's written description and enablement rejections. Withdrawal of the rejections is respectfully requested.

Double Patenting Rejections

At page 9 of the Office Action, claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of co-pending Application No. 13/246,718. In conjunction with this paper, Applicants have filed a terminal disclaimer with respect to the '718 application. The filing of this terminal disclaimer is not an admission that such is required. Withdrawal of this rejection is therefore respectfully requested.

At page 9 of the Office Action, claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,358 (issued from U.S. Application No. 13/228,342). In conjunction with this paper, Applicants have filed a terminal disclaimer with respect to the '358 patent. The filing of this terminal disclaimer is not an admission that such is required. Withdrawal of this rejection is therefore respectfully requested.

At page 10 of the Office Action, claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,017,376 (issued from U.S. Application No. 12/953,884). In conjunction with this paper, Applicants have filed a terminal disclaimer with respect to the '376 patent. The filing of this terminal disclaimer is not an admission that such is required. Withdrawal of this rejection is therefore respectfully requested.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that no further impediments exist to the allowance of this application and, therefore, request an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to **Deposit Account No. 50-1283.**

Dated: May 8, 2012

Respectfully submitted,
COOLEY LLP

By: _____
Angie S. Mah
Reg. No. 64,902

CUSTOMER NO. 58249

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PTO/SB/25 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) GEVO-041/13US 310142-2263

In re Application of: Catherine Asleson DUNDON et al.

Application No.: 13/246,693

Filed: September 27, 2011

For: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

The owner*, Gevo, Inc., of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number 13/246,718, filed on September 27, 2011, as such term is defined in 35 U.S.C. 154 and 173, and as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of any patent granted on said **reference** application, "as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application," in the event that: any such patent: granted on the pending **reference** application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. ☑ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☐ The undersigned is an attorney or agent of record. Reg. No. _____

Signature — 05-02-2012 Date

Brett Lund, J.D., M.B.A., Executive VP, General Counsel
Typed or printed name

(303) 858-8358
Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this statement. See MPEP § 324.

PTO/SB/26 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) GEVO-041/13US 310142-2263

In re Application of: Catherine Asleson DUNDON et al.

Application No.: 13/246,693

Filed: September 27, 2011

For: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

The owner*, Gevo, Inc., of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term **prior patent** No. 8,071,358 as the term of said prior patent is defined in 35 U.S.C. 154 and 173, and as the term of said **prior patent** is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

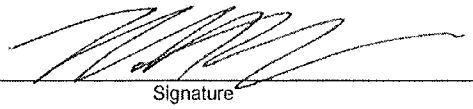
expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☑ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☐ The undersigned is an attorney or agent of record. Reg. No._____



Signature — 05-02-2012 Date

Brett Lund, J.D., M.B.A.
Typed or printed name

(303) 858-8358
Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

PTO/SB/26 (07-09)
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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) GEVO-041/13US 310142-2263

In re Application of: Catherine Asleson DUNDON et al.

Application No.: 13/246,693

Filed: September 27, 2011

For: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

The owner*, Gevo, Inc., of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term **prior patent** No. 8,017,376 as the term of said prior patent is defined in 35 U.S.C. 154 and 173, and as the term of said **prior patent** is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

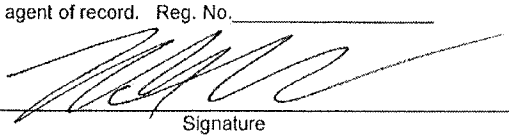
expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☑ For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☐ The undersigned is an attorney or agent of record. Reg. No._____



Signature — 05-02-2012 Date

Brett Lund, J.D., M.B.A.
Typed or printed name

(303) 858-8358
Telephone Number

☑ Terminal disclaimer fee under 37 CFR 1.20(d) included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

PTO/SB/96 (07-09)
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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Gevo, Inc.

Application No./Patent No.: 13/246,693 Filed/Issue Date: September 27, 2011

Titled: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

Gevo, Inc. , a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in
(The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made)

the patent application/patent identified above, by virtue of either:

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 027126, Frame 0834, or for which a copy therefore is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☐ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature	May 2, 2012 Date
Brett Lund, J.D., M.B.A. Printed or Typed Name	Exec. VP, General Counsel Title

In re App'n of: Catherine Asleson DUNDON *et al.* Confirmation No.: 5847

Serial No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Kim, Alexander D.

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §1.97(d)

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98.

[x] Pursuant to 37 C.F.R. §1.98, a copy of document 2 cited in the attached Form PTO/SB/08a is enclosed.

[x] No copies of any U.S. patents or U.S. patent application publications listed on the attached Form PTO/SB/08a are being provided pursuant to 37 C.F.R. §1.98.

This Information Disclosure Statement is filed after the period specified in 37 C.F.R. § 1.97(c):

In accordance with 37 C.F.R. §1.97(d) also enclosed is:

[x] Fee under 37 C.F.R. §1.17(p) in the amount of $180.00; and

[x] Statement as specified in 37 C.F.R. §1.97(e):

[ ] Each item of information contained in the Information Disclosure Statement cited herein was first cited in a communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing date of the Information Disclosure Statement; or

[x] No item of information contained in the Information Disclosure Statement submitted herewith was cited in a communication from a foreign patent office in a

counterpart foreign application, and, to the knowledge of the undersigned, having made a reasonable inquiry, no item of information contained in the Information Disclosure Statement was known to any individual designated in 37 C.F.R. §1.56(c) more than three months prior to the filing date of the Information Disclosure Statement.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Forms PTO/SB/08a to the undersigned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-1283.

Dated: May 8, 2012

Respectfully submitted,
COOLEY LLP

By: _____
Angie S. Mah
Reg. No. 64,902

USPTO Customer No. 58249
COOLEY LLP
ATTN: Patent Group
777, 6th Street NW, Suite 1100
Washington, DC 20001
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PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code2 (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US 2012/0064561 A1	03-15-2012	Flint et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS

Examiner's Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	2.	Determination for *Inter Partes* Reexamination Control No. 95/001,870, mailed March 23, 2012	

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

58249 7590 05/01/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER	
KIM, ALEXANDER D	

ART UNIT	PAPER NUMBER
1656	

MAIL DATE	DELIVERY MODE
05/01/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *16 February 2012*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1-19* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-19* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *02/16/2012*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Application Status

1. In response to the previous Office action, a non-Final rejection (mailed on 12/13/2011), Applicants filed a response and amendment received on 02/16/2012. In said amendment, claims 1, and 13-16 are amended.

Thus, Claims 1-19 will be examined herein.

Information Disclosure Statement

2. The information disclosure statement (IDS) submitted on 02/16/2012 was filed after the mailing date of the first Office action on the merits on 12/13/2011. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statements are being considered by the examiner. A copy of Form PTO/SB/08 is attached to the instant Office action.

Withdrawn-Claim Rejections - 35 USC § 112

3. The previous rejection of Claim 1 (Claims 2-19 dependent therefrom) under of 35 U.S.C. 112, second paragraph, for reciting “the ... activity” for Grx3 and Grx4 protein, is withdrawn by virtue of applicants' amendment (i.e., deletion of the term or phrase at issue).

4. The previous rejection of Claim 1 (Claims 2-19 dependent therefrom) under of 35 U.S.C. 112, second paragraph, for reciting "increase the expression or activity"; and

Claims 2, 4, and 5 (Claim 3 and 6 dependent therefrom) for reciting the term "increased"; in referring to the Aft protein's activity or its expression, is withdrawn by virtue of applicants' amendment (i.e., deletion of the term or phrase at issue).

5. The previous rejection of Claim 5 under of 35 U.S.C. 112, second paragraph, for reciting "the activity or expression of an endogenous protein", is withdrawn by virtue of reconsideration by the Examiner (Claim 5 do not recites "the activity or expression an endogenous protein".

6. The previous rejection of Claim 15 under of 35 U.S.C. 112, second paragraph, for reciting "overexpress" in referring to the Aft proteins, is withdrawn by virtue of applicants' amendment.

7. The previous rejection of Claims 13-16, under 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends, is withdrawn by virtue of applicants' amendment.

Claim Rejections - 35 USC § 112

8. The previous rejection of Claims 1-19 under 35 U.S.C. § 112, first paragraph, **written description**, is maintained for reasons below.

Applicants notes that, in view of instant amendment, amended claims specifies that recombinant yeast is engineered to have at least one modification to an endogenous gene by deletion of one or more nucleotides of said endogenous gene or regulatory region thereof, or insertion of one or more nucleotides into said endogenous gene of GRX3 and GRX4 or regulatory region thereof; and combination thereof; and argue specification teaches engineering microorganism by alteration, disruption or knock-out of gene such as GRX3 and/or GRX4 (see bottom of page 9, Remarks filed on 2/16/2012). Applicants argue deletion or insertions is well known by those skilled in the art, wherein GRX3 and GRX4 is well known in view of Pujol-Carrion et al.; thus, what is well known in the art need not be provided by specification (see top of page 10, Remarks filed on 2/16/2012).

Applicants' arguments have been fully considered but are not deemed persuasive for the following reasons. The Examiner acknowledges there is no need to describe what is well known in the art such as mutating gene sequence of known gene, and also acknowledges yeast GRX3 and GRX4 genes are already known in the art as described above. However, as noted in the previous office action, and as acknowledged by applicants, instant claims also encompasses genetic mutation in very broad genus of any regulatory region which regulating GRX3 and GRX4 expression and/or activity. As noted above, even considering recombinant DNA technology is well known, instant specification describes inactivation of GRX3 gene and/or GRX4 gene themselves, but not regulatory region(s) to inactivate GRX3 gene and/or GRX4 gene. As similarly noted in the previous office action, instant specification and prior art do not

describe correlation of structure of regulatory region to be mutated for function of inactivating GRX3 and/or GRX4. For example, said genus of any regulatory region can be near and/or far from GRX3, 4 genes; or they can be even located totally unrelated site, anywhere within the genome of yeast but may regulate GRX3, 4 by metabolic pathways. Without the sufficient correlation between structure of genus regulatory region and its function, one skilled in the art cannot possess the alteration, deletion, insertion and/or knock-out of said genus regulatory region when they are not described; thus, not being able to possess inactivation of regulatory region of GRX3 and/or GRX4, in turn unable to possess inactivation of endogenous GRX3 and/or GRX4 gene. For all reasons above and reasons noted in the previous office action mailed out on 2/16/2012, instant rejection is maintained.

9. The previous rejection of Claims 1-19 under 35 U.S.C. 112, first paragraph, **scope of enablement**, is maintained for reasons below.

Applicants have provided the same reasons noted above for traversal of instant rejection. Applicants notes that, in view of instant amendment, amended claims specifies that recombinant yeast is engineered to have at least one modification to an endogenous gene by deletion of one or more nucleotides of said endogenous gene or regulatory region thereof, or insertion of one or more nucleotides into said endogenous gene of GRX3 and GRX4 or regulatory region thereof; and combination thereof; and argue specification teaches engineering microorganism by alteration, disruption or knock-out of gene such as GRX3 and/or GRX4 (see bottom of page 9, Remarks filed on

2/16/2012). Applicants argue deletion or insertions is well known by those skilled in the art, wherein GRX3 and GRX4 is well known in view of Pujol-Carrion et al.; thus, what is well known in the art need not be provided by specification (see top of page 10, Remarks filed on 2/16/2012).

Applicants' arguments have been fully considered but are not deemed persuasive for the following reasons. As noted above, the Examiner acknowledges there is no need to describe what is well known in the art such as mutating gene sequence of known gene, and also acknowledges yeast GRX3 and GRX4 genes are already known in the art as described above. However, as noted in the previous office action, and as acknowledged by applicants, instant claims also encompasses genetic mutation in very broad genus of any regulatory region which regulating GRX3 and GRX4 expression and/or activity. As noted above, even considering recombinant DNA technology is well known, instant specification describes inactivation of GRX3 gene and/or GRX4 gene themselves, but not regulatory region(s) to inactivate GRX3 gene and/or GRX4 gene. As similarly noted in the previous office action, instant specification and prior art do not disclose sufficient direction and guidance to make and use of inactivating GRX3 and/or GRX4 gene by altering, disrupting, deletion and/or knocking out genus of any regulatory gene which inactivating GRX3, GRX4 expression and/or their activities. For example, said genus of any regulatory region can be near and/or far from GRX3, 4 genes; or they can be even located totally unrelated site, anywhere within the genome of yeast but may regulate GRX3, 4 by metabolic pathways. Thus, unpredictability of inactivating GRX3, GRX4 expression and/or their activities by

mutating their regulatory region thereof is very high, and would require undue experimentation for one skilled in the art to make mutation(s) in said genus regulatory region for use in inactivating GRX3, GRX4 expression and/or their activity.

Withdrawn-Claim Rejections - 35 USC § 102

10. The previous rejection of Claims 1-12 and 15-19 under 35 U.S.C. 102(e) as being anticipated by Anthony et al. (12/569,069 filed on 9/29/2009; published as USPAP 2010/0081179 on 4/1/2010; as cited in IDS filed on 11/18/2011) as evidenced by Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011) and Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS), is withdrawn by virtue of applicants' amendment.

Withdrawn-Claim Rejections - 35 USC § 103

11. The previous rejection of Claims 1-19 under 35 U.S.C. 103(a) as being unpatentable over Anthony et al. (12/569,069 filed on 9/29/2009; published as USPAP 2010/0081179 on 4/1/2010; as cited in IDS filed on 11/18/2011) **in view of** Li et al. (US Patent Application Pub. No: US 2009/0163376 A1; filed on 12/18/2008) as evidenced by Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011) and Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS), is withdrawn by virtue of applicants' amendment.

12. The previous rejection of Claims 1-12 and 15-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Liao et al. (US Patent Application Pub. No: US 2009/0081746 A1; published on Mar. 26, 2009) in view of Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS) as evidenced by John Imsande (Iron-sulfur cluster: Formation perturbation, and physiological functions., Plant Physiol. Biochem., 1999, Vol. 37, pages 87-97) and Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011); and KEGG Enzyme 4.2.1.9 (last viewed on 12/6/2011) is withdrawn by virtue of applicants' argument in view of instant amendment (see page 12 - top of page 16, Remarks filed on 2/16/2012).

13. The previous rejection of Claims 1-19 under 35 U.S.C. 103(a) as being unpatentable over Liao et al. (US Patent Application Pub. No: US 2009/0081746 A1; published on Mar. 26, 2009) in view of Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS), and **in further view of** Li et al. (US Patent Application Pub. No: US 2009/0163376 A1; filed on 12/18/2008) and Anthony et al. (2010/0129886 which is USPAP of 12/617,017 filed on 11/12/2009, with effective priority date of Nov. 13, 2008; as cited in IDS); as evidenced by KEGG Enzyme 4.2.1.9 (last viewed on 12/6/2011), John Imsande (Iron-sulfur cluster: Formation perturbation, and physiological functions., Plant Physiol. Biochem., 1999, Vol. 37, pages 87-97) and Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS) is

withdrawn by virtue of applicants' argument in view of instant amendment (see page 12 - top of page 16, Remarks filed on 2/16/2012).

Maintained-Double Patenting

14. The previous rejection of Claims 1-19 rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of copending Application No. 13/246,718, is maintained for reasons below.

Applicants requested that the rejection be held in abeyance until allowable subject matter is found (see page 16, Remarks filed on 2/16/2012). Thus, instant rejection is maintained for same reasons noted in the previous non-final office action mailed out on 12/13/2011.

15. The previous rejection of Claims 1-19 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,358 (from US application 13/228,342) is maintained for reasons below.

Applicants argue the instant amendment makes clear distinction compared to claims 1-20 of U.S. Patent No. 8,071,358 (from US application 13/228,342). Applicants requested that the rejection be held in abeyance until allowable subject matter is found. Applicants' arguments have been fully considered but are not deemed persuasive for the following reasons. The claims 1-20 of U.S. Patent No. 8,071,358 (from US application 13/228,342) encompasses the inactivation of GRX3 and/or GRX4 for

reasons noted in the previous non-final office action mailed out on 12/13/2011, Thus, instant claims are obvious and instant rejection is maintained.

16. The previous rejection of Claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,376 (from US application 12/953,884), is maintained for reasons below.

Applicants requested that the rejection be held in abeyance until allowable subject matter is found (see page 16, Remarks filed on 2/16/2012). Thus, instant rejection is maintained for same reasons noted in the previous non-final office action mailed out on 12/13/2011.

Conclusion

17. Claims 1-19 are not allowed for the reasons identified in the numbered sections of this Office action. Applicants must respond to the objections/rejections in each of the numbered section in this Office action to be fully responsive in prosecution.

Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the

shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Alexander D. Kim whose telephone number is (571) 272-5266. The examiner can normally be reached on 10AM-6:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on (571) 272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Alexander D Kim/
Primary Examiner, Art Unit 1656

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

58249 7590 02/16/2012

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER	
KIM, ALEXANDER D	
ART UNIT	**PAPER NUMBER**
1656	

MAIL DATE	DELIVERY MODE
02/16/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

All participants (applicant, applicant's representative, PTO personnel):

(1) *ALEXANDER KIM*.

(3) *Andrew Hawkins*.

(2) *Paul Wickman*.

(4)_____.

Date of Interview: *14 February 2012*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☒112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1*.

Identification of prior art discussed: *Pujol-Carrion et al. and Ihring et al* .

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicants' representative and the Examiner discussed claim language to overcome rejection under 35 USC 112, first paragraph. Applicants' representative also presented why instant invention is unobvious over the prior art reference of Pujol-Carrion et al. and Ihring et al. The Examiner noted the argument and/or evidence why instant invention is unobvious need consideration in view of teachings of prior art and will be fully considered in view of claim amendment when it is filed in reply to the previous non-final office action.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Alexander D Kim/ Primary Examiner, Art Unit 1656	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Catherine Asleson Dundon *et al.* Confirmation No.: 5847

Application No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Alexander D. Kim

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window, **Mail Stop Amendment**
Randolph Building
401 Dulany Street
Alexandria, VA 22314

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §1.97(c)

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98.

[x] Pursuant to 37 C.F.R. §1.98, copies of documents 1-4 cited in the attached Form PTO/SB/08a are enclosed.

This Information Disclosure Statement is filed after the period specified in 37 C.F.R. § 1.97(b), but before the mailing of:

[x] a final action under 37 C.F.R. §1.113;

[ ] a notice of allowance under 37 C.F.R. §1.311; or

[ ] an action that otherwise closes prosecution in this application.

In accordance with 37 C.F.R. §1.97(c) also enclosed is:

[x] Fee under 37 C.F.R. §1.17(p) in the amount of $180.00; or

[ ] Statement as specified in 37 C.F.R. §1.97(e):

[ ] Each item of information contained in the Information Disclosure Statement cited herein was first cited in a communication from a foreign patent office in a counterpart foreign application not more than three months

prior to the filing date of the Information Disclosure Statement; or

[ ] No item of information contained in the Information Disclosure Statement submitted herewith was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the undersigned, having made a reasonable inquiry, no item of information contained in the Information Disclosure Statement was known to any individual designated in 37 C.F.R. §1.56(c) more than three months prior to the filing date of the Information Disclosure Statement.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Forms PTO/SB/08a to the undersigned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-1283.

Dated: February 16, 2012

Respectfully submitted,
COOLEY LLP

USPTO Customer No. 58249
COOLEY LLP
ATTN: Patent Group
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By: /Paul A. Wickman/
Paul A. Wickman
Reg. No. 61,242

PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code2 (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	1.	WO 2011/103300 A2	08-25-2011	BUTAMAX(TM) ADVANCED BIOFUELS LLC		

NON PATENT LITERATURE DOCUMENTS

Examiner's Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	2.	Mühlenhoff, U., et al., "Cytosolic Monothiol Glutaredoxins Function in Intracellular Iron Sensing and Trafficking via Their Bound Iron-Sulfur Cluster," Cell Metabolism 12: 373-385 (October 6, 2010)	

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08a (09-08)
Approved for use through 10/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT LIST

(Use as many sheets as necessary)

Complete if Known	
Application Number	13/246,693
Filing Date	September 27, 2011
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Alexander D. Kim
Attorney Docket Number	GEVO-041/13US 310142-2263

NON PATENT LITERATURE DOCUMENTS

Examiner's Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[6]
	3.	Puig, S., et al., "Coordinated Remodeling of Cellular Metabolism during Iron Deficiency through Targeted mRNA Degradation," Cell 120:99-110 (2005)	
	4.	"Request for *Inter Partes* Reexamination of U.S. Patent No. 8,017,376 Under 35 U.S.C. § 311 and 37 C.F.R. § 1.913," 153 pages, U.S. 95/001,870 (filed January 10, 2012)	

Examiner Signature:		Date Considered	

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re App'n of: Catherine Asleson DUNDON *et al.* Confirmation No.: 5847

Serial No.: 13/246,693 Group Art Unit: 1656

Filed: September 27, 2011 Examiner: Kim, Alexander D.

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT UNDER 37 CFR § 1.111

This paper responds to the Non-Final Office Action dated December 13, 2011. Applicants respectfully request reconsideration of this application in view of the following amendments and remarks.

Amendments to the Claims are reflected on the listing of the claims which begins on page 2 of this paper.

Remarks begin on page 6 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior listings in the application. Please amend the claims as follows.

1. (Currently Amended) A recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to ~~delete or attenuate the expression or activity of one or more endogenous glutathione-dependent oxidoreductases selected from the group consisting of Grx3 and Grx4~~ comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated endogenous gene results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin or regulatory region thereof, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin or a regulatory region thereof, and combinations thereof.

2. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, said isobutanol producing metabolic pathway comprising the following substrate to product conversions:

(a) pyruvate to acetolactate;

(b) acetolactate to 2,3-dihydroxyisovalerate;

(c) 2,3-dihydroxyisovalerate to α-ketoisovalerate;

(d) α-ketoisovalerate to isobutyraldehyde; and

(e) isobutyraldehyde to isobutanol;

and wherein said DHAD catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate.

3. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of pyruvate to acetolactate is an acetolactate synthase.

4. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of acetolactate to 2,3-dihydroxyisovalerate is a ketol-acid reductoisomerase.

5. (Previously Presented) The recombinant yeast microorganism of claim 4, wherein said ketol-acid reductoisomerase is an NADH-dependent ketol-acid reductoisomerase.

6. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the cytosol.

7. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the mitochondria.

8. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Lactococcus lactis.*

9. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Streptococcus mutans*.

10. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde is a 2-keto acid decarboxylase.

11. (Previously Presented) The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol is an alcohol dehydrogenase.

12. (Previously Presented) The recombinant yeast microorganism of claim 11, wherein said alcohol dehydrogenase is an NADH-dependent alcohol dehydrogenase.

13. (Currently Amended) The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is further engineered to inactivate one or more endogenous pyruvate decarboxylase (PDC) genes.

14. (Currently Amended) The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is further engineered to inactivate one or more endogenous glycerol-3-phosphate dehydrogenase (GPD) genes.

15. (Currently Amended) The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is further engineered ~~to overexpress~~ to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins as compared to the corresponding yeast microorganism that has not been engineered to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins.

16. (Currently Amended) The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is further engineered to express one or more polynucleotides encoding one or more constitutively active activator of ferrous transport (Aft) proteins.

17. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following genera: *Saccharomyces*, *Kluyveromyces*, *Pachysolen*, *Zygosaccharomyces*, *Debaryomyces*, *Pichia*, *Schizosaccharomyces*, *Candida*, *Issatchenkia*, *Hansenula*, *Yarrowia*, *Tricosporon*, *Rhodotorula,* and *Myxozyma.*

18. (Previously Presented) The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following species: *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces bayanus*, *Saccharomyces paradoxus*, *Saccharomyces castelli*, *Saccharomyces kluyveri*, *Kluyveromyces thermotolerans*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces waltii*, *Pachysolen tannophilis*, *Zygosaccharomyces bailli*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Debaromyces carsonii*, *Pichia pastorius*, *Pichia anomala*, *Pichia stipitis*, *Pichia castillae*, *Schizosaccharomyces pombe*, *Candida utilis*, *Candida glabrata*, *Candida tropicalis*, *Candida xestobii*, *Issatchenkia orientalis*, *Issatchenkia occidentalis*, *Issatchenkia scutulata*, *Hansenula anomala*, and *Yarrowia lipolytica*.

19. (Previously Presented) A method of producing isobutanol comprising: (a) providing the recombinant yeast microorganism of claim 2; and (b) cultivating the recombinant yeast microorganism of claim 2 in a culture medium containing a feedstock providing a carbon source, until a recoverable quantity of the isobutanol is produced.

Remarks

Status of Claims and Claim Amendments

In this Amendment, claims 1 and 13-16 have been amended. After entry of this Amendment, claims 1-19 will be pending.

Claim 1 has been amended to specify that the "recombinant yeast microorganism is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated endogenous gene results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin or regulatory region thereof, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin or a regulatory region thereof, and combinations thereof." Support for this amendment can be found in original claim 1 and throughout the specification as originally filed, *e.g.*, at paragraphs [0058], [00119]-[00121], and [00211].

The amendments to dependent claims 13-16 are formalistic in nature.

Applicants respectfully submit that no new matter has been introduced by way of these amendments.

The Office Action dated December 13, 2011 has been carefully reviewed and the following reply is made in response thereto. In view of the following remarks, Applicants respectfully request reconsideration of this application and the timely allowance of the pending claims.

Examiner Interview

Applicants thank the Examiner for the courtesy extended to the Applicants and Applicants' agent during the telephonic interview of February 14, 2012. Potential amendments to the claims as they relate to the rejections under 35 U.S.C. § 102 and 35 U.S.C. § 103 were

discussed. Applicants additionally presented evidence concerning the surprising benefits of Grx inactivation on DHAD activity in light of the Ojeda and Mühlenhoff references discussed below.

Claim Objections

At pages 2-3 of the Office Action, the Office objected to claim 1 for the recitation of the abbreviation "Grx". Without acquiescing to the propriety of this objection, Applicants have amended claim 1 to specify that GRX3 and GRX4 correspond to "monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4)," respectively. Therefore, Applicants respectfully request that this objection be removed.

Claim Rejections under 35 USC § 112, Second Paragraph

At page 3 of the Office Action, the Office rejected claims 1-19 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

Specifically, the Office rejected claim 1 (and claims 2-19 as being dependent thereon) as allegedly being indefinite in the recitation of "the . . . activity" for Grx3 and Grx4. Without acquiescing to the propriety of this rejection, Applicants have removed any reference to Grx3 and/or Grx4 activity from claim 1. Therefore, Applicants respectfully request that this grounds of rejection be removed.

At page 4 of the Office Action, the Office rejected claim 5 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite in the recitation of "overexpress" in referring to the Aft proteins. Specifically, the Office alleges that the term "overexpress" is a relative term, which renders the claim indefinite without the point of reference. Without acquiescing to the propriety of this rejection, Applicants have amended claim 15 to recite that the "recombinant yeast microorganism is further engineered to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins as compared to the corresponding yeast microorganism that has not been engineered to comprise increased expression of one or more polynucleotides encoding one or more activator of ferrous transport

(Aft) proteins." Claim 15, as amended, now provides the point of reference as requested by the Office. Therefore, Applicants respectfully request that this grounds of rejection be removed.

Claim Rejections under 35 USC § 112, Fourth Paragraph

At pages 4-5 of the Office Action, the Office rejected claims 13-16 under 35 U.S.C. § 112, fourth paragraph, as allegedly being of improper dependent form for failing to further limit the subject matter of the claim. Without acquiescing to the propriety of this rejection, Applicants have amended claims 13-16 to specify that the recombinant yeast microorganism is "*further* engineered" as requested by the Office. Therefore, Applicants respectfully request that this grounds of rejection be removed.

Claim Rejections under 35 USC § 112, First Paragraph, Written Description and Enablement

At pages 6-9 of the Office Action, the Office rejected claims 1-19 as allegedly failing to comply with the written description requirement. Specifically, the Office alleges that the breadth of the previous claims encompassed an overly broad genus of recombinant yeast cells which have been engineered to delete or attenuate the expression or activity of endogenous Grx3 or Grx4, wherein said engineering encompasses (a) mutations in any Grx3-4 regulatory gene(s) or protein(s) related to expression or activity of said Grx3 or Grx4; and (b) any mutation (*i.e.*, substitution, deletion, insertion and/or combination thereof) in said Grx3 or Grx4 proteins themselves such that the mutated Grx3 or Grx4 has attenuated expression or attenuated activity.

At pages 9-13 of the Office Action, the Office rejected claims 1-19 under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement. The Office acknowledges that the specification is enabling for recombinant yeast microorganisms engineered to delete the expression of endogenous Grx3 and/or Grx4. *See* the Office Action, p. 9, paragraph 8 (emphasis added). The Office alleges, however, that the specification does not reasonably provide enablement for recombinant yeast microorganisms which have been engineered to comprise (a) mutations in any Grx3-4 regulatory gene(s) or protein(s) related to expression or activity of said

Grx3 or Grx4; and (b) any mutation (*i.e.*, substitution, deletion, insertion and/or combination thereof) in said Grx3 or Grx4 proteins themselves such that the mutated Grx3 or Grx4 has attenuated expression or attenuated activity.

Without acquiescing to the propriety of this rejection, Applicants respectfully submit that claim 1 has been amended to specify that the "recombinant yeast microorganism is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), and wherein said inactivated endogenous gene results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin or regulatory region thereof, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin or a regulatory region thereof, and combinations thereof." Support for this amendment can be found in original claim 1 and throughout the specification as originally filed, *e.g.*, at paragraphs [0058], [00119]-[00121], and [00211].

Applicants respectfully submit that claim 1, as amended, specifies that the recombinant yeast microorganism is engineered to comprise at least one modification to an endogenous gene encoding a monothiol glutaredoxin (*e.g.* Grx3 and/or Grx4), and that the modification is selected from the group consisting of a deletion of one or more nucleotides of said endogenous gene or regulatory region thereof (*e.g.*, a partial or complete gene deletion), an insertion of one or more nucleotides into an endogenous gene or a regulatory region thereof (*e.g.*, a gene disruption using, for instance, a selectable antibiotic resistance marker), and combinations thereof. Accordingly, amended claim 1 (and claims 2-19 depending therefrom) now specifies the types of modifications (*e.g.*, deletions, insertions, and combinations thereof) which may be made to an endogenous gene encoding a monothiol glutaredoxin. Applicants respectfully submit that methods of gene disruptions and gene deletions are well-known in the art and are described in the specification. For example, the specification teaches at paragraph [00211] that an engineered microorganism can include an alteration, disruption, deletion or knock-out of a gene (*e.g.*, GRX3 and/or GRX4) to alter the cellular physiology. Furthermore, paragraph [0058] teaches that modifications can include deletions or insertions of single or multiple residues in a protein

encoded by a polynucleotide sequence. Moreover, paragraph [0058] teaches that modifications may constitute an insertion and/or a deletion of part or all of a gene.

Applicants further submit that those skilled in the art were well aware at the time of filing of particular deletions and insertions that could be made to an endogenous gene encoding a monothiol glutaredoxin (*e.g.*, Grx3 and/or Grx4). For example, Pujol-Carrion *et al.* teach methods of disrupting Grx3 and/or Grx4 through the use of gene disruption. *See* Pujol Carrion *et al.*, 2006, *J. Cell Sci.* 119: 4554-4564 at p. 4562. What is well known in the art need not be provided by the specification. Therefore, one of skill in the art would recognize from the disclosed that the Applicant was in possession of the claimed invention in amended claim 1. And Applicants note that the instantly pending claim 1 – which the Office has rejected herein as lacking enablement – now recites the subject matter which the Office has found to be enabled. Accordingly, Applicants submit that claim 1, and claims 2-19 depending therefrom, should no longer be subject to the Office's written description and enablement rejections.

Claim Rejections under § 35 USC 102

At pages 14-16 of the Office Action, claims 1-12 and 15-19 are rejected under 35 U.S.C. § 102(e) as allegedly anticipated by Anthony *et al.* (US 2010/0081179, hereinafter "Anthony") as evidenced by Ihrig *et al.* (*Eukaryotic Cell*, Dec. 11, 2009, 9: 460-471, hereinafter "Ihrig") and Pujol-Carrion *et al.* (*J. Cell Sci.* 119: 4554-4564, hereinafter "Pujol-Carrion"). Specifically, the Office alleges that Anthony teaches recombinant yeast microorganisms comprising an overexpressed DHAD and methods of using said recombinant yeast microorganisms to produce isobutanol. The Office further alleges that since yeast inherently express Grx3-4 and the attenuation level of claim 1 is not limited to any specific condition, the yeast microorganisms of Anthony have lower expression as compared to a corresponding yeast cell transformed with Grx3 and/or Grx4 by an overexpression vector.

Applicants respectfully traverse this rejection. Specifically, it is noted that:

> A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a

> single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987); MPEP § 2131.

In this case, Anthony is silent with respect to engineering a recombinant yeast cell to delete or attenuate the expression or activity of Grx3 and/or Grx4. Moreover, there is no evidence from Anthony showing that the yeast cells of Anthony would inherently have deleted or attenuated activity of Grx3 and/or Grx4. Indeed, "in relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original). Thus, Applicants respectfully submit that Anthony does not anticipate claim 1 under 35 U.S.C. § 102(e).

Nevertheless, to expedite prosecution, claim 1 has been amended to specify that the recombinant yeast microorganism is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4).

Applicants respectfully point out that the element of amended claim 1 specifying that the recombinant yeast microorganism "is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4)" is not disclosed by Anthony expressly or inherently. Further, amended claim 1 specifies that the inactivated endogenous gene encoding a monothiol glutaredoxin "results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin or regulatory region thereof, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin or a regulatory region thereof, and combinations thereof." Again, this element is not disclosed by Anthony expressly or inherently. Thus, in view of the amendment to claim 1 and the above remarks, the rejection of claims 1-12 and 15-19 should be reconsidered and withdrawn.

Claim Rejections under § 35 USC 103

At pages 17-18 of the Office Action, claims 1-19 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Anthony in view of Li (US 2009/0163376, hereinafter "Li") as evidenced by Ihrig and Pujol-Carrion.

As noted above, claim 1 has been amended to specify that the recombinant yeast microorganism is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4), wherein said inactivated endogenous gene results from the deletion of one or more nucleotides of said endogenous gene encoding a monothiol glutaredoxin or regulatory region thereof, the insertion of one or more nucleotides into said endogenous gene encoding a monothiol glutaredoxin or a regulatory region thereof, and combinations thereof.

As described above, Anthony fails to expressly or inherently teach a recombinant yeast microorganism which is engineered to comprise at least one inactivated endogenous gene encoding a monothiol glutaredoxin selected from the group consisting of monothiol glutaredoxin-3 (GRX3) and monothiol glutaredoxin-4 (GRX4). This deficiency of Anthony is not cured by Li, which provides no teaching or suggestion for engineering yeast to comprise an inactivated GRX3 gene and/or GRX4 gene. Accordingly, Applicants respectfully submit that the pending claims are not obvious over Anthony in view of Li.

At pages 18-20 of the Office Action, claims 1-12 and 15-19 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Liao *et al.* (US 2009/0081746, hereinafter "Liao") in view of Pujol-Carrion as evidenced by John Imsande (1999, *Plant Physiol. Biochem.* 37: 87-97, hereinafter "Imsande"), Ihrig, and KEGG Enzyme 4.2.1.9.

At pages 20-22 of the Office Action, claims 1-19 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Liao in view of Pujol Carrion, and further in view of Li and Anthony *et al.* (US 2010/0129886) as evidenced by KEGG Enzyme 4.2.1.9, Imsande, and Ihrig.

Specifically, the Office asserts that Liao teaches an isobutanol biosynthetic pathway in yeast comprising a DHAD – this enzyme is correctly noted in the Action as requiring a Fe/S cofactor for catalytic activity. *See* the Office Action at p. 19, citing KEGG Enzyme 4.2.1.9 and Imsande. The Office alleges it would have been obvious to modify the yeast of Liao by deleting endogenous GRX3 and/or GRX4, because deletion of GRX3 and/or GRX4 has been shown to increase iron accumulation in yeast. *See* the Office Action, p. 20, citing Pujol-Carrion. Applicants respectfully traverse the rejection for the reasons described below.

Importantly, the Office has presented no evidence teaching or suggesting that the increased accumulation of iron in Grx3- and/or Grx4-deficient cells would improve DHAD activity. As the Office correctly notes, DHAD is an iron-sulfur (Fe/S) protein, meaning that it requires a Fe/S cofactor for activity. Notably, studies of Grx3 and/or Grx4 depletion in yeast indicate that Grx3 and/or Grx4 do not impact the activity of several Fe/S proteins other than DHAD, including aconitase, sulfite reductase, and isopropylmalate isomerase (Leu1). *See, e.g.*, Ojeda *et al.*, 2006, *J. Biol. Chem.* 281(26): 17661-69 at p. 17667, Col. 1.[1] For instance, Ojeda "addressed whether Grx3 and Grx4 . . . contributed to Fe-S maturation" and observed that "***in the absence of either Grx3 or Grx4, the activities of mitochondrial and cytosolic Fe-S clusters enzymes were unaffected.***" Ojeda at p. 17668, Col. 2 (emphasis added). Applicants respectfully submit that these results, if anything, teach away from modifying a yeast comprising DHAD to disrupt or delete an endogenous GRX3 and/or GRX4 gene. Indeed, based upon these results, a skilled artisan would not have had a reasonable expectation that disruption or deletion of an endogenous GRX3 and/or GRX4 gene would result in increased DHAD activity.

In fact, Applicants respectfully submit that one skilled in the art might have expected that Grx3- and/or Grx4-deficiency would diminish DHAD activity, thus teaching away from the proposed combination of Liao and Pujol-Carrion. Indeed, the art teaches that "***[d]epletion of Grx3/4 specifically impaired all iron-requiring reactions in the cytosol, mitochondria, and nucleus, including the synthesis of Fe/S clusters.***" *See* Mühlenhoff, 2010, *Cell Metabolism* 12:

[1] Hereinafter referred to as "Ojeda" and cited in the information disclosure statement of November 18, 2011.

373-385, Abstract (emphasis added).[2] Moreover, the art teaches that although GRX3 and/or GRX4 deletion leads to increased iron accumulation (*See, e.g.*, Pujol-Carrion), this "surplus iron in Grx3/4-deficient cells is not bioavailable for efficient funneling into iron-dependent processes." *Id.* at p. 383, Col. 1. In other words, while Grx3/4-deficient cells may accumulate more iron as compared to wild-type cells, they are unable to use it biologically.

In the context of Fe/S proteins, the art teaches that in Grx3/4-deficient cells, "***the activities of the mitochondrial Fe/S protein aconitase and cytosolic catalase, a heme-containing protein, drastically decreased, despite the presumed sufficient cellular iron supply.***" *Id.* at p. 374, Col. 1 (emphasis added), under the heading, "Deficiency in Grx3/4 is Associated with Defects in Iron-Dependent Enzymes." Thus, based on these results, it can be reasonably concluded that the skilled artisan would expect similar inhibitory effects on the activity of DHAD, also a Fe/S-requiring protein. This is particularly true because the art teaches that "a general impairment in the de novo assembly of Fe/S proteins [occurs] upon depletion of Grx3/4." *Id.* at p. 374, Col. 2, under the heading, "Deficiency in Grx3/4 Impairs the De Novo Synthesis of Cellular Fe/S Clusters and Heme." Applicants respectfully submit that based upon the aforementioned information, the skilled artisan would have been dissuaded from disrupting or deleting an endogenous GRX3 and/or GRX4 gene in a DHAD-requiring biosynthetic pathway – indeed, the skilled artisan would have reasonably concluded that these modifications would likely failed to produce the desired effects on DHAD activity and/or result in reduced DHAD activity.

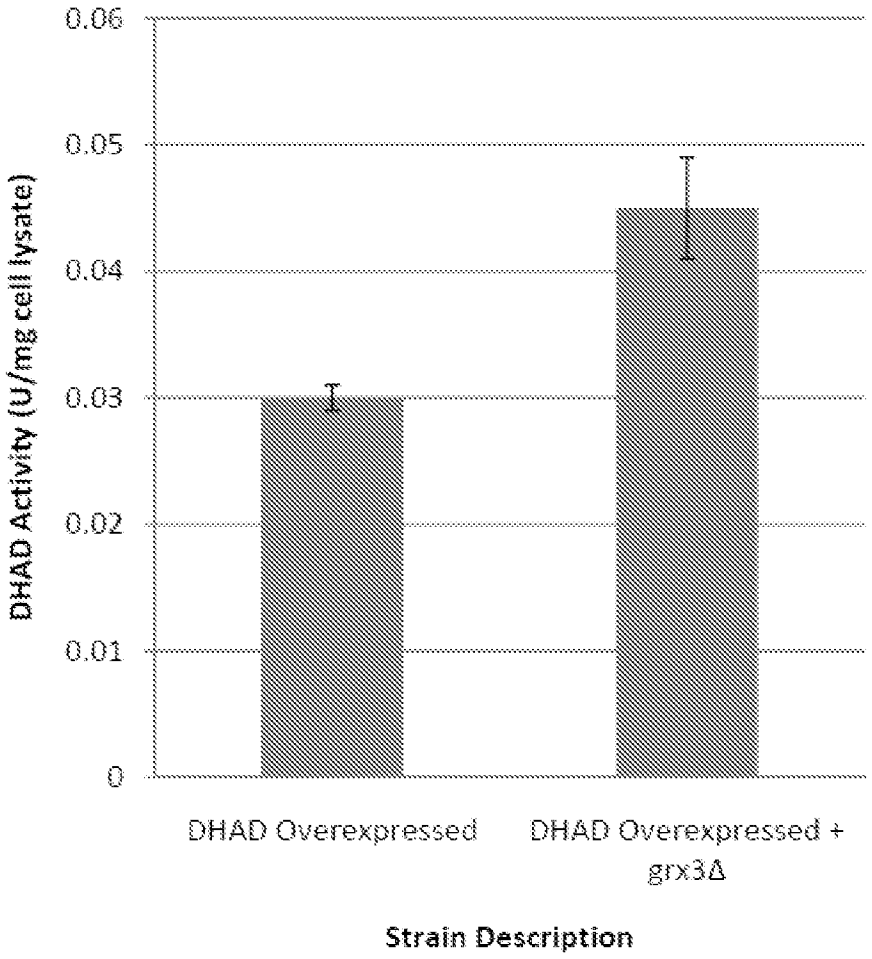
Accordingly, for at least this reason, Applicants respectfully submit that pending claims 1-19 are not obvious over the cited art. Reconsideration and withdrawal of the obviousness rejection is respectfully requested.

Surprising Results

Applicants further submit that in light of the above information, it is surprising that the Applicants have observed increased DHAD activity in strains with disrupted Grx activity.

[2] Hereinafter referred to as "Mühlenhoff" and cited in the accompanying information disclosure statement.

Specifically, the data presented below shows that yeast with a disrupted Grx protein surprisingly exhibit ~ 50% more DHAD activity as compared to yeast where Grx is unmodified.



It is unexpected that DHAD activity would increase in yeast strains where Grx is disrupted or deleted. This is particularly true given the teachings of Ojeda and Muhlenhoff, which explain how the activities of other Fe/S-requiring enzymes were either reduced or unaffected by the absence of Grx3 and/or Grx4. Therefore, the Applicants' observation that the activity of DHAD, also a Fe/S-requiring enzyme, increases in the absence of Grx is surprising. This surprising property is also of practical significance, as the specification teaches how DHAD is an essential enzyme in several biosynthetic pathways and that high DHAD activity is

important to sustaining commercially viable productivities, yields, and titers within these biosynthetic pathways. *See, e.g.,* the specification at paragraph [0006].

Accordingly, Applicants respectfully submit that they have shown the presence of a surprising property in the claimed yeast microorganism. This surprising result was not suggested by the prior art and therefore the cited art does not render the claims obvious.

Double Patenting Rejections

At pages 22-23 of the Office Action, claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of co-pending Application No. 13/246,718. Applicants respectfully request that the rejection be held in abeyance until allowable subject matter is found in one of the pending applications.

At page 23 of the Office Action, claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,358 (issued from U.S. Application No. 13/228,342). Applicants respectfully submit that the current amendments to claim 1, the lone independent claim of the present application, create a clear distinction compared to claims 1-20 of U.S. Patent No. 8,071,358. Nevertheless, if the Examiner concludes that an issue of obviousness-type double patenting remains, Applicants will consider filing a Terminal Disclaimer once the presently pending claims are otherwise held to be allowable.

At page 24 of the Office Action, claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,017,376 (issued from U.S. Application No. 12/953,884). Applicants respectfully submit that the current amendments to claim 1, the lone independent claim of the present application, create a clear distinction compared to claims 1-20 of U.S. Patent No. 8,017,376. Nevertheless, if the Examiner concludes that an issue of obviousness-type double patenting remains, Applicants will consider filing a Terminal Disclaimer once the presently pending claims are otherwise held to be allowable.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that no further impediments exist to the allowance of this application and, therefore, request an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to **Deposit Account No. 50-1283**.

Dated: February 16, 2012

Respectfully submitted,
COOLEY LLP

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263

CONFIRMATION NO. 5847

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PUBLICATION NOTICE

Title:METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

Publication No.US-2012-0028322-A1
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NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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EXAMINER	
KIM, ALEXANDER D	

ART UNIT	PAPER NUMBER
1656	

MAIL DATE	DELIVERY MODE
12/13/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	13/246,693	DUNDON ET AL.	
	Examiner	Art Unit	
	ALEXANDER KIM	1656	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *18 November 2011*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1-19* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-19* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *27 September 2011* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *11/18/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Application Status

1. Claims 1-19 are pending in this instant Office action.

Priority

2. Applicant's claim for the benefit of a divisional application of prior application 13/228,342 filed on 9/8/2011 (now US Patent 8,071,358), which claims benefit of divisional application of 12/953,884 filed on 11/24/2010 (now US Patent 8,017,376) which claims benefit of provisional application 61/350,209 (filed on 6/1/2010) and 61/263,952 (filed on 11/24/2009) under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, or 365(c) is acknowledged.

Applicant's claim no foreign priority under 35 U.S.C. 119(a)-(d).

Information Disclosure Statement

3. The information disclosure statement (IDS) filed on 11/18/2011 has been reviewed, and its references have been considered except for those which have been lined through. A copy of Form PTO/SB/08 is attached to the instant Office action.

Claim Objections

4. Claims 1-19 are objected to because of the following informalities:

Claim 1 (Claims 2-19 dependent therefrom) recites an abbreviation "Grx". Abbreviations, unless otherwise obvious and/or commonly used in the art, *e.g.,* "DNA",

should not be recited in the claims without at least once reciting the entire phrase for which the abbreviation is used in its first appearance in the claims.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

5. Claims 1-19 are rejected under of 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

a. Claim 1 (Claims 2-19 dependent therefrom) recites "the ... activity" for Grx3 and Grx4 protein. There is insufficient antecedent basis for this limitation of "the activity" of said Grx3 and Grx4 protein in the claim. It is unclear if the claims are limited to the one function disclosed in the specification or to any other measurable activity which includes, but not limited to any catalytic activity, immunological activity, binding activity.

b. Claim 1 (Claims 2-19 dependent therefrom) recites "increase the expression or activity"; and Claims 2, 4, and 5 (Claim 3 and 6 dependent therefrom) recite the term "increased"; in referring to the Aft protein's activity or its expression. However, the term "increase" or "increased" is a relative term, which renders the claim indefinite without the point of reference. The term

"increase" or "increased" are not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention.

c. Claim 5 recite "the activity or expression of an endogenous protein" (emphasis added). The meets and bounds of the activity of Aft protein is unclear if the claim is limited to a one particular activity of endogenous protein. For example, the activity of any endogenous protein can includes, but not limited to, any catalytic activity, immunological activity, binding activity, or an activity that can be measured in an assay.

d. Claim 15 recites term "overexpress" in referring to the Aft proteins. However, the term "overexpress" is a relative term, which renders the claim indefinite without the point of reference. The specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention.

Appropriate clarification is required.

The following is a quotation of the fourth paragraph of 35 U.S.C. 112:

> Subject to the [fifth paragraph of 35 U.S.C. 112], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

6. Claims 13-16 are rejected under 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon

which it depends, or for failing to include all the limitations of the claim upon which it depends.

Claims 13-16, which depends from claim 1, disclose that said recombinant yeast microorganism is engineered to inactivate PDC or GPD genes, or said recombinant yeast microorganism is engineered to overexpress Aft proteins, or constitutively active Aft proteins; which do not further limit limitation of claim 1 which requires that "recombinant yeast microorganism is engineered to delete or attenuate the expression or activity of endogenous Grx3 or Grx4. Reciting “further comprises” or “further engineered” in claims 13-16 would overcome instant rejection.

Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements. The U.S. Court of Appeals for the Federal Circuit indicated that although the requirements of 35 U.S.C. 112, 4th paragraph, are related to matters of form, noncompliance with 35 U.S.C. 112, 4th paragraph, renders the claim unpatentable just as non-compliance with other paragraphs of 35 U.S.C. 112 would. See Pfizer, Inc. v. Ranbaxy Labs., Ltd., 457 F.3d 1284, 1291-92 (Fed. Cir. 2006) (holding a dependent claim in a patent invalid for failure to comply with 35 U.S.C. 112, 4th paragraph). Therefore, if a dependent claim does not comply with the requirements of 35 U.S.C. 112, 4th paragraph, the dependent claim should be rejected under 35 U.S.C. 112, 4th paragraph, as unpatentable rather than objecting to the claim. See also MPEP § 608.01(n), Section III, “Infringement Test” for dependent claims.

Appropriate clarification is required.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 1-19 are rejected under 35 U.S.C. § 112, first paragraph, **written description**, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Court of Appeals for the Federal Circuit has recently held that a "written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as be structure, formula [or] chemical name,' of the claimed subject matter sufficient to distinguish it from other materials." University of California v. Eli Lilly and Co., 1997 U.S. App. LEXIS 18221, at *23, quoting Fiers v. Revel, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993) (bracketed material in original). To fully describe a genus of genetic material, which is a chemical compound, applicants must (1) fully describe at least one species of the claimed genus sufficient to represent said genus whereby a skilled artisan, in view of the prior art, could predict the structure of other species encompassed by the claimed genus and (2) identify the common characteristics of the claimed molecules, e.g., structure, physical and/or chemical characteristics, functional characteristics when coupled with a known or disclosed

correlation between function and structure, or a combination of these (Enzo Biochem 63 USPQ2d 1609 (CAFC 2002)).

University of Rochester v. G.D. Searle & Co. (69 USPQ2d 1886 (2004)) specifically points to the applicability of both Lily and Enzo Biochemical to methods of using products, wherein said products lack adequate written description. While in University of Rochester v. G.D. Searle & Co. the methods were held to lack written description because not a single example of the product used in the claimed methods was described, the same analysis applies wherein the product, used in the claimed methods, must have adequate written description as noted from Enzo Biochemical (see above).

The instant specification teaches recombinantly overexpressing known Aft protein in yeast host cell by transforming with overexpression vector comprising Aft gene. Also, instant specification discloses "Enhancing DHAD Activity by increased GRX3/GRX4 Activity and/or Expression" which contradicting the instant claims which requires deletion or attenuation of endogenous Grx3 and/or Grx4 (see page 32 of instant specification). However, the breath of claims encompassed overly broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered to delete or attenuate the expression or activity of endogenous Grx3 or Grx4, wherein said engineering encompasses change or mutations in any Grx3-4 regulatory gene(s) or protein(s) related to expression or activity of said Grx3 or Grx4. The breath of claims 15-16 also encompassed overly broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered

to overexpress or express Aft proteins, wherein said engineering encompasses change or mutations in any regulatory gene(s) or protein(s) related to the expression of Aft proteins. Also, said engineering includes, but not limited to introducing any mutation (i.e., substitution, deletion, insertion and/or combination thereof) in said Grx3, or Grx4 protein themselves such that mutated Grx3 or Grx4 has attenuated expression or attenuated any activity. The prior art by Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS) teach that deletion of endogenous yeast Grx3 and/or Grx4 translocate Aft protein to nucleus of said yeast which is considered to be constitutively active transcriptional factor. To fully describe a genus of mutant recombinant nucleic acid molecules, applicants must (1) fully describe at least one species of the claimed genus sufficient to represent said genus whereby a skilled artisan, in view of the prior art, could predict the structure of other species encompassed by the claimed genus and (2) identify the common characteristics of the claimed molecules, e.g., structure, physical and/or chemical characteristics, functional characteristics when coupled with a known or disclosed correlation between function and structure, or a combination of these. However, the instant specification or the prior art do not describe any structure of regulatory gene(s) or protein(s) or any mutant thereof in correlation with the function of decreasing (e.g., attenuate) expression or activity of Grx3 or Grx4; or for the function of overexpressing Aft proteins or expressing constitutively active Aft proteins other than recombinantly overexpressing Aft encoding gene by transforming with an overexpression vector having Aft gene. Also, instant specification or the prior art do not describe any mutant Grx3, Grx4 structure

(comprising substitution, deletion, addition, and combination thereof, for example) correlated with function of being reduced any activity or expression of endogenous Grx3 and Grx4. The instant specification do not teach any engineering of any regulatory gene(s) or protein to express or overexpress Aft proteins other than using an overexpression vector recombinantly. Because claims encompasses overly broad yeast with very widely varying structure by engineering any Grx3-4 and/or Aft regulatory gene and/or proteins, for example; and also having unlimited mutations thereof with no functional relationship of attenuating expression or deletion of Grx3,4 or overexpressing Aft or constitutively active Aft, without sufficient structure of said regulatory agent with correlation to said functions of less expression or activity; one skilled in the art would not be in possession of full scope of the claimed genus recombinant yeast or a method thereof for producing isobutanol by the instant specification.

8. Claims 1-19 are rejected under 35 U.S.C. 112, first paragraph, **scope of enablement**, because the specification, while being enabling for a recombinant yeast with delete the expression of endogenous Grx3 and Grx4 by homologous recombination which inherently induce Aft activation as transcriptional activator by preventing export of Aft from nucleus; however, **does not** reasonably provide enablement for very broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered to delete or attenuate the expression or activity of endogenous Grx3 or Grx4, wherein said engineering encompasses change or mutations in any Grx3-4 regulatory gene(s) or protein(s) related to expression or activity

of said Grx3 or Grx4; overly broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered to overexpress or express Aft proteins in claims 15-16, wherein said engineering encompasses change or mutations in any regulatory gene(s) or protein(s) related to the expression of Aft proteins; or very broad engineered yeast cell wherein said engineering includes, but not limited to introducing any mutation (i.e., substitution, deletion, insertion and/or combination thereof) in said Grx3, or Grx4 protein themselves such that mutated Grx3 or Grx4 has attenuated expression or attenuated any activity.

The factors to be considered in determining whether undue experimentation is required are summarized In re Wands 858 F.2d 731, 8 USPQ2nd 1400 (Fed. Cir, 1988). The Court in Wands states: "Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue,' not 'experimentation.' " (Wands, 8 USPQ2d 1404). Clearly, enablement of a claimed invention cannot be predicated on the basis of quantity of experimentation required to make or use the invention. "Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations." (Wands, 8 USPQ2d 1404). The factors to be considered in determining whether undue experimentation is required include: (1) the quantity of experimentation necessary, (2) the amount or direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or

unpredictability of the art, and (8) the breadth of the claims. While all of these factors are considered, a sufficient amount for a prima facie case are discussed below.

The nature of the invention is drawn to a recombinant yeast with deleted endogenous Grx3-4 encoding gene by homologous recombination, and optionally overexpressing Aft protein in yeast host cell by transforming with overexpression vector comprising previously known Aft gene.

However, the breath of claims encompassed overly broad genus of the breath of claims encompassed overly broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered to delete or attenuate the expression or activity of endogenous Grx3 or Grx4, wherein said engineering encompasses change or mutations in any Grx3-4 regulatory gene(s) or protein(s) related to expression or activity of said Grx3 or Grx4. The breath of claims 15-16 also encompassed overly broad genus of recombinant yeast cell (or method of using said cell for isobutanol production) which comprises engineered to overexpress or express Aft proteins, wherein said engineering encompasses change or mutations in any regulatory gene(s) or protein(s) related to the expression of Aft proteins. Also, said engineering includes, but not limited to introducing any mutation (i.e., substitution, deletion, insertion and/or combination thereof) in said Grx3, or Grx4 protein themselves such that mutated Grx3 or Grx4 has attenuated expression or attenuated any activity. Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS) teach that deletion of endogenous yeast Grx3 and/or Grx4 translocate Aft protein to nucleus of said yeast which induced to be constitutively active transcriptional factor.

Although, recombinant DNA techniques for making mutant from known polypeptide is well known in the art, generating a function of interest (i.e., increasing Aft activity) is unpredictable. Even if the unmodified Aft protein is limited to a previously known Aft protein or any known regulator protein for Aft transcriptional activator is known; making any mutation (e.g., substitution, addition, deletion and/or any combination thereof) in said Aft protein is unpredictable while maintaining Aft protein's transcriptional factor activity. For example, **Guo** et al. (2004, Proc. Natl. Acad. Sci. USA 101: 9205-9210) teach that while proteins are fairly tolerant to mutations resulting in single amino acid changes, increasing the number of substitutions additively increases the probability that the protein will be inactivated (pg. 9209, right column, §2). Also, **Lazar** et al. (1988, Mol. Cell. Biol. 8:1247-1252) showed that the "conservative" substitution of glutamic acid for aspartic acid at position 47 reduced biological function of transforming growth factor alpha while "nonconservative" substitutions with alanine or asparagines had no effect (abstract). Similarly, **Hill** et al. (1998, Biochem. Biophys. Res. Comm. 244:573-577) teach that when three histidines that are maintained in ADP-glucose pyrophosphorylase across several species are substituted with the "nonconservative" amino acid glutamine, there is little effect on enzyme activity, while the substitution of one of those histidines with the "conservative" amino acid arginine drastically reduced enzyme activity (see Table 1). **Wacey** et al. (Hum Genet, 1999, Volume 104, pages 15-22) disclose the "mutation-induced functional/structural perturbation of a given protein" by substitution and teach that "the biophysical parameters in question are inextricable associated with the highly complex micro-environment of the mutated residue, it has not

proved possible to extrapolate insights obtained from the replacement of specific amino acid in a particular protein fold to similar substitutions in other proteins" (see middle of right column, page 15). Furthermore, any change in amino acid sequence(s) in any given Grx3-4 protein (or Grx3-4 regulator gene and/or protein thereof) while maintaining low activity (e.g., attenuated or decreased activity) is unpredictable by lack of sufficient guidance and direction in instant specification and prior art. Furthermore, instant specification teaches "Enhancing DHAD Activity by increased GRX3/GRX4 Activity and/or Expression" (see page 32 of instant specification) which contradicting the instant claims which requires deletion or attenuation of endogenous Grx3 and/or Grx4. Because the prior art and the instant specification do not teach sufficient direction and guidance how to make any engineering of any regulatory gene(s) and/or protein(s) of Grx3-4, or a regulator gene and/or protein which regulate or enhance the Aft protein expression; the unpredictability of making claimed recombinant yeast with said any genetic engineering and a method of producing isobutanol using said yeast is very high. For all of the above reason, it would require undue experimentation necessary to practice the full scope of claimed yeast cell and method thereof for producing isobutanol.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent

> granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

9. Claims 1-12 and 15-19 are rejected under 35 U.S.C. 102(e) as being anticipated by Anthony et al. (12/569,069 filed on 9/29/2009; published as USPAP 2010/0081179 on 4/1/2010; as cited in IDS filed on 11/18/2011) as evidenced by Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011) and Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS).

The 12/569,069 has been published as noted above. Thus, page numbers and lines will be recited according to the USPAP 2010/0081179.

Anthony et al. teach an "increased expression of DHAD activity is desired for enhanced microbial production of branched chain amino acids .... isobutanol" (see The first paragraph of Background of the Invention). Anthony et al. also teach a recombinant yeast host comprising heterologous Fe-S cluster protein (see claim 1) which includes "dihydroxy-acid dehydratase" (see claim 8) by an expression vector, for example, and method of using the yeast cell to produce isobutanol (see claim 15, for example); thus, meeting the limitation of overexpressing DHAD resulting in increasing the dehydratase activity of DHAD. Since yeast cell inherently expresses Grx3-4 (See Ihrig et al.) and the attenuation level is not limited to any specific condition in claim 1, the yeast cell of Anthony et al. above have lower expression or activity compared to the yeast cell transformed with Grx3 and/or Grx4 by an overexpression vector. An yeast cell inherently expresses endogenous Aft1 and/or Aft2 which acts as transcriptional

activator which shuttle between the cytosol and nucleus and once it is in the nucleus it becomes an active transcriptional activator (i.e., constitutively active) in yeast (see right column, lines 13-14, page 460) maintaining homeostasis of iron. As noted above, the increase in activity and expression is not limited to any condition, the recombinant yeast of Anthony et al. grown during production of isobutanol has increased expression (an overexpression, for example) and activity of Aft protein compared to the yeast cell in stationary, for example (at 0º C or lower). The increased expression of Aft protein also meets the limitation of "overexpressed" since the level of overexpression is not limited to any degree of overexpression. Thus, the yeast of Anthony et al. or method of producing isobutanol in culture medium meets all limitations of instant claim **1**. Anthony et al. also teach the recombinant yeast comprises and isobutanol biosynthetic pathway which refers to an enzyme pathway to produce isobutanol from pyruvate (see paragraph 0047) which includes all enzymes requires for the pathway shown in Figure 1 which comprises step of “pyruvate to acetolactate (FIG. 1 pathway step a) as catalyzed for example by acetolactate synthase; [0114] acetolactate to 2,3-dihydroxyisovalerate (FIG. 1 pathway step b) as catalyzed for example by acetohydroxy acid isomeroreductase; [0115] 2,3-dihydroxyisovalerate to α-ketoisovalerate (FIG. 1 pathway step c) as catalyzed for example by acetohydroxy acid dehydratase also called DHAD; [0116] α-ketoisovalerate to isobutyraldehyde (FIG. 1 pathway step d) as catalyzed for example by branched-chain α-keto acid decarboxylase; and [0117] isobutyraldehyde to isobutanol (FIG. 1 pathway step e) as catalyzed for example by branched-chain alcohol dehydrogenase.” (see paragraph 0112), wherein alcohol dehydrogenase inherently

uses NADH as cofactor; meeting the limitations of instant claims **2-5 and 10-12**. Anthony et al. teach that heterologous DHAD can be expressed in the cytosol (see paragraph 0013 or 0075). Anthony et al. also teach S. cerevisiae DHAD contains mitochondrial targeting signal, thus, when transformed into yeast host, it would reside in mitochondria, meeting the limitations of claims **6-7**. The yeast cell inherently having Aft1 translocate to nucleus becoming a constitutively active (since it is transcriptional activator) for maintaining homeostasis of iron (see bottom right column, page 4558 of Pujol-Carrion et al.); and in view of unclear level of overexpression as noted above, the recombinant yeast cell of Anthony et al. inherently expressing functional Aft protein meets the limitations of instant claims **15-16**. Anthony et al. also teach Saccharomyces cerevisiae as host cell can be used for isobutanol production using the disclosed biosynthetic pathways (see paragraph 0120 or Example 1); thus, meeting the limitations of claims **17-18**. In view of broad and reasonable interpretation of the recited term "derived" (for example, having no limitation of its source or any specific amino acid sequence of DHAD), the Kluyveromyces lactis DHAD used in Example 1 meets the limitations of claims **8-9**. Anthony et al. teach, in claim 15, for example, a method of producing isobutanol by growing the recombinant yeast cell inherently have a carbon source containing material in culture medium "under conditions wherein isobutanol is produced", meeting the limitation of instant claim **19**.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 1-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Anthony et al. (12/569,069 filed on 9/29/2009; published as USPAP 2010/0081179 on 4/1/2010; as cited in IDS filed on 11/18/2011) **in view of** Li et al. (US Patent Application Pub. No: US 2009/0163376 A1; filed on 12/18/2008) as evidenced by Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011) and Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS).

The teachings of Anthony et al. as evidenced by Ihrig et al. and Pujol-Carrion et al. is noted above.

Anthony et al. does not teach inactivation of endogenous PDC and/or GPD genes.

Li et al. teach glyceraldehye-3-phosphate dehydrogense reaction which is a reaction wasteful use of NADH produced which is required in synthesis of isobutanol from pyruvate (see paragraph 0082). Li et al. further teach a process of increasing production of isobutanol form pyruvate (see Figure 1) and teach "To prevent misdirection of pyruvate away from isobutanol production, a decarboxylase with decreased affinity for pyruvate is desired." (see paragraph 0135).

Therefore, it would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to modify the recombinant yeast cell producing

isobutanol by further deleting endogenous PDC and/or GPD genes with a reasonable expectation of success. The motivation to do so is provided by Li et al. who teach the usefulness of redirecting intermediate and cofactor for increasing an isobutanol production. Thus, the claimed invention as a whole was prima facie obvious over the combined teachings of the prior art.

11. Claims 1-12 and 15-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Liao et al. (US Patent Application Pub. No: US 2009/0081746 A1; published on Mar. 26, 2009) in view of Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS) as evidenced by John Imsande (Iron-sulfur cluster: Formation perturbation, and physiological functions., Plant Physiol. Biochem., 1999, Vol. 37, pages 87-97) and Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS filed on 11/18/2011); and KEGG Enzyme 4.2.1.9 (last viewed on 12/6/2011).

Liao et al. teaches a biosynthetic pathway of producing isobutanol from pyruvate (see Figure 4 and Figure 7) which includes all enzymes including, but not limited to, dihydroxy acid dehydratase (DHAD) which converts 2,3-dihydroxy-isovalerate to α-keto-isovalerate. According to the isobutanol pathway in Figure 4, Liao et al. teaches enzymes in bioproduction of isobutanol by "transforming a microorganism with one or more recombinant polynucleotides encoding polypeptide that includes, for example, acetohydroxy acid synthase (e.g., ilvIH operon), acetohydroxy acid isomeroreductase (e.g., ilvC), dihydroxy-acid dehydratase (e.g., **ilvD**), 2-keto-acid decarboxylase (e.g.,

PDC6, ARO10, THI3, kivd, or pdc), ...and alcohol dehydrogenase activity." (see paragraph 0154). Liao et al. also discloses "These strategies can also be readily implemented in yeast or other industrial microorganisms. In the case of isobutanol production, the complete pathway is CoA-independent and requires only pyruvate as a precursor." (see paragraph 0244). Since yeast cell inherently expresses Grx3-4 (See Ihrig et al.) and the attenuation level is not limited to any specific condition in claim 1, the yeast cell of Anthony et al. above have lower expression or activity compared to the yeast cell transformed with Grx3 and/or Grx4 by an overexpression vector. An yeast cell inherently expresses endogenous Aft1 and/or Aft2 which acts as transcriptional activator which shuttle between the cytosol and nucleus and once it is in the nucleus it becomes an active transcriptional activator (i.e., constitutively active) in yeast (see right column, lines 13-14, page 460) maintaining homeostasis of iron. As noted above, the increase in activity and expression is not limited to any condition, the recombinant yeast of Anthony et al. grown during production of isobutanol has increased expression (an overexpression, for example) and activity of Aft protein compared to the yeast cell in stationary, for example (at 0º C or lower). Thus, the recombinant yeast of Liao et al. for producing isobutanol meets the limitation of having less Grx3 and/or Grx4 in instant claim 1; and Aft proteins in instant claims 15-16. DHAD is well known enzyme (see KEGG Enzyme 4.2.1.9) requires Fe/S cofactor for its catalytic activity (see enzyme 4.2.1.9 in Table I, page 90 of John Imsande). Ihrig et al. teach that yeast contains two iron-dependent enzymes in branched-chain amino acid biosynthesis, and the activity of Ilv3 (mitochondrial dihydroxy-acid dehydratase) was virtually undetectable in wild-type

upon iron depletion (Fig. 3D) (see left column, lines 30-32). Liao et al. teach the suitable yeast host includes "Saccharomyces cerevisiae" (see paragraph 0083). Also, other species in claims 17 and 18 as yeast host for bioengineering is well known in the art.

Pujol-Carrion et al. teach deletion of endogenous grx3 and grx4 gene increase total iron accumulation in yeast compared to the wild type (see Figure 6, page 4558). Pujol-Carrion et al. also discloses Aft1 are constitutively induced (see bottom of left column, page 4555).

Therefore, it would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to modify the yeast cell by overexpressing DHAD and deleting activity of Grx3 and/or Grx4 with a reasonable expectation of success. The motivation to do so is provided by Pujol-Carrion et al. who teaches deletion of grx3 and/or grx4 increases iron accumulation which is required by DHAD in isobutanol production pathway. The step of claim 19 is obvious for culturing the recombinant yeast above for producing isobutanol. Thus, the claimed invention as a whole was prima facie obvious over the combined teachings of the prior art.

12. Claims 1-19 are rejected under 35 U.S.C. 103(a) as being unpatentable over Liao et al. (US Patent Application Pub. No: US 2009/0081746 A1; published on Mar. 26, 2009) in view of Pujol-Carrion et al. (J. Cell Science, 2006, Vol. 119, pages 4554-4564; as cited in IDS), and **in further view of** Li et al. (US Patent Application Pub. No: US 2009/0163376 A1; filed on 12/18/2008) and Anthony et al. (2010/0129886 which is

USPAP of 12/617,017 filed on 11/12/2009, with effective priority date of Nov. 13, 2008; as cited in IDS); as evidenced by KEGG Enzyme 4.2.1.9 (last viewed on 12/6/2011), John Imsande (Iron-sulfur cluster: Formation perturbation, and physiological functions., Plant Physiol. Biochem., 1999, Vol. 37, pages 87-97) and Ihrig et al. (Eukaryotic Cell, Dec. 11, 2009, Vol. 9, pages 460-471; as cited in IDS).

The teachings of Liao et al., Pujol-Carrion et al. are disclosed above.

Liao et al. and Pujol-Carrion et al. do not teach inactivation of PDC or GPD genes.

Li et al. teach glyceraldehye-3-phosphate dehydrogense reaction which is a reaction wasteful use of NADH produced which is required in synthesis of isobutanol from pyruvate (see paragraph 0082). Li et al. further teach a process of increasing production of isobutanol form pyruvate (see Figure 1) and teach "To prevent misdirection of pyruvate away from isobutanol production, a decarboxylase with decreased affinity for pyruvate is desired." (see paragraph 0135).

It is well known in the art to target proteins into mitochondria or cytosol using a mitochondrial targeting signal sequence (or without it) in yeast cell as shown by Anthony et al. having recombinant DHAD having mitochondrial targeting signal sequence. expressed in mitochondria or cytosol as noted above (see Figure 1 or paragraph 0026).

Therefore, it would have been obvious to one of ordinary skill in the art at the time the claimed invention was made to modify the recombinant yeast cell producing isobutanol by further deleting endogenous PDC and/or GPD genes with a reasonable expectation of success. The motivation to do so is provided by Li et al. who teach the

usefulness of redirecting intermediate and cofactor for increasing an isobutanol production. Thus, the claimed invention as a whole was prima facie obvious over the combined teachings of the prior art.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

13. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of copending Application No. 13/246,718. Although the conflicting claims are not identical, they are not patentably distinct from each other for reasons below.

The claims 1-20 of copending Application No. 13/246,718 are also drawn to a recombinant yeast comprising recombinantly overexpressed DHAD and deletion or

attenuation of the expression or activity of Grx3 and/or Grx4; and optionally having further overexpression of Aft proteins, or further limiting by having isobutanol producing metabolic pathway identical to exogenous enzymes which carries out reactions identical to instant claims 2 a) to e) by identical enzymes. Claim 20 of copending Application No. 13/246,718 is drawn to method of producing isobutanol using yeast of claim 2. Thus, instant claims 1-19 are obvious and/or anticipated by the claims 1-20 of copending Application No. 13/246,718.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

14. Claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,358 (from US application 13/228,342). Although the conflicting claims are not identical, they are not patentably distinct from each other because claims 1-19 are drawn to a recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a DHAD and expressing a mutant Aft protein which increases the dehydratase activity of DHAD, wherein Aft protein is constitutively active. Claim 20 is drawn to method of making isobutanol using said recombinant yeast in claim 2. The deletion of endogenous Grx3 and/or Grx4 is also included in view of claim 56 of priority document 61/263,952. Thus, instant claims 1-19 are anticipated and/or obvious over the claims of 1-20 of U.S. Patent No. 8,071,358.

15. Claims 1-19 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-20 of U.S. Patent No. 8,071,376 (from US application 12/953,884). Although the conflicting claims are not identical, they are not patentably distinct from each other because claims 1-19 are drawn to a recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a DHAD and recombinantly overexpressed polynucleotides encoding one or more Aft protein which increases the dehydratase activity of DHAD, wherein Aft protein also mutant constitutively active Aft protein (see claims 15-16). Claim 20 is drawn to method of making isobutanol using said recombinant yeast in claim 2. The deletion of endogenous Grx3 and/or Grx4 is also included in view of claim 56 of priority document 61/263,952. Thus, instant claims 1-19 are anticipated and/or obvious over the claims of 1-20 of U.S. Patent No. 8,071, 376.

Conclusion

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to ALEXANDER KIM whose telephone number is (571)272-5266. The examiner can normally be reached on 9AM-5:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on (571) 272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Alexander D Kim/
Primary Examiner, Art Unit 1656

Notice of References Cited	Application/Control No. 13/246,693	Applicant(s)/Patent Under Reexamination DUNDON ET AL.	
	Examiner ALEXANDER KIM	Art Unit 1656	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2009/0081746	03-2009	Liao et al.	435/160
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Guo et al., Protein tolerance to random amino acid change, 2004, Proc. Natl. Acad. Sci. USA 101: 9205-9210.
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	X	Wacey et al., Disentangling the perturbational effects of amino acid substitutions in the DNA-binding domain of p53., Hum Genet, 1999, Volume 104, pages 15-22.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 13/246,693	Applicant(s)/Patent Under Reexamination DUNDON ET AL.	
	Examiner ALEXANDER KIM	Art Unit 1656	Page 2 of 2

U.S. PATENT DOCUMENTS

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	A	US-			
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	C	US-			
	D	US-			
	E	US-			
	F	US-			
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	I	US-			
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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	John Imsande, Iron-sulfur cluster: Formation perturbation, and physiological functions., Plant Physiol. Biochem., 1999, Vol. 37, pages 87-97.
	V	KEGG Enzyme 4.2.1.9 (last viewed on 12-6-2011).
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of DUNDON *et al.*	Confirmation No.: 5847
Application No.: 13/246,693	Group Art Unit: 1656
Filed: September 27, 2011	Examiner: Kim, Alexander D.

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

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INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §1.97(b)

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant(s) hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98.

[x] No copies of any U.S. patents or U.S. patent application publications listed on the attached Form PTO/SB/08a are being provided pursuant to 37 C.F.R. §1.98.

[x] Copies of the remaining publications listed on the attached Form PTO/SB/08a are not being provided pursuant to 37 C.F.R. §1.98(d) because the publications were previously cited by or submitted to the Office in prior Application Serial Nos. 12/953,884 and/or 13/228,342 to which the above-identified application claims priority under 35 U.S.C. §120.

[x] The Examiner's attention is directed to related United States Patent Application Serial No. 12/953,884, filed November 24, 2010, and issued as US Patent No. 8,017,376 on September 13, 2011.

[x] The Examiner's attention is directed to related United States Patent Application Serial No. 13/228,342, filed September 8, 2011.

This Information Disclosure Statement is filed within any one of the following time periods:

[ ] within three months from the filing date of this national application other than a CPA under 37 C.F.R. § 1.53(d);

[ ] within three months from the date of entry of the national stage as set forth in 37 C.F.R. §1.491 in this international application;

[x] before the mailing date of a first office action on the merits; or

[ ] before the mailing of a first office action after the filing of a request for continued examination under 37 C.F.R. § 1.114.

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Forms PTO/SB/08a to the undersigned. The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 50-1283.

Dated: November 18, 2011

Respectfully submitted,
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Approved for use through 07/31/2012. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	13246693
Filing Date	2011-09-27
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Kim, Alexander D.
Attorney Docket Number	GEVO-041/13US 310142-2263

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	7851188		2010-12-14	Donaldson et al.	
	2	8017376		2011-09-13	Dundon et al.	

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	1	20110020889		2011-01-27	Feldman et al.	
	2	20090305363		2009-12-20	Anthony et al.	
	3	20090215137		2009-08-27	Hawkins et al.	
	4	20090226990		2009-09-10	Hawkins et al.	

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	1	WO2009076480	WO		2009-06-17	Synthetic Genomics, Inc.		☐
	2	WO2010075504	WO		2010-07-01	Gevo, Inc.		☐
	3	WO2011019894	WO		2011-02-17	Gevo, Inc.		☐

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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	45	PCT/US10/57957, Applicant: Gevo, Inc. Int'l Search Report-Written Opinion (Apr. 8, 2011)	☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	13246693
	Filing Date	2011-09-27
	First Named Inventor	Catherine Asleson Dundon
	Art Unit	1656
	Examiner Name	Kim, Alexander D.
	Attorney Docket Number	GEVO-041/13US 310142-2263

If you wish to add additional non-patent literature document citation information please click the Add button Add			
EXAMINER SIGNATURE			
Examiner Signature		Date Considered	
*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.			

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	13246693
Filing Date	2011-09-27
First Named Inventor	Catherine Asleson Dundon
Art Unit	1656
Examiner Name	Kim, Alexander D.
Attorney Docket Number	GEVO-041/13US 310142-2263

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Paul A. Wickman/	Date (YYYY-MM-DD)	2011-11-18
Name/Print	Paul A. Wickman	Registration Number	61242

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/246,693	09/27/2011	Catherine Asleson Dundon	GEVO-041/13US 310142-2263	5847

58249 7590 11/04/2011

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

EXAMINER	
KIM, ALEXANDER D	

ART UNIT	PAPER NUMBER
1656	

MAIL DATE	DELIVERY MODE
11/04/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES PATENT AND TRADEMARK OFFICE

NOV 0 4 2011

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON DC 20001

In re Application of :

DUNDON, Catherine *et al.*	:	DECISION ON PETITION
Application No. 13/246693	:	TO MAKE SPECIAL UNDER
Filed: September 27, 2011	:	THE GREEN TECHNOLOGY
Attorney Docket No. VO-041/13US 310142-2263	:	PILOT PROGRAM

This is a decision on the petition under 37 CFR 1.102, filed September 27, 2011, to make the above-identified application special under the pilot program for applications pertaining to Green Technologies as set forth in 74 Federal Register Notice 64666 (December 8, 2009) and amended by 75 Federal Register Notice 28554 (May 21, 2010) and 75 Federal Register Notice 69049 (November 10, 2010).

The petition is **GRANTED**.

A grantable petition to make an application special under 37 CFR 1.102 and the pilot program as set forth in 74 FR 64666 must be directed to a nonprovisional application filed under 35 USC 111(a) or be a national stage entry under 35 USC 371, exclusive of any reissue applications.

In order to qualify for special status, the following requirements must be met. 1) The application must have no more than 3 independent claims and no more than 20 total claims. 2) The application must not contain any multiple dependent claims. 3) The petition must state the basis for seeking special status, i.e., the claimed invention either: A) materially enhances the quality of the environment or B) materially contributes to: i) the discovery or development of renewable energy resources, ii) the more efficient utilization and conservation of energy resources, or iii) greenhouse gas emission reduction. 4) If the disclosure is not clear on its face that the claimed invention materially contributes under category (A) or (B), the petition must be accompanied by a statement by the applicant, assignee, or an attorney/agent registered to practice before the Office explaining how the materiality standard is met. 5) A statement that applicant will agree to make an election without traverse in a telephonic interview if a restriction requirement is made by the examiner. 6) The petition to make special must be filed electronically. 7) The petition must be filed at least one day prior to the date that a first Office Action appears in the Patent Application Information Retrieval (PAIR) system. 8) The petition must be accompanied by a request for early publication in compliance with 37 CFR 1.219 and include the publication fee as set forth in 37 CFR 1.18(d).

The requirement for a fee for consideration of the petition to make special for applications pertaining to Green Technologies has been waived.

The instant petition complies with items 1 – 8 above. Accordingly, the above-identified application has been accorded "special" status.

Telephone inquires concerning this decision should be directed to Manjunath Rao at 571-272-0939.

The application is being forwarded to the Technology Center Art Unit 1656 for action on the merits commensurate with this decision.

/Manjunath Rao/

Manjunath Rao
Supervisory Patent Examiner &
POC for TC 1600 Green Tech Petitions
Technology Center 1600

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/246,693	09/27/2011	1652	830	GEVO-041/13US 310142-000	19	1

CONFIRMATION NO. 5847

58249
COOLEY LLP
ATTN: Patent Group
Suite 1100
777 - 6th Street, NW
WASHINGTON, DC 20001

FILING RECEIPT

OC00000050517186

Date Mailed: 10/24/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Catherine Asleson Dundon, Englewood, CO;
Aristos Aristidou, Highlands Ranch, CO;
Andrew Hawkins, Parker, CO;
Doug Lies, Parker, CO;
Lynne H. Albert, Golden, CO;

Assignment For Published Patent Application

GEVO, INC., Englewood, CO

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a DIV of 13/228,342 09/08/2011
and is a DIV of 12/953,884 11/24/2010 PAT 8,017,376
which claims benefit of 61/263,952 11/24/2009
and claims benefit of 61/350,209 06/01/2010

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 10/17/2011

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/246,693**

Projected Publication Date: 02/02/2012

Non-Publication Request: No

Early Publication Request: Yes

**** SMALL ENTITY ****

Title

METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PTO/SB/05 (08-08)
Approved for use through 09/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office. U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	GEVO-041/13US 310142-000
First Inventor	DUNDON, Catherine Asleson
Title	METHODS OF INCREASING...
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☑ **Fee Transmittal Form** (e.g., PTO/SB/17)
2. ☑ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☑ **Specification** [*Total Pages* 123]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☑ **Drawing(s)** (*35 U.S.C. 113*) [*Total Sheets* 7]
5. **Oath or Declaration** [*Total Sheets* 14]
   a. ☐ Newly executed (original or copy)
   b. ☑ A copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 18 completed)
   i. ☑ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) name in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☑ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a. – c. are required)
   a. ☑ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503)
    (*Should be specifically itemized)*
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☑ Other: Petition to Make Special Under Green Technology Pilot Program

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☐ Continuation ☑ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 13/228,342

Prior application information: *Examiner* Not Yet Assigned *Art Unit:* 1656

19. CORRESPONDENCE ADDRESS

☑ The address associated with Customer Number: 58249 ***OR*** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Paul A. Wickman/		Date	September 27, 2011	
Name (Print/Type)	Paul A. Wickman			Registration No. (Attorney/Agent)	61,242

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re App'n of: Catherine Asleson DUNDON *et al.* Confirmation No.: *To Be Assigned*

Serial No.: *To Be Assigned* (Div. of 13/228,342) Group Art Unit: *To Be Assigned*

Filed: September 27, 2011 Examiner: *To Be Assigned*

For: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

VIA EFS-WEB

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO MAKE SPECIAL

STATEMENT OF SPECIAL STATUS AND REQUEST TO PARTICIPATE IN THE GREEN TECHNOLOGY PILOT PROGRAM

Dear Sir,

Submitted herewith is the completed USPTO form PTO/SB/420 and a Petition to Make Special Pursuant to the Federal Register Notice of December 8, 2009, Vol. 74, No. 234, pp. 64666-8, to participate in the Green Technology Pilot Program as described in said Notice.

The above-reference application has been not yet been published. In accordance with the requirements for the Pilot Program, please charge Deposit Account No. 50-1283 for the publication fee set forth in 37 CFR 1.18(d).

Applicants submit that the application is directed to a single invention that relates to the development of renewable components for fuel blends, and as such, the claimed invention materially contributes to the discovery or development of renewable energy resources, enhances the quality of the environment and results in a more efficient utilization and conservation of energy resources.

If participation in the Green Technology Pilot Program is granted, Applicants hereby agree to make an election without traverse in a telephonic interview and elect an invention that meets the eligibility requirement if the Office determines that the claims are not directed to a single invention.

Filed concurrently with this petition is a divisional application which claims priority to US Patent Application No. 13/228,342 under 35 U.S.C. §120. The divisional application submitted herewith contains no more than three independent claims and twenty total claims and does not contain any multiple dependent claims.

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283.

Dated: September 27, 2011

Respectfully submitted,
COOLEY LLP

CUSTOMER NO. 58249

By: /Paul A. Wickman/
Paul A. Wickman
Reg. No. 61,242

COOLEY LLP
ATTN: Patent Group
777 6th Street NW, Suite 1100
Washington, DC 20001
Tel: (720) 566-4250
Fax: (202) 842-7899

Doc Code: PET.GREEN
Document Description: Petition for Green Tech Pilot

PTO/SB/420 (11-10)
Approved for use through 01/31/2011. OMB 0651-0062
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE

PETITION TO MAKE SPECIAL UNDER THE GREEN TECHNOLOGY PILOT PROGRAM

Attorney Docket Number: GEVO-041/13US	Application Number (if known): Not Yet Assigned	Filing date: September 27, 2011

First Named Inventor: Catherine Asleson DUNDON

Title: METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND...

APPLICANT HEREBY REQUESTS TO PARTICIPATE IN THE GREEN TECHNOLOGY PILOT PROGRAM FOR THE ABOVE-IDENTIFIED APPLICATION. See Instruction Sheet on page 2.

This petition must be timely filed electronically using the USPTO electronic filing system, EFS-Web.

1. By filing this petition:

   Applicant is requesting early publication: Applicant hereby requests early publication under 37 CFR 1.219 and the publication fee set forth in 37 CFR 1.18(d) accompanies this request.

2. By filing this petition: applicant is agreeing to make an election without traverse in a telephonic interview and elect an invention that meets the eligibility requirements for the Green Technology Pilot Program, if the Office determines that the claims are not obviously directed to a single invention. See Instruction Sheet.

3. This request is accompanied by statements of special status for the eligibility requirement.

4. The application contains no more than three (3) independent claims and twenty (20) total claims.

5. The application does not contain any multiple dependent claims.

6. Other attachments: Statement of Special Status

Signature /Paul A. Wickman/	Date September 27, 2011
Name (Print/Typed) Paul A. Wickman	Registration Number 61,242

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 11.18. Please see 37 CFR 1.4(d) for the form of the signature. If necessary, submit multiple forms for more than one signature see below*.*

☒ *Total of 1 forms are submitted.

PTO/SB/420 (11-10)
Approved for use through 01/31/2011. OMB 0651-0062
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE

Instruction Sheet for Petition to Make Special Under the Green Technology Pilot Program

(Not to be Submitted to the USPTO)

The following is a summary of the requirements (for more information see the notices (i) "Pilot Program for Green Technologies Including Greenhouse Gas Reduction," (ii) "Elimination of Classification Requirement in the Green Technology Pilot Program," and (iii) "Expansion and Extension of the Green Technology Pilot Program," available on the USPTO web site at http://www.uspto.gov/patents/init_events/green_tech.jsp):

1) The application must be a non-reissue, non-provisional utility application filed under 35 U.S.C. 111 (a), or an international application that has entered the national stage in compliance with 35 U.S.C. 371, irrespective of the filing date of the application. Reexamination proceedings are excluded from this pilot program.

2) The application must contain three or fewer independent claims and twenty or fewer total claims. The application must not contain any multiple dependent claims. For an application that contains more than three independent claims or twenty total claims, or multiple dependent claims, applicant must file a preliminary amendment in compliance with 37 CFR 1.121 to cancel the excess claims and/or the multiple dependent claims at the time the petition to make special is filed.

3) The claims must be directed to a single invention that materially enhances the quality of the environment, or that materially contributes to: (1) the discovery or development of renewable energy resources; (2) the more efficient utilization and conservation of energy resources; or (3) green house gas emission reduction (see the eligibility requirements of sections II and III of the notice (i) cited above). The petition must include a statement that, if the USPTO determines that the claims are directed to multiple inventions (e.g., in a restriction requirement), applicant will agree to make an election without traverse in a telephonic interview, and elect an invention that meets the eligibility requirements in section II or III of the notice (i) cited above.

4) The petition to make special must be timely filed electronically using the USPTO electronic filing system, EFS-Web, and selecting the document description of "Petition for Green Tech Pilot" on the EFS-Web screen. Applicant should use form PTO/SB/420, which is available as a Portable Document Format (PDF) fillable form in EFS-Web and on the USPTO Web site.

5) The petition to make special must be filed at least one day prior to the date that a first Office action (which may be an Office action containing only a restriction requirement) appears in the Patent Application Information Retrieval (PAIR) system. Applicant may check the status of the application using PAIR.

6) The petition to make special must be accompanied by a request for early publication in compliance with 37 CFR 1.219 and the publication fee set forth in 37 CFR 1.18(d).

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

DECLARATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

the specification of which:

(check one)

☐ is attached hereto;

☒ was filed as United States Application Serial No. 12/953,884 on November 24, 2010, and was amended on _____ (if applicable);

☐ was filed as PCT International Application No. _____on _____ and was amended under PCT Article 19 or Article 34 on_____ (if applicable);

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information which is known to me to be material to the patentability of said invention in accordance with 37 C.F.R. §1.56;

I hereby claim foreign priority benefits under 35 U.S.C. §119 and/or §365 of any foreign application(s) for patent, any foreign application(s) for inventor's certificate, or any PCT international application(s) designating at least one country other than the United States of America listed below; I have also identified below any foreign application(s) for patent, any foreign application(s) for inventor's certificate, or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Prior Foreign Application(s)

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			☐ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

61/263,952 (Application Number) November 24, 2009 (Filing Date) (day, month, year)

61/350,209 (Application Number) June 1, 2010 (Filing Date) (day, month, year)

I hereby claim the benefit under 35 U.S.C. §120 and/or §365 of any United States application(s) or of any international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Application(s) or PCT International Applications Designating the U.S. for benefit under 35 U.S.C. §120

U.S. APPLICATIONS			**STATUS (*check one*)**		
U.S. APPLICATION NO.	U.S. FILING DATE (day, month, year)		Pending	Patented	Abandoned
			☐	☐	☐
			☐	☐	☐
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE (day, month, year)	U.S. APPLICATION NOS. (if any)			
			☐	☐	☐
			☐	☐	☐

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature _____ Date 2/16/2011

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Full name of eleventh inventor: SMITH, Christopher

Inventor's signature Chris Smith Date 3/1/2011
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Full name of twelfth inventor: **ALBERT, Lynne H.**

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Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS
First Named Inventor/Applicant Name:	Catherine Asleson Dundon
Filer:	Paul A. Wickman/Sherry Bitler
Attorney Docket Number:	GEVO-041/13US 310142-000

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility filing Fee (Electronic filing)	4011	1	95	95
Utility Search Fee	2111	1	310	310
Utility Examination Fee	2311	1	125	125
Pages:				
Claims:				
Miscellaneous-Filing:				
Publ. Fee- early, voluntary, or normal	1504	1	300	300
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**830**

Electronic Acknowledgement Receipt

EFS ID:	11062877
Application Number:	13246693
International Application Number:	
Confirmation Number:	5847
Title of Invention:	METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS
First Named Inventor/Applicant Name:	Catherine Asleson Dundon
Customer Number:	58249
Filer:	Paul A. Wickman/Sherry Bitler
Filer Authorized By:	Paul A. Wickman
Attorney Docket Number:	GEVO-041/13US 310142-000
Receipt Date:	27-SEP-2011
Filing Date:	
Time Stamp:	19:36:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$830
RAM confirmation Number	6618
Deposit Account	501283
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		GEVO04113US-NonProvisional_Application.pdf	1064414 93582761a68dbb59946191cd5de7d8216444bb2c	yes	130

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Specification	1	119
Claims	120	122
Abstract	123	123
Drawings-only black and white line drawings	124	130

Warnings:

Information:

2	Application Data Sheet	GEVO04113US-ads.pdf	139549 1540e984695221ce1d4da6923d798c45420b86d1	no	4

Warnings:

Information:

This is not an USPTO supplied ADS fillable form

3	Miscellaneous Incoming Letter	GEVO04113US-Inventor_Deletion.pdf	84130 4de921f39be28c5e05deb437481cc3ce98fe2a10	no	2

Warnings:

Information:

4	Transmittal of New Application	GEVO04113US-UtilityTransmittal.pdf	276115 907880912c84a2cae62b2753be808fccfba9f5aa	no	2

Warnings:

Information:

5	Green Tech Petition under 37 CFR 1.102	GEVO04113US-Green_Tech_Pilot_Program_Petition.pdf	91273 a07fd6116c6ff2f5f506251fe928d41465751c2a	no	2

Warnings:

Information:

6	Green Tech Petition under 37 CFR 1.102	GEVO04113US-SB0420_Petition.pdf	941150 559880eeba2fc24db8c5f60c46fbea6fcd7b6cb1	no	3

Warnings:

Information:					
7	Oath or Declaration filed	GEVO04113US-declaration.pdf	312137 a90211a9b20d569cdb58dc9e16409960bf47ca04	no	14
Warnings:					
Information:					
8	Sequence Listing (Text File)	GEVO04113US-Seqlist_ST25.txt	672813	no	0
Warnings:					
Information:					
9	Fee Worksheet (SB06)	fee-info.pdf	37004 314a7d7bcc803a641084ea6873a7023d0653df5b	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	3618585	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional of U.S. Application Serial No. 13/228,342, filed September 8, 2011, which is a divisional application of U.S. Application Serial No. 12/953,884, filed November 24, 2010, now U.S. Patent No. 8,017,376, which claims the benefit of U.S. Provisional Application Serial No. 61/263,952, filed November 24, 2009, and U.S. Provisional Application Serial No. 61/350,209, filed June 1, 2010, each of which are herein incorporated by reference in their entireties for all purposes.

ACKNOWLEDGMENT OF GOVERNMENTAL SUPPORT

[0002] This invention was made with government support under Contract No. IIP-0823122, awarded by the National Science Foundation, and under Contract No. EP-D-09-023, awarded by the Environmental Protection Agency. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] Recombinant microorganisms and methods of producing such organisms are provided. Also provided are methods of producing beneficial metabolites including fuels, chemicals, and amino acids by contacting a suitable substrate with recombinant microorganisms and enzymatic preparations therefrom.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[0004] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: GEVO_041_13US_SeqList_ST25.txt, date recorded: September 27, 2011, file size: 658 kilobytes).

BACKGROUND

[0005] Dihydroxyacid dehydratase (DHAD) is an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate and of 2,3-dihydroxy-3-

methylvalerate to 2-keto-3-methylvalerate. This enzyme plays an important role in a variety of biosynthetic pathways, including pathways producing valine, isoleucine, leucine and pantothenic acid (vitamin B5). DHAD also catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate as part of isobutanol biosynthetic pathways disclosed in commonly owned and co-pending US Patent Publication Nos. 2009/0226991 and 2010/0143997. In addition, biosynthetic pathways for the production of 3-methyl-1-butanol and 2-methyl-1-butanol use DHAD to convert 2,3-dihydroxyisovalerate to α-ketoisovalerate and 2,3-dihydroxy-3-methylvalerate to 2-keto-3-methylvalerate, respectively (Atsumi *et al.*, 2008, Nature 451(7174): 86-9).

[0006] DHAD is an essential enzyme in all of these biosynthetic pathways, hence, it is desirable that recombinant microorganisms engineered to produce the above-mentioned compounds exhibit optimal DHAD activity. The optimal level of DHAD activity will typically have to be at levels that are significantly higher than those found in non-engineered microorganisms in order to sustain commercially viable productivities, yields, and titers. The present application addresses this need by engineering recombinant microorganisms to improve their DHAD activity.

SUMMARY OF THE INVENTION

[0007] The present inventors have discovered that overexpression of the transcriptional activator genes *AFT1* and/or *AFT2* or homologs thereof in a recombinant yeast microorganism improves DHAD activity. Thus, the invention relates to recombinant yeast cells engineered to provide increased heterologous or native expression of *AFT1* and/or *AFT2* or homologs thereof. In general, cells that overexpress *AFT1* and/or *AFT2* or homologs thereof exhibit an enhanced ability to produce beneficial metabolites such as isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid.

[0008] One aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism is engineered to overexpress one or more polynucleotides encoding one or more Aft proteins or homologs thereof. In one embodiment, the Aft protein is selected from SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 209, SEQ ID NO: 211,

SEQ ID NO: 213, SEQ ID NO: 215, SEQ ID NO: 217, SEQ ID NO: 219, SEQ ID NO: 221, SEQ ID NO: 223, and SEQ ID NO: 225. In another embodiment, one or more of the polynucleotides encoding said one or more Aft proteins or homologs thereof is a native polynucleotide. In yet another embodiment, one or more of the polynucleotides encoding said one or more Aft proteins or homologs thereof is a heterologous polynucleotide.

[0009] In a specific embodiment according to this aspect, the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism has been engineered to overexpress a polynucleotide encoding Aft1 (SEQ ID NO: 2) and/or Aft2 (SEQ ID NO: 4) or a homolog thereof. In one embodiment, the polynucleotide encoding the Aft protein or homolog thereof is native to the recombinant microorganism. In another embodiment, the polynucleotide encoding the Aft protein or homolog thereof is heterologous to the recombinant microorganism.

[0010] Another aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein the activity of one or more Aft proteins or homologs thereof is increased. In one embodiment, the Aft protein is selected from SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 209, SEQ ID NO: 211, SEQ ID NO: 213, SEQ ID NO: 215, SEQ ID NO: 217, SEQ ID NO: 219, SEQ ID NO: 221, SEQ ID NO: 223, and SEQ ID NO: 225. In one embodiment, the polynucleotide encoding the Aft protein or homolog thereof is native to the recombinant microorganism. In another embodiment, the polynucleotide encoding the Aft protein or homolog thereof is heterologous to the recombinant microorganism.

[0011] Another aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism has been engineered to overexpress one or more polynucleotides encoding one or more proteins or homologs thereof regulated by an Aft protein or homolog thereof. In one embodiment, the proteins regulated by an Aft protein or homolog thereof are selected from *FET3, FET4, FET5, FTR1, FTH1, SMF3, MRS4, CCC2, COT1, ATX1, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FIT1, FIT2, FIT3, ARN1, ARN2, ARN3, ARN4, ISU1, ISU2, TIS11, HMX1, AKR1, PCL5, YOR387C,*

YHL035C, YMR034C, ICY2, PRY1, YDL124W, BNA2, ECM4, LAP4, YOL083W, YGR146C, BIO5, YDR271C, OYE3, CTH1, CTH2, MRS3, MRS4, HSP26, YAP2, VMR1, ECL1, OSW1, NFT1, ARA2, TAF1/TAF130/TAF145, YOR225W, YKR104W, YBR012C, and *YMR041C* or homologs thereof. In a specific embodiment, the protein regulated by an Aft protein or homolog thereof is ENB1. In another specific embodiment, the protein regulated by an Aft protein or homologs thereof is FET3. In yet another specific embodiment, the protein regulated by an Aft protein or homolog thereof is SMF3. In one embodiment, all genes demonstrated to increase DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway are overexpressed. Where none of the *AFT* regulon genes expressed alone are effective in increasing DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway, then 1, 2, 3, 4, 5, or more of the genes in the *AFT* regulon may be overexpressed together.

[0012] In various embodiments described herein, the DHAD-requiring biosynthetic pathway may be selected from isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and/or pantothenic acid biosynthetic pathways. In various embodiments described herein, the DHAD enzyme which acts as part of an isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and/or pantothenic acid biosynthetic pathway may be localized to the cytosol. In alternative embodiments, the DHAD enzyme which acts as part of an isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and/or pantothenic acid biosynthetic pathway may be localized to the mitochondria. In additional embodiments, a DHAD enzyme which acts as part of an isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and/or pantothenic acid biosynthetic pathway is localized to the cytosol and the mitochondria.

[0013] In one embodiment, the invention is directed to a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway and wherein said microorganism is engineered to overexpress one or more polynucleotides encoding one or more Aft proteins or homologs thereof. In one embodiment, the Aft protein is selected from SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 209, SEQ ID

NO: 211, SEQ ID NO: 213, SEQ ID NO: 215, SEQ ID NO: 217, SEQ ID NO: 219, SEQ ID NO: 221, SEQ ID NO: 223, and SEQ ID NO: 225.

[0014] In a specific embodiment, the invention is directed to a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway and wherein said microorganism is engineered to overexpress a polynucleotide encoding Aft1 (SEQ ID NO: 2) or a homolog thereof. In another specific embodiment, the invention is directed to a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway and wherein said microorganism is engineered to overexpress a polynucleotide encoding Aft2 (SEQ ID NO: 4) or a homolog thereof. In yet another embodiment, the invention is directed to a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway and wherein said microorganism is engineered to overexpress a polynucleotide encoding Aft1 (SEQ ID NO: 2) or a homolog thereof and Aft2 (SEQ ID NO: 4) or a homolog thereof.

[0015] In each of the aforementioned aspects and embodiments, the Aft protein may be a constitutively active Aft protein or a homolog thereof. In one embodiment, the constitutively active Aft protein or homolog thereof comprises a mutation at a position corresponding to the cysteine 291 residue of the native *S. cerevisiae* Aft1 (SEQ ID NO: 2). In a specific embodiment, the cysteine 291 residue is replaced with a phenylalanine residue. In another embodiment, the constitutively active Aft protein or homolog thereof comprises a mutation at a position corresponding to the cysteine 187 residue of the native *S. cerevisiae* Aft2 (SEQ ID NO: 2). In a specific embodiment, the cysteine 187 residue is replaced with a phenylalanine residue.

[0016] In another embodiment, the invention is directed to a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, wherein said microorganism has been engineered to overexpress one or more polynucleotides encoding one or more proteins or homologs thereof regulated by an Aft protein or homolog thereof. In one embodiment, the proteins regulated by Aft or a homolog thereof are selected from *FET3, FET4, FET5, FTR1, FTH1, SMF3, MRS4, CCC2, COT1, ATX1, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FIT1, FIT2, FIT3, ARN1, ARN2, ARN3, ARN4, ISU1, ISU2, TIS11, HMX1, AKR1, PCL5, YOR387C, YHL035C, YMR034C, ICY2, PRY1, YDL124W, BNA2, ECM4, LAP4, YOL083W, YGR146C, BIO5, YDR271C,*

OYE3, CTH1, CTH2, MRS3, MRS4, HSP26, YAP2, VMR1, ECL1, OSW1, NFT1, ARA2, TAF1/TAF130/TAF145, YOR225W, YKR104W, YBR012C, and *YMR041C* or homologs thereof. In a specific embodiment, the protein regulated by an Aft protein or homolog thereof is ENB1. In another specific embodiment, the protein regulated by an Aft protein or homologs thereof is FET3. In yet another specific embodiment, the protein regulated by an Aft protein or homolog thereof is SMF3. In one embodiment, all genes demonstrated to increase DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway are overexpressed. Where none of the *AFT* regulon genes expressed alone are effective in increasing DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway, then 1, 2, 3, 4, 5, or more of the genes in the *AFT* regulon may be overexpressed together.

[0017] In one embodiment, the isobutanol producing metabolic pathway comprises at least one exogenous gene that catalyzes a step in the conversion of pyruvate to isobutanol. In another embodiment, the isobutanol producing metabolic pathway comprises at least two exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least three exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least four exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at five exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol.

[0018] In one embodiment, one or more of the isobutanol pathway genes encodes an enzyme that is localized to the cytosol. In one embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least one isobutanol pathway enzyme localized in the cytosol. In another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least two isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least three isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least four isobutanol pathway enzymes localized in the cytosol. In an exemplary embodiment, the recombinant microorganisms comprise an isobutanol producing

metabolic pathway with five isobutanol pathway enzymes localized in the cytosol. In a further exemplary embodiment, at least one of the pathway enzymes localized to the cytosol is a cytosolically active DHAD enzyme as disclosed herein.

[0019] In various embodiments described herein, the isobutanol pathway genes encodes enzyme(s) selected from the group consisting of acetolactate synthase (ALS), ketol-acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), 2-keto-acid decarboxylase (KIVD), and alcohol dehydrogenase (ADH).

[0020] Another aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism has been engineered to overexpress a polynucleotide encoding Grx3 and/or Grx4 or a homolog thereof. In one embodiment, the polynucleotide encoding the Grx protein or homolog thereof is native to the recombinant microorganism. In another embodiment, the polynucleotide encoding the Grx protein or homolog thereof is heterologous to the recombinant microorganism.

[0021] In various embodiments described herein, the recombinant microorganism may be engineered reduce the concentration of reactive oxygen species (ROS) in the recombinant microorganism. Thus, the recombinant microorganisms may be engineered to express one or more proteins that reduce the concentration of reactive oxygen species (ROS) in said cell. The proteins to be expressed for reducing the concentration of reactive oxygen species may be selected from catalases, superoxide dismutases, metallothioneins, and methionine sulphoxide reductases. In a specific embodiment, said catalase may be encoded by one of more of the genes selected from the group consisting of the *E. coli* genes *katG* and *katE*, the *S. cerevisiae* genes *CTT1* and *CTA1*, or homologs thereof. In another specific embodiment, said superoxide dismutase is encoded by one of more of the genes selected from the group consisting of the *E. coli* genes *sodA*, *sodB*, *sodC*, the *S. cerevisiae* genes *SOD1* and *SOD2*, or homologs thereof. In another specific embodiment, said metallothionein is encoded by one of more of the genes selected from the group consisting of the *S. cerevisiae CUP1-1* and *CUP1-2* genes or homologs thereof. In another specific embodiment, said metallothionein is encoded by one or more genes selected from the group consisting of the *Mycobacterium tuberculosis MymT* gene and the *Synechococcus* PCC 7942 *SmtA* gene or homologs thereof. In another specific embodiment, said methionine sulphoxide reductase is encoded by one or more genes selected from the group consisting of the *S. cerevisiae* genes *MXR1* and *MXR2*, or homologs thereof.

[0022] In some embodiments, the recombinant microorganism may be engineered to increase the level of available glutathione in the recombinant microorganism. Thus, the recombinant microorganisms may be engineered to express one or more proteins that increase the level of available glutathione in the cell. In one embodiment, the proteins are selected from glutaredoxin, glutathione reductase, glutathione synthase, and combinations thereof. In a specific embodiment, said glutaredoxin is encoded by one of more of the genes selected from the group the *S. cerevisiae* genes *GRX2, GRX4, GRX6,* and *GRX7*, or homologs thereof. In another specific embodiment, said glutathione reductase is encoded by the *S. cerevisiae* genes *GLR1* or homologs thereof. In another specific embodiment, said glutathione synthase is encoded by one of more of the genes selected from the *S. cerevisiae* genes *GSH1* and *GSH2*, or homologs thereof. In some embodiments, two enzymes are expressed to increase the level of available glutathione in the cell. In one embodiment, the enzymes are γ-glutamyl cysteine synthase and glutathione synthase. In a specific embodiment, said glutathione synthase is encoded by one of more of the genes selected from the group the *S. cerevisiae* genes *GSH1* and *GSH2*, or homologs thereof.

[0023] In some embodiments, it may be desirable to overexpress one or more functional components of the thioredoxin system, as overexpression of the functional components of the thioredoxin system can increase the amount of bioavailable thioredoxin. In one embodiment, the functional components of the thioredoxin system may be selected from a thioredoxin and a thioredoxin reductase. In a specific embodiment, said thioredoxin is encoded by the *S. cerevisiae TRX1* and *TRX2* genes or homologs thereof. In another specific embodiment, said thioredoxin reductase is encoded by S. cerevisiae *TRR1* gene or homologs thereof. In additional embodiments, the recombinant microorganism may further be engineered to overexpress the mitochondrial thioredoxin system. In one embodiment, the mitochondrial thioredoxin system is comprised of the mitochondrial thioredoxin and mitochondrial thioredoxin reductase. In a specific embodiment, said mitochondrial thioredoxin is encoded by the *S. cerevisiae TRX3* gene or homologs thereof. In another specific embodiment, said mitochondrial thioredoxin reductase is encoded by the *S. cerevisiae TRR2* gene or homologs thereof.

[0024] In various embodiments described herein, it may be desirable to engineer the recombinant microorganism to overexpress one or more mitochondrial export proteins. In a specific embodiment, said mitochondrial export protein may be

selected from the group consisting of the *S. cerevisiae ATM1*, the *S. cerevisiae ERV1*, and the *S. cerevisiae BAT1*, or homologs thereof.

[0025] In addition, the present invention provides recombinant microorganisms that have been engineered to increase the inner mitochondrial membrane electrical potential, $\Delta\Psi_M$. In one embodiment, this is accomplished via overexpression of an ATP/ADP carrier protein, wherein said overexpression increases ATP^{4-} import into the mitochondrial matrix in exchange for ADP^{3-}. In a specific embodiment, said ATP/ADP carrier protein is encoded by the *S. cerevisiae AAC1*, *AAC2*, and/or *AAC3* genes or homologs thereof. In another embodiment, the inner mitochondrial membrane electrical potential, $\Delta\Psi_M$ is increased via a mutation in the mitochondrial ATP synthase complex that increases ATP hydrolysis activity. In a specific embodiment, said mutation is an ATP1-111 suppressor mutation or a corresponding mutation in a homologous protein.

[0026] In various embodiments described herein, it may further be desirable to engineer the recombinant microorganism to express one or more enzymes in the cytosol that reduce the concentration of reactive nitrogen species (RNS) and/or nitric oxide (NO) in said cytosol. In one embodiment, said one or more enzymes are selected from the group consisting of nitric oxide reductases and glutathione-S-nitrosothiol reductase. In a specific embodiment, said nitric oxide reductase is encoded by one of more of the genes selected from the group consisting of the *E. coli* gene *norV* and the *Fusarium oxysporum* gene *P-450dNIR*, or homologs thereof. In another specific embodiment, said glutathione-S-nitrosothiol reductase is encoded by the *S. cerevisiae* gene *SFA1* or homologs thereof. In one embodiment, said glutathione-S-nitrosothiol reductase gene *SFA1* is overexpressed. In another specific embodiment, said one or more enzymes is encoded by a gene selected from the group consisting of the *E. coli* gene *ytfE*, the *Staphylococcus aureus* gene *scdA*, and *Neisseria gonorrhoeae* gene *dnrN*, or homologs thereof.

[0027] Also provided herein are recombinant microorganisms that demonstrate increased the levels of sulfur-containing compounds within yeast cells, including the amino acid cysteine, such that this sulfur is more available for the production of iron-sulfur cluster-containing proteins in the yeast cell. In one embodiment, the recombinant microorganism has been engineered to overexpress one or more of the genes selected from the *S. cerevisiae* genes *MET1, MET2, MET3, MET5, MET8, MET10, MET14, MET16, MET17, HOM2, HOM3, HOM6, CYS3, CYS4, SUL1*, and *SUL2*, or homologs thereof. The recombinant microorganism may additionally or

optionally also overexpress one or more of the genes selected from the *S. cerevisiae* genes *YCT1, MUP1, GAP1, AGP1, GNP1, BAP1, BAP2, TAT1*, and *TAT2*, or homologs thereof.

[0028] In various embodiments described herein, the recombinant microorganism may exhibit at least about 5 percent greater dihydroxyacid dehydratase (DHAD) activity as compared to the parental microorganism. In another embodiment, the recombinant microorganism may exhibit at least about 10 percent, at least about 15 percent, about least about 20 percent, at least about 25 percent, at least about 30 percent, at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 100 percent, at least about 200 percent, or at least about 500 percent greater dihydroxyacid dehydratase (DHAD) activity as compared to the parental microorganism.

[0029] In various embodiments described herein, the recombinant microorganisms may be microorganisms of the Saccharomyces clade, *Saccharomyces sensu stricto* microorganisms, Crabtree-negative yeast microorganisms, Crabtree-positive yeast microorganisms, post-WGD (whole genome duplication) yeast microorganisms, pre-WGD (whole genome duplication) yeast microorganisms, and non-fermenting yeast microorganisms.

[0030] In some embodiments, the recombinant microorganisms may be yeast recombinant microorganisms of the Saccharomyces clade.

[0031] In some embodiments, the recombinant microorganisms may be *Saccharomyces sensu stricto* microorganisms. In one embodiment, the *Saccharomyces sensu stricto* is selected from the group consisting of *S. cerevisiae, S. kudriavzevii, S. mikatae, S. bayanus, S. uvarum. S. carocanis* and hybrids thereof.

[0032] In some embodiments, the recombinant microorganisms may be Crabtree-negative recombinant yeast microorganisms. In one embodiment, the Crabtree-negative yeast microorganism is classified into a genera selected from the group consisting of *Kluyveromyces*, *Pichia*, *Issatchenkia, Hansenula*, or *Candida*. In additional embodiments, the Crabtree-negative yeast microorganism is selected from *Kluyveromyces lactis, Kluyveromyces marxianus, Pichia anomala, Pichia stipitis, Hansenula anomala, Candida utilis* and *Kluyveromyces waltii*.

[0033] In some embodiments, the recombinant microorganisms may be Crabtree-positive recombinant yeast microorganisms. In one embodiment, the Crabtree-

positive yeast microorganism is classified into a genera selected from the group consisting of *Saccharomyces, Kluyveromyces, Zygosaccharomyces, Debaryomyces, Candida, Pichia* and *Schizosaccharomyces*. In additional embodiments, the Crabtree-positive yeast microorganism is selected from the group consisting of *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces bayanus, Saccharomyces paradoxus, Saccharomyces castelli, Saccharomyces kluyveri, Kluyveromyces thermotolerans, Candida glabrata, Z. bailli, Z. rouxii, Debaryomyces hansenii, Pichia pastorius, Schizosaccharomyces pombe,* and *Saccharomyces uvarum.*

[0034] In some embodiments, the recombinant microorganisms may be post-WGD (whole genome duplication) yeast recombinant microorganisms. In one embodiment, the post-WGD yeast recombinant microorganism is classified into a genera selected from the group consisting of *Saccharomyces* or *Candida*. In additional embodiments, the post-WGD yeast is selected from the group consisting of *Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces bayanus, Saccharomyces paradoxus, Saccharomyces castelli,* and *Candida glabrata.*

[0035] In some embodiments, the recombinant microorganisms may be pre-WGD (whole genome duplication) yeast recombinant microorganisms. In one embodiment, the pre-WGD yeast recombinant microorganism is classified into a genera selected from the group consisting of *Saccharomyces, Kluyveromyces, Candida, Pichia, Issatchenkia, Debaryomyces, Hansenula, Pachysolen, Yarrowia* and *Schizosaccharomyces.* In additional embodiments, the pre-WGD yeast is selected from the group consisting of *Saccharomyces kluyveri, Kluyveromyces thermotolerans, Kluyveromyces marxianus, Kluyveromyces waltii, Kluyveromyces lactis, Candida tropicalis, Pichia pastoris, Pichia anomala, Pichia stipitis, Isstachenkia orientalis, Issatchenkia occidentalis, Debaryomyces hansenii, Hansenula anomala, Pachysolen tannophilis, Yarrowia lipolytica,* and *Schizosaccharomyces pombe.*

[0036] In some embodiments, the recombinant microorganisms may be microorganisms that are non-fermenting yeast microorganisms, including, but not limited to those, classified into a genera selected from the group consisting of *Tricosporon*, *Rhodotorula*, *Myxozyma*, or *Candida*. In a specific embodiment, the non-fermenting yeast is *C. xestobii*.

[0037] In another aspect, the present invention provides methods of producing beneficial metabolites including fuels, chemicals, and amino acids using a

recombinant microorganism as described herein. In one embodiment, the method includes cultivating the recombinant microorganism in a culture medium containing a feedstock providing the carbon source until a recoverable quantity of the metabolite is produced and optionally, recovering the metabolite. In one embodiment, the microorganism produces the metabolite from a carbon source at a yield of at least about 5 percent theoretical. In another embodiment, the microorganism produces the metabolite at a yield of at least about 10 percent, at least about 15 percent, about least about 20 percent, at least about 25 percent, at least about 30 percent, at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 85 percent, at least about 90 percent, at least about 95 percent, or at least about 97.5 percent theoretical. The metabolite may be derived from any DHAD-requiring biosynthetic pathway, including, but not limited to, biosynthetic pathways for the production of isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid.

[0038] In one embodiment, the recombinant microorganism is grown under aerobic conditions. In another embodiment, the recombinant microorganism is grown under microaerobic conditions. In yet another embodiment, the recombinant microorganism is grown under anaerobic conditions.

BRIEF DESCRIPTION OF DRAWINGS

[0039] Illustrative embodiments of the invention are illustrated in the drawings, in which:

[0040] Figure 1 illustrates an exemplary embodiment of an isobutanol pathway.

[0041] Figure 2 illustrates a phylogenetic tree of DHAD proteins. Numbers at nodes indicate bootstrap values. Ec_ilvD is a known 4Fe-4S DHAD enzyme from *Escherichia coli*.

[0042] Figure 3 illustrates a *S. cerevisiae AFT1-1^{UP}* allelic exchange construct.

[0043] Figure 4 illustrates a *S. cerevisiae AFT2-1^{UP}* allelic exchange construct.

[0044] Figure 5 illustrates a linear DNA fragment containing the *K. marxianus AFT*, the *L. lactis* DHAD, and a G418 resistance marker.

[0045] Figure 6 illustrates a linear DNA fragment containing the *L. lactis DHAD* and a G418 resistance marker.

DETAILED DESCRIPTION

[0046] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polynucleotide" includes a plurality of such polynucleotides and reference to "the microorganism" includes reference to one or more microorganisms, and so forth.

[0047] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

[0048] Any publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0049] The term "microorganism" includes prokaryotic and eukaryotic microbial species from the Domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

[0050] The term “genus” is defined as a taxonomic group of related species according to the Taxonomic Outline of Bacteria and Archaea (Garrity, G.M., Lilburn, T.G., Cole, J.R., Harrison, S.H., Euzeby, J., and Tindall, B.J. (2007) The Taxonomic Outline of Bacteria and Archaea. TOBA Release 7.7, March 2007. Michigan State University Board of Trustees.

[0051] The term “species” is defined as a collection of closely related organisms with greater than 97% 16S ribosomal RNA sequence homology and greater than 70% genomic hybridization and sufficiently different from all other organisms so as to be recognized as a distinct unit.

[0052] The terms "recombinant microorganism," “modified microorganism’” and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or over-express endogenous polynucleotides, or to express heterologous polynucleotides, such as those included in a vector, or which have an alteration in expression of an endogenous gene. By

"alteration" it is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more polypeptides or polypeptide subunits, or activity of one or more polypeptides or polypeptide subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the alteration. For example, the term "alter" can mean "inhibit," but the use of the word "alter" is not limited to this definition.

[0053] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired product encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantitated by qRT-PCR or by Northern hybridization (see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Protein encoded by a selected sequence can be quantitated by various methods, *e.g.,* by ELISA, by assaying for the biological activity of the protein, or by employing assays that are independent of such activity, such as western blotting or radioimmunoassay, using antibodies that recognize and bind the protein. See Sambrook *et al.,* 1989, *supra*. The polynucleotide generally encodes a target enzyme involved in a metabolic pathway for producing a desired metabolite. It is understood that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0054] The term “overexpression” refers to an elevated level (*e.g.*, aberrant level) of mRNAs encoding for a protein(s) (*e.g.* an Aft protein or homolog thereof), and/or to elevated levels of protein(s) (*e.g.* Aft) in cells as compared to similar corresponding unmodified cells expressing basal levels of mRNAs (*e.g.,* those encoding Aft proteins) or having basal levels of proteins. In particular embodiments, Aft1 and/or Aft2, or homologs thereof, or Aft regulon proteins, or homologs thereof, may be overexpressed by at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, 10-fold,

12-fold, 15-fold or more in microorganisms engineered to exhibit increased Aft1 and/or Aft2, or Aft regulon mRNA, protein, and/or activity.

[0055] The term "wild-type microorganism" describes a cell that occurs in nature, *i.e.* a cell that has not been genetically modified. A wild-type microorganism can be genetically modified to express or overexpress a first target enzyme. This microorganism can act as a parental microorganism in the generation of a microorganism modified to express or overexpress a second target enzyme. In turn, the microorganism modified to express or overexpress a first and a second target enzyme can be modified to express or overexpress a third target enzyme.

[0056] Accordingly, a “parental microorganism” functions as a reference cell for successive genetic modification events. Each modification event can be accomplished by introducing a nucleic acid molecule in to the reference cell. The introduction facilitates the expression or overexpression of a target enzyme. It is understood that the term "facilitates" encompasses the activation of endogenous polynucleotides encoding a target enzyme through genetic modification of *e.g.*, a promoter sequence in a parental microorganism. It is further understood that the term "facilitates" encompasses the introduction of heterologous polynucleotides encoding a target enzyme in to a parental microorganism.

[0057] The term "engineer" refers to any manipulation of a microorganism that results in a detectable change in the microorganism, wherein the manipulation includes but is not limited to inserting a polynucleotide and/or polypeptide heterologous to the microorganism and mutating a polynucleotide and/or polypeptide native to the microorganism.

[0058] The term "mutation" as used herein indicates any modification of a nucleic acid and/or polypeptide which results in an altered nucleic acid or polypeptide. Mutations include, for example, point mutations, deletions, or insertions of single or multiple residues in a polynucleotide, which includes alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence, such as, but not limited to, regulatory or promoter sequences. A genetic alteration may be a mutation of any type. For instance, the mutation may constitute a point mutation, a frame-shift mutation, an insertion, or a deletion of part or all of a gene. In addition, in some embodiments of the modified microorganism, a portion of the microorganism genome has been replaced with a heterologous polynucleotide. In some embodiments, the mutations are naturally-occurring. In other embodiments, the mutations are the results of artificial selection

pressure. In still other embodiments, the mutations in the microorganism genome are the result of genetic engineering.

[0059] The term "biosynthetic pathway", also referred to as "metabolic pathway", refers to a set of anabolic or catabolic biochemical reactions for converting one chemical species into another. Gene products belong to the same "metabolic pathway" if they, in parallel or in series, act on the same substrate, produce the same product, or act on or produce a metabolic intermediate (*i.e.*, metabolite) between the same substrate and metabolite end product.

[0060] As used herein, the term “isobutanol producing metabolic pathway” refers to an enzyme pathway which produces isobutanol from pyruvate.

[0061] The term “heterologous” as used herein with reference to molecules and in particular enzymes and polynucleotides, indicates molecules that are expressed in an organism other than the organism from which they originated or are found in nature, independently of the level of expression that can be lower, equal or higher than the level of expression of the molecule in the native microorganism. The term “heterologous” is also used synonymously herein with the term "exogenous."

[0062] On the other hand, the term “native” or “endogenous” as used herein with reference to molecules, and in particular enzymes and polynucleotides, indicates molecules that are expressed in the organism in which they originated or are found in nature, independently of the level of expression that can be lower equal or higher than the level of expression of the molecule in the native microorganism. It is understood that expression of native enzymes or polynucleotides may be modified in recombinant microorganisms.

[0063] The term “feedstock” is defined as a raw material or mixture of raw materials supplied to a microorganism or fermentation process from which other products can be made. For example, a carbon source, such as biomass or the carbon compounds derived from biomass are a feedstock for a microorganism that produces a biofuel in a fermentation process. However, a feedstock may contain nutrients other than a carbon source.

[0064] The term "substrate" or "suitable substrate" refers to any substance or compound that is converted or meant to be converted into another compound by the action of an enzyme. The term includes not only a single compound, but also combinations of compounds, such as solutions, mixtures and other materials which contain at least one substrate, or derivatives thereof. Further, the term "substrate" encompasses not only compounds that provide a carbon source suitable for use as a

starting material, such as any biomass derived sugar, but also intermediate and end product metabolites used in a pathway associated with a recombinant microorganism as described herein.

[0065] The term "C2-compound" as used as a carbon source for engineered yeast microorganisms with mutations in all pyruvate decarboxylase (PDC) genes resulting in a reduction of pyruvate decarboxylase activity of said genes refers to organic compounds comprised of two carbon atoms, including but not limited to ethanol and acetate.

[0066] The term "fermentation" or "fermentation process" is defined as a process in which a microorganism is cultivated in a culture medium containing raw materials, such as feedstock and nutrients, wherein the microorganism converts raw materials, such as a feedstock, into products.

[0067] The term "volumetric productivity" or "production rate" is defined as the amount of product formed per volume of medium per unit of time. Volumetric productivity is reported in gram per liter per hour (g/L/h).

[0068] The term "specific productivity" or "specific production rate" is defined as the amount of product formed per volume of medium per unit of time per amount of cells. Specific productivity is reported in gram or milligram per liter per hour per OD (g/L/h/OD).

[0069] The term "yield" is defined as the amount of product obtained per unit weight of raw material and may be expressed as g product per g substrate (g/g). Yield may be expressed as a percentage of the theoretical yield. "Theoretical yield" is defined as the maximum amount of product that can be generated per a given amount of substrate as dictated by the stoichiometry of the metabolic pathway used to make the product. For example, the theoretical yield for one typical conversion of glucose to isobutanol is 0.41 g/g. As such, a yield of isobutanol from glucose of 0.39 g/g would be expressed as 95% of theoretical or 95% theoretical yield.

[0070] The term "titer" is defined as the strength of a solution or the concentration of a substance in solution. For example, the titer of a biofuel in a fermentation broth is described as g of biofuel in solution per liter of fermentation broth (g/L).

[0071] "Aerobic conditions" are defined as conditions under which the oxygen concentration in the fermentation medium is sufficiently high for an aerobic or facultative anaerobic microorganism to use as a terminal electron acceptor.

[0072] In contrast, "anaerobic conditions" are defined as conditions under which the oxygen concentration in the fermentation medium is too low for the

microorganism to use as a terminal electron acceptor. Anaerobic conditions may be achieved by sparging a fermentation medium with an inert gas such as nitrogen until oxygen is no longer available to the microorganism as a terminal electron acceptor. Alternatively, anaerobic conditions may be achieved by the microorganism consuming the available oxygen of the fermentation until oxygen is unavailable to the microorganism as a terminal electron acceptor.

[0073] "Aerobic metabolism" refers to a biochemical process in which oxygen is used as a terminal electron acceptor to make energy, typically in the form of ATP, from carbohydrates. Aerobic metabolism occurs e.g. via glycolysis and the TCA cycle, wherein a single glucose molecule is metabolized completely into carbon dioxide in the presence of oxygen.

[0074] In contrast, "anaerobic metabolism" refers to a biochemical process in which oxygen is not the final acceptor of electrons contained in NADH. Anaerobic metabolism can be divided into anaerobic respiration, in which compounds other than oxygen serve as the terminal electron acceptor, and substrate level phosphorylation, in which the electrons from NADH are utilized to generate a reduced product via a "fermentative pathway."

[0075] In "fermentative pathways", NAD(P)H donates its electrons to a molecule produced by the same metabolic pathway that produced the electrons carried in NAD(P)H. For example, in one of the fermentative pathways of certain yeast strains, NAD(P)H generated through glycolysis transfers its electrons to pyruvate, yielding ethanol. Fermentative pathways are usually active under anaerobic conditions but may also occur under aerobic conditions, under conditions where NADH is not fully oxidized via the respiratory chain. For example, above certain glucose concentrations, Crabtree-positive yeasts produce large amounts of ethanol under aerobic conditions.

[0076] The term "byproduct" means an undesired product related to the production of a biofuel or biofuel precursor. Byproducts are generally disposed as waste, adding cost to a production process.

[0077] The term "non-fermenting yeast" is a yeast species that fails to demonstrate an anaerobic metabolism in which the electrons from NADH are utilized to generate a reduced product via a fermentative pathway such as the production of ethanol and CO_2 from glucose. Non-fermentative yeast can be identified by the "Durham Tube Test" (J.A. Barnett, R.W. Payne, and D. Yarrow. 2000. Yeasts Characteristics and Identification. 3rd edition. p. 28-29. Cambridge University Press,

Cambridge, UK.) or by monitoring the production of fermentation productions such as ethanol and CO_2.

[0078] The term “polynucleotide” is used herein interchangeably with the term “nucleic acid” and refers to an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof, including but not limited to single stranded or double stranded, sense or antisense deoxyribonucleic acid (DNA) of any length and, where appropriate, single stranded or double stranded, sense or antisense ribonucleic acid (RNA) of any length, including siRNA. The term “nucleotide” refers to any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or a pyrimidine base and to a phosphate group, and that are the basic structural units of nucleic acids. The term “nucleoside” refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. The term “nucleotide analog” or “nucleoside analog” refers, respectively, to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or with a different functional group. Accordingly, the term polynucleotide includes nucleic acids of any length, DNA, RNA, analogs and fragments thereof. A polynucleotide of three or more nucleotides is also called nucleotidic oligomer or oligonucleotide.

[0079] It is understood that the polynucleotides described herein include "genes" and that the nucleic acid molecules described herein include "vectors" or "plasmids." Accordingly, the term "gene", also called a "structural gene" refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. The transcribed region of the gene may include untranslated regions, including introns, 5'-untranslated region (UTR), and 3'-UTR, as well as the coding sequence.

[0080] The term "operon" refers to two or more genes which are transcribed as a single transcriptional unit from a common promoter. In some embodiments, the genes comprising the operon are contiguous genes. It is understood that transcription of an entire operon can be modified (*i.e.*, increased, decreased, or eliminated) by modifying the common promoter. Alternatively, any gene or combination of genes in an operon can be modified to alter the function or activity of the encoded polypeptide. The modification can result in an increase in the activity of

the encoded polypeptide. Further, the modification can impart new activities on the encoded polypeptide. Exemplary new activities include the use of alternative substrates and/or the ability to function in alternative environmental conditions.

[0081] A "vector" is any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are "episomes," that is, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine -conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

[0082] "Transformation" refers to the process by which a vector is introduced into a host cell. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including chemical transformation (e.g. lithium acetate transformation), electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or agrobacterium mediated transformation.

[0083] The term "enzyme" as used herein refers to any substance that catalyzes or promotes one or more chemical or biochemical reactions, which usually includes enzymes totally or partially composed of a polypeptide, but can include enzymes composed of a different molecule including polynucleotides.

[0084] The term “protein,” “peptide,” or “polypeptide” as used herein indicates an organic polymer composed of two or more amino acidic monomers and/or analogs thereof. As used herein, the term “amino acid” or “amino acidic monomer” refers to any natural and/or synthetic amino acids including glycine and both D or L optical isomers. The term “amino acid analog” refers to an amino acid in which one or more individual atoms have been replaced, either with a different atom, or with a different functional group. Accordingly, the term polypeptide includes amino acidic polymer of any length including full length proteins, and peptides as well as analogs and fragments thereof. A polypeptide of three or more amino acids is also called a protein oligomer or oligopeptide

[0085] The term "homolog," used with respect to an original enzyme or gene of a first family or species, refers to distinct enzymes or genes of a second family or species which are determined by functional, structural or genomic analyses to be an enzyme or gene of the second family or species which corresponds to the original enzyme or gene of the first family or species. Most often, homologs will have functional, structural or genomic similarities. Techniques are known by which homologs of an enzyme or gene can readily be cloned using genetic probes and PCR. Identity of cloned sequences as homolog can be confirmed using functional assays and/or by genomic mapping of the genes.

[0086] A protein has "homology" or is "homologous" to a second protein if the amino acid sequence encoded by a gene has a similar amino acid sequence to that of the second gene. Alternatively, a protein has homology to a second protein if the two proteins have "similar" amino acid sequences. (Thus, the term "homologous proteins" is defined to mean that the two proteins have similar amino acid sequences).

[0087] The term “analog” or “analogous” refers to nucleic acid or protein sequences or protein structures that are related to one another in function only and are not from common descent or do not share a common ancestral sequence. Analogs may differ in sequence but may share a similar structure, due to convergent evolution. For example, two enzymes are analogs or analogous if the enzymes catalyze the same reaction of conversion of a substrate to a product, are unrelated in sequence, and irrespective of whether the two enzymes are related in structure.

Enhancing DHAD Activity by Altering Aft1/Aft2 Activity and/or Expression

[0088] The present inventors have found that altering the expression of the *AFT1* and/or *AFT2* genes of *S. cerevisiae* surprisingly increases DHAD activity and contributes to increased isobutanol titers, productivity, and yield in strains comprising DHAD as part of an isobutanol-producing metabolic pathway. The observed increases in DHAD activity resulting from the increased expression of *AFT1* and/or *AFT2* therefore has broad applicability to any DHAD-requiring biosynthetic pathway, as DHAD activity is often a rate-limiting component of such pathways.

[0089] Accordingly, one aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism is engineered to overexpress one or more polynucleotides encoding one or more Aft proteins or homologs thereof.

[0090] As used herein, a "DHAD-requiring biosynthetic pathway" refers to any metabolic pathway which utilizes DHAD to convert 2,3-dihydroxyisovalerate to α-ketoisovalerate or 2,3-dihydroxy-3-methylvalerate to 2-keto-3-methylvalerate. Examples of DHAD-requiring biosynthetic pathways include, but are not limited to, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid (vitamin B5) metabolic pathways. The metabolic pathway may naturally occur in a microorganism (*e.g.*, a natural pathway for the production of valine) or arise from the introduction of one or more heterologous polynucleotides through genetic engineering. In one embodiment, the recombinant microorganisms expressing the DHAD-requiring biosynthetic pathway are yeast cells. Engineered biosynthetic pathways for synthesis of isobutanol are described in commonly owned and co-pending applications US 12/343,375 (published as US 2009/0226991), US 12/696,645, US 12/610,784 (published as US 2010/0143997), US 12/855,276, PCT/US09/62952 (published as WO/2010/051527), and PCT/US09/69390 (published as WO/2010/075504), all of which are herein incorporated by reference in their entireties for all purposes. Additional DHAD-requiring biosynthetic pathways have been described for the synthesis of valine, leucine, and isoleucine (See, *e.g.*, WO/2001/021772, and McCourt *et al.*, 2006, *Amino Acids* 31: 173-210), pantothenic acid (See, *e.g.*, WO/2001/021772), 3-methyl-1-butanol (See, *e.g.*, WO/2008/098227, Atsumi *et al.*, 2008, *Nature* 451: 86-89, and Connor *et al.*, 2008, *Appl. Environ. Microbiol.* 74: 5769-5775), and 2-methyl-1-butanol (See, *e.g.*, WO/2008/098227, WO/2009/076480, and Atsumi *et al.*, 2008, *Nature* 451: 86-89).

[0091] As used herein, the terms "DHAD" or "DHAD enzyme" or "dihydroxyacid dehydratase" are used interchangeably herein to refer to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to ketoisovalerate and/or the conversion of 2,3-dihydroxy-3-methylvalerate to 2-keto-3-methylvalerate. DHAD sequences are available from a vast array of microorganisms, including, but not limited to, *L. lactis*, *E. coli*, *S. cerevisiae*, *B. subtilis*, *Streptococcus pneumoniae*, and *Streptococcus mutans*. A representative list of DHAD enzymes that can benefit from the methods described herein, such as the increased expression of *AFT1* and/or *AFT2* or homologs thereof, include, but are not limited to those, disclosed in 2010/0081154, as well as those disclosed in commonly owned and co-pending U.S. Patent Application Serial Nos. 12/855,276 and 61/407,815. Such DHAD enzymes may be cytosolically localized or mitochondrially localized. A representative listing of DHAD enzymes exhibiting cytosolic localization and activity are disclosed in commonly

owned and co-pending U.S. Patent Application Serial No. 12/855,276.

[0092] Without being bound by any theory, it is believed that altered expression of an *AFT* gene (*e.g.* the *AFT1* and/or *AFT2* genes) enhances cellular iron availability, which leads to an improvement in the activity of the iron-sulfur (FeS) cluster-containing protein, DHAD. The observation that increased expression of the *AFT* genes improves DHAD activity is surprising, particularly in light of recently published findings by Ihrig *et al.* (2010, *Eukaryotic Cell* 9: 460-471). Notably, Ihrig *et al.* observed that the increased expression of Aft1 in *S. cerevisiae* had little to no effect on the activity of another FeS cluster-containing protein, Leu1 (isopropylmalate isomerase of the leucine biosynthesis pathway). In contrast to observations made by Ihrig *et al.* with respect to the FeS protein, Leu1, the present inventors unexpectedly observed that increased expression of Aft1 and/or Aft2 resulted in a significant increase in the activity of DHAD, also an iron-sulfur (FeS) cluster-containing protein. Moreover, in strains comprising DHAD as part of an isobutanol-producing metabolic pathway, the increased expression of Aft1 produced significant increases in isobutanol titer, productivity, and yield.

[0093] In *S. cerevisiae*, *AFT1* and *AFT2* encode for the transcription factors, Aft1 and Aft2 ("activator of ferrous transport"), respectively. It is hypothesized that Aft1 and Aft2 activate gene expression when iron is scarce in wild-type *S. cerevisiae*. Consequently, strains lacking both Aft1 and Aft2 exhibit reduced expression of the iron regulon. As with many other paralogous genes, *AFT1* and *AFT2* code for proteins that have significant regions of identity and overlapping functions. The DNA-binding domain of each protein is in a highly conserved N-terminal region, and a conserved cysteine-to-phenylalanine mutation in either protein generates a factor that activates the high expression of the iron regulon irrespective of iron concentrations.

[0094] In yeast, homeostatic regulation of iron uptake occurs (Eide *et al.*, 1992, *J. Biol Chem.* 267: 20774-81). Iron deprivation induces activity of a high affinity iron uptake system. This induction is mediated by increased transcript levels for genes involved in the iron uptake system, and *AFT1* is hypothesized to play a critical role in this process (Yamaguchi-Iwai *et al.*, 1995, *The EMBO Journal* 14: 1231-9). Yamaguchi-Iwai *et al.* observed that mutant strains lacking *AFT1*, due to gene deletion, are unable to induce the high-affinity iron uptake system. On the other hand, mutant strains carrying the $AFT1^{UP}$ allele exhibit a gain-of-function phenotype in which iron uptake cannot be repressed by available iron in the environment. The

AFT1^{UP} and *AFT2*^{UP} alleles described above act as gain of function point mutations. *AFT1*^{UP} is due to the mutation Cys^{291}Phe (Rutherford *et al.*, 2005, *Journal of Biological Chemistry* 281: 10135-40). *AFT2*^{UP} is due to the mutation Cys^{187}Phe (Rutherford *et al.*, 2001, *PNAS* 98: 14322-27).

[0095] There are clear phenotypic differences in strains that separately lack *AFT1* or *AFT2*. An *aft1* null strain exhibits low ferrous iron uptake and grows poorly under low-iron conditions or on a respiratory carbon source. No phenotype has been attributed to an *aft2* null strain. An *aft1 aft2* double null strain is, however, more sensitive to low-iron growth than a single *aft1* null strain, which is consistent with the functional similarity of these factors. The partial redundancy of these factors allows *AFT2* to complement an *aft1* null strain when it is overexpressed from a plasmid. The properties of Aft1 and Aft2 that distinguish them from each other have not been fully elucidated. Both factors mediate gene regulation via an iron-responsive element that contains the core sequence 5'-CACCC-3'. Without being bound to any theory, it is likely that sequences adjacent to this element influence the ability of each factor to mediate regulation via a particular iron-responsive element. The differential regulation of individual genes by Aft1 and Aft2 results in each factor generating a distinct global transcriptional profile (Table 1) (Rutherford *et al.*, 2004, *Eukaryotic Cell* 3: 1-13; Conde e Silva *et al.*, 2009, *Genetics* 183: 93-106).

Table 1. Genes Regulated by Metal-Responsive Transcription Factors.

Transcription Factor	Description	Gene Name(s)
Aft1	Transporters	*FET4, FET5, FTR1, FTH1, SMF3, MRS3, MRS4, CCC2, COT1*
	Cu chaperone	*ATX1*
	Ferroxidase	*FET3, FET5*
	Metalloreductases	*FRE1, FRE2, FRE3, FRE4, FRE5, FRE6*
	Cell wall proteins	*FIT1, FIT2, FIT3*
	Siderophore transport	*ARN1, ARN2, ARN3, ARN4*
	Fe-S biosynthesis	*ISU1, ISU2*
	Other	*TIS11, HMX1, AKR1, PCL5, YOR387c, YHL035c, YMR034c, ICY2, PRY1, YDL124w, CTH1, CTH2,*
Aft2	Transporters	*SMF3, MRS4, FTR1, COT1*
	Cu chaperone	*ATX1*
	Ferroxidase	*FET3, FET5*
	Metalloreductases	*FRE1*
	Cell wall proteins	*FIT1, FIT3, FIT2*
	Fe-S biosynthesis	*ISU1*
	Other	*BNA2, ECM4, LAP4, TIS11, YOL083w, YGR146c, YHL035c*

[0096] In *S. cerevisiae*, the Aft1 regulon consists of many genes that are involved

in the acquisition, compartmentalization, and utilization of iron. These include genes involved in iron uptake (*FET3*, *FTR1*, and *FRE1*, *FRE2*), siderophore uptake (*ARN1-4* and *FIT1-3*), iron transport across the vacuole membrane (*FTH1*), and iron-sulfur cluster formation (*ISU1 and ISU2*). Aft1 binds to a conserved promoter sequence in an iron-dependent manner and activates transcription under low-iron conditions. The Aft2 regulator controls the expression of several distinct genes (Table 2) (Rutherford *et al.*, 2004, *Eukaryotic Cell* 3: 1-13). The initial step in iron acquisition requires reduction of ferric iron chelates in the environment by externally directed reductases encoded by the *FRE1* and *FRE2* genes, thereby generating the ferrous iron substrate for the transport process (Dancis *et al.*, 1992, *PNAS* 89: 3869-73; Georgatsou and Alexandraki, 1994, *Mol. Cell. Biol.* 14: 3065-73). *FET3* encodes a multi-copper oxidase (Askwith *et al.*, 1994, *Cell* 76: 403-10; De Silva *et al.*, 1995, *J. Biol. Chem.* 270: 1098-1101) that forms a molecular complex with the iron permease encoded by *FTR1*. This complex, located in the yeast plasma membrane, mediates the high-affinity transport of iron into the cell (Stearman *et al.*, 1996, *Science* 271: 1552-7). *AFT* genes may be found in yeast strains other than *S. cerevisiae*. For example, in *K. lactis*, a homolog of the *S. cerevisiae AFT1* has been found and designated *Kl_AFT* (Conde e Silva *et al.*, 2009, *Genetics* 183: 93-106). In this fungus, Kl_Aft has been found to activate transcription of genes regulated by Aft1 in *S. cerevisiae*. Thus, altering the regulation, activity, and/or expression of *AFT* homologs in fungal strains other than *S. cerevisiae*, is also within the scope of this invention. A person skilled in the art will be able to utilize publicly available sequences to construct relevant recombinant microorganisms with altered expression of *AFT* homologs. A listing of a representative number of *AFT* homologs known in the art and useful in the construction of recombinant microorganisms engineered for increased DHAD activity are listed Table 2. One skilled in the art, equipped with this disclosure, will appreciate other suitable homologs for the generation of recombinant microorganisms with increased DHAD activity. Sequences of *AFT* genes found in sub-species or variants of a given species may not be identical (See, *e.g.*, > 98% identity amongst *S. cerevisiae AFT1* genes of SEQ ID NOs: 1, 208, 210, 212, 214, 216, 218, 220, 222, and 224). While it is preferred to overexpress an *AFT* gene native to the subspecies or variant, *AFT* genes may be interchangeably expressed across subspecies or variants of the same species.

Table 2. Representative Aft Homologs of Yeast Origin

Species Origin (Gene Name)	Nucleic Acid Sequence (SEQ ID NO)	Amino Acid Sequence (SEQ ID NO)
Saccharomyces cerevisiae S288c (AFT1)	1	2
Saccharomyces cerevisiae S288c (AFT2)	3	4
Candida glabrata (AFT1)	5	6
Candida glabrata (AFT2)	7	8
Zygosaccharomyces rouxii (AFT)	9	10
Ashbya gossypii (AFT)	11	12
Kluyveromyces lactis (AFT)	13	14
Vanderwaltozyma polyspora (AFT)	15	16
Lachancea thermotolerans (AFT)	17	18
Debaromyces hanseii (AFT)	19	20
*Saccharomyces bayanus**	21	22
*Saccharomyces castelli**	23	24
*Kluyveromyces waltii**	25	26
*Saccharomyces kluyveri**	27	28
Kluyveromyces marxianus	29	30
Issatchenkia orientalis (AFT1-1)	31	32
Issatchenkia orientalis (AFT1-2)	33	34
Saccharomyces bayanus (AFT2)	35	36
Saccharomyces castelli (AFT2)	37	38
S. cerevisiae W303 (AFT1)	208	209
S. cerevisiae DBVPG1106 (AFT1)	210	211
S. cerevisiae NCYC361 (AFT1)	212	213
S. cerevisiae Y55 (AFT1)	214	215
S. cerevisiae YJM981 (AFT1)	216	217
S. cerevisiae RM11_1A (AFT1)	218	219
S. cerevisiae UWOPS87_2421 (AFT1)	220	221
S. cerevisiae SK1 (AFT1)	222	223
S. cerevisiae YPS606 (AFT1)	224	225

* Byrne K.P., Wolfe, K.H. (2005) The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. Genome Research, 15(10):1456-61

[0097] Without being bound by any theory, it is believed that increasing the expression of the gene *AFT1* or a homolog thereof will modulate the amount and availability of iron in the host cell. Since Aft1 activates the expression of target genes in response to changes in iron availability, overexpression of *AFT1* increases the machinery to import more iron into the cytosol and/or mitochondria. A person skilled in the art, equipped with this disclosure, will appreciate suitable methods for

increasing the expression (*i.e.* overexpressing) *AFT1*. For instance, in one embodiment, *AFT1* or homolog thereof may be overexpressed from a plasmid. In another embodiment, one or more copies of the *AFT1* gene or a homolog thereof is inserted into the chromosome under the control of a constitutive promoter. In addition, a skilled person in the art, equipped with this disclosure, will recognize that the amount of *AFT1* overexpressed may vary from one yeast to the next. For example, the optimal level of overexpression may be one, two, three, four or more copies in a given yeast.

[0098] In additional embodiments, the native Aft1 or homolog thereof may be replaced with a mutant version that is constitutively active. In one embodiment, the native Aft1 is replaced with a mutant version that comprises a modification or mutation at a position corresponding to amino acid cysteine 291 of the *S. cerevisiae* Aft1 (SEQ ID NO: 2). In an exemplary embodiment, the cysteine 291 residue of the native *S. cerevisiae* Aft1 (SEQ ID NO: 2) or homolog thereof is replaced with a phenylalanine residue.

[0099] As will be understood by one of ordinary skill in the art, modified Aft1 proteins and homologs thereof may be obtained by recombinant or genetic engineering techniques that are routine and well-known in the art. For example, mutant Aft1 proteins and homologs thereof, can be obtained by mutating the gene or genes encoding Aft1 or the homologs of interest by site-directed mutagenesis. Such mutations may include point mutations, deletion mutations and insertional mutations. For example, one or more point mutations (*e.g.*, substitution of one or more amino acids with one or more different amino acids) may be used to construct mutant Aft1 proteins of the invention. The corresponding cysteine position of Aft1 homologs may be readily identified by one skilled in the art. Thus, given the defined region and the examples described in the present application, one with skill in the art can make one or a number of modifications which would result in the constitutive expression of Aft1.

[00100] Without being bound by any theory, it is believed that increasing the expression of the gene *AFT2* or a homolog thereof will modulate the amount and availability of iron in the host cell. *AFT2* overexpression is predicted to result in increased expression of the machinery to import more iron into the cytosol and/or mitochondria. A person skilled in the art, equipped with this disclosure, will appreciate suitable methods for increasing the expression (*i.e.* overexpression) of *AFT2*. For instance, in one embodiment, *AFT2* or homolog thereof may be

overexpressed from a plasmid. In another embodiment, one or more copies of the *AFT2* gene or a homolog thereof is inserted into the chromosome under the control of a constitutive promoter. In addition, a skilled person in the art, equipped with this disclosure, will recognize that the amount of *AFT2* overexpressed may vary from one yeast to the next. For example, the optimal level of overexpression may be one, two, three, four or more copies in a given yeast. Moreover, the expression level may be tuned by using a promoter that achieves the optimal expression level in a given yeast

[00101] In another embodiment, the native Aft2 or homolog thereof may be replaced with a mutant version that is constitutively active. In one embodiment, the native Aft2 is replaced with a mutant version that comprises a modification or mutation at a position corresponding to amino acid cysteine 187 of the *S. cerevisiae* Aft2 (SEQ ID NO: 4). In an exemplary embodiment, the cysteine 187 residue of the native *S. cerevisiae* Aft2 (SEQ ID NO: 4) or homolog thereof is replaced with a phenylalanine residue.

[00102] As will be understood by one of ordinary skill in the art, modified Aft2 proteins and homologs thereof may be obtained by recombinant or genetic engineering techniques that are routine and well-known in the art. For example, mutant Aft2 proteins and homologs thereof, can be obtained by mutating the gene or genes encoding Aft2 or the homologs of interest by site-directed. Such mutations may include point mutations, deletion mutations and insertional mutations. For example, one or more point mutations (*e.g.*, substitution of one or more amino acids with one or more different amino acids) may be used to construct mutant Aft2 proteins of the invention. The corresponding cysteine position of Aft2 homologs may be readily identified by one skilled in the art. Thus, given the defined region and the examples described in the present application, one with skill in the art can make one or a number of modifications which would result in the constitutive expression of Aft2.

[00103] In various exemplary embodiments, increasing the expression of both *AFT1* and/or *AFT2* will increase DHAD activity and the production of beneficial metabolites from DHAD-requiring biosynthetic pathways.

[00104] Embodiments in which the regulation, activity, and/or expression of *AFT1* and/or *AFT2* are altered can also be combined with increases in the extracellular iron concentration to provide increased iron in the cytosol and/or mitochondria of the cell. Increase in iron in either the cytosol or the mitochondria by this method appears to

make iron more available for the FeS cluster-containing protein, DHAD. Without being bound by any theory, it is believed that such an increase in iron leads to a corresponding increase in DHAD activity.

[00105] As described herein, the increased activity of DHAD in a recombinant microorganism is a favorable characteristic for the production of beneficial metabolites including isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid derived from DHAD-requiring biosynthetic pathways. Without being bound by any theory, it is believed that the increase in DHAD activity as observed by the present inventors results from enhanced cellular iron levels as mediated by the altered regulation, expression, and/or activity of *AFT1* and/or *AFT2*. Thus, in various embodiments described herein, the present invention provides recombinant microorganisms with increased DHAD activity as a result of alterations in *AFT1* and/or *AFT2* regulation, expression, and/or activity. In one embodiment, the alteration in *AFT1* and/or *AFT2* regulation, expression, and/or activity increases the activity of a cytosolically-localized DHAD. In another embodiment, the alteration in *AFT1* and/or *AFT2* regulation, expression, and/or activity increases the activity of a mitochondrially-localized DHAD.

[00106] While particularly useful for the biosynthesis of isobutanol, the altered regulation, expression, and/or activity of *AFT1* and/or *AFT2* is also beneficial to any other fermentation process in which increased DHAD activity is desirable, including, but not limited to, the biosynthesis of isoleucine, valine, leucine, pantothenic acid (vitamin B5), 2-methyl-1-butanol, and 3-methyl-1-butanol.

[00107] As described herein, the present inventors have observed increased isobutanol titers, productivity, and yields in recombinant microorganisms exhibiting increased expression of *AFT1* and/or *AFT2*. Without being bound by any theory, it is believed that the increases in isobutanol titer, productivity, and yield are due to the observed increases in DHAD activity. Thus, in one embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *AFT1* or a homolog thereof is increased. In another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *AFT2* or a homolog thereof is increased. In yet another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said

recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *AFT1* and *AFT2* or homologs thereof is increased.

[00108] In alternative embodiments, nucleic acids having a homology to *AFT1* and/or *AFT2* of at least about 50%, of at least about 60%, of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

[00109] In one embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Aft1 or a homolog thereof is increased. In another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Aft2 or a homolog thereof is increased. In yet another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Aft1 and Aft2 or homologs thereof is increased.

[00110] In alternative embodiments, proteins having a homology to Aft1 and/or Aft2 of at least about 50%, of at least about 60%, of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

[00111] In one embodiment, the isobutanol producing metabolic pathway comprises at least one exogenous gene that catalyzes a step in the conversion of pyruvate to isobutanol. In another embodiment, the isobutanol producing metabolic pathway comprises at least two exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least three exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at least four exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol. In yet another embodiment, the isobutanol producing metabolic pathway comprises at five exogenous genes that catalyze steps in the conversion of pyruvate to isobutanol.

[00112] In one embodiment, one or more of the isobutanol pathway genes encodes an enzyme that is localized to the cytosol. In one embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least one isobutanol pathway enzyme localized in the cytosol. In another embodiment, the recombinant microorganisms comprise an isobutanol producing

metabolic pathway with at least two isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least three isobutanol pathway enzymes localized in the cytosol. In yet another embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with at least four isobutanol pathway enzymes localized in the cytosol. In an exemplary embodiment, the recombinant microorganisms comprise an isobutanol producing metabolic pathway with five isobutanol pathway enzymes localized in the cytosol. In a further exemplary embodiment, at least one of the pathway enzymes localized to the cytosol is a cytosolically active DHAD enzyme as disclosed herein.

[00113] In various embodiments described herein, the isobutanol pathway genes encodes enzyme(s) selected from the group consisting of acetolactate synthase (ALS), ketol-acid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), 2-keto-acid decarboxylase (KIVD), and alcohol dehydrogenase (ADH).

[00114] As described above, the transcription factors Aft1 and Aft2 regulate genes involved in the acquisition, compartmentalization, and utilization of iron. Thus, in additional aspects, the present invention provides methods of increasing DHAD activity and the production of beneficial metabolites produced from DHAD-requiring biosynthetic pathways as a result of alterations in the regulation, expression, and/or activity of genes regulated by Aft1 and Aft2. In one embodiment, the gene(s) regulated by Aft1 and Aft2 is selected from the group consisting of *FET3, FET4, FET5, FTR1, FTH1, SMF3, MRS4, CCC2, COT1, ATX1, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FIT1, FIT2, FIT3, ARN1, ARN2, ARN3, ARN4, ISU1, ISU2, TIS11, HMX1, AKR1, PCL5, YOR387C, YHL035C, YMR034C, ICY2, PRY1, YDL124W, BNA2, ECM4, LAP4, YOL083W, YGR146C, BIO5, YDR271C, OYE3, CTH1, CTH2, MRS3, MRS4, HSP26, YAP2, VMR1, ECL1, OSW1, NFT1, ARA2, TAF1/TAF130/TAF145, YOR225W, YKR104W, YBR012C,* and *YMR041C* or a homolog thereof. While particularly useful for the biosynthesis of isobutanol, the altered regulation, expression, and/or activity of genes regulated by Aft1 and Aft2 is also beneficial to any other fermentation process in which increased DHAD activity is desirable, including, but not limited to, the biosynthesis of isoleucine, valine, leucine, pantothenic acid (vitamin B5), 1-butanol, 2-methyl-1-butanol, and 3-methyl-1-butanol.

[00115] In one embodiment, all genes demonstrated to increase DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway

are overexpressed. Where none of the *AFT* regulon genes expressed alone are effective in increasing DHAD activity and/or the production of a metabolite from a DHAD-requiring biosynthetic pathway, then 1, 2, 3, 4, 5, or more of the genes in the *AFT* regulon are overexpressed together.

[00116] As described herein, the present inventors have observed increased isobutanol titers, productivity, and yields in recombinant microorganisms exhibiting increased expression of the transcription factors *AFT1* and/or *AFT2*, which regulate the expression of genes involved in the acquisition, compartmentalization, and utilization of iron. Thus, in one embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression and/or activity of one or more genes selected from the group consisting of *FET3, FET4, FET5, FTR1, FTH1, SMF3, MRS4, CCC2, COT1, ATX1, FRE1, FRE2, FRE3, FRE4, FRE5, FRE6, FIT1, FIT2, FIT3, ARN1, ARN2, ARN3, ARN4, ISU1, ISU2, TIS11, HMX1, AKR1, PCL5, YOR387C, YHL035C, YMR034C, ICY2, PRY1, YDL124W, BNA2, ECM4, LAP4, YOL083W, YGR146C, BIO5, YDR271C, OYE3, CTH1, CTH2, MRS3, MRS4, HSP26, YAP2, VMR1, ECL1, OSW1, NFT1, ARA2, TAF1/TAF130/TAF145, YOR225W, YKR104W, YBR012C,* and *YMR041C* or a homolog thereof is increased.

Enhancing DHAD Activity by Increased GRX3/GRX4 Activity and/or Expression

[00117] As described herein, increasing the expression of the genes *GRX3* and/or *GRX4* will generally modulate the amount and availability of iron in the yeast cytosol or mitochondria. Accordingly, one aspect of the invention is directed to a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway, wherein said microorganism has been engineered to overexpress a polynucleotide encoding Grx3 and/or Grx4 or a homolog thereof. In one embodiment, the polynucleotide encoding the Grx protein or homolog thereof is native to the recombinant microorganism. In another embodiment, the polynucleotide encoding the Grx protein or homolog thereof is heterologous to the recombinant microorganism.

[00118] Grx3 and Grx4 are monothiol glutaredoxins that have been shown to be involved in cellular Fe content modulation and delivery in yeast. Glutaredoxins are glutathione-dependent thiol-disulfide oxidoreductases that function in maintaining the cellular redox homeostasis. *S. cerevisiae* has two dithiol glutaredoxins (Grx1 and

Grx2) and three monothiol glutaredoxins (Grx3, Grx4, and Grx5). The monothiol glutaredoxins are believed to reduce mixed disulfides formed between a protein and glutathione in a process known as deglutathionylation. In contrast, dithiol glutaredoxins can participate in deglutathionylation as well as in the direct reduction of disulfides. Grx5, the most studied monothiol glutaredoxin, is localized to the mitochondrial matrix, where it participates in the maturation of Fe-S clusters. Grx3 and Grx4 are predominantly localized to the nucleus. These proteins can substitute for Grx5 when overexpressed and targeted to the mitochondrial matrix; no information on their natural function has been reported. In addition to the reported interaction between Grx3 and Aft1, iron inhibition of Aft1 requires glutathione. It has been shown that iron sensing is dependent on the presence of the redundant Grx3 and Grx4 proteins. One report indicated that removal of both Grx3 and Grx4 resulted in constitutive expression of the genes regulated by Aft1/Aft2. This result suggested that the cells accumulated Fe at levels greater than normal.

[00119] In one embodiment, Grx3 is overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx4 is overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx3 and Grx4 are overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx3, Grx4, or Grx3 and Grx4 are deleted or attenuated. In another embodiment, Grx3 and Aft1 are overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx4 and Aft1 are overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx3 and Aft2 are overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. In another embodiment, Grx4 and Aft2 are overexpressed from a plasmid or by inserting multiple copies of the gene into the chromosome under the control of a constitutive promoter. These embodiments can also be combined with increases in the extracellular iron concentration to provide increased iron in the cytosol or mitochondria of the cell. One or both of: Aft1, Aft2 is overexpressed either alone or in combination with: Grx3 or Grx4. Such overexpression can be accomplished by plasmid or by inserting multiple copies of

the gene into the chromosome under the control of a constitutive promoter.

[00120] As described herein, the increased activity of DHAD in a recombinant microorganism is a favorable characteristic for the production of beneficial metabolites including isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid from DHAD-requiring metabolic pathways. Thus, in various embodiments described herein, the present invention provides recombinant microorganisms with increased DHAD activity as a result of alterations in *GRX3* and/or *GRX4* regulation, expression, and/or activity. In one embodiment, the alteration in *GRX3* and/or *GRX4* regulation, expression, and/or activity increases the activity of a cytosolically-localized DHAD. In another embodiment, the alteration in *GRX3* and/or *GRX4* regulation, expression, and/or activity increases the activity of a mitochondrially-localized DHAD.

[00121] While particularly useful for the biosynthesis of isobutanol, the altered regulation, expression, and/or activity of *GRX3* and/or *GRX4* is also beneficial to any other fermentation process in which increased DHAD activity is desirable, including, but not limited to, the biosynthesis of isoleucine, valine, leucine, pantothenic acid (vitamin B5), 1-butanol, 2-methyl-1-butanol, and 3-methyl-1-butanol.

[00122] In one embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *GRX3* or a homolog thereof is increased. In another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *GRX4* or a homolog thereof is increased. In yet another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the expression of *GRX3* and *GRX4* or homologs thereof is increased.

[00123] In alternative embodiments, nucleic acids having a homology to *GRX3* and/or *GRX4* of at least about 50%, of at least about 60%, of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

[00124] In one embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Grx3 or a homolog thereof is increased. In another embodiment, the present

invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Grx4 or a homolog thereof is increased. In yet another embodiment, the present invention provides a recombinant microorganism for producing isobutanol, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, and wherein the activity of Grx3 and Grx4 or homologs thereof is increased.

[00125] In alternative embodiments, proteins having a homology to Grx3 and/or Grx4 of at least about 50%, of at least about 60%, of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

Altering the Iron-Sulfur Cluster Domain and/or Redox Active Domain

[00126] In general, the yeast cytosol demonstrates a different redox potential than a bacterial cell, as well as the yeast mitochondria. As a result, isobutanol pathway enzymes such as DHAD which exhibit an iron sulfur (FeS) domain and/or redox active domain, may require the redox potential of the native environments to be folded or expressed in a functional form. Expressing the protein in the yeast cytosol, which can harbor unfavorable redox potential, has the propensity to result in an inactive protein, even if the protein is expressed. The present inventors have identified a number of different strategies to overcome this problem, which can arise when an isobutanol pathway enzyme such as DHAD which is suited to a particular environment with a specific redox potential is expressed in the yeast cytosol.

[00127] In one embodiment, the present invention provides DHAD enzymes that exhibit a properly folded iron-sulfur cluster domain and/or redox active domain in the cytosol. Such DHAD enzymes may either be native or heterologous DHAD homologs or functional analogs or comprise a mutated or modified iron-sulfur cluster domain and/or redox active domain, allowing for a DHAD enzyme to be expressed in the yeast cytosol in a functional form. Thus, if an enzyme in the isobutanol production pathway was identified that was fully soluble and active in the cytosol of said recombinant microorganism, such enzyme can be used without addition of chaperone proteins not already present in the cytosol or without increased expression of chaperone proteins already present in the cytosol. However, some DHAD proteins may need the assistance of additional chaperones or increased chaperone levels to exhibit optimal cytosolic activity.

[00128] Therefore, in various embodiments described herein, the recombinant microorganisms may further comprise a nucleic acid encoding a chaperone protein, wherein said chaperone protein assists the folding of a protein exhibiting cytosolic activity. Addition of the chaperone protein can lead to improved activity, solubility, and/or correct folding of the DHAD enzyme. In one embodiment, the chaperone may be a native protein. In another embodiment, the chaperone protein may be an exogenous protein. In some embodiments, the chaperone protein may be selected from the group consisting of: endoplasmic reticulum oxidoreductin 1 (Ero1, accession no. NP_013576.1), including variants of Ero1 that have been suitably altered to reduce or prevent its normal localization to the endoplasmic reticulum; thioredoxins (which includes Trx1, accession no. NP_013144.1; and Trx2, accession no. NP_011725.1), thioredoxin reductase (Trr1, accession no. NP_010640.1); glutaredoxins (which includes Grx1, accession no. NP_009895.1; Grx2, accession no. NP_010801.1; Grx3, accession no. NP_010383.1; Grx4, accession no. NP_01101.1; Grx5, accession no. NP_015266.1; Grx6, accession no. NP_010274.1; Grx7, accession no. NP_009570.1; Grx8, accession no. NP_013468.1); glutathione reductase Glr1 (accession no. NP_015234.1); Jac1 (accession no. NP_011497.1), including variants of Jac1 that have been suitably altered to reduce or prevent its normal mitochondrial localization; Hsp60 and Hsp10 proteins (*e.g.,* yeast Hsp 60 and Hsp10 proteins, or other eukaryotic Hsp60 and Hsp10 homologs), bacterial chaperonin homologs (*e.g.*, GroEL and GroES proteins from *Lactococcus lactis*); homologs or active variants thereof, and combinations thereof.

[00129] As described herein, it is preferred that the DHAD enzymes are properly assembled and folded, thus allowing for said DHADs to exhibit maximal activity in the cytosol. In yeast, the DHAD Ilv3 is involved in biosynthesis of the amino acids leucine, isoleucine and valine. Ilv3 is typically localized to the mitochondria, where the chaperonin proteins Hsp60 and Hsp10 aid in the proper folding of the protein (Dubaquie *et. al.* The EMBO Journal 1998 17: 5868-5876). In wild-type yeast cells, Ilv3 is found in the soluble fraction of cell lysates. In extracts from an *hsp60* temperature-sensitive mutant, at the non-permissive temperature, there is no detectable soluble Ilv3. All of the protein is found in the insoluble fraction, in a presumably inactivated state. In an *hsp10* temperature-sensitive mutant, at the non-permissive temperature, about half of the Ilv3 is found in the insoluble portion, indicating that Hsp10 is also important for proper folding of Ilv3, but that Hsp60 is required. (Dubaquie *et. al.* The EMBO Journal 1998 17: 5868-5876).

[00130] Thus, in one embodiment of the present invention, wherein the yeast DHAD encoded by *ILV3* gene is used in the cytosol of a isobutanol-producing recombinant microorganism (*e.g.*, a yeast microorganism), Hsp60 and/or Hsp10 from the same yeast, homologs thereof from other microorganisms, or active variants thereof can be overexpressed in said microorganism to increase the activity, solubility, and/or correct folding of DHAD encoded by *ILV3* gene to increase the productivity, titer, and/or yield of isobutanol produced. Alternatively, if said microorganism is a yeast and it naturally expresses chaperonin proteins homologous to Hsp60 and/or Hsp10 in its cytosol, DHAD encoded by *ILV3* can be expressed in said yeast without the overexpression of the Hsp60 and/or the Hsp10 proteins. In another embodiment, wherein the DHAD derived from an organism other than yeast is used for isobutanol production, chaperonin homologs, or active variants thereof derived from said non-yeast organism or related non-yeast organism can be overexpressed together with the DHAD derived from said non-yeast organism. In one embodiment, said non-yeast organism is an eukaryotic organism. In another embodiment, said non-yeast organism is a prokaryotic organism. In a further embodiment, said non-yeast organism is a bacterium (*e.g.*, *E. coli.*, or *Lactococcus lactis*). For example, the *Lactococcus lactis* GroEL and GroES chaperonin proteins are expressed in the yeast cytosol in conjunction with the IlvD from *Lactococcus lactis*. Overexpression of these genes may be accomplished by methods as described herein.

[00131] Also disclosed herein are recombinant microorganisms comprising one or more genes encoding an iron-sulfur cluster assembly protein. Iron-sulfur cluster assembly for insertion into yeast apo-iron-sulfur proteins begins in yeast mitochondria. To assemble in yeast the active iron-sulfur proteins containing the cluster, either the apo-iron-sulfur protein is imported into the mitochondria from the cytosol and the iron-sulfur cluster is inserted into the protein and the active protein remains localized in the mitochondria; or the iron-sulfur clusters or precursors thereof are exported from the mitochondria to the cytosol and the active protein is assembled in the cytosol or other cellular compartments.

[00132] Targeting of yeast mitochondrial iron-sulfur proteins or non-yeast iron-sulfur proteins to the yeast cytosol can result in such proteins not being properly assembled with their iron-sulfur clusters. This present invention overcomes this problem by co-expression and cytosolic targeting in yeast of proteins for iron-sulfur cluster assembly and cluster insertion into apo-iron-sulfur proteins, including iron-

sulfur cluster assembly and insertion proteins from organisms other than yeast, together with the apo-iron-sulfur protein to provide assembly of active iron-sulfur proteins in the yeast cytosol.

[00133] In some embodiments, the present invention provides methods of using Fe-S cluster containing protein in the eukaryotic cytosol for improved isobutanol production in a microorganism, comprising overexpression of a Fe-S cluster-containing protein in the isobutanol production pathway in an microorganism. In a preferred embodiment, said microorganism is a yeast microorganism. In one embodiment, said Fe-S cluster-containing protein is a endogenous protein. In another embodiment, said Fe-S cluster-containing protein is an exogenous protein. In one embodiment, said Fe-S cluster-containing protein is derived from a eukaryotic organism. In another embodiment, said Fe-S cluster-containing protein is derived from a prokaryotic organism. In one embodiment, said Fe-S cluster-containing protein is DHAD. In one embodiment, said Fe-S cluster is a 2Fe-2S cluster. In another embodiment, said Fe-S cluster is a 4Fe-4S cluster.

[00134] All known DHAD enzymes contain an iron sulfur cluster, which is assembled *in vivo* by a multi-component pathway. DHADs contain one of at least two types of iron sulfur clusters, a 2Fe-2S cluster as typified by the spinach enzyme (Flint and Emptage, *JBC* 1988 263(8): 3558) or a 4Fe-4S cluster as typified by the *E. coli* enzyme (Flint *et. al., JBC* 1993 268(20): 14732). In eukaryotic cells, iron-sulfur cluster proteins can be found in either the cytosol or, more commonly, in the mitochondria. Within the mitochondria, a set of proteins, collectively similar to the ISC and/or SUF systems of *E. coli*, are present and participate in the assembly, maturation, and proper insertion of Fe-S clusters into mitochondrial target proteins. (Lill and Mühlenhoff, 2008, *Annu. Rev. Biochem.*, 77:669-700). In addition, a cytosolic iron sulfur assembly system is present and is collectively termed the CIA machinery. The CIA system promotes proper Fe-S cluster maturation and loading into cytosolically-localized iron sulfur proteins such as Leu1. Importantly, function of the CIA system is dependent on a critical (but still uncharacterized) factor exported from the mitochondria. In the yeast *S.cerevisiae*, the native DHAD, encoded by *ILV3*, is a mitochondrially-localized protein, where it is presumably properly recognized and activated by Fe-S cluster insertion by the endogenous machinery. Accordingly, ectopic expression of a DHAD in the yeast cytosol might be not expected to be functional due to its presence in a non-native compartment and the concomitant lack of appropriate Fe-S cluster assembly machinery.

[00135] The *E. coli* DHAD (encoded by *ilvD*) is sensitive to oxygen, becoming quickly inactivated when isolated under aerobic conditions (Flint *et. al., JBC* 1993 268(20): 14732; Brown *et. al. Archives Biochem. Biophysics* 1995 319(1): 10). It is thought that this oxygen sensitivity is due to the presence of a labile 4Fe-4S cluster, which is unstable in the presence of oxygen and reactive oxygen species, such as oxygen radicals and hydrogen peroxide. In yeast and other eukaryotes, the mitochondrial environment is reducing, *i.e.* it is a low oxygen environment, in contrast to the more oxygen-rich environment of the cytosol. The redox state of the cytosol is thus expected to be a problem for expressing mitochondrially localized DHADs, which are natively located in the mitochondria, or in expressing DHADs from many bacterial species which typically have an intracellular reducing environment. The spinach DHAD has been shown to be more oxygen resistant than the *E. coli* enzyme in *in vitro* assays (Flint and Emptage, JBC 1988 263(8):3558), which may be due to its endogenous localization to the plastid, where it would normally encounter a relatively high-oxygen environment. It has been suggested that DHADs with 2Fe-2S clusters are inherently more resistant to oxidative damage and they are therefore an attractive possibility for inclusion in the cytosolically localized isobutanol pathway.

[00136] An additional complication to the oxygen sensitivity of DHADs is that the iron sulfur clusters must be properly assembled and inserted into the enzyme such that an active enzyme results. There are several types of machinery that produce iron sulfur clusters and properly assemble them into proteins, including the NIF system found in bacteria and in some eukaryotes, the ISC system found in bacteria and mitochondria, the SUF system found in bacteria and plastids, and the CIA system found in the cytosol of eukaryotes.

[00137] Thus, the methods of using Fe-S cluster in the eukaryotic cytosol for improved enzymatic activity in isobutanol production pathway as described above may further comprise the co-expression a heterologous Fe-S cluster-containing DHAD with the NIF assembly system in the yeast cytosol to aid in assembling said heterologous DHADs. The NIF system found in the parasite *Entamoeba histolytica* has been shown to complement the double deletion of the *E. coli* ISC and SUF assembly systems (Ali et. al. JBC 2004 279(16): 16863) . The critical components of the *Entamoeba* assembly system comprise only two genes, NifS and NifU. In one embodiment, these two components are overexpressed in the yeast cytosol to increase activity and/or stability of cytosolic DHADs. In one embodiment, the NIF system is the *E. hisotlytica* NIF system; in another embodiment, the NIF system is

from other organisms (e.g. *Lactococcus lactis*). An advantage of using the *E. hisotlytica* assembly system is that it has already been demonstrated to work in a heterologous organism, *E. coli*.

[00138] A 2Fe-2S cluster-containing DHAD can be used in the present invention. In one embodiment, the 2Fe-2S cluster DHADs includes all known 2Fe-2S cluster dehydratase enzymes identified biochemically. In another embodiment, the 2Fe-2S cluster DHADs include those predicted to be 2Fe-2S cluster dehydratases containing some version of the consensus motif for 2Fe-2S cluster proteins, *e.g.*, the motif $CX_4CX_2CX_{\sim 30}C$ (SEQ ID NO: 39, Lill and Mühlenhoff, 2008, *Annu. Rev. Biochem.*, 77:669-700). For example, based on the extremely highly conserved DHAD gene sequences shared amongst plant species, the inventors have synthesized a likely 2Fe-2S DHAD from *Arabidopsis* (and rice, *Oryza sativa japonica*) which can be used to improve isobutanol production *in vivo* in the cytosolic isobutanol pathway.

[00139] Alternatively, a DHAD may be determined to be a 2Fe-2S protein or a 4Fe-4S protein based on a phylogenetic tree, such as Figure 2. Sequences not present on the example phylogenetic tree disclosed here could be added to the tree by one skilled in the art. Furthermore, once a new sequence was added to the DHAD phylogenetic tree, one skilled in the art may be able to determine if it is a 2Fe-2S or a 4Fe-4S cluster containing protein based on the phylogenetic relationship to known 2Fe-2S or a 4Fe-4S cluster containing DHADs.

[00140] In another embodiment, a 4Fe-4S cluster-containing DHAD could substitute for the 2Fe-2S cluster-containing DHAD in the cytosol. In one embodiment, said 4Fe-4S cluster DHAD is engineered to be oxygen resistant, and therefore more active in the cytosol of cells grown under aerobic conditions.

[00141] In one embodiment of this invention, the apo-iron-sulfur protein DHAD enzyme encoded by the *E. coli ilvD* gene is expressed in yeast together with *E. coli* iron-sulfur cluster assembly and insertion genes comprising either the *cyaY*, *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx* and *isuX* genes or the *sufA*, *sufB*, *sufC*, *sufD*, *sufS* and *sufE* genes. This strategy allows for both the apo-iron-sulfur protein (DHAD) and the iron-sulfur cluster assembly and insertion components (the products of the *isc* or *suf* genes) to come from the same organism, causing assembly of the active DHAD iron-sulfur protein in the yeast cytosol. As a modification of this embodiment, for those *E. coli* iron-sulfur cluster assembly and insertion components that localize to or are predicted to localize to the yeast mitochondria upon expression in yeast, the genes for these components are engineered to eliminate such targeting signals to ensure

localization of the components in the yeast cytoplasm. Thus, in some embodiments, one or more genes encoding an iron-sulfur cluster assembly protein may be mutated or modified to remove a signal peptide, whereby localization of the product of said one or more genes to the mitochondria is prevented. In certain embodiments, it may be preferable to overexpress one or more genes encoding an iron-sulfur cluster assembly protein.

[00142] In additional embodiments, iron-sulfur cluster assembly and insertion components from other than *E. coli* can be co-expressed with the *E. coli* DHAD protein to provide assembly of the active DHAD iron-sulfur cluster protein. Such iron-sulfur cluster assembly and insertion components from other organisms can consist of the products of the *Helicobacter pylori nifS* and *nifU* genes or the *Entamoeba histolytica nifS* and *nifU* genes. As a modification of this embodiment, for those non-*E. coli* iron-sulfur cluster assembly and insertion components that localize to or are predicted to localize to the yeast mitochondria upon expression in yeast, the genes for these components can be engineered to eliminate such targeting signals to ensure localization of the components in the yeast cytoplasm.

[00143] As a further modification of this embodiment, in addition to co-expression of these proteins in aerobically-grown yeast, these proteins may be co-expressed in anaerobically-grown yeast to lower the redox state of the yeast cytoplasm to improve assembly of the active iron-sulfur protein.

[00144] In another embodiment, the above iron-sulfur cluster assembly and insertion components can be co-expressed with DHAD apo-iron-sulfur enzymes other than the *E. coli* IlvD gene product to generate active DHAD enzymes in the yeast cytoplasm. As a modification of this embodiment, for those DHAD enzymes that localize to or are predicted to localize to the yeast mitochondria upon expression in yeast, then the genes for these enzymes can be engineered to eliminate such targeting signals to ensure localization of the enzymes in the yeast cytoplasm.

[00145] In additional embodiments, the above methods used to generate active DHAD enzymes localized to yeast cytoplasm may be combined with methods to generate active acetolactate synthase, KARI, KIVD and ADH enzymes in the same yeast for the production of isobutanol by yeast.

[00146] In another embodiment, production of active iron-sulfur proteins other than DHAD enzymes in yeast cytoplasm can be accomplished by co-expression with iron-sulfur cluster assembly and insertion proteins from organisms other than yeast, with

proper targeting of the proteins to the yeast cytoplasm if necessary and expression in anaerobically growing yeast if needed to improve assembly of the active proteins.

[00147] In another embodiment, the iron-sulfur cluster assembly protein encoding genes may be derived from eukaryotic organisms, including, but not limited to yeasts and plants. In one embodiment, the iron-sulfur cluster protein encoding genes are derived from a yeast organism, including, but not limited to *S. cerevisiae*. In specific embodiments, the yeast-derived genes encoding iron-sulfur cluster assembly proteins are selected from the group consisting of Cfd1 (accession no. NP_012263.1), Nbp35 (accession no. NP_011424.1), Nar1 (accession no. NP_014159.1), Cia1 (accession no. NP_010553.1), and homologs or variants thereof. In a further embodiment, the iron-sulfur cluster assembly protein encoding genes may be derived from plant nuclear genes which encode proteins translocated to chloroplasts or plant genes found in the chloroplast genome itself.

[00148] In certain embodiments described herein, it may be desirable to reduce or eliminate the activity and/or proteins levels of one or more iron-sulfur cluster containing cytosolic proteins. This modification increases the capacity of a yeast to incorporate [Fe-S] clusters into cytosolically expressed proteins wherein said proteins can be native proteins that are expressed in a non-native compartment or heterologous proteins. This is achieved by deletion of a highly expressed native cytoplasmic [Fe-S]-dependent protein. More specifically, the gene *LEU1* is deleted coding for the 3-isopropylmalate dehydratase which catalyses the conversion of 3-isopropylmalate into 2-isopropylmaleate as part of the leucine biosynthetic pathway in yeast. Leu1p contains an 4Fe-4S cluster which takes part in the catalysis of the dehydratase. Some DHAD enzymes also contain a 4Fe-4S cluster involved in its dehydratase activity. Therefore, although the two enzymes have different substrate preferences the process of incorporation of the Fe-S cluster is generally similar for the two proteins. Given that Leu1p is present in yeast at 10000 molecules per cell (Ghaemmaghami S. *et al. Nature* 2003 425: 737), deletion of *LEU1* therefore ensures that the cell has enough spare capacity to incorporate [Fe-S] clusters into at least 10000 molecules of cytosolically expressed DHAD. Taking into account the specific activity of DHAD (*E. coli* DHAD is reported to have a specific activity of 63 U/mg (Flint, D.H. *et al., JBC* 1993 268: 14732), the *LEU1* deletion yeast strain would generally exhibit an increased capacity for DHAD activity in the cytosol as measured in cell lysate.

[00149] In alternative embodiments, it may be desirable to further overexpress an

additional enzyme that converts 2,3-dihydroxyisovalerate to ketoisovalerate in the cytosol. In a specific embodiment, the enzyme may be selected from the group consisting of 3-isopropylmalate dehydratase (Leu1p) and imidazoleglycerol-phosphate dehydrogenase (His3p) or other dehydratases listed in Table 3.

Table 3. Dehydratases with putative activity towards 2,3-dihydroxyisovalerate.

Gene	Species	Native Substrate	Comments
dgoD	*E. coli*	D-galactonate	Acid-sugar dehydratases
rspA	*E. coli*	D-mannonate, D-altronate	
yfaW	*E. coli*	L-rhamnonate	
fucD	*X. campestris*	L-fuconate	
LGD1	*H. jecorina*	L-galactonate	
pdd	*K. oxytoca*	diols	Other non-Fe-S dehydratases
ENO1/2, ERR1/2/3	*S. cerevisiae*	2-phosphoglycerate	
HIS3	*S. cerevisiae*	Imidazoleglycerol-phosphate	

[00150] Because in some embodiments, DHAD activity may be limited in the cytosol, alternative dehydratases that convert dihydroxyisovalerate (DHIV) to 2-ketoisovalerate (KIV) and are physiologically localized to the yeast cytosol may be utilized. Leu1p and His3p and other enzymes encoded by genes listed in Table 3 are dehydratases that potentially may exhibit affinity for DHIV. Leu1p is an Fe-S binding protein that is involved in leucine biosynthesis and is also normally localized to the cytosol. His3p is involved in histidine biosynthesis and is similar to Leu1p, it is generally localized to the cytosol or predicted to be localized to the cytosol. This modification overcomes the problem of a DHAD that is limiting isobutanol production in the cytosol of yeast. The use of an alternative dehydratase that has activity in the cytosol with a low activity towards DHIV may thus be used in place of the DHAD in the isobutanol pathway. As described herein, such enzyme may be further engineered to increase activity with DHIV.

Increased Mitochondrial Export of Essential Components for Iron Sulfur Protein Assembly in the Cytosol

[00151] As noted herein, the third step in an exemplary isobutanol biosynthetic pathway is the conversion of dihydroxyisovalerate (DHIV) to ketoisovalerate (KIV) by

a dihydroxyacid dehydratase (DHAD). DHADs often require iron sulfur clusters for activity, and the native yeast DHAD acquires its iron sulfur cluster via the mitochondrial ISC machinery, remaining within the mitochondria as an active enzyme. However, isobutanol production by the engineered pathway requires DHAD to be functionally expressed within the cytosol, and such a DHAD presumably requires iron sulfur clusters to be added in the cytosol. One of the inventions disclosed herein addresses possible genetic or chemical approaches to increase the functional activity of cytosol DHADs. The present invention provides ways to increase the export of an essential compound that is generated in mitochondria, thereby increasing the amount of the compound available for use by the cytosolic iron sulfur assembly machinery (e.g. CIA) to effectively increase the functional expression of cytosolic DHADs.

Overexpressing Mitochondrial Iron Sulfur Cluster (ISC) Machinery

[00152] The compound generated within the mitochondrial matrix that is essential for iron sulfur protein assembly in the cytosol is subsequently exported through the ABC transporter, Atm1, and is chaperoned across the intermembrane space of the mitochondria to the cytosol by Erv1 (reviewed in Lill and Mühlenhoff, 2008, *Annu. Rev. Biochem.*, 77:669-700). *Sc*_BAT1 was identified as a third putative component of the mitochondrial export machinery required for the export of an unknown compound essential for cytosolic iron-sulfur cluster biosynthesis from the mitochondrial matrix to the cytosol by a genetic selection of suppressors of a *Sc_atm1* temperature sensitive allele (Kispal *et al*, 1996, *JBC*, 271:24458-24464). It is also suggested that a further strong indication for a direct functional relationship between Atm1p and Bat1p is the leucine auxotrophy associated with the deletion of the *ATM1* gene.

[00153] To facilitate export of the essential compound, the present invention provides in an embodiment recombinant microorganisms that have been engineered to overexpress one or more mitochondrial export proteins. In various embodiments described herein, the mitochondrial export protein may be selected from the group consisting of the *S. cerevisiae ATM1*, the *S. cerevisiae ERV1*, and the *S. cerevisiae BAT1*, or homologs thereof. Such manipulations can increase the export of the essential compound out of the mitochondria to increase the amount available for use by the cytosolic iron sulfur assembly machinery (e.g. CIA) to effectively increase the functional expression of cytosolic DHADs.

Increasing Inner Mitochondrial Membrane Electrical Potential

[00154] In one embodiment, the present invention provides recombinant microorganisms that have further been engineered to increase inner mitochondrial membrane potential, $\Delta\Psi_M$. As described herein, although yeast cells require a function mitochondrial compartment, they are viable without the mitochondrial genome (mtDNA). However, loss of mtDNA has been linked to destabilization of the nuclear genome (Veatch *et al.*, 2009, *Cell*, 137(7):1179-1181). Nuclear genome stability was restored in yeast lacking mtDNA when a suppressor mutation (*ATP1-111*) was introduced (Veatch *et al.*, 2009, *Cell*, 137(7):1179-1181, Francis *et al,,* 2007, *J. Bioenerg. Biomembr.* 39(2):149-157). The mutation has been shown to increase ATP hydrolysis activity of the mitochondrial ATP synthase, and similar mutations in the ATP synthase complex have also been shown to increase the electrical potential across the inner membrane of mitochondria, $\Delta\Psi_M$, in cells lacking mtDNA (Smith *et al.*, 2005, *Euk Cell*, 4(12):2057-2065; Kominsky *et al.*, 2002, *Genetics*, 162:1595-1604). Generation of $\Delta\Psi_M$ is required for efficient import of proteins into the mitochondrial matrix, including those involved in assembly and export of a complex required for the assembly of iron sulfur clusters into proteins in the cytosol. The link between $\Delta\Psi_M$ and iron sulfur cluster assembly in the cytosol is supported by microarray data that indicate that the transcriptional profile of cells lacking mtDNA (decreased $\Delta\Psi_M$) is similar to yeast grown under iron depletion conditions (Veatch *et al.*, 2009, *Cell*, 137(7):1179-1181). Introduction of the *ATP1-111* suppressor mutation restores the transcriptional profile to one resembling a wild-type cell's transcriptional profile (Veatch *et al.*, 2009, *Cell*, 137(7):1179-1181). Taken together, these data indicate that $\Delta\Psi_M$ must be sufficient to support assembly of cytosolic iron sulfur proteins, particularly those involved in nuclear genome stability (Veatch et al., *Cell* 2009, 137(7):1247-1258).

[00155] Thus, the present invention aims to generate the highest possible $\Delta\Psi_M$ in a yeast with an intact mitochondrial genome, allowing for the maximization the export of the complex required for assembly of cytosolic iron sulfur proteins, which can in turn increase the amount available for use by the cytosolic iron sulfur assembly machinery (*e.g.* CIA) to effectively increase the functional expression of cytosolic DHADs. $\Delta\Psi_M$ can be maximized several different ways, including, but not limited to: (1) Introducing mutations in the mitochondrial ATP synthase complex that increase ATP hydrolysis activity, or active variants thereof; (2) Overexpressing an ATP/ADP

carrier protein that leads to an increase ATP^{4-} import into the mitochondrial matrix in exchange for ADP^{3-}, contributing to generation of $\Delta\Psi_M$; (3) Removal and/or overexpression of additional gene(s) involved in generation of $\Delta\Psi_M$; and (4) Addition of chemical reagents that lead to an increase in $\Delta\Psi_M$.

[00156] In various embodiments described herein, the recombinant microorganism may comprise a mutation in the mitochondrial ATP synthase complex that increases ATP hydrolysis activity. In one embodiment, said mutant mitochondrial is an ATP synthase which can increase ATP hydrolysis activity is from a eukaryotic organism (*e.g.*, a yeast ATP1, ATP2, ATP3). In another embodiment, said mutant mitochondrial ATP synthase is from a prokaryotic organism (*e.g.,* bacteria). Non-limiting examples of said mutant mitochondrial ATP synthase include, mutant ATPase from the *ATP1-111* strain in Francis *et al., J Bioenerg Biomembr,* 2007, 39(2):127-144), a mutant ATPase from the *atp2-227* strain in Smith *et al.*, 2005, *Euk Cell*, 4(12):2057-2065, or a mutant ATPase from the *yme1* strain in Kominsky *et al.*, 2002, *Genetics*, 162:1595-1604). In another embodiment, active variants, or homologs of the mutant mitochondrial ATP synthases described above can be applied. In one embodiment, an ATP synthase having a homology to any of ATP1, ATP2, and ATP3 of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

[00157] In one embodiment, the inner mitochondrial membrane electrical potential can be increased by overexpressing an ATP/ADP carrier protein. Overexpression of the ATP/ADP carrier protein increases ATP^{4-} import into the mitochondrial matrix in exchange for ADP^{3-}. Non-limiting examples of ATP/ADP carrier proteins include the *S. cerevisiae*_AAC1 or the *S. cerevisiae*_AAC3, and active variants or homologs thereof. In one embodiment, an ATP/ADP carrier protein having a homology to either the *S. cerevisiae*_AAC1 or *S. cerevisiae*_AAC3 of at least about 70%, at least about 80%, or at least about 90% similarity can be used for a similar purpose.

[00158] In another embodiment, the inner mitochondrial membrane electrical potential can be increased by removal and/or overexpression of additional gene(s) involved in the generation of $\Delta\Psi_M$. A person skilled in the art will be familiar with proteins encoded by such genes. Non-limiting examples include the protein complexes in the mitochondrial electron transport chain which are responsible for establishing H^+ ions gradient. For examples, complexes on the inner membrane of mitochondria that are involved in conversion of NADH to NAD^+ (Complex I, NADH dehydrogenase), succinate to fumarate (Complex II, cytochrome bc_1 complex), and

oxygen to water (Complex IV, cytochrome c oxidase), which are responsible for the transfer of H^+ ions. In another embodiment, enzymes in the citric acid cycle in the matrix of mitochondria can be overexpressed to increase NADH and succinate production, such that more H^+ ions are available. These enzymes include, citrate synthase, aconitase, isocitrate dehydrogenase, α-Ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase.

[00159] In yet another embodiment, the inner mitochondrial membrane electrical potential can be increased by the addition of chemical reagents that lead to an increase in $\Delta\Psi_M$. In one embodiment, said chemical reagents are substrates in the citric acid cycle in the matrix of mitochondria, wherein when added into the culture, more NADH and succinate can be produced which in turn increase $\Delta\Psi_M$ in the mitochondria. Non-limiting examples of said substrates include, oxaloacetate, acetyl CoA,citrate, cis-Aconitate, isocitrate, oxalosuccinate, α-Ketoglutarate, succinyl-CoA, succinate, fumarate and L-Malate.

Enhancing Cytosolic DHADs Activity by Increasing Cytosol Sulfur Levels

[00160] Also provided herein are methods of increasing the levels of sulfur-containing compounds within yeast cells, including the amino acid cysteine, such that this sulfur is more available for the production of iron-sulfur cluster-containing proteins in the yeast cytosol or mitochondria. Specifically, by increasing the concentration of sulfur-containing compounds in the cell such, the activity of a functional DHAD is enhanced in the yeast cytosol or mitochondria.

[00161] Accordingly, the present invention provides in an embodiment recombinant microorganisms that have been engineered to overexpress one or more genes to increase biosynthesis of cysteine or uptake of exogenous cysteine by the cell in order to increase the amount and availability of sulfur-containing compounds for the production of active iron-sulfur cluster-containing proteins in the yeast cytosol or mitochondria. In one embodiment, the recombinant microorganisms have been engineered to increase the expression of one or more proteins to increase cysteine biosynthesis by the cell, including, but not limited to *MET3, MET14, MET16, MET10, MET5, MET1, MET8, MET2, MET17, HOM3, HOM2, HOM6, CYS3, CYS4, SUL1, SUL2,* active variants thereof, homologs thereof, and combination thereof, to increase cysteine biosynthesis by the cell. In another embodiment, the recombinant microorganisms have been engineered to increase the expression of one or more

transport proteins, including, but not limited to *YCT1, MUP1, GAP1, AGP1, GNP1, BAP1, BAP2, TAT1*, active variants thereof, homologs thereof, and combination thereof.

[00162] As noted above, increasing uptake of exogenous cysteine by the cell will increase the amount and availability of sulfur-containing compounds for the production of active iron-sulfur cluster containing proteins in the cytosol or mitochondria of the cell. Addition of increased exogenous cysteine to yeast cells, separately from or in addition to increased expression of the transport protein-encoding genes as described above, can also increase the level and availability of sulfur-containing compounds within the cell such that the sulfur is more available for the production of iron-sulfur cluster-containing proteins in the cell cytosol or mitochondria.

[00163] Sulfur is a necessary element for the biogenesis of iron-sulfur cluster (FeS cluster)-containing protein *in vivo*. Sulfur is a component of the FeS clusters that are incorporated into such proteins and is also a component of compounds such as glutathiones, which are essential for FeS cluster biogenesis in many organisms as well as being involved in cellular redox homeostasis. The direct source of the sulfur for these processes in many organisms is the amino acid cysteine. The sulfur from cysteine is mobilized into FeS clusters during FeS cluster biogenesis using cysteine desulfurase proteins identified in many organisms such as IscS, SufS (together with SufE), NifS and Nfs1 (together with Isd11). Additionally, glutathione biosynthesis requires cysteine.

[00164] Increased expression of Fe-S cluster-containing proteins in organisms such as the budding yeast *S. cerevisiae* results in an increased demand for sulfur, in the form of cysteine, in the cell. Such an increased demand for cysteine may possibly be met by natural induction of the endogenous cysteine biosynthetic pathway but maximal natural induction of this pathway may be insufficient to provide enough cysteine for the proper assemble and maintenance of increased levels of FeS cluster-containing proteins in the cell. Such cells with an increased demand for cysteine may also induce cysteine and/or sulfate transport pathways to bring in exogenous cysteine for or sulfate, which is the sulfur donor for cysteine biosynthesis. However, maximal natural induction of these transport systems may also be insufficient to meet the sulfur requirement of such cells.

[00165] Assembly of active FeS cluster-containing proteins in the native yeast cytosol requires the production and export to the cytosol by the mitochondria of an

unidentified sulfur-containing compound derived from the mitochondrial FeS cluster biogenesis pathway and the amino acid cysteine and requiring glutathione for export. Overexpression of an FeS cluster-containing protein in the yeast cytosol or the localization of a previously non-cytosolic FeS cluster-containing protein to the yeast cytosol may result in the decreased availability of this unidentified sulfur-containing compound in the yeast cytosol and low activity of the cytosolic FeS cluster-containing protein or proteins. Increased availability of cysteine to the cell may prevent this limitation by providing increased sulfur for the biosynthesis of this compound and sufficient glutathione for its export from the mitochondria.

[00166] Sulfur for the assembly of FeS cluster-containing proteins expressed in the yeast cytosol may also be provided by localization of cysteine desulfurase proteins to the yeast cytosol. Expression of such proteins in the yeast cytosol may result in an increased demand for cysteine by such cells, especially in the cytosol. Additionally, damage to the FeS cluster of FeS cluster-containing proteins expressed in the yeast cytosol, due to the oxic nature of the yeast cytosol or due to reactive oxygen or nitrogen species, may require additional sulfur derived from cysteine for repair or regeneration of the damaged clusters. As well, additional sulfur derived from cysteine may modulate the redox balance of the yeast cytosol through the production of increased levels of compounds such as glutathione which may positively affect the assembly or activity of FeS cluster-containing proteins in the yeast cytosol.

[00167] Increased cellular sulfur in the form of cysteine can be provided by increasing the biosynthesis of cysteine in the cell or by increasing cellular uptake of exogenous cysteine. Increasing the cellular level of cysteine in these ways is expected to increase the level of other sulfur-containing compounds in the cell that derive their sulfur from cysteine or the cysteine biosynthesis pathway. Cysteine biosynthesis in *S. cerevisiae* involves the uptake of exogenous sulfate by transport proteins encoded by the *SUL1* and/or *SUL2* genes and the action of the proteins encoded by the *MET3, MET14, MET16, MET10, MET5, MET1, MET8, MET2, MET17, HOM3, HOM2, HOM6, CYS4* and *CYS4* genes. Exogenous cysteine is taken up into *S. cerevisiae* by the high-affinity transport system encoded by the *YCT1* gene but also by the broader-specificity transport proteins encoded by the *MUP1, GAP1, AGP1, GNP1, BAP1, BAP2, TAT1* and *TAT2* genes.

[00168] Thus, in an additional aspect, the invention is directed to methods of increasing the levels of sulfur-containing compounds within the yeast cytosol and/or mitochondria, such that sulfur is more available for the production of iron-sulfur

cluster-containing proteins in the cytosol or mitochondria. In one embodiment, the levels of sulfur-containing compounds within the yeast cytosol and/or mitochondria are increased. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol or mitochondria leads to an increase in activity of a cytosolically expressed FeS cluster-containing protein DHAD, which catalyzes the reaction of 2,3-dihydroxyisovalerate to 2-ketoisovalerate. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol or mitochondria leads to an increase in activity of a cytosolically expressed DHAD. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol and/or mitochondria leads to an increase in activity of a cytosolically expressed DHAD and a subsequent increase in the productivity, titer, and/or yield of isobutanol produced by the DHAD-containing strain. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol or mitochondria leads to an increase in activity of a mitochondrially expressed FeS cluster-containing protein DHAD, which catalyzes the reaction of 2,3-dihydroxyisovalerate to 2-ketoisovalerate. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol or mitochondria leads to an increase in activity of a mitochondrially expressed DHAD. In another embodiment, an increase in sulfur-containing compounds in the yeast cytosol and/or mitochondria leads to an increase in activity of a mitochondrially expressed DHAD and a subsequent increase in the productivity, titer, and/or yield of isobutanol produced by the DHAD-containing strain.

[00169] In another embodiment, the genes *YCT1, MUP1, GAP1, AGP1, GNP1, BAP1, BAP2, TAT1*, and *TAT2*, active variants thereof, homologs thereof or combination thereof are overexpressed from a plasmid or by inserting multiple copies of the gene or genes into the chromosome under the control of a constitutive promoter. This embodiment can also be combined with providing increased extracellular cysteine to the yeast cells to provide increased sulfur-containing compounds in the cytosol and/or mitochondria of the cells. Overexpression of these genes may be accomplished by methods as described above.

[00170] In another embodiment, providing increased extracellular cysteine to the yeast cells in the absence of any additional engineered expression of transport proteins will provide increased sulfur containing compounds in the cytosol and/or mitochondria of the cells for the improved production of active FeS cluster-containing proteins in the yeast cytosol or mitochondria, which leads to increased isobutanol productivity, titer, and/or yield by the cell.

Enhancing Cytosolic DHAD Activity by Mitigating Oxidative Species or Oxidative Stress

[00171] The present application also describes methods of protecting enzymes in a DHAD-requiring biosynthetic pathway (specifically DHAD) in a microorganism to increase the production of beneficial metabolites by mitigating oxidative species or oxidative stress induced damage in the cytosol of said microorganism. Non-limiting examples of oxidative species include, nitric oxide (NO), reactive nitrogen species (RNS), reactive oxygen species (ROS), hydroxyl radical species, organic hydroperoxide, hypochlorous acids, and combinations thereof. As used herein, the phrase "reactive oxygen species" or "ROS" refers to free radicals that contain the oxygen atom. ROS are very small molecules that include oxygen ions and peroxides and can be either inorganic or organic. They are highly reactive due to the presence of unpaired valence shell electrons. During times of environmental stress (e.g. UV or heat exposure) ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation.

[00172] Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.

[00173] In chemical terms, oxidative stress is a large rise (becoming less negative) in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis.

[00174] A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides, and/or other reactive species. Some of the less reactive of these species (such as superoxide)

can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage. The major portion of long term effects is inflicted by damage on DNA. Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart. Non-limiting example of oxidants include, superoxide anion ($\bullet O_2^-$, formed in many autoxidation reactions and by the electron transport chain), hydrogen peroxide (H_2O_2, formed by disputation of $\bullet O_2^-$ or by direct reduction of O_2) , organic hydroperoxide (ROOH, formed by radical reactions with cellular components such as lipids and/or nucleobases), oxygen centered organic radicals (e.g., RO• alkoxy and ROO•, peroxy radicals, formed in the presence of oxygen by radical addition to double bonds or hydrogen abstraction), hypochlorous acid (HOCl, formed from H_2O_2 by myeloperoxidase, and peroxynitrite (ONOO-, formed in a rapid reaction between $\bullet O_2^-$ and NO•).

[00175] Biological defenses against oxidative damage include protective proteins that remove reactive oxygen species, molecules that sequester metal ions, and enzymes that repair damaged cellular components. Oxidative stress can be defined as a disturbance in the prooxidant-antioxidant balance in favor of prooxidants. One such class of prooxidants are reactive oxygen species, or ROS. ROS are highly reactive species of oxygen, such as superoxide ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), produced within the cell, usually as side products of aerobic respiration. By some reports, as much as 2% of the oxygen that enters the respiratory chain is converted to superoxide through a one-electron reduction of oxygen. A small amount of superoxide radical is always released from the enzyme when oxygen is reduced by electron carriers such as flavoproteins or cytochromes. This is because the electrons are transferred to oxygen one at a time. The hydroxyl radical and hydrogen peroxide are derived from the superoxide radical.

[00176] Many microbes possess native enzymes to detoxify these ROS. One example of such a system is superoxide dismutase (SOD) plus catalase. SOD catalyzes a reaction where one superoxide radical transfers its extra electron to the second radical, which is then reduced to hydrogen peroxide. Catalase catalyzes the transfer of two electrons from one hydrogen peroxide molecule to the second, oxidizing the first to oxygen and reducing the second to two molecules of water. If

the hydrogen peroxide is not disposed of, then it can oxidize transition metals, such as free iron(II) in the Fenton reaction, and form the free hydroxyl radical, OH·. No known mechanisms exists to detoxify hydroxyl radicals, and thus protection from toxic forms of oxygen must rely on eliminating superoxide and hydrogen peroxide.

[00177] In yeast, to counteract damage of oxidative stress, there are several antioxidant systems with an apparent functional redundancy. For example, there are detoxifying enzymes such as catalases, cytochrome c peroxidase, glutathione peroxidases, glytaredoxins and peroxiredoxins, and many isoforms in distinct cellular compartments (Jamieson *et al.*, 1998, *Yeast.* 14:1511-1527; Grant *et al.*, 2001, *Mol. Microbiol* 39:533-541; Collinson *et al.*, 2003, *J. Biol. Chem.* 278:22492-22497; Park *et al.*, 2000, *J. Biol. Chem.* 275:5723-5732).

[00178] As described above, an enzyme involved in the isobutanol production pathway, dihydroxyacid dehydratase (DHAD), contains an iron-sulfur (FeS) cluster domain. This iron-sulfur (FeS) cluster domain is sensitive to damage by ROS, which can lead to inactive enzyme. Both 2Fe-2S and 4Fe-4S DHAD enzymes may be susceptible to inactivation by ROS, however direct evidence exists for inactivation of 4Fe-4S cluster containing proteins, such as homoaconitase and isopropylmalate dehydratase in yeast and DHAD and fumarase from *E. coli*. Therefore, to achieve a functional DHAD expressed in the yeast cytosol in an environment where a substantial amount of ROS may exist from respiration, it may be beneficial to protect the DHAD enzyme from ROS inactivation or oxidative stress through expression of on or more enzymes that reduce or eliminate ROS from the cell.

[00179] To mitigate the potential harmful effects of reactive oxygen species (ROS) or oxidative stress on DHAD in the yeast cytosol, the present inventors have devised several strategies to protect or repair the DHAD from ROS damage. In various embodiments described herein, the invention provides recombinant microorganisms that have been engineered to express one or more proteins in the cytosol that reduce the concentration of reactive oxygen species (ROS) in said cytosol.

[00180] In one embodiment, enzymes that reduce or eliminate the amount of ROS in the cytosol are expressed and targeted to the yeast cytosol. Specifically, enzymes such as catalase, superoxide dismutase (SOD), cytochrome *c* peroxidase, glutathione peroxidases, glytaredoxins, peroxiredoxins, metallothioneins, and methionine sulphoxide reductases, or any isoforms thereof are expressed, such that they lead to reduction in ROS such as hydrogen peroxide, superoxide, peroxide radicals, and other ROS in the yeast cytosol.

[00181] In one embodiment, a catalase is expressed to reduce the concentration of ROS in the cytosol. In another embodiment, a superoxide dismutase (SOD) is expressed to reduce the concentration of ROS in the cytosol. Usually, microbes that grow by aerobic respiration possess one or both of SOD and catalase. For example, the bacterium *E. coli* and the yeast *Saccharomyces cerevisiae* each possesses at least one native SOD and catalase (e.g., SOD1 or SOD2 from yeast). In *E. coli*, the genes *kat*G and *kat*E encode catalase enzymes, and the genes *sod*A, *sod*B and *sod*C encode SodA, SodB, and SodC superoxide dismutase enzymes. respectively. In *S. cerevisiae*, the genes *CTT1* and *CTA1* encode catalase CTT1 and CTA1 enzymes, and the genes *SOD1* and *SOD2* encode SOD1 and SOD2 superoxide dismutase enzymes. Many other organisms possess catalase and SOD enzymes and these genes may also be useful for reduction of ROS in the yeast cytosol. In one embodiment, SOD homologs from species other than *E. coli* or yeast can be expressed in yeast cytosol to reduce oxidative stress. In one embodiment, said other species is a plant or a fungus. For example, *SOD*1 from *N. crassa* (fungus) may be functionally expressed in the yeast cytosol. In various embodiments described herein, active variants or homologs of the above-described catalases and SODs can be functionally expressed in the yeast cytosol. In another embodiment, protein having a homology to any one of the catalases or SODs described above possessing at least about 70%, at least about 80%, or at least about 90% similarity can be functionally expressed in the yeast cytosol.

[00182] In one embodiment, the catalase genes from *E. coli* are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, the catalase genes from *S. cerevisiae* are overexpressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In one embodiment, the SOD genes from *E. coli* are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, the *SOD* genes from *S. cerevisiae* are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, promoters of native genes are altered, such that the level of SOD or catalase in the *S. cerevisiae* cytosol is increased. In yet another embodiment, expression of SOD or catalase in the yeast

cytosol is mediated by a plasmid. In yet another embodiment, expression of SOD or catalase in the yeast cytosol is mediated by expression of one or more copies of the gene from the chromosome. Other homologs of catalase or SOD may be identified by one skilled in the art through tools such as BLAST and sequence alignment. These other homologs may be expressed in a similar manner described above to achieve a functional catalase or SOD in the yeast cytosol.

[00183] In another embodiment, a methionine sulphoxide reductase enzyme is expressed to reduce the amount of ROS and protect DHAD from ROS damage and inactivation. In one embodiment, the methionine sulphoxide reductase may be derived from a eukaryotic organism (*e.g.,* a yeast, fungus, or plant). In another embodiment, the methionine sulphoxide reductases may be derived from a prokaryotic organism (e.g., *E. coli*). The principal enzymatic mechanism for reversing protein oxidation acts on the oxidation product of just one amino acid residue, methionine. This specificity for Met reflects the fact that Met is particularly susceptible to oxidation compared with other amino acids. Methionine sulphoxide reductases (MSRs) are conserved across nearly all organisms from bacteria to humans, and have been the focus of considerable attention in recent years. Two MSR activities have been characterized in the yeast *Saccharomyces cerevisiae*: MsrA (encoded by MXR1) reduces the S stereoisomer of methionine sulphoxide (MetO), while MsrB (encoded by the YCL033c ORF), which we term here MXR2) reduces the R stereoisomer of MetO. Consistent with defense against oxidative damage, mutants deficient in MSR activity are hypersensitive to pro-oxidants such as H_2O_2, paraquat and Cr, while MSR overexpression enhances resistance. Besides methionine residues, iron–sulfur (FeS) clusters are exquisitely ROS-sensitive components of many cellular proteins. It has been reported that MSR activity helps to preserve the function of cellular FeS clusters.

[00184] In one embodiment, the methionine sulphoxide reductase genes from *S. cerevisiae* are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. Specifically, the *S. cerevisiae* methionine sulphoxide reductase genes MsrA (encoded by *MXR1*) and MsrB (encoded by the *YCL033c* ORF) are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. The resulting methionine sulphoxide reductase expressing strain will generally demonstrate improved isobutanol productivity, titer, and/or yield compared to the

parental strain that does not comprise methionine sulphoxide reductase genes that are expressed in and targeted to the cytosol. Methionine sulphoxide reductases from other organisms, such as bacteria, may be identified by sequence homology using tools such as BLAST and pairwise sequence alignments by one skilled in the art.

[00185] In yet another embodiment, expression or overexpression of glutathione synthesis enzymes, for example GSH1, leads to increased glutathione in the cell and protection of the DHAD enzyme in the yeast cytosol. In one embodiment, said enzymes are derived from a bacteria (*e.g.*, *E. coli.*). In another embodiment, said enzymes are derived from yeast (*e.g.*, *S.cerevisiae*). In yet another embodiment, said enzymes are derived from a yeast species different from the yeast used for isobutanol production.

[00186] In one embodiment, one or more metallothionein proteins are expressed in the yeast cytosol to mitigate oxidative stress. Metallothioneins are a family of proteins found in many organisms including yeast and mammals. The biologic function of metallothionein (MT) has been a perplexing topic ever since the discovery of this protein. Many studies have suggested that MT plays a role in the homeostasis of essential metals such as zinc and copper, detoxification of toxic metals such as cadmium, and protection against oxidative stress. MT contains high levels of sulfur. The mutual affinity of sulfur for transition metals makes the binding of these metals to MT thermodynamically stable. Under physiologic conditions, zinc-MT is the predominant form of the metal-binding protein. However, other metals such as copper (Cu) are also bound by MT. Oxidation of the thiolate cluster by a number of mild cellular oxidants causes metal release and formation of MT-disulfide (or thionin if all metals are released from MT, but this is unlikely to occur in vivo), which have been demonstrated *in vivo*. MT-disulfide can be reduced by glutathione in the presence of selenium catalyst, restoring the capacity of the protein to bind metals like Zn and Cu. This MT redox cycle may play a crucial role in MT biologic function. It may link to the homeostasis of essential metals, detoxification of toxic metals and protection against oxidative stress. In fact, MT has been shown to substitute for superoxide dismutase in yeast cells in the presence of Cu to protect cells and proteins from oxidative stress.

[00187] In one embodiment, said metallothuineins are derived from a eukaryotic organism (e.g., a yeast, fungus, or plant). In another embodiment, said metallothuineins are derived from a prokaryotic organism (e.g., *E. coli,*

Mycobacterium tuberculosis). For example, the metallothionein genes *CUP1-1* and *CUP1-2* encoding metallothionein CUP1 from *S. cerevisiae*, active variants thereof, homologs thereof, or combination thereof are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, *S. cerevisiae* metallothionein genes *CUP1-1* and *CUP1-2* are expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, Mycobacterium tuberculosis metallothionein gene *MymT* encoding metallothionein is expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD that is also expressed in and targeted to the yeast cytosol. In another embodiment, *Synechococcus PCC 7942* metallothionein gene *SmtA* is expressed in and targeted to the cytosol of yeast to reduce the amount of ROS and increase the activity of DHAD that is also expressed in and targeted to the yeast cytosol. The resulting metallothionein expressing strain has improved isobutanol productivity, titer, and/or yield compared to the parental strain. Metallothioneins from other organisms, such as bacteria, may be identified by sequence homology using tools such as BLAST and pairwise sequence alignments by one skilled in the art.

[00188] In another embodiment, one or more proteins in the thioredoxin system and/or the glutathione/glutaredoxin system, active variants thereof, homologs thereof, or combination thereof are expressed in the yeast cytosol to mitigate oxidative stress. In one embodiment, said proteins in the thioredoxin system and/or the glutathione/glutaredoxin system are derived from a eukaryotic organism (e.g., a yeast, fungus, or plant). In another embodiment, said proteins in the thioredoxin system and/or the glutathione/glutaredoxin system are derived from a prokaryotic organism (e.g., *E. coli*). The thioredoxin system and the glutathione/glutaredoxin system help maintain the reduced environment of the cell and play significant roles in defending the cell against oxidative stress. Glutathione is the major protective small molecule against oxidative stress in *Saccharomyces cerevisiae*. Glutathione, the tripeptide γ-glutamyl-cysteinyl-glycine, makes up the major free thiol pool present in millimolar concentrations in aerobic cells. The biosynthesis of glutathione requires γ-glutamyl cysteine synthase (termed Gsh1p) glutathione synthase (Gsh2p) and ATP. Glutathione is essential for viability of yeast but not of bacteria such as *E. coli*. Yeast cells lacking Gsh1p (genotype *gsh1Δ*) are able to survive in the presence of an

external source of glutathione. Deletion of the GSH1 gene encoding the enzyme that catalyzes the first step of glutathione biosynthesis leads to growth arrest, which can be relieved by either glutathione or reducing agents such as dithiothreitol. Evidence suggests that glutathione, in addition to its protective role against oxidative damage, performs a novel and specific function in the maturation of cytosolic Fe/S proteins. Therefore, increasing the levels of glutathione in the yeast cytosol is predicted to protect or increase the steady-state levels of active FeS cluster containing proteins expressed in the yeast cytosol. Specifically, increasing glutathione within the yeast cytosol may increase the amount of active DHAD enzyme expressed in the yeast cytosol, thereby leading to an increase in the titer, productivity, and/or yield of isobutanol produced from the pathway within which DHAD participates (*e.g.* the isobutanol pathway in Figure 1).

[00189] Thioredoxins and glutaredoxins are small heat-stable proteins with redox-active cysteines that facilitate the reduction of other proteins by catalyzing cysteine thiol-disulfide exchange reactions. The glutathione/glutaredoxin system consists of glutaredoxin, glutathione (produced by glutathione synthase), glutathione reductase and NADPH (as an electron donor). Thus, to increase the effective levels of available glutathione, one or a combination of each of the following enzymes is functionally overexpressed in the yeast cytosol: glutaredoxin (encoded in *S.cerevisiae* by *GRX2*, *GRX4*, *GRX6*, and *GRX7*), glutathione reductase (encoded in *S.cerevisiae* by *GLR1*); and glutathione synthase (encoded in *S.cerevisiae* by *GSH1* and *GSH2*). In one embodiment, homologs thereof, active variants thereof, or combination thereof can be expressed in the yeast cytosol to mitigate oxidative stress.

[00190] In another embodiment, the γ-glutamyl cysteine synthase and glutathione synthase genes from *S. cerevisiae* are expressed in and targeted to the cytosol of yeast to increase the amount of glutathione and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. In another embodiment, *S. cerevisiae* γ-glutamyl cysteine synthase and glutathione synthase genes *Gsh1* and *Gsh2* are expressed in and targeted to the cytosol of yeast to increase the amount of glutathione and increase the activity of DHAD also expressed in and targeted to the yeast cytosol. The resulting γ-glutamyl cysteine synthase and glutathione synthase expressing strain has improved isobutanol productivity, titer, and/or yield compared to the parental strain. Homologous genes encoding γ-glutamyl cysteine synthase

and glutathione synthase from other organisms, such as other yeast strains, may be identified by sequence homology using tools such as BLAST and pairwise sequence alignments by one skilled in the art.

[00191] Thioredoxins contain two conserved cysteines that exist in either a reduced form as in thioredoxin-$(SH)_2$) or in an oxidized form as in thioredoxin-S_2) when they form an intramolecular disulfide bridge. Thioredoxins donate electrons from their active center dithiol to protein disulfide bonds (Protein-S_2) that are then reduced to dithiols (Protein-$(SH)_2$). The resulting oxidized thioredoxin disulfide is reduced directly by thioredoxin reductase with electrons donated by NADPH. Hence the thioredoxin reduction system consists of thioredoxin, thioredoxin reductase, and NADPH. Oxidized glutaredoxins, on the other hand, are reduced by the tripeptide glutathione (gamma-Glu-Cys-Gly, known as GSH) using electrons donated by NADPH. Hence the glutathione/glutaredoxin system consists of glutaredoxin, glutathione, glutathione reductase and NADPH.

[00192] *S. cerevisiae* contains a cytoplasmic thioredoxin system comprised of the thioredoxins Trx1p and Trx2p and the thioredoxin reductase Trr1p, and a complete mitochondrial thioredoxin system comprised of the thioredoxin Trx3p and the thioredoxin reductase Trr2p. Evidence suggests that the cytoplasmic thioredoxin system may have overlapping function with the glutathione/glutaredoxin system. The mitochondrial thioredoxin system, on the other hand, does not appear to be able to substitute for either the cytoplasmic thioredoxin or glutathione/glutaredoxin systems. Instead, the mitochondrial thioredoxin proteins, thioredoxin (Trx3p) and thioredoxin reductase (Trr2p) have been implicated in the defense against oxidative stress generated during respiratory metabolism.

[00193] Overexpression of the essential cytosolic functional components of the thioredoxin system is thus predicted to increase the amount of bioavailable cytosolic thioredoxin, resulting in a significant increase in cellular redox buffering potential and concomitant increase in stable, active cytosolic FeS clusters and DHAD activity. Thus, one or more of the following genes are expressed either singly or in combination, thereby resulting in a functional increase in available thioredoxin: a thioredoxin (encoded in *S.cerevisiae* by *TRX1* and *TRX2*) and a thioredoxin reductase (encoded in *S.cerevisiae* by *TRR1*). Separately, or in combination with the aforementioned genes, the mitochondrial thioredoxin system (encoded by thioredoxin gene *TRX3* and thioredoxin reductase gene *TRR2*) are overexpressed, and, although functional in the mitochondria, provide an added or synergistic effect

on FeS cluster assembly or stability, as assayed by increased DHAD activity and/or output of isobutanol in a fermentation. Overexpression of these genes may be accomplished by methods as described above. In one embodiment, active variants of any one of the aforementioned thioredoxins or thioredoxin reductases, homologs thereof, or combination thereof are expressed in the yeast cytosol to mitigate oxidative stress.

Enhancing Cytosolic DHAD Activity by Mitigating Stress Mediated by Reactive Nitrogen Species (RNS)

[00194] Nitric oxide and reactive nitrogen species are highly reactive, short-lived molecules that can be generated during periods of cellular stress. The exact mechanisms by which these molecules are created, or their downstream targets, is not completely understood and is the subject of intense investigation. However, the functional groups present in many proteins -- for example, FeS clusters -- are readily attacked and inactivated by NO/RNS. Loss of these labile functional groups usually results in an inactive enzyme.

[00195] Nitric oxide and reactive nitrogen species are highly reactive, short-lived molecules that can be generated during normal cellular function, respiration, and during periods of cellular or redox stress. RNS are produced in eukaryotic cells starting with the reaction of nitric oxide (•NO) with superoxide (O2•−) to form peroxynitrite (ONOO−):

•NO (nitric oxide) + O2•− (super oxide) → ONOO− (peroxynitrite)

[00196] Peroxynitrite itself is a highly reactive species which can directly react with various components of the cell. Alternatively peroxynitrite can react with other molecules to form additional types of RNS including nitrogen dioxide ($•NO_2$) and dinitrogen trioxide (N_2O_3) as well as other types of chemically reactive radicals. Important reactions involving RNS include:

ONOO− + H+ → ONOOH (peroxynitrous acid) → $•NO_2$ (nitrogen dioxide) + •OH (hydroxyl radical)

ONOO− + CO_2 (carbon dioxide) → $ONOOCO_2$− (nitrosoperoxycarbonate)

$ONOOCO_2$− → $•NO_2$ (nitrogen dioxide) + O=C(O•)O− (carbonate radical)

•NO + $•NO_2$ is in equilibrium with N_2O_3 (dinitrogen trioxide)

[00197] NO exhibits other types of interaction that are candidates for mediating aspects of its physiological action. Notably, in a process known as nitrosylation, or nitrosation, NO can modify free sulfydryl (thiol) groups of cysteines in proteins to produce nitrosothiols, SNOs. Transfer of the NO adduct from one sulfydryl to another transnitrosylation) is likely to play a signal transduction role (reviewed in Stamler *et al.,*2001). Study of this post-translational modification, which is proposed to be a widespread mediator of signaling, is a relatively new field, and the list of proteins that are modified through nitrosylation is expanding rapidly. Because NO is highly reactive, transport of an NO signal in tissues can be facilitated through reaction with glutathione and movement of the resulting *S*-nitrosoglutathione (GSNO), which can subsequently signal by modifying thiol groups on target proteins by transnitrosylation (Lipton *et al.,* 2001; Foster *et al.,* 2003). The discovery of GSNO reductase (GSNOR), which reduces GSNO to restore GSH and to eliminate the NO adduct as NH^{4+} (Jensen *et al.,* 1998), revealed the importance of the control of this NO metabolite.

[00198] The exact mechanisms by which the aforementioned molecules are generated, or their downstream targets, are not completely understood and are the subject of intense investigation. However, the functional groups present in many proteins -- for example, FeS clusters -- are readily attacked by NO/RNS. The enzyme dihydroxyacid dehydratase (DHAD) contains an iron-sulfur (FeS) cluster cofactor that is sensitive to damage by NO or RNS. As an example of the biological sensitivity of this class of enzyme to attack by NO/RNS, inactivation of the *E.coli* DHAD (encoded by *ilvD*) and subsequent bacterial cell death resulting from macrophage-generated NO is a major component of the mammalian humoral immune response.

[00199] The present invention provides methods of mitigating the potentially harmful effects of oxidative and nitrosative stress (e.g., NO and/or or RNS) on enzymes involved in the production of isobutanol in the yeast cytosol. Specifically, the enzyme dihydroxyacid dehydratase (DHAD) contains an iron-sulfur (Fe-S) cluster that is sensitive to damage by NO and/or RNS, leading to inactive enzyme. Strategies of mitigating such harmful effects include, but are not limited to, increasing repair of iron-sulfur clusters damaged by oxidative and nitrosative stress conditions; reducing nitric oxide levels by introduction of a nitric oxide reductase (NOR) activity in the cell; reducing the levels of SNO's by overexpression of a GSNO-reductase; or

combination thereof.

[00200] Strategies disclosed herein are intended to protect or repair DHAD from NO/RNS damage. Accordingly, in one embodiment, the present invention provides recombinant microorganisms that have been engineered to express one or more enzymes in the cytosol that reduce the concentration of reactive nitrogen species (RNS) and/or nitric oxide in said cytosol.

[00201] In one embodiment, the present invention provides recombinant microorganisms that have been engineered to express a nitric oxide reductase that reduce the concentration of reactive nitrogen species (RNS) and/or nitric oxide in said cytosol. To reduce nitric oxide levels in the yeast cytosol, one or more nitric oxide reductases (NORs) or active variants thereof can be introduced into the cell by overexpression. Genes present in several microbial species have been shown to encode a nitric oxide reductase activity. For example, in *E.coli* the gene for a flavorubredoxin, *norV*, encodes a flavo-diiron NO reductase that is one of the most highly induced genes when *E.coli* cells are exposed to NO or GSNO. Previous work has identified a gene present in the microbe *Fusarium oxysporum* as encoding a cytochrome P-450 55A1 (P-450dNIR) that encodes a nitric oxide reductase (Nakahara *et al.*, 1993, *J. Biol. Chem. 268*:8350–8355). When expressed in a eukaryotic cell, this gene product appears to be cytosolically localized and exhibits effects consistent with its reducing intracellular NO levels (Dijkers *et al.,* 2009, *Molecular Biology of the Cell*, 20: 4083-4090). Thus, in one embodiment, homologs of any above-described nitric oxide reductases, active variants thereof, or combinations thereof are expressed in the yeast cytosol to mitigate nitric oxide.

[00202] In contrast to *E. coli* and *F. oxysporum*, *S. cerevisiae* lacks an endogenous NOR activity (and no homologs of either NOR protein is found in the *S. cerevisiae* genome). Thus, to provide such an activity, the *F. oxysporum* NOR gene is synthesized or amplified from genomic DNA, or the *E. coli norV* gene is amplified from genomic DNA, and either (or both) cloned into a suitable yeast expression vector. Such a vector could either be high copy (e.g., 2micron origin) or low copy (CEN/ARSH), or a single or multiple copies of the gene could be stably integrated into the genome of a host organism, specifically a yeast containing a cytosolic isobutanol pathway. In each case, methods to clone a gene into a plasmid so that it is expressed at a desired level under the control of a known yeast promoter (including those steps required to transform a host yeast cell) are well known to those skilled in the art. In those cases where the NOR gene is expressed from an

episomal plasmid, it can be advantageous to simultaneous overexpress a desired DHAD gene, either from the same or from another plasmid, thereby allowing one to assay the resulting output in DHAD activity. Similar approaches are undertaken to express the NOR gene in the presence of a plasmid(s) encoding an isobutanol production pathway, where the results of NOR expression are manifested in changes in isobutanol productivity, titer, or yield. It is understood by one skilled in the art that expression of all genes, both NOR and genes encoding the isobutanol pathway may be integrated into the genome of a host organism in a single or multiple copies of the gene(s), specifically a yeast containing a cytosolic isobutanol pathway.

[00203] In another embodiment, the present invention provides recombinant microorganisms that have been engineered to express a glutathione-S-nitrosothiol reductase (GSNO-reductase) that reduces the concentration of reactive nitrogen species (RNS) and/or nitric oxide in said cytosol. To reduce the levels of SNO's, one or more GSNO-reductases or active variants thereof can be introduced into the cell by overexpression. In *S. cerevisiae*, the gene *SFA1* has been shown to encode a formaldehyde dehydrogenase that possesses GSNO reductase activity (Liu *et al.*, 2001, *Nature 410*:490–494). Sfa1p is a member of the class III alcohol dehydrogenases (EC:1.1.1.284), which are bifunctional enzymes containing both alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase activities. The glutathione-dependent formaldehyde dehydrogenase activity of Sfa1p is required for the detoxification of formaldehyde, and the alcohol dehydrogenase activity of Sfa1p can catalyze the final reactions in phenylalanine and tryptophan degradation. Sfa1p is also able to act as a hydroxymethylfurfural (HMF) reductase and catabolize HMF, a compound formed in the production of certain biofuels. Sfa1p has been localized to the cytoplasm and the mitochondria, and can act on a variety of substrates, including S-hydroxymethylglutathione, phenylacetaldehyde, indole acetaldehyde, octanol, 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, and S-nitrosoglutathione.

[00204] Sfa1 protein levels are reported as being low-to-moderate from proteome-wide analyses (Ghaemmaghami *et al.*, 2003, *Nature 425*(6959):737-41). Thus, in an analogous fashion to the approach described for overexpression of NOR, the gene *SFA1* is overexpressed, thereby decoupling it from its normal regulatory control and permitting significant increase in Sfa1 activity in the cell, which results in measureable increases in DHAD activity and/or fermentation output, as described above. Overexpression of these genes may be accomplished by methods as

described above. In one embodiment, homologs of *SFA1*, active variants thereof, or combinations thereof are expressed in the yeast cytosol to mitigate stresses brought on by reactive nitrogen species.

[00205] In additional embodiments, alternative enzymes may be expressed and targeted to the yeast cytosol containing the isobutanol pathway to mitigate the effects of reactive nitrogen species. Specifically, the enzyme YtfE encoded by *E.coli ytfE*, homologs thereof, active variants thereof, may be expressed, such that they lead to reduction in NO/RNS in the yeast cytosol and/or a concomitant increase in DHAD function. Such an increase is detected by in vitro assay of DHAD activity, and/or by an increase in productivity, titer, or yield of isobutanol produced by isobutanol pathway-containing cells.

[00206] To increase repairment of iron-sulfur clusters, in one embodiment, the gene *ytfE* from *E.coli* is expressed in the yeast cytosol which contains a functional isobutanol pathway and DHAD such that DHAD activity and/or isobutanol productivity, titer, or yield are increased from the yeast cells. In *E. coli*, the gene *ytfE* has been shown to play an important role in maintaining active Fe-S clusters. A recent report (Justino et al., (2009). Escherichia coli Di-iron YtfE protein is necessary for the repair of stress-damaged Iron-Sulfur Clusters. *JBC* 282(14): 10352-10359) showed that Δ*ytfE* strains have several phenotypes, including enhanced susceptibility to nitrosative stress and are defective in the activity of several iron-sulfur-containing proteins. For example, the damage of the $[4Fe\text{-}4S]^{2+}$ clusters of aconitase B and fumarase A caused by exposure to hydrogen peroxide and nitric oxide stress occurs at higher rates in the absence of *ytfE*. The *ytfE* null mutation also abolished the recovery of aconitase and fumarase activities, which is observed in wild-type *E. coli* once the stress is scavenged. Notably, upon the addition of purified holo-YtfE protein to mutant cell extracts, the enzymatic activities of fumarase and aconitase were fully recovered, and at rates similar to the wild-type strain. Thus, YtfE is critical for the repair of iron-sulfur clusters damaged by oxidative and nitrosative stress conditions, and presents an attractive candidate for overexpression in a host cell that normally lacks this activity, such as *S. cerevisiae*, where Fe-S cluster proteins are also being overexpressed as part of the isobutanol pathway.

[00207] To provide such an activity, the *E.coli ytfE* gene can be amplified from genomic DNA by PCR with appropriate primers, and cloned into a suitable yeast expression vector. Such a vector could either be high copy (e.g., 2micron origin) or

low copy (CEN/ARS), or a single or multiple copies of the gene could be stably integrated into the genome of a host organism. In each case, methods to clone a gene into a plasmid so that it is expressed at a desired level under the control of a known yeast promoter (including those steps required to transform a host yeast cell) are well known to those skilled in the art. In those cases where the *ytfE* gene is expressed from an episomal plasmid, it can be advantageous to simultaneous overexpress a desired DHAD gene, either from the same or from another plasmid, thereby allowing one to assay the resulting output in DHAD activity. Similar approaches are undertaken to express the *ytfE* gene in the presence of a plasmid(s) encoding an isobutanol production pathway, where the results of *ytfE* expression are manifested in changes in isobutanol productivity, titer, or yield. More specifically, ytfE is expressed in the yeast cytosol which contains a functional isobutanol pathway and DHAD such that DHAD activity and/or isobutanol productivity, titer, or yield are increased from the yeast cells.

[00208] In addition, functional homologs of *E.coli* ytfE have been identified and characterized. For example, genes from two pathogenic prokaryotes—*scdA* from *Staphylococcus aureus*, and *dnrN* from *Neisseria gonorrhoeae*, have been shown to have properties similar to that of ytfE (Overton, T.W., et al (2008). Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulfur centers. *J. Bacteriology* 190(6): 2004-2013). Thus, similar approaches to overexpress either of these genes are employed, as described for *E.coli ytfE*, above. Overexpression of these genes may be accomplished by methods as described above.

The Microorganism in General

[00209] The recombinant microorganisms provided herein can express a plurality of heterologous and/or native target enzymes involved in pathways for the production of beneficial metabolites such as isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid from a suitable carbon source.

[00210] Accordingly, "engineered" or "modified" microorganisms are produced via the introduction of genetic material into a host or parental microorganism of choice and/or by modification of the expression of native genes, thereby modifying or altering the cellular physiology and biochemistry of the microorganism. Through the introduction of genetic material and/or the modification of the expression of native

genes the parental microorganism acquires new properties, *e.g.* the ability to produce a new, or greater quantities of, an intracellular metabolite. As described herein, the introduction of genetic material into and/or the modification of the expression of native genes in a parental microorganism results in a new or modified ability to produce beneficial metabolites such as isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid from a suitable carbon source. The genetic material introduced into and/or the genes modified for expression in the parental microorganism contains gene(s), or parts of genes, coding for one or more of the enzymes involved in a biosynthetic pathway for the production of one or more metabolites selected from isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid and may also include additional elements for the expression and/or regulation of expression of these genes, *e.g.* promoter sequences.

[00211] In addition to the introduction of a genetic material into a host or parental microorganism, an engineered or modified microorganism can also include alteration, disruption, deletion or knocking-out of a gene or polynucleotide to alter the cellular physiology and biochemistry of the microorganism. Through the alteration, disruption, deletion or knocking-out of a gene or polynucleotide the microorganism acquires new or improved properties (e.g., the ability to produce a new metabolite or greater quantities of an intracellular metabolite, improve the flux of a metabolite down a desired pathway, and/or reduce the production of byproducts).

[00212] Recombinant microorganisms provided herein may also produce metabolites in quantities not available in the parental microorganism. A "metabolite" refers to any substance produced by metabolism or a substance necessary for or taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material (*e.g.*, glucose or pyruvate), an intermediate (*e.g.*, 2-ketoisovalerate), or an end product (*e.g.*, isobutanol) of metabolism. Metabolites can be used to construct more complex molecules, or they can be broken down into simpler ones. Intermediate metabolites may be synthesized from other metabolites, perhaps used to make more complex substances, or broken down into simpler compounds, often with the release of chemical energy.

[00213] The disclosure identifies specific genes useful in the methods, compositions and organisms of the disclosure; however it will be recognized that absolute identity to such genes is not necessary. For example, changes in a particular gene or polynucleotide comprising a sequence encoding a polypeptide or

enzyme can be performed and screened for activity. Typically such changes comprise conservative mutations and silent mutations. Such modified or mutated polynucleotides and polypeptides can be screened for expression of a functional enzyme using methods known in the art.

[00214] Due to the inherent degeneracy of the genetic code, other polynucleotides which encode substantially the same or functionally equivalent polypeptides can also be used to clone and express the polynucleotides encoding such enzymes.

[00215] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms typically use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons. Codons can be substituted to reflect the preferred codon usage of the host, a process sometimes called "codon optimization" or "controlling for species codon bias."

[00216] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al*., 1989, *Nucl Acids Res.* 17: 477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, typical stop codons for *S. cerevisiae* and mammals are UAA and UGA, respectively. The typical stop codon for monocotyledonous plants is UGA, whereas insects and *E. coli* commonly use UAA as the stop codon (Dalphin *et al*., 1996, *Nucl Acids Res.* 24: 216-8). Methodology for optimizing a nucleotide sequence for expression in a plant is provided, for example, in U.S. Pat. No. 6,015,891, and the references cited therein.

[00217] Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given enzyme of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described above are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss

or significant loss of a desired activity. The disclosure includes such polypeptides with different amino acid sequences than the specific proteins described herein so long as they modified or variant polypeptides have the enzymatic anabolic or catabolic activity of the reference polypeptide. Furthermore, the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

[00218] In addition, homologs of enzymes useful for generating metabolites are encompassed by the microorganisms and methods provided herein.

[00219] As used herein, two proteins (or a region of the proteins) are substantially homologous when the amino acid sequences have at least about 30%, 40%, 50% 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In one embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, typically at least 40%, more typically at least 50%, even more typically at least 60%, and even more typically at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00220] When "homologous" is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (*e.g.*, charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences

differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (See, *e.g.*, Pearson W.R., 1994, *Methods in Mol Biol* 25: 365-89.

[00221] The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[00222] Sequence homology for polypeptides, which is also referred to as percent sequence identity, is typically measured using sequence analysis software. See commonly owned and co-pending application US 2009/0226991. A typical algorithm used comparing a molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST. When searching a database containing sequences from a large number of different organisms, it is typical to compare amino acid sequences. Database searching using amino acid sequences can be measured by algorithms described in commonly owned and co-pending application US 2009/0226991.

[00223] It is understood that a range of microorganisms can be modified to include a recombinant metabolic pathway suitable for the production of beneficial metabolites from DHAD-requiring biosynthetic pathways. In various embodiments, microorganisms may be selected from yeast microorganisms. Yeast microorganisms for the production of a metabolite such as isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid may be selected based on certain characteristics:

[00224] One characteristic may include the property that the microorganism is selected to convert various carbon sources into beneficial metabolites such as isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid. The term “carbon source” generally refers to a substance suitable to be used as a source of carbon for prokaryotic or eukaryotic cell growth. Examples of suitable carbon sources are described in commonly owned and co-pending application US 2009/0226991. Accordingly, in one embodiment, the recombinant microorganism herein disclosed can convert a variety of carbon sources to products, including but not limited to glucose, galactose, mannose, xylose, arabinose, lactose, sucrose, and mixtures thereof.

[00225] The recombinant microorganism may thus further include a pathway for the production of isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and/or pantothenic acid from five-carbon (pentose) sugars including xylose. Most yeast species metabolize xylose via a complex route, in which xylose is first reduced to xylitol via a xylose reductase (XR) enzyme. The xylitol is then oxidized to xylulose via a xylitol dehydrogenase (XDH) enzyme. The xylulose is then phosphorylated via an xylulokinase (XK) enzyme. This pathway operates inefficiently in yeast species because it introduces a redox imbalance in the cell. The xylose-to-xylitol step uses NADH as a cofactor, whereas the xylitol-to-xylulose step uses NADPH as a cofactor. Other processes must operate to restore the redox imbalance within the cell. This often means that the organism cannot grow anaerobically on xylose or other pentose sugar. Accordingly, a yeast species that can efficiently ferment xylose and other pentose sugars into a desired fermentation product is therefore very desirable.

[00226] Thus, in one aspect, the recombinant is engineered to express a functional exogenous xylose isomerase. Exogenous xylose isomerases functional in yeast are known in the art. See, e.g., Rajgarhia *et al,* US2006/0234364, which is herein incorporated by reference in its entirety. In an embodiment according to this aspect, the exogenous xylose isomerase gene is operatively linked to promoter and terminator sequences that are functional in the yeast cell. In a preferred embodiment, the recombinant microorganism further has a deletion or disruption of a native gene that encodes for an enzyme (e.g. XR and/or XDH) that catalyzes the conversion of xylose to xylitol. In a further preferred embodiment, the recombinant microorganism also contains a functional, exogenous xylulokinase (XK) gene operatively linked to promoter and terminator sequences that are functional in the yeast cell. In one embodiment, the xylulokinase (XK) gene is overexpressed.

[00227] In one embodiment, the microorganism has reduced or no pyruvate decarboxylase (PDC) activity. PDC catalyzes the decarboxylation of pyruvate to acetaldehyde, which is then reduced to ethanol by ADH via an oxidation of NADH to NADH+. Ethanol production is the main pathway to oxidize the NADH from glycolysis. Deletion of this pathway increases the pyruvate and the reducing equivalents (NADH) available for the DHAD-requiring biosynthetic pathway. Accordingly, deletion of *PDC* genes can further increase the yield of desired metabolites.

[00228] In another embodiment, the microorganism has reduced or no glycerol-3-

phosphate dehydrogenase (GPD) activity. GPD catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) via the oxidation of NADH to NAD+. Glycerol is then produced from G3P by Glycerol-3-phosphatase (GPP). Glycerol production is a secondary pathway to oxidize excess NADH from glycolysis. Reduction or elimination of this pathway would increase the pyruvate and reducing equivalents (NADH) available for the DHAD-requiring biosynthetic pathway. Thus, deletion of *GPD* genes can further increase the yield of desired metabolites.

[00229] In yet another embodiment, the microorganism has reduced or no PDC activity and reduced or no GPD activity. PDC-minus/GPD-minus yeast production strains are described in co-pending applications US 12/343,375 (published as US 2009/0226991), US 12/696,645, and US 12/820,505, which claim priority to US Provisional Application 61/016,483, all of which are herein incorporated by reference in their entireties for all purposes.

[00230] In one embodiment, the yeast microorganisms may be selected from the *"Saccharomyces* Yeast Clade", as described in commonly owned and co-pending application US 2009/0226991.

[00231] The term "Saccharomyces *sensu stricto*" taxonomy group is a cluster of yeast species that are highly related to *S. cerevisiae* (Rainieri *et al.*, 2003, *J. Biosci Bioengin* 96: 1-9). Saccharomyces *sensu stricto* yeast species include but are not limited to *S. cerevisiae, S. cerevisiae*, *S. kudriavzevii, S. mikatae, S. bayanus, S. uvarum, S. carocanis* and hybrids derived from these species (Masneuf *et al.*, 1998, *Yeast* 7: 61-72).

[00232] An ancient whole genome duplication (WGD) event occurred during the evolution of the hemiascomycete yeast and was discovered using comparative genomic tools (Kellis *et al.*, 2004, *Nature* 428: 617-24; Dujon *et al.*, 2004, *Nature* 430:35-44; Langkjaer *et al.*, 2003, *Nature* 428: 848-52; Wolfe *et al.*, 1997, *Nature* 387: 708-13). Using this major evolutionary event, yeast can be divided into species that diverged from a common ancestor following the WGD event (termed "post-WGD yeast" herein) and species that diverged from the yeast lineage prior to the WGD event (termed "pre-WGD yeast" herein).

[00233] Accordingly, in one embodiment, the yeast microorganism may be selected from a post-WGD yeast genus, including but not limited to Saccharomyces and Candida. The favored post-WGD yeast species include: *S. cerevisiae, S. uvarum, S. bayanus, S. paradoxus, S. castelli,* and *C. glabrata.*

[00234] In another embodiment, the yeast microorganism may be selected from a pre-whole genome duplication (pre-WGD) yeast genus including but not limited to Saccharomyces, Kluyveromyces, Candida, Pichia, Issatchenkia, Debaryomyces, Hansenula, Yarrowia and, Schizosaccharomyces. Representative pre-WGD yeast species include: *S. kluyveri, K. thermotolerans, K. marxianus, K. waltii, K. lactis, C. tropicalis, P. pastoris, P. anomala, P. stipitis, I. orientalis, I. occidentalis, I. scutulata, D. hansenii, H. anomala, Y. lipolytica,* and *S. pombe.*

[00235] A yeast microorganism may be either Crabtree-negative or Crabtree-positive as described in described in commonly owned and co-pending application US 2009/0226991. In one embodiment the yeast microorganism may be selected from yeast with a Crabtree-negative phenotype including but not limited to the following genera: Kluyveromyces, Pichia, Issatchenkia, Hansenula, and Candida. Crabtree-negative species include but are not limited to: *K. lactis, K. marxianus, P. anomala, P. stipitis, I. orientalis, I. occidentalis, I. scutulata, H. anomala,* and *C. utilis.* In another embodiment, the yeast microorganism may be selected from a yeast with a Crabtree-positive phenotype, including but not limited to Saccharomyces, Kluyveromyces, Zygosaccharomyces, Debaryomyces, Pichia and Schizosaccharomyces. Crabtree-positive yeast species include but are not limited to: *S. cerevisiae, S. uvarum, S. bayanus, S. paradoxus, S. castelli, S. kluyveri, K. thermotolerans, C. glabrata, Z. bailli, Z. rouxii, D. hansenii, P. pastorius,* and *S. pombe*.

[00236] Another characteristic may include the property that the microorganism is that it is non-fermenting. In other words, it cannot metabolize a carbon source anaerobically while the yeast is able to metabolize a carbon source in the presence of oxygen. Nonfermenting yeast refers to both naturally occurring yeasts as well as genetically modified yeast. During anaerobic fermentation with fermentative yeast, the main pathway to oxidize the NADH from glycolysis is through the production of ethanol. Ethanol is produced by alcohol dehydrogenase (ADH) via the reduction of acetaldehyde, which is generated from pyruvate by pyruvate decarboxylase (PDC). In one embodiment, a fermentative yeast can be engineered to be non-fermentative by the reduction or elimination of the native PDC activity. Thus, most of the pyruvate produced by glycolysis is not consumed by PDC and is available for the isobutanol pathway. Deletion of this pathway increases the pyruvate and the reducing equivalents available for the DHAD-requiring biosynthetic pathway. Fermentative pathways contribute to low yield and low productivity of desired metabolites such as

isobutanol. Accordingly, deletion of *PDC* may increase yield and productivity of desired metabolites such as isobutanol.

[00237] In some embodiments, the recombinant microorganisms may be microorganisms that are non-fermenting yeast microorganisms, including, but not limited to those, classified into a genera selected from the group consisting of *Tricosporon*, *Rhodotorula*, *Myxozyma*, or *Candida*. In a specific embodiment, the non-fermenting yeast is *C. xestobii*.

Isobutanol-Producing Yeast Microorganisms

[00238] As described herein, in one embodiment, a yeast microorganism is engineered to convert a carbon source, such as glucose, to pyruvate by glycolysis and the pyruvate is converted to isobutanol via an isobutanol producing metabolic pathway (See, *e.g.*, WO/2007/050671, WO/2008/098227, and Atsumi *et al., 2008, Nature* 45: 86-9). Alternative pathways for the production of isobutanol have been described in WO/2007/050671 and in Dickinson *et al.*, 1998, *J Biol Chem* 273:25751-6.

[00239] Accordingly, in one embodiment, the isobutanol producing metabolic pathway to convert pyruvate to isobutanol can be comprised of the following reactions:

1. 2 pyruvate → acetolactate + CO_2
2. acetolactate + NAD(P)H → 2,3-dihydroxyisovalerate + $NAD(P)^+$
3. 2,3-dihydroxyisovalerate → alpha-ketoisovalerate
4. alpha-ketoisovalerate → isobutyraldehyde + CO_2
5. isobutyraldehyde +NAD(P)H → isobutanol + NADP

[00240] These reactions are carried out by the enzymes 1) Acetolactate Synthase (ALS), 2) Keto-acid Reducto-Isomerase (KARI), 3) Dihydroxy-acid dehydratase (DHAD), 4) Keto-isovalerate decarboxylase (KIVD), and 5) an Alcohol dehydrogenase (ADH) (Figure 1). In another embodiment, the yeast microorganism is engineered to overexpress these enzymes. For example, these enzymes can be encoded by native genes. Alternatively, these enzymes can be encoded by heterologous genes. For example, ALS can be encoded by the *alsS* gene of *B. subtilis*, *alsS* of *L. lactis*, or the *ilvK* gene of *K. pneumonia*. For example, KARI can be encoded by the *ilvC* genes of *E. coli, C. glutamicum, M. maripaludis, or Piromyces sp E2*. For example, DHAD can be encoded by the *ilvD* genes of *E. coli*,

C. glutamicum, or L. lactis. For example, KIVD can be encoded by the *kivD* gene of *L. lactis*. ADH can be encoded by *ADH2, ADH6,* or ADH7 of *S. cerevisiae.*

[00241] In one embodiment, pathway steps 2 and 5 may be carried out by KARI and ADH enzymes that utilize NADH (rather than NADPH) as a co-factor. Such enzymes are described in commonly owned and co-pending applications US 12/610,784 and PCT/US09/62952 (published as WO/2010/051527), which are herein incorporated by reference in their entireties for all purposes. The present inventors have found that utilization of NADH-dependent KARI and ADH enzymes to catalyze pathway steps 2 and 5, respectively, surprisingly enables production of isobutanol under anaerobic conditions. Thus, in one embodiment, the recombinant microorganisms of the present invention may use an NADH-dependent KARI to catalyze the conversion of acetolactate (+NADH) to produce 2,3-dihydroxyisovalerate. In another embodiment, the recombinant microorganisms of the present invention may use an NADH-dependent ADH to catalyze the conversion of isobutyraldehyde (+NADH) to produce isobutanol. In yet another embodiment, the recombinant microorganisms of the present invention may use both an NADH-dependent KARI to catalyze the conversion of acetolactate (+NADH) to produce 2,3-dihydroxyisovalerate, and an NADH-dependent ADH to catalyze the conversion of isobutyraldehyde (+NADH) to produce isobutanol.

[00242] In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to isobutanol. In one embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to isobutyraldehyde. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to keto-isovalerate. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to 2,3-dihydroxyisovalerate. In another embodiment, the yeast microorganism may be engineered to have increased ability to convert pyruvate to acetolactate.

[00243] Furthermore, any of the genes encoding the foregoing enzymes (or any others mentioned herein (or any of the regulatory elements that control or modulate expression thereof)) may be optimized by genetic/protein engineering techniques, such as directed evolution or rational mutagenesis, which are known to those of ordinary skill in the art. Such action allows those of ordinary skill in the art to optimize the enzymes for expression and activity in yeast.

[00244] In addition, genes encoding these enzymes can be identified from other fungal and bacterial species and can be expressed for the modulation of this pathway. A variety of organisms could serve as sources for these enzymes, including, but not limited to, *Saccharomyces* spp., including *S. cerevisiae* and *S. uvarum*, *Kluyveromyces* spp., including *K. thermotolerans*, *K. lactis*, and *K. marxianus*, *Pichia* spp., *Hansenula* spp., including *H. polymorpha*, *Candida* spp., *Trichosporon* spp., *Yamadazyma* spp., including *Y.* spp. *stipitis*, *Torulaspora pretoriensis*, *Schizosaccharomyces* spp., including *S. pombe*, *Cryptococcus* spp., *Aspergillus* spp., *Neurospora* spp., or *Ustilago* spp. Sources of genes from anaerobic fungi include, but not limited to, *Piromyces* spp., *Orpinomyces* spp., or *Neocallimastix* spp. Sources of prokaryotic enzymes that are useful include, but not limited to, *Escherichia. coli*, *Zymomonas mobilis*, *Staphylococcus aureus*, *Bacillus* spp., *Clostridium* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Lactococcus* spp., *Enterobacter* spp., and *Salmonella* spp.

Methods in General

Identification of an Aft Protein in a Microorganism

[00245] Any method can be used to identify genes that encode for proteins with Aft activity. Aft1 and Aft2 enhance cellular iron availability. Generally, genes that are homologous or similar to a known *AFT* gene, e.g. *S. cerevisiae AFT1* (encoding for SEQ ID NO: 2) or *S. cerevisiae AFT2* (encoding for SEQ ID NO: 4) can be identified by functional, structural, and/or genetic analysis. In most cases, homologous or similar *AFT* genes and/or homologous or similar Aft proteins will have functional, structural, or genetic similarities. Techniques known to those skilled in the art may be suitable to identify homologous genes and homologous enzymes. Generally, analogous genes and/or analogous enzymes can be identified by functional analysis and will have functional similarities. Techniques known to those skilled in the art may be suitable to identify analogous genes and analogous enzymes. For example, to identify homologous or analogous genes, proteins, or enzymes, techniques may include, but not limited to, cloning a *AFT* gene by PCR using primers based on a published sequence of a gene/enzyme or by degenerate PCR using degenerate primers designed to amplify a conserved region among *AFT* genes. Further, one skilled in the art can use techniques to identify homologous or analogous genes, proteins, or enzymes with functional homology or similarity. For instance, the computer program BLAST may be used for such a purpose. To identify homologous

or similar genes and/or homologous or similar proteins, analogous genes and/or analogous proteins, techniques also include comparison of data concerning a candidate gene or enzyme with databases such as BRENDA, KEGG, or MetaCYC. The candidate gene or enzyme may be identified within the above mentioned databases in accordance with the teachings herein.

Identification of PDC and GPD in a Yeast Microorganism

[00246] Any method can be used to identify genes that encode for enzymes with pyruvate decarboxylase (PDC) activity or glycerol-3-phosphate dehydrogenase (GPD) activity. Suitable methods for the identification of PDC and GPD are described in co-pending applications US 12/343,375 (published as US 2009/0226991), US 12/696,645, and US 12/820,505, which claim priority to US Provisional Application 61/016,483, all of which are herein incorporated by reference in their entireties for all purposes.

Genetic Insertions and Deletions

[00247] Any method can be used to introduce a nucleic acid molecule into yeast and many such methods are well known. For example, transformation and electroporation are common methods for introducing nucleic acid into yeast cells. *See*, *e.g.*, Gietz *et al*., 1992, *Nuc Acids Res*. 27: 69-74; Ito *et al.*, 1983, *J. Bacteriol*. 153: 163-8; and Becker *et al.*, 1991, *Methods in Enzymology* 194: 182-7.

[00248] In an embodiment, the integration of a gene of interest into a DNA fragment or target gene of a yeast microorganism occurs according to the principle of homologous recombination. According to this embodiment, an integration cassette containing a module comprising at least one yeast marker gene and/or the gene to be integrated (internal module) is flanked on either side by DNA fragments homologous to those of the ends of the targeted integration site (recombinogenic sequences). After transforming the yeast with the cassette by appropriate methods, a homologous recombination between the recombinogenic sequences may result in the internal module replacing the chromosomal region in between the two sites of the genome corresponding to the recombinogenic sequences of the integration cassette. (Orr-Weaver *et al*., 1981, *PNAS USA* 78: 6354-58).

[00249] In an embodiment, the integration cassette for integration of a gene of interest into a yeast microorganism includes the heterologous gene under the control of an appropriate promoter and terminator together with the selectable marker flanked by recombinogenic sequences for integration of a heterologous gene into the

yeast chromosome. In an embodiment, the heterologous gene includes an appropriate native gene desired to increase the copy number of a native gene(s). The selectable marker gene can be any marker gene used in yeast, including but not limited to, *HIS3*, *TRP1*, *LEU2*, *URA3*, *bar*, *ble*, *hph*, and *kan*. The recombinogenic sequences can be chosen at will, depending on the desired integration site suitable for the desired application.

[00250] In another embodiment, integration of a gene into the chromosome of the yeast microorganism may occur via random integration (Kooistra *et al.*, 2004, *Yeast* 21: 781-792).

[00251] Additionally, in an embodiment, certain introduced marker genes are removed from the genome using techniques well known to those skilled in the art. For example, URA3 marker loss can be obtained by plating *URA3* containing cells in FOA (5-fluoro-orotic acid) containing medium and selecting for FOA resistant colonies (Boeke *et al.*, 1984, *Mol. Gen. Genet* 197: 345-47).

[00252] The exogenous nucleic acid molecule contained within a yeast cell of the disclosure can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state that can stably be passed on ("inherited") to daughter cells. Such extra-chromosomal genetic elements (such as plasmids, mitochondrial genome, *etc.*) can additionally contain selection markers that ensure the presence of such genetic elements in daughter cells. Moreover, the yeast cells can be stably or transiently transformed. In addition, the yeast cells described herein can contain a single copy, or multiple copies of a particular exogenous nucleic acid molecule as described above.

Reduction of Enzymatic Activity

[00253] Yeast microorganisms within the scope of the invention may have reduced enzymatic activity such as reduced glycerol-3-phosphate dehydrogenase activity. The term "reduced" as used herein with respect to a particular enzymatic activity refers to a lower level of enzymatic activity than that measured in a comparable yeast cell of the same species. The term reduced also refers to the elimination of enzymatic activity than that measured in a comparable yeast cell of the same species. Thus, yeast cells lacking glycerol-3-phosphate dehydrogenase activity are considered to have reduced glycerol-3-phosphate dehydrogenase activity since most, if not all, comparable yeast strains have at least some glycerol-3-phosphate

dehydrogenase activity. Such reduced enzymatic activities can be the result of lower enzyme concentration, lower specific activity of an enzyme, or a combination thereof. Many different methods can be used to make yeast having reduced enzymatic activity. For example, a yeast cell can be engineered to have a disrupted enzyme-encoding locus using common mutagenesis or knock-out technology. In addition, certain point-mutation(s) can be introduced which results in an enzyme with reduced activity.

[00254] Alternatively, antisense technology can be used to reduce enzymatic activity. For example, yeast can be engineered to contain a cDNA that encodes an antisense molecule that prevents an enzyme from being made. The term "antisense molecule" as used herein encompasses any nucleic acid molecule that contains sequences that correspond to the coding strand of an endogenous polypeptide. An antisense molecule also can have flanking sequences (*e.g.*, regulatory sequences). Thus antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA.

[00255] Yeast having a reduced enzymatic activity can be identified using many methods. For example, yeast having reduced glycerol-3-phosphate dehydrogenase activity can be easily identified using common methods, which may include, for example, measuring glycerol formation via liquid chromatography.

Overexpression of Heterologous Genes

[00256] Methods for overexpressing a polypeptide from a native or heterologous nucleic acid molecule are well known. Such methods include, without limitation, constructing a nucleic acid sequence such that a regulatory element promotes the expression of a nucleic acid sequence that encodes the desired polypeptide. Typically, regulatory elements are DNA sequences that regulate the expression of other DNA sequences at the level of transcription. Thus, regulatory elements include, without limitation, promoters, enhancers, and the like. For example, the exogenous genes can be under the control of an inducible promoter or a constitutive promoter. Moreover, methods for expressing a polypeptide from an exogenous nucleic acid molecule in yeast are well known. For example, nucleic acid constructs that are used for the expression of exogenous polypeptides within Kluyveromyces and Saccharomyces are well known (*see*, *e.g.*, U.S. Pat. Nos. 4,859,596 and 4,943,529, for Kluyveromyces and, *e.g.*, Gellissen *et al.*, Gene 190(1):87-97 (1997)

for *Saccharomyces*). Yeast plasmids have a selectable marker and an origin of replication. In addition certain plasmids may also contain a *cen*tromeric sequence. These *cen*tromeric plasmids are generally a single or low copy plasmid. Plasmids without a *cen*tromeric sequence and utilizing either a 2 micron (*S. cerevisiae*) or 1.6 micron (*K. lactis*) replication origin are high copy plasmids. The selectable marker can be either prototrophic, such as *HIS3*, *TRP1*, *LEU2*, *URA3* or *ADE2*, or antibiotic resistance, such as, *bar*, *ble*, *hph*, or *kan*.

[00257] In another embodiment, heterologous control elements can be used to activate or repress expression of endogenous genes. Additionally, when expression is to be repressed or eliminated, the gene for the relevant enzyme, protein or RNA can be eliminated by known deletion techniques.

[00258] As described herein, any yeast within the scope of the disclosure can be identified by selection techniques specific to the particular enzyme being expressed, over-expressed or repressed. Methods of identifying the strains with the desired phenotype are well known to those skilled in the art. Such methods include, without limitation, PCR, RT-PCR, and nucleic acid hybridization techniques such as Northern and Southern analysis, altered growth capabilities on a particular substrate or in the presence of a particular substrate, a chemical compound, a selection agent and the like. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of the encoded polypeptide. For example, an antibody having specificity for an encoded enzyme can be used to determine whether or not a particular yeast cell contains that encoded enzyme. Further, biochemical techniques can be used to determine if a cell contains a particular nucleic acid molecule encoding an enzymatic polypeptide by detecting a product produced as a result of the expression of the enzymatic polypeptide. For example, transforming a cell with a vector encoding acetolactate synthase and detecting increased acetolactate con*cen*trations compared to a cell without the vector indicates that the vector is both present and that the gene product is active. Methods for detecting specific enzymatic activities or the presence of particular products are well known to those skilled in the art. For example, the presence of acetolactate can be determined as described by Hugenholtz and Starrenburg, 1992, *Appl. Micro. Biot.* 38:17-22.

Methods for the Overexpression of AFT Genes

[00259] Overexpression of the *AFT1 and AFT2* genes may be accomplished by

any number of methods. In one embodiment, overexpression of the *AFT1 and AFT2* genes may be accomplished with the use of plasmid vectors that function in yeast. In exemplary embodiments, the expression of *AFT1*, *AFT2*, and/or homologous genes may be increased by overexpressing the genes on a CEN plasmid or alternative plasmids with a similar copy number. In one embodiment, *AFT1* or a homolog thereof is overexpressed on a CEN plasmid or alternative plasmids with a similar copy number. In another embodiment, *AFT2* or a homolog thereof is overexpressed on a CEN plasmid or alternative plasmids with a similar copy number. In yet another embodiment, *AFT1* and *AFT2* or homologs thereof are overexpressed on a CEN plasmid or alternative plasmids with a similar copy number.

[00260] In further embodiments, expression of genes from single or multiple copy integrations into the chromosome of the cell may be useful. Use of a number of promoters, such as *TDH3, TEF1, CCW12, PGK1*, and *ENO2*, may be utilized. As would be understood in the art, the expression level may be fine-tuned by using a promoter that achieves the optimal expression (*e.g.* optimal overexpression) level in a given yeast. Different levels of expression of the genes may be achieved by using promoters with different levels of activity, either in single or multiple copy integrations or on plasmids. An example of such a group of promoters is a series of truncated *PDC1* promoters designed to provide different strength promoters. Alternatively promoters that are active under desired conditions, such as growth on glucose, may be used. For example a promoter from one of the glycolytic genes, the *PDC1* promoter, and a promoter from one of the *ADH* genes in *S. cerevisiae* may all be useful. Also, embodiments are exemplified using the yeast *S. cerevisiae*. However, other yeasts, such as those from the genera listed herein may also be used.

[00261] As described herein, overexpression of the Aft1 protein or a homolog thereof may be obtained by expressing a constitutively active Aft1 or a homolog thereof. In one embodiment, the constitutively active Aft1 or a homolog thereof comprises a mutation at a position corresponding to the cysteine 291 residue of the native *S. cerevisiae* Aft1 (SEQ ID NO: 2). In a specific embodiment, the cysteine 291 residue is replaced with a phenylalanine residue.

[00262] As described herein, overexpression of the Aft2 protein or a homolog thereof may be obtained by expressing a constitutively active Aft2 or a homolog thereof. In one embodiment, the constitutively active Aft2 or a homolog thereof comprises a mutation at a position corresponding to the cysteine 187 residue of the

native *S. cerevisiae* Aft2 (SEQ ID NO: 2). In a specific embodiment, the cysteine 187 residue is replaced with a phenylalanine residue.

Increase of Enzymatic Activity

[00263] Yeast microorganisms of the invention may be further engineered to have increased activity of enzymes. The term "increased" as used herein with respect to a particular enzymatic activity refers to a higher level of enzymatic activity than that measured in a comparable yeast cell of the same species. For example, overexpression of a specific enzyme can lead to an increased level of activity in the cells for that enzyme. Increased activities for enzymes involved in glycolysis or the isobutanol pathway would result in increased productivity and yield of isobutanol.

[00264] Methods to increase enzymatic activity are known to those skilled in the art. Such techniques may include increasing the expression of the enzyme by increased copy number and/or use of a strong promoter, introduction of mutations to relieve negative regulation of the enzyme, introduction of specific mutations to increase specific activity and/or decrease the Km for the substrate, or by directed evolution. See, e.g., Methods in Molecular Biology (vol. 231), ed. Arnold and Georgiou, Humana Press (2003).

Methods of Using Recombinant Microorganisms for High-Yield Fermentations

[00265] For a biocatalyst to produce a beneficial metabolite most economically, it is desirable to produce said metabolite at a high yield. Preferably, the only product produced is the desired metabolite, as extra products (*i.e.* by-products) lead to a reduction in the yield of the desired metabolite and an increase in capital and operating costs, particularly if the extra products have little or no value. These extra products also require additional capital and operating costs to separate these products from the desired metabolite.

[00266] In one aspect, the present invention provides a method of producing a beneficial metabolite derived from a DHAD-requiring biosynthetic pathway. In one embodiment, the method includes cultivating a recombinant microorganism comprising a DHAD-requiring biosynthetic pathway in a culture medium containing a feedstock providing the carbon source until a recoverable quantity of the beneficial metabolite is produced and optionally, recovering the metabolite. In an exemplary embodiment, said recombinant microorganism has been engineered to overexpress a polynucleotide encoding Aft1 (SEQ ID NO: 2) and/or Aft2 (SEQ ID NO: 4) or a homolog thereof. The beneficial metabolite may be derived from any DHAD-

requiring biosynthetic pathway, including, but not limited to, biosynthetic pathways for the production of isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, valine, isoleucine, leucine, and pantothenic acid. In a specific embodiment, the beneficial metabolite is isobutanol.

[00267] In a method to produce a beneficial metabolite from a carbon source, the yeast microorganism is cultured in an appropriate culture medium containing a carbon source. In certain embodiments, the method further includes isolating the beneficial metabolite from the culture medium. For example, isobutanol may be isolated from the culture medium by any method known to those skilled in the art, such as distillation, pervaporation, or liquid-liquid extraction

[00268] In one embodiment, the recombinant microorganism may produce the beneficial metabolite from a carbon source at a yield of at least 5 percent theoretical. In another embodiment, the microorganism may produce the beneficial metabolite from a carbon source at a yield of at least about 10 percent, at least about 15 percent, about least about 20 percent, at least about 25 percent, at least about 30 percent, at least about 35 percent, at least about 40 percent, at least about 45 percent, at least about 50 percent, at least about 55 percent, at least about 60 percent, at least about 65 percent, at least about 70 percent, at least about 75 percent, at least about 80 percent, at least about 85 percent, at least about 90 percent, at least about 95 percent, or at least about 97.5% theoretical. In a specific embodiment, the beneficial metabolite is isobutanol.

[00269] This invention is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference for all purposes.

EXAMPLES

General Materials and Methods for Examples

[00270] Media: Media used were standard yeast medium (for example Sambrook, J., Russel, D.W. Molecular Cloning, A Laboratory Manual. 3rd ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press and Guthrie, C. and Fink, G.R. eds. Methods in Enzymology Part B: Guide to Yeast Genetics and Molecular and Cell Biology 350:3-623 (2002)). YP medium contains 1% (w/v) yeast extract, 2% (w/v) peptone. YPD is YP containing 2% (w/v) glucose.

[00271] *S. cerevisiae* Transformations: The yeast strain of interest was grown on

YPD medium. The strain was re-suspended in 100 mM lithium acetate. Once the cells were re-suspended, a mixture of DNA (final volume of 15 µL with sterile water), 72 µL 50% w/v PEG, 10 µL 1 M lithium acetate, and 3 µL of denatured salmon sperm DNA (10 mg/mL) was prepared for each transformation. In a 1.5 mL tube, 15 µL of the cell suspension was added to the DNA mixture (100 µL), and the transformation suspension was vortexed for 5 short pulses. The transformation was incubated for 30 min at 30°C, followed by incubation for 22 min at 42°C. The cells were collected by centrifugation (18,000 rcf, 10 sec, 25°C). The cells were resuspended in 1 mL YPD and after an overnight recovery shaking at 30°C and 250 rpm, the cells were spread over YPD + 0.2 g/L G418 + 0.1 g/L hygromycin selective plates. Transformants were then single colony purified onto selective plates containing appropriate antibiotics.

[00272] Preparation of Yeast Lysate: Cells were thawed on ice and resuspended in lysis buffer (50 mM Tris pH 8.0, 5 mM $MgSO_4$) such that the result was a 20% cell suspension by mass. 1000 µL of glass beads (0.5 mm diameter) were added to a 1.5 mL microcentrifuge tube and 875 µL of cell suspension was added. Yeast cells were lysed using a Retsch MM301 mixer mill (Retsch Inc. Newtown, PA), mixing 6 X 1 min each at full speed with 1 min incubations on ice between each bead-beating step. The tubes were centrifuged for 10 min at 23,500 rcf at 4°C and the supernatant was removed for use. The lysates were held on ice until assayed.

[00273] DHAD Assay: Each sample was diluted in DHAD assay buffer (50 mM Tris pH 8, 5 mM $MgSO_4$) to a 1:10 and a 1:40 to 1:100 dilution. Three samples of each lysate were assayed, along with no lysate controls. 10 µL of each sample (or DHAD assay buffer) was added to 0.2 mL PCR tubes. Using a multi-channel pipette, 90 µL of the substrate was added to each tube (substrate mix was prepared by adding 4 mL DHAD assay buffer to 0.5 mL 100 mM DHIV). Samples were put in a thermocycler (Eppendorf Mastercycler) at 35°C for 30 min followed by a 5 min incubation at 95°C. Samples were cooled to 4°C on the thermocycler, then centrifuged at 3000 rcf for 5 min. Finally, 75 µL of supernatant was transferred to new PCR tubes and submitted to analytics for analysis by Liquid Chromatography, method 2. DHAD activity units were calculated as µmol KIV produced/min/mg total cell lysate protein in the assay.

[00274] Protein Concentration Determination: Yeast lysate protein concentration was determined using the BioRad Bradford Protein Assay Reagent Kit (Cat# 500-0006, BioRad Laboratories, Hercules, CA) and using BSA for the standard curve.

Briefly, 10 µL standard or lysate were added into a microcentrifuge tube. The samples were diluted to fit in the linear range of the standard curve (1:40). 500 µL of 1:4 diluted and filtered Bio-Rad protein assay dye was added to the blank and samples and then vortexed. Samples were incubated at room temperature for 6 min, transferred into cuvettes and the OD_{595} was determined in a spectrophotometer. The linear regression of the standards was then used to calculate the protein concentration in each sample.

[00275] Gas Chromatography: Analysis of volatile organic compounds including isobutanol, was performed on a HP 5890/6890/7890 gas chromatograph fitted with an HP 7673 Autosampler, a ZB-FFAP column (Phenomenex; 30 m length, 0.32 mm ID, 0.25 µM film thickness) or equivalent connected to a flame ionization detector (FID). The temperature program was as follows: 200°C for the injector, 300°C for the detector, 100°C oven for 1 min, 70°C/min gradient to 230°C, and then hold for 2.5 min. Analysis was performed using authentic standards (>99%, obtained from Sigma-Aldrich) and a 5-point calibration curve with 1-pentanol as the internal standard.

[00276] Liquid Chromatography, Method 1: Analysis of organic acid metabolites, specifically pyruvate, acetate, 2,3-dihydroxy-isovalerate, and 2,3-butanediol, was performed on an HP-1200 High Performance Liquid Chromatography system equipped with two Rezex RFQ 150 x 4.6 mm columns in series. Organic acid metabolites were detected using an HP-1100 UV detector (210 nm) and refractive index. The column temperature was 60°C. This method was isocratic with 0.0180 N H_2SO_4 in Milli-Q water as mobile phase. Flow was set to 1.1 mL/min. Injection volume was 20 µL and run time was 16 min. Analysis was performed using authentic standards (>99%, obtained from Sigma-Aldrich, with the exception of DHIV (2,3-dihidroxy-3-methyl-butanoate, CAS 1756-18-9), which was custom synthesized at Caltech (Cioffi, E. *et al.* Anal Biochem 104 pp.485 (1980)), and a 5-point calibration curve.

[00277] Liquid Chromatography, Method 2: Analysis of 2-keto-isovalerate (KIV), the product indicating DHAD activity, was measured using liquid chromatography. DNPH reagent (12 mM 2,4 - Dinitrophenyl Hydrazine, 20 mM Citric Acid pH 3.0, 80% Acetonitrile, 20% MilliQ H_2O) was added to each sample in a 1:1 ratio. Samples were incubated for 30 min at 70°C in a thermo-cycler (Eppendorf, Mastercycler). Analysis of KIV was performed on an HP-1200 High Performance Liquid Chromatography system equipped with an Eclipse XDB C-18 reverse phase column (Agilent) and a C-

18 reverse phase column guard (Phenomenex). KIV was detected using an HP-1100 UV detector (360 nm). The column temperature was 50°C. This method was isocratic with 70% acetonitrile 2.5% phosphoric acid (4%), 27.5% water as mobile phase. Flow was set to 3 mL/min. Injection size was 10 µL and run time was 2 min.

Example 1: Overexpression of *AFT1* Increases DHAD Activity and Isobutanol Productivity, Titer, and Yield in Fermentation Vessels

[00278] The purpose of this example is to demonstrate that overexpression of *AFT1* increases DHAD activity, isobutanol titer, productivity, and yield.

[00279] Media: Medium used for the fermentation was YP + 80 g/L glucose + 0.2 g/L G418 + 0.1 g/L hygromycin + 100µM $CuSO_4 \cdot 5H_2O$ + 1% v/v ethanol. The medium was filter sterilized using a 1L bottle top Corning PES 0.22µm filter (431174). Medium was pH adjusted to 6.0 in the fermenter vessels using 6N KOH.

[00280] Vessel Preparation and Operating Conditions: Batch fermentations were conducted using six 2 L top drive motor DasGip vessels with a working volume of 0.9 L per vessel. Vessels were sterilized, along with the appropriate dissolved oxygen probes and pH probes, for 60 min at 121°C. pH probes were calibrated prior to sterilization, however, dissolved oxygen probes were calibrated post sterilization in order to allow for polarization.

[00281] Process Control Parameters: Initial volume, 900 mL. Temperature, 30°C. pH 6.0, pH was controlled using 6N KOH and 2N H_2SO_4 (Table 4).

Table 4. Process control parameters.

Growth phase	Oxygen transfer rate	10 mM/h
	Air overlay	5.0slph
	Agitation	700 rpm
	Dissolved oxygen	Not controlled
Fermentation phase	Oxygen transfer rate	0.5 mM/h to 1.8mM/h*
	Air overlay	5.0slph
	Agitation	300 rpm/400 rpm*
	Dissolved oxygen	Not controlled

*Oxygen transfer rate increased from 0.5 mM/h to 1.8 mM/h by increase in agitation from 300 rpm to 400 rpm 56 h post inoculation.

[00282] Fermentation: The fermentation was run for 119 h. Vessels were sampled 3 times daily. Sterile 5 mL syringes were used to collect 3 mL of fermenter culture via a sterile sample port. The sample was placed in a 2 mL microfuge tube and a portion was used to measure cell density (OD_{600}) on a Genesys 10 spectrophotometer (Thermo Scientific). The remaining sample was filtered through a

0.22 µm pore-size Corning filter. The supernatant from each vessel was refrigerated in a 96-well, deep well plate, and stored at 4ºC prior to gas and liquid chromatography analysis (see General Methods).

[00283] Off-gas Measurements: On-line continuous measurement of each fermenter vessel off-gas by mass spectrometry analysis was performed for oxygen, isobutanol, ethanol, carbon dioxide, and nitrogen throughout the experiment. Fermentor off-gas was analyzed by Prima dB mass spectrometer (Thermo, Waltham, MA) for nitrogen, oxygen, argon, carbon dioxide, isobutanol, ethanol, and isobutyraldehyde. A reference stream of similar composition to the inlet fermentor air was also analyzed. The mass spectrometer cycles through the reference air and fermentor off-gas streams (one by one) and measures percent concentration of these gases after an 8.3 min settling time to ensure representative samples. Equation 1 is a derived value expression input into the mass spectrometer software to determine OTR using percent oxygen and percent nitrogen from the reference air (% O_{2in} and % N_{2in}) and fermentor off-gas (% O_{2out} and % N_{2out}). Nitrogen is not involved in cellular respiration, and therefore, can be used to compensate for outlet oxygen dilution caused by the formation of CO_2. The inlet flow is calculated from Equation 2 based on the ideal gas law and is standardized to 1.0 sLph flow rate and 1.0 L fermentor working volume to yield a derived value OTR in mmol/L/h from the mass spectrometer. This derived value OTR is then multiplied by actual inlet flow rate (sLph) and divided by actual working volume (L) in fermentation spreadsheets to obtain an OTR for specific operating conditions.

Equation 1.

$$OTR = \left[\%\,O2_{in} - \left(\%\,O2_{out} * \frac{\%\,N2_{in}}{\%\,N2_{out}}\right)\right] * Flow_{in}$$

Equation 2.

$$Flow_{in} = \frac{1\,L}{h} * \left[\frac{0.83\,atm}{0.08206\frac{L\,atm}{mol\,K} * 294\,K * 1\,L}\right] * \frac{1000\,mmol}{mol}$$

[00284] See the General Methods for a description of how the yeast transformations were performed, as well as a description of how the yeast lysate was prepared. The DHAD assay and protein concentration assay are also described in the general methods section. Strains, plasmids, and the gene/protein sequences

used in Example 1 are described in Tables 5, 6, and 7, respectively.

Table 5. Genotype of strain disclosed in Example 1.

GEVO Number	Genotype
GEVO2843	*S.cerevisiae CEN.PK2, MATa ura3 leu2 his3 trp1 pdc1Δ::[P_{CUP1}:Bs_alsS1_coSc:T_{CYC1}: P_{PGK1}: Ll_kivD2: P_{ENO2}: Sp_HIS5] pdc5Δ::[LEU2-bla-P_{TEF1}: ILV3ΔN: P_{TDH3}: Ec_ilvC_coSc^{Q110V}] pdc6Δ::[URA3: bla; P_{TEF1}: Ll_kivD2: P_{TDH3}: Dm_ADH]* {evolved for C2 supplement-independence, glucose tolerance and faster growth}

Table 6. Plasmids disclosed in Example 1.

Plasmid Name	Relevant Genes/Usage	Genotype
pGV2227	Plasmid pGV2227 is a 2micron plasmid expressing KARI, DHAD, KIVD, and ADH	P_{TDH3}:Ec_ilvC_coSc^{Q110V} P_{TEF1}:Ll_ilvD_coSc P_{PGK1}-Ll_kivD2_coEc P_{ENO2}_ Ll_adhA 2μ ori, bla, G418R
pGV2196	Empty CEN plasmid	P_{TDH3}: empty P_{TEF1}: empty P_{PGK1}: empty CEN ori, bla, HygroR
pGV2472	CEN plasmid expressing AFT1	P_{TDH3}:Sc_AFT1 P_{TEF1}: empty P_{PGK1}:empty CEN ori, bla, HygroR

Table 7. Nucleotide and amino acid sequences of genes and proteins disclosed in Examples.

Protein	Source	Gene (SEQ ID NO)	Protein (SEQ ID NO)
AFT	*S. cerevisiae*	*Sc_AFT1* (SEQ ID NO: 1)	Sc_Aft1 (SEQ ID NO: 2)
	S. cerevisiae	*Sc_AFT2* (SEQ ID NO: 3)	Sc_Aft2 (SEQ ID NO: 4)
	K. lactis	*Kl_AFT* (SEQ ID NO: 13)	Kl_Aft (SEQ ID NO: 14)
	K. marxianus	*Km_AFT* (SEQ ID NO: 29)	Km_Aft (SEQ ID NO: 30)
	I. orientalis	*Io_AFT1-2* (SEQ ID NO: 33)	Io_Aft1-2 (SEQ ID NO: 34)
ALS	*B. subtilis*	*Bs_alsS1_coSc* (SEQ ID NO: 40)	Bs_AlsS1 (SEQ ID NO: 41)
KARI	*E. coli*	*Ec_ilvC_coSc*^{Q110V} (SEQ ID NO: 42)	Ec_IlvC^{Q110V} (SEQ ID NO: 43)
	E. coli	*Ec_ilvC_coSc*^{P2D1A1} (SEQ ID NO: 44)	Ec_IlvC^{P2D1A1} (SEQ ID NO: 45)
KIVD	*L. lactis*	*Ll_kivd2_coEc* (SEQ ID NO: 46)	Ll_Kivd2 (SEQ ID NO: 47)
DHAD	*L. lactis*	*Ll_ilvD_coSc* (SEQ ID NO: 48)	Ll_IlvD (SEQ ID NO: 49)
	S. cerevisiae	*Sc_ILV3ΔN20* (SEQ ID NO: 50)	Sc_Ilv3ΔN20 (SEQ ID NO: 51)
	S. mutans	*Sm_ilvD_coSc* (SEQ ID NO: 52)	Sm_IlvD (SEQ ID NO: 53)
	N. crassa	*Nc_ILVD2_coSc*(SEQ ID NO: 54)	Nc_IlvD2 (SEQ ID NO: 55)
ADH	*D. melanogaster*	*Dm_ADH* (SEQ ID NO: 56)	Dm_Adh (SEQ ID NO: 57)
	L. lactis	*Ll_adhA* (SEQ ID NO: 58)	Ll_AdhA (SEQ ID NO: 59)
	L. lactis	*Ll_adhA*^{RE1} (SEQ ID NO: 60)	Ll_AdhA^{RE1} (SEQ ID NO: 61)
TFC1	*S. cerevisiae*	*TFC1* (SEQ ID NO: 202)	Tfc1 (SEQ ID NO: 203)

[00285] GEVO2843 was co-transformed with two plasmids (Table 8). GEVO3342 contains plasmids pGV2227 and pGV2196; GEVO3343 contains plasmids pGV2227

and pGV2472.

Table 8. Indicates the strains containing plasmids transformed together into strain GEVO2843.

GEVO	Plasmid 1	Plasmid 2
3342	pGV2227 (DHAD)	pGV2196 (no AFT1)
3343	pGV2227 (DHAD)	pGV2472 (AFT1)

[00286] DHAD Assay Results: The *in vitro* DHAD enzymatic activity of lysates from the microaerobic fermentation of GEVO3342 and GEVO3343 were carried out as described above. Overexpression of *AFT1* from a CEN plasmid resulted in a three-fold increase in specific DHAD activity (U/mg total cell lysate protein). Data is presented as specific DHAD activity (U/mg total cell lysate protein) averages from technical triplicates with standard deviations. DHAD activity for GEVO3342 (control) was 0.066 ± 0.005 U/mg and DHAD activity for GEVO3343 (*AFT*1 over-expressed) was 0.215 ± 0.008 U/mg at the end of the fermentation (119 h).

[00287] Isobutanol Results: Isobutanol titers, rates and yields were calculated based on the experiment run in batch fermentors. Table 9 shows the increase in isobutanol titer, rate and yield in the strain overexpressing the *AFT*1 gene. The overexpression of *AFT1* from a CEN plasmid (GEVO3343) resulted in an increase in isobutanol titer, an increase in isobutanol yield, and an increase in isobutanol rate.

Table 9. Isobutanol titer, rate and yield for replicate fermentation experiments.

	GEVO3342	GEVO3342	GEVO3343	GEVO3343
	control plasmid		Aft1 gene on a CEN plasmid	
Titer (g/L)	3.66	3.96	5.69	5.80
Rate (g/L/h)	0.03	0.03	0.05	0.05
Yield (% theor.)	19	20	34	34

[00288] Change in metabolic by-products: The strain transformed with the *AFT1* gene expressed on the CEN plasmid (GEVO3343) produced less pyruvate, acetate, DHIV (dihydroxyisovalerate)/DH2MB (2,3-dihydroxy-2-methylbutanoic acid), and 2,3-butanediol than the strain with the control plasmid (GEVO3342) during the fermentation. There was a six fold decrease in pyruvate, one fold decrease in acetate, one and a half fold decrease in DHIV/DH2MB, and six fold decrease in 2,3-butanediol.

Example 2: Overexpression of *AFT2* Increases DHAD Activity

[00289] The purpose of this example is to demonstrate that overexpression of

AFT2 increases DHAD activity. Methods of strain construction and cloning techniques are described in Example 1. Strain GEVO2843 is described in Table 5.

Table 10. Plasmids disclosed in Example 2.

Plasmid Name	Relevant Genes/Usage	Genotype
pGV2247	Plasmid pGV2247 is a 2micron plasmid expressing KARI, DHAD, KIVD, and ADH	P_{TDH3}:*Ec_ilvC_coSc*^{P2D1A1} P_{TEF1}:*Ll_ilvD_coSc* P_{PGK1}-*Ll_kivD2_coEc* P_{ENO2}: *Ll_adhA* *2μ ori, bla,* G418R
pGV2196	Empty CEN plasmid	P_{TDH3}: *empty* P_{TEF1}: *empty* P_{PGK1}: *empty* *CEN ori, bla,* HygroR
pGV2627	CEN plasmid expressing AFT2	P_{TDH3}:*empty* P_{TEF1}: empty P_{PGK1}:*Sc_AFT2* *CEN ori, bla,* HygroR

Methods

[00290] Methods for yeast transformations and the preparation of yeast lysates are described in the general methods. The DHAD assay, the liquid chromatography, method 2, assay, and assays for measuring protein concentration are described in the general methods.

[00291] Results for DHAD Activity: Data is presented as specific DHAD activity (U/mg total cell lysate protein) averages from biological and technical triplicates with standard deviations. DHAD activity in GEVO2843 (Table 5) transformed with pGV2247 + pGV2196 (no *AFT2*) was 0.358 ± 0.009 U/mg, DHAD activity for pGV2247 + pGV2627 (contains *AFT2*) was 0.677 ± 0.072 U/mg. The overexpression of *AFT2* increased the amount of DHAD activity in the strain.

Example 3: Overexpression of *AFT1* Increases DHAD Activity for DHAD Enzymes from Multiple Organisms

[00292] The purpose of this example is to demonstrate that overexpression of *AFT1* increases DHAD activity for DHAD enzymes from multiple organisms.

[00293] Strains and plasmids used in Example 4 are described in Tables 11 and 12, respectively.

Table 11. Genotype of strains disclosed in Example 3.

GEVO Number	Genotype	Plasmid
GEVO3626	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}-P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	None
GEVO3873	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2603
GEVO3874	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2603
GEVO3875	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2607
GEVO3876	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608
GEVO3877	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608
GEVO3878	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608
GEVO3879	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2603 + pGV2472
GEVO3880	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{Kl_URA3} gpd2::T_{Kl_URA3} pdc1::P_{PDC1}:Ll_kivD2_coSc5:P_{FBA1}:LEU2:T_{LEU2}:P_{ADH1}:Bs_alsS1_coSc:T_{CYC1}:P_{PGK1}:Ll_kivD2_coEc:P_{ENO2}:Sp_HIS5 pdc5::$T_{Kl\_URA3\_short}$:P_{FBA1}:Kl_URA3:T_{Kl_URA3} pdc6::P_{TEF}:Ll_ilvD_coSc_P_{TDH3}:Ec_ilvC_coSc$^{P2D1\text{-}A1}$:P_{ENO2}:Ll_adhA:P_{FBA1}:Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2603 + pGV2472

GEVO3881	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2603 + pGV2472
GEVO3928	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2607 + pGV2472
GEVO3929	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2607 + pGV2472
GEVO3930	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608 + pGV2472
GEVO3931	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608 + pGV2472
GEVO3932	*Saccharomyces cerevisiae MATa ura3 leu2 his3 trp1 gpd1::T_{KI_URA3} gpd2::T_{KI_URA3} pdc1::P_{PDC1}:*LI_kivD2_coSc5*:P_{FBA1}:*LEU2*:T_{LEU2}:P_{ADH1}:*Bs_alsS1_coSc*:T_{CYC1}:P_{PGK1}:*LI_kivD2_coEc*:P_{ENO2}:*Sp_HIS5 pdc5*::$T_{KI\_URA3\_short}$:P_{FBA1}:*KI_URA3*:T_{KI_URA3} pdc6::P_{TEF}:*LI_ilvD_coSc*_P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1-A1}$:P_{ENO2}:*LI_adhA*:P_{FBA1}:*Sc_TRP1* {evolved for C2 supplement-independence, glucose tolerance and faster growth}	pGV2608 + pGV2472

Table 12. Plasmids disclosed in Example 3.

Plasmid Name	Relevant Genes/Usage	Genotype
pGV2603	Plasmid pGV2603 is a 2 micron plasmid expressing KARI, LI_IlvD DHAD, KIVD, and ADH	P_{TDH3}:*Ec_ilvC_coSc*$^{P2D1A1\text{-}his*}$ P_{TEF1}:*LI_ilvD_coSc* P_{ENO2}_ *LI_adhA*^{RE1} *2μ ori, bla,* G418R
pGV2607	Plasmid pGV2607 is a 2 micron plasmid expressing KARI, Nc_IlvD2 DHAD, KIVD, and ADH	P_{TDH3}:*Ec_ilvC_coSc*^{P2D1A1} P_{TEF1}:*Nc_ilvD2_coSc* P_{ENO2}_ *LI_adhA*^{RE1} *2μ ori, bla,* G418R
pGV2608	Plasmid pGV2608 is a 2 micron plasmid expressing KARI, Sm_IlvD DHAD, KIVD, and ADH	P_{TDH3}:*Ec_ilvC_coSc*^{P2D1A1} P_{TEF1}:*Sm_ilvD_coSc* P_{ENO2}_ *LI_adhA*^{RE1} *2μ ori, bla,* G418R
pGV2472	CEN plasmid expressing AFT1	P_{TDH3}: *Sc_AFT1* P_{TEF1}: empty P_{PGK1}: *empty* *CEN ori, bla,* HygroR

* - Contains 6-his tags as compared to *Ec_ilvC_coSc*^{P2D1A1}

[00294] Shake Flask Fermentations: Fermentations were performed to compare the DHAD enzyme activity of strains GEVO3879, GEVO3880, GEVO3881, GEVO3928, GEVO3929, GEVO3930, GEVO3931 and GEVO3932, which overexpress *AFT1* from *S. cerevisiae* from plasmid pGV2472, with strains GEVO3873, GEVO3874, GEVO3875, GEVO3876, GEVO3877, and GEVO3878, which do not overexpress *AFT1*. Strains GEVO3873, GEVO3874, GEVO3879, GEVO3880 and GEVO3881 express the *Lactococcus lactis* IlvD protein (Ll_IlvD) from the *Ll_ilvD* gene on pGV2603. Strains GEVO3875, GEVO3928 and GEVO3929 express the *Neurospora crassa* IlvD2 protein (Nc_IlvD2) from the *Nc_ilvD2* gene on pGV2607. Strains GEVO3876, GEVO3877, GEVO3878, GEVO3930, GEVO3931 and GEVO3932 express the *Streptococcus mutans* IlvD protein (Sm_IlvD) from the *Sm_ilvD* gene on pGV2608. These plasmids were all present in the same host background strain, GEVO3626.

[00295] Strains containing plasmid pGV2472 were maintained and grown in media containing both 0.2 g/L G418 and 0.1 g/L hygromycin while strains lacking pGV2472 were maintained and grown in media containing 0.2 g/L G418. Yeast strains were inoculated from cell patches or from purified single colonies from YPD supplemented with 0.2 g/L G418 medium agar plates or from YPD supplemented with 0.2 g/L G418 and 0.1 g/L hygromycin medium agar plates into 3 mL of growth medium in 14 mL round-bottom snap-cap tubes to provide three replicates of strains carrying each plasmid or plasmid combination. The growth media used were YPD + 0.2 g/L G418 + 1% v/v ethanol medium for strains lacking pGV2472 and YPD + 0.2 g/L G418 + 0.1 g/L hygromycin + 1% v/v ethanol medium for strains containing pGV2472. The cultures were incubated for up to 24 h shaking at an angle at 250 rpm at 30°C. Separately for each tube culture, these overnight cultures were used to inoculate 50 mL of medium in a 250 mL baffled flask with a sleeve closure to an OD_{600} of 0.1. The media used were YP + 50 g/L glucose + 0.2 g/L G418 + 1% v/v ethanol medium for strains lacking pGV2472 and YP + 50 g/L glucose + 0.2 g/L G418 + 0.1 g/L hygromycin + 1% v/v ethanol medium for strains containing pGV2472. These flask cultures were incubated for up to 24 h shaking at 250 rpm at 30°C. The cells from these flask cultures were harvested separately for each flask culture by centrifugation at 3000 rcf for 5 min and each cell pellet was resuspended separately in 5 mL of YP medium supplemented with 80 g/L glucose, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, 200 mM MES buffer, pH 6.5, and 0.2 g/L G418. Each cell suspension was used to inoculate 50 mL of YP

medium supplemented with 80 g/L glucose, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, 200 mM MES buffer, pH 6.5, and 0.2 g/L G418 in a 250 mL non-baffled flask with a vented screw-cap to an OD_{600} of approximately 5. These fermentations were incubated shaking at 250 rpm at 30°C. After 73 h of incubation, the cells from half of each fermentation culture were harvested by centrifugation at 3000 rcf for 5 min at 4°C. Each cell pellet was resuspended in 25 mL of cold MilliQ water and then harvested by centrifugation at 3000 rcf for 5 min at 4°C. The supernatant was removed from each pellet and the tubes containing the pellets were frozen at -80°C.

[00296] Cell lysate production, total protein quantification, DHAD assays and liquid chromatography, method 2, were performed as described in the general methods.

[00297] Overexpression of *S. cerevisiae AFT1* Increased the DHAD Activity of Strains Expressing Different DHAD Enzymes: Overexpression of *S. cerevisiae AFT1* increased the DHAD enzyme activity of strains expressing the *L. lactis* IlvD, *N. crassa* IlvD2 and *S. mutans* IlvD DHADs by at least 2.5-fold (Table 13). DHAD enzyme activities of the strains expressing the different DHADs were similar in the absence of *AFT1* overexpression but were at different increased enzyme activity levels in the strains expressing the different DHADs together with *AFT1* overexpression. This demonstrates that *AFT1* overexpression increases the activity of multiple DHAD enzymes from several different organisms.

Table 13. DHAD enzyme activity results from shake flask fermentations demonstrating increased DHAD activity from *S. cerevisiae* expressing DHAD enzymes from *L. lactis, N. crassa and S. mutans* and overexpressing *AFT1*.

Expressed DHAD	DHAD Enzyme Activity (µmol KIV/min/mg lysate)	
	No AFT1 Overexpression	AFT1 Overexpression
Ll_IlvD	0.27 ± 0.02	1.26 ± 0.16
Nc_IlvD2	0.29 ± 0.05	1.14 ± 0.15
Sm_IlvD	0.34 ± 0.05	0.85 ± 0.08

Example 4: Simultaneous Overexpression of *AFT1* and *AFT2* Increases DHAD Activity

[00298] The purpose of this example is to demonstrate that overexpression of *S. cerevisiae AFT1* (*Sc_AFT1)* and *S. cerevisiae AFT2* (*Sc_AFT2)* increases DHAD activity.

[00299] Standard molecular biology methods for cloning and plasmid construction were generally used, unless otherwise noted (Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold

Spring Harbor Laboratory Press). Cloning techniques included gel purification of DNA fragments (using the Zymoclean Gel DNA Recovery Kit, Cat# D4002, Zymo Research Corp, Orange, CA).

[00300] *S. cerevisiae* Transformations: Co-transformations with the CEN and 2μ plasmids into *S. cerevisiae* strains are described below. Briefly, the *S. cerevisiae* strain GEVO2843 (Table 5) was grown on YPD medium. From the plate, the strain was re-suspended in 100 mM lithium acetate. Once the cells were re-suspended, a mixture of DNA (final volume of 15 μL with sterile water), 72 μL 50% w/v PEG, 10 μL 1 M lithium acetate, and 3 μL of denatured salmon sperm DNA (10 mg/mL) was prepared for each transformation. In a 1.5 mL tube, 15 μL of the cell suspension was added to the DNA mixture (100 μL), and the transformation suspension was vortexed for 5 short pulses. The transformation was incubated for 30 min at 30°C, followed by incubation for 22 min at 42°C. The cells were collected by centrifugation (18,000 rcf, 10 sec, 25°C). The cells were resuspended in 1 mL YPD and after an overnight recovery shaking at 30°C and 250 rpm, the cells were spread over YPD supplemented with 0.2 g/L G418 and 0.1 g/L hygromycin selective plates. Transformants were then single colony purified onto G418 and hygromycin selective plates.

[00301] Shake Flask Fermentation: Fermentations for the *AFT1*/*AFT2* transformant strains were performed. Starter cultures with each transformed strain were inoculated into 3 mL YPD with 0.1 g/L hygromycin, 0.2 g/L G418, 1% v/v EtOH and incubated shaking at 250 rpm at 30°C. Pre-cultures for the fermentations were inoculated to 0.05 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.1 g/L hygromycin, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20μM $CuSO_4$ at pH 6.5 in 250 mL baffled flasks, shaking at 250 rpm at 30°C. Fermentation cultures were inoculated to 4.0 - 5.0 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.1 g/L hygromycin, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20μM $CuSO_4$ at pH 6.5 in 250 mL unbaffled flasks, shaking at 75 rpm at 30°C. All cultures were done in biological triplicate.

[00302] Preparation of Yeast Lysate: 50 mL of cells were spun down at 4°C, 3000 rcf for 5 min from the 72hr timepoint of the fermentation. The medium was decanted and the cells were resuspended in 10 mL of cold MilliQ water. The cells were centrifuged a second time at 4°C, 3000 rcf for 5 min. The medium was again decanted and the cells were centrifuged at 4°C, 3000 rcf for 5 min. Remaining media

was removed and the cell pellet was frozen at -80°C. Cells were thawed on ice and resuspended in lysis buffer (50 mM Tris pH 8.0, 5 mM $MgSO_4$) such that the result was a 20% cell suspension by mass. 1000 µL of glass beads (0.5 mm diameter) were added to a 1.5 mL microcentrifuge tube and 875 µL of cell suspension was added. Yeast cells were lysed using a Retsch MM301 mixer mill (Retsch Inc. Newtown, PA), mixing 6 X 1 min each at full speed with 1 min incubations on ice between each bead-beating step. The tubes were centrifuged for 10 min at 23,500 rcf at 4°C and the supernatant was removed for use. The lysates were held on ice until assayed.

[00303] DHAD Assay: each sample was diluted in DHAD assay buffer (50 mM Tris pH 8, 5 mM $MgSO_4$) to a 1:10 and 1:100 dilution. Three samples of each lysate were assayed, along with no lysate controls. 10 µL of each sample (or DHAD assay buffer) was added to 0.2 mL PCR tubes. Using a multi-channel pipette, 90 µL of the substrate was added to each tube (substrate mix was prepared by adding 4 mL DHAD assay buffer to 0.5 mL 100 mM DHIV). Samples were put in a thermocycler (Eppendorf Mastercycler) at 35°C for 30 min followed by a 5 min incubation at 95°C. Samples were cooled to 4°C on the thermocycler, then centrifuged at 3000 rcf for 5 min. Finally, 75 µL of supernatant was transferred to new PCR tubes and submitted to analytics for analysis by Liquid Chromatography, method 2. Yeast lysate protein concentration was determined as described under General Methods.

[00304] Liquid Chromatography, method 2: DNPH reagent (4:1 of 15 mM 2,4 - Dinitrophenyl Hydrazine:100 mM Citric Acid pH 3.0) was added to each sample in a 1:1 ratio. Samples were incubated for 30 min at 70°C in a thermo-cycler (Eppendorf, Mastercycler). Analysis of keto-isovalerate and isobutyraldehyde was performed on an Agilent 1200 High Performance Liquid Chromatography system equipped with an Eclipse XDB C-18 reverse phase column (Agilent) and a C-18 reverse phase column guard (Phenomenex). Ketoisovalerate and isobutyraldehyde were detected using an Agilent 1100 UV detector (360 nm). The column temperature was 50°C. This method was isocratic with 70% acetonitrile 2.5% phosphoric acid (0.4%), 27.5% water as mobile phase. Flow was set to 3 mL/min. Injection size was 10 µL and run time was 2 min.

[00305] Results for DHAD Activity: Data is presented as specific DHAD activity (U/mg total cell lysate protein) averages from biological and technical triplicates with standard deviations. DHAD activity in GEVO2843 transformed with pGV2247 (Table 10) + pGV2196 (empty vector, Table 6) was 0.358 ± 0.009 U/mg. DHAD activity for

GEVO2843 transformed with pGV2247 + pGV2626 (CEN plasmid that contains Sc_AFT1 and Sc_AFT2; Genotype: P_{TDH3}:*Sc_AFT1*, P_{TEF1}: empty, P_{PGK1}:*Sc_AFT2, CEN ori*, *bla,* HygroR) was 0.902 ± 0.032 U/mg. The simultaneous overexpression of *Sc_AFT1* and *Sc_AFT2* increased the amount of DHAD activity in the strain.

Example 5: *AFT1* Expression Increases DHAD Activity Independently of DHAD Protein Levels

[00306] The following example illustrates that overexpression of the *AFT1* gene in *Saccharomyces cerevisiae* leads to increased DHAD activity independently of DHAD protein levels.

Table 14. Genotype of strains disclosed in Example 5.

GEVO No.	Genotype
GEVO3882	*MATa ura3 leu2 his3 trp1 gpd1::*T_{KI_URA3} *gpd2::*T_{KI_URA3} *tma29::*T_{KI_URA3} *pdc1::*P_{PDC1}*:LI_kivD2_coSc5:*P_{FBA1}*:LEU2:*T_{LEU2}*:*P_{ADH1}*:Bs_alsS1_coSc:*T_{CYC1}*:*P_{PGK1}*:LI_kivD 2_coEc:*P_{ENO2}*:Sp_HIS5 pdc5::*T_{KI_URA3} *pdc6::*$T_{KI\_URA3\_short}$*:*P_{FBA1}*:KI_URA3:*T_{KI_URA3} {evolved for C2 supplement-independence, glucose tolerance and faster growth} [pGV2603]
GEVO3901	*MATa ura3 leu2 his3 trp1 gpd1::*T_{KI_URA3} *gpd2::*T_{KI_URA3} *tma29::*T_{KI_URA3} *pdc1::*P_{PDC1}*:LI_kivD2_coSc5:*P_{FBA1}*:LEU2:*T_{LEU2}*:*P_{ADH1}*:Bs_alsS1_coSc:*T_{CYC1}*:*P_{PGK1}*:LI_kivD 2_coEc:*P_{ENO2}*:Sp_HIS5 pdc5::*T_{KI_URA3} *pdc6::*P_{TDH3}*:Sc_AFT1:*P_{ENO2}*:LI_adhA*^{RE1}*:*$T_{KI\_URA3\_short}$*:*P_{FBA1}*-KI_URA3:*T_{KI_URA3} {evolved for C2 supplement-independence, glucose tolerance and faster growth} [pGV2603]

[00307] Media: Medium used was standard yeast medium (for example Sambrook, J., Russel, D.W. Molecular Cloning, A Laboratory Manual. 3rd ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press and Guthrie, C. and Fink, G.R. eds. Methods in Enzymology Part B: Guide to Yeast Genetics and Molecular and Cell Biology 350:3-623 (2002)). YP medium contains 1% (w/v) yeast extract, 2% (w/v) peptone. YPD is YP containing 2% (w/v) glucose.

[00308] Fermentations in benchtop fermentors: Fermentations in benchtop fermentors were performed to compare the DHAD enzyme activity and DHAD protein level of GEVO3882 (no *AFT1* overexpression) to GEVO3901 (*AFT1* overexpression). For these fermentations, 1 mL from thawed frozen stocks of the strains were transferred to 500 mL baffled flasks containing 80 mL of YP medium supplemented with 80 g/L glucose, 5 g/L ethanol, 0.5 g/L $MgSO_4$ and 0.2 g/L G418 and incubated for 24 h at 30°C in an orbital shaker at 250 rpm. The flask culture for each strain was transferred to duplicate 2-L top drive motor fermentor vessels with a working volume of 0.9 L of YP medium supplemented with 80 g/L glucose, 5 g/L ethanol, 0.5 g/L $MgSO_4$ and 0.2 g/L G418 per vessel for a starting OD_{600} of 0.5. Fermentors were operated at 30°C and pH 6.0 controlled with 6N KOH and 2N

H_2SO_4 in a 2-phase aerobic condition based on oxygen transfer rate (OTR). Initially, fermentors were operated at a growth phase OTR of 10 mM/h by fixed agitation of 700 rpm and an air overlay of 5 sL/h. Cultures were grown for 20 h to approximately 10-13 OD_{600} then immediately switched to a production aeration OTR = 0.5 mM/h by reducing agitation from 700 rpm to 300 rpm for the period of 20 h to 70.5 h.

[00309] Sample Collection: Samples from each fermentor were collected at 15.5 h, 20 h, 27 h, 48.5 h and 70.5 h to measure optical density at 600 nm (OD_{600}). A volume of culture equal to 150 OD600 was then collected from each fermentor at each time point using 60 mL sterile syringes via a sterile sample port on each vessel and placed on ice in 500 mL centrifuge bottles. The samples were centrifuged at 4000 rcf for 10 min at 4°C to pellet the cells. The cell pellets were then separately resuspended in 60 mL cold deionized water for DHAD enzyme assays or cold deionized water containing Yeast/Fungal Protease Arrest (GBiosciences) for DHAD protein quantification and separated into 10 mL aliquots which were centrifuged at 4000 rcf for 10 min at 4°C to pellet the cells. The supernatant was removed from each pellet and the resulting cell pellets were stored frozen at -80°C until used to prepare cell lysates.

[00310] Cell Lysate Production: Cell lysates were prepared for each frozen sample pellet in lysis buffer U1, which contains 0.1 M sodium phosphate, pH 7.0, 5 mM $MgCl_2$ and 1 mM DTT, for DHAD enzyme assays or lysis buffer U1 containing Yeast/Fungal Protease Arrest (GBiosciences) for DHAD protein quantification. Each cell pellet was individually suspended to 20% (w/v) in the appropriate lysis buffer and 1 mL of that cell suspension was added together with 1000 µL of 0.5 mm diameter glass beads to a 1.5 mL microcentrifuge tube. The yeast cells were lysed using a Retsch MM301 mixer mill (Retsch Inc., Newtown, PA) by mixing for six 1-min cycles at full speed with 1-min incubations on ice between each cycle. The tubes were then centrifuged for 10 min at 23,500 rcf at 4°C and the supernatant was removed. Samples for DHAD enzyme assays were held on ice until assayed on the same day and samples for DHAD protein quantification were frozen at -20°C. Yeast lysate protein concentration was determined as described under General Methods.

[00311] DHAD Assay: Each cell lysate sample was diluted 1:10 in DHAD assay buffer (50 mM Tris, pH 8, 5 mM $MgSO_4$). Three samples of diluted lysate were assayed, along with three controls of DHAD assay buffer containing no lysate. 10 µL of each sample or control was added to 0.2 mL PCR tubes. Using a multi-channel pipette, 90 µL of substrate mix, prepared by adding 4 mL DHAD assay buffer to 0.5

mL 100 mM DHIV, was added to each tube. These tubes were placed in an Eppendorf Mastercycler thermocycler and incubated at 35°C for 30 min followed by incubation at 95°C for 5 min then cooled to 4°C in the thermocycler and centrifuged at 3000 rcf for 5 min. 75 µL of supernatant from each tube was transferred to separate new PCR tubes and submitted for liquid chromatography analysis for keto-isovalerate quantification. The DHAD activity was calculated as µmol KIV produced/min/mg total cell lysate protein in the assay.

[00312] Liquid Chromatography for Keto-Isovalerate Quantification: 100 µL of DNPH reagent, containing 12 mM 2,4-dinitrophenyl hydrazine, 10 mM citric acid, pH 3.0, 80% Acetonitrile and 20% MilliQ H_2O, was added to 100 µL of each sample. The mixtures were then incubated for 30 min at 70°C in an Eppendorf Mastercycler thermocycler. Analysis of keto-isovalerate (KIV) was performed on an HP-1200 High Performance Liquid Chromatography system equipped with an Eclipse XDB C-18 reverse phase column (Agilent) and a C-18 reverse phase column guard (Phenomenex). Keto-isovalerate (KIV) was detected using an HP-1100 UV detector at 210 nm. The column temperature was 50°C. This method was isocratic with 70% acetonitrile to water as mobile phase with 2.5% dilute phosphoric acid (4%). Flow was set to 3 mL/min. Injection size was 10 µL and the run time was 2 min.

[00313] DHAD Protein Quantification: Cell lysate samples were prepared for gel electrophoresis by mixing with appropriate volumes of 4X LDS loading buffer (Invitrogen) and 10X reducing agent solution (Invitrogen) and MilliQ water, followed by incubation at 70°C for 10 min. Prepared samples were run on 4-12% acrylamide Bis-Tris gels (Invitrogen) at 200V for 55 min on the Novex Gel Midi System (Invitrogen) and protein was subsequently transferred from the gel to PVDF membrane with the Novex Semi-Dry Blotter (Invitrogen). Gel electrophoresis and protein transfer were performed according to the manufacturer's recommendations. PVDF membranes with transferred proteins were blocked in 2% ECL Advance Blocking Agent (GE Healthcare) diluted in filtered TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% v/v Tween 20) for 1 h at room temperature under mild agitation. Membranes were then probed with a 1:500 dilution of rabbit anti-Ll_IlvD or a 1:500 dilution of rabbit anti-Sc_Ilv3 serum for 1 h at room temperature under mild agitation. Membranes were washed with filtered TBST for 15 min, followed by three 5 min washes with additional filtered TBST. Membranes were then incubated with a 1:5000 dilution of goat anti-rabbit AlexaFluor 633-tagged secondary antibody (Invitrogen) for 1 h at room temperature under mild agitation while protected from

light. Membranes were washed with TBST as described above while protected from light and then were dried and scanned on a Storm 860 fluorescence imaging system (Molecular Dynamics) using the 635 nm laser at 300V and 100µm resolution. ImageQuant software (GE Healthcare) was used to perform standardized densitometry to quantify relative levels of protein expression, reported as integrated band intensity from the blots.

[00314] Overexpression of *AFT1* Increases DHAD Activity Without Increasing DHAD Protein Levels: DHAD enzyme activity and DHAD protein levels from benchtop fermentor fermentations are summarized in Tables 15 and 16. *AFT1*-overexpressing strain GEVO3901 contains at least 1.5-fold higher DHAD enzyme activity at all fermentation sample time points compared with strain GEVO3882 with no *AFT1* overexpression (Table 15). The ratio of DHAD enzyme activity in GEVO3901 overexpressing *AFT1* compared to DHAD enzyme activity in strain GEVO3882 with no *AFT1* overexpression was higher during the growth phase of the fermentation (3.7 at 15.5 h, 3.8 at 20 h) than during the production phase of the fermentation (2.8 at 27 h, 1.5 at 48.5 h and 1.8 at 70.5 h).

[00315] DHAD protein levels from *AFT1*-overexpressing strain GEVO3901 were not substantially different from strain GEVO3882 with no *AFT1* overexpression at any of the fermentation sample time points (Table 16). Neither the Ll_IlvD nor the Sc_Ilv3 DHAD protein levels were substantially different from GEVO3901 overexpressing *AFT1* compared with GEVO3882 without *AFT1* overexpression at any fermentation sample time point.

Table 15. DHAD enzyme activity results from fermentation samples demonstrating increased DHAD activity with *AFT1* overexpression.

Time of Sample	DHAD Enzyme Activity (µmol KIV/min/mg lysate protein)	
	No AFT1 Overexpression (GEVO3882)	AFT1 Overexpression (GEVO3901)
15.5 h	0.060 ± 0.007	0.224 ± 0.009
20.5 h	0.076 ± 0.003	0.286 ± 0.064
27 h	0.119 ± 0.049	0.338 ± 0.020
48.5 h	0.262 ± 0.026	0.386 ± 0.078
70.5 h	0.367 ± 0.021	0.652 ± 0.083

Table 16. DHAD protein level determinations from fermentation samples demonstrating no increase in DHAD protein levels with *AFT1* overexpression.

Time of Sample	Ll_IlvD DHAD Protein Level (Integrated Band Intensity)		Sc_Ilv3 DHAD Protein Level (Integrated Band intensity)	
	No AFT1 Overexpression	AFT1 Overexpression	No AFT1 Overexpression	AFT1 Overexpression

15.5 h	11941 ± 870	11144 ± 821	206 ± 47	227 ± 20
20.5 h	10339 ± 830	10634 ± 749	225 ± 108	260 ± 52
27 h	10057 ± 636	10065 ± 816	256 ± 37	244 ± 74
48.5 h	9803 ± 114	9956 ± 273	158 ± 6	180 ± 41
70.5 h	10010 ± 341	11212 ± 1922	181 ± 15	268 ± 25

Example 6: Mutating *Sc_AFT1* or *Sc_AFT2* to *Sc_AFT1*^{UP} or *Sc_AFT2*^{UP} Alleles

[00316] A point mutation in Sc_Aft1 and Sc_Aft2 causes derepression of transcriptional activation in the presence of iron. Sc_Aft1-1^{UP} mutation changes Cys291Phe (Yamaguchi-Iwia *et al.* 1995 *EMBO Journal* 14: 1231-9). The Sc_Aft2-1^{UP} mutation changes Cys187Phe (Rutherford *et al*. 2001 *PNAS* 98: 14322-7). The purpose of this example is to demonstrate that mutating the endogenous copy of *Sc_AFT1* or *Sc_AFT2* into the *Sc_AFT1-1*^{up} or *Sc_AFT2-1*^{up} mutant alleles generally mimics the overexpression of *Sc_AFT1* or *Sc_AFT2* by increasing DHAD activity and isobutanol titers in yeast strains carrying an isobutanol producing metabolic pathway.

[00317] In this example, *Sc_AFT1* and *Sc_AFT2* are replaced in the genome by *Sc_AFT1-1*^{UP} and *Sc_AFT2-1*^{UP} alleles, either individually or together. Figures 3 and 4 show the constructs for the allelic replacement for *Sc_AFT1-1*^{UP} (SEQ ID NO: 62) and *Sc_AFT2-1*^{UP} (SEQ ID NO: 63). These constructs are synthesized by DNA2.0. The constructs are transformed into GEVO2843 (Table 5) either with pGV2227 (Table 6) or pGV2196 (empty vector control, Table 6) to yield GEVO6209 and GEVO6210 (Table 17).

[00318] Yeast Transformations: Transformations of either the linear *Sc_AFT1-1*^{UP} or the *Sc_AFT2-1*^{UP} constructs or pGV2227(or pGV2196) into GEVO2483 are described below. Briefly, the *S. cerevisiae* strain GEVO2843 is grown on YPD medium. The strain is re-suspended in 100 mM lithium acetate. Once the cells are re-suspended, a mixture of DNA (final volume of 15 µL with sterile water), 72 µL 50% w/v PEG, 10 µL 1 M lithium acetate, and 3 µL of denatured salmon sperm DNA (10 mg/mL) is prepared for each transformation. In a 1.5 mL tube, 15 µL of the cell suspension is added to the DNA mixture (100 µL), and the transformation suspension is vortexed for 5 short pulses. The transformation is incubated for 30 min at 30°C, followed by incubation for 22 min at 42°C. The cells are collected by centrifugation (18,000 rcf, 10 sec, 25°C). The cells are resuspended in 1 mL YPD and after an overnight recovery shaking at 30°C and 250 rpm, the transformants are spread over YPD supplemented with 0.2 g/L G418 selective plates. Transformants are then single colony purified onto G418 selective plates. GEVO2483 containing

pGV2227 or pGV2196 and transformed with the linear AFT^{UP} constructs are plated onto YPD with 0.2 g/L G418 and 0.1 g/L hygromycin.

Table 17. Genotype of strains disclosed in Example 6.

GEVO Number	Genotype
GEVO6209	*S.cerevisiae CEN.PK2, MATa ura3 leu2 his3 trp1 pdc1Δ::P_{CUP1}:[Bs_alsS1_coSc:T_{CYC1}: P_{PGK1}: Ll_kivD2_coEc: P_{ENO2}: Sp_HIS5] pdc5Δ::[LEU2:bla:P_{TEF1}: ILV3ΔN20: P_{TDH3}: Ec_ilvC_coSc^{Q110V}] pdc6Δ::[URA3: bla; P_{TEF1}: Ll_kivD2_coEC: P_{TDH3}: Dm_ADH] aft1 Δ::[P_{AFT1}:AFT1-1^{UP}:P_{ENO2}:G418]* {evolved for C2 supplement-independence, glucose tolerance and faster growth}.
GEVO6210	*S.cerevisiae CEN.PK2, MATa ura3 leu2 his3 trp1 pdc1Δ::P_{CUP1}:[Bs_alsS1_coSc:T_{CYC1}: P_{PGK1}: Ll_kivD2_coEc: P_{ENO2}: Sp_HIS5] pdc5Δ::[LEU2:bla:P_{TEF1}: ILV3ΔN20: P_{TDH3}: Ec_ilvC_coSc^{Q110V}] pdc6Δ::[URA3: bla; P_{TEF1}: Ll_kivD2_coEC: P_{TDH3}: Dm_ADH] aft2 Δ::[P_{AFT2}:AFT2-1^{UP}: P_{ENO2}:G418]* {evolved for C2 supplement-independence, glucose tolerance and faster growth}

[00319] Strains that grow on 0.2 g/L G418 and 0.1 g/L hygromycin are further screened by PCR to determine if the integration has replaced *Sc_AFT1* or *Sc_AFT2*.

[00320] For AFT1: The primer AFT1UP forward (SEQ ID NO: 64) is used with the primer pENO2R (SEQ ID NO: 65) to yield a 599 base pair product that will not be present in the parental strain. The primer AFT1UP forward is used with primer AFT1termR (SEQ ID NO: 66) to ensure that the parental *Sc_AFT1* does not remain in the strain. If integrated correctly, these primers give an approximately 2210 base pair product; if the parental *Sc_AFT1* remains in the strain the product size is 584 base pairs. Finally, the *Sc_AFT1-1^{UP}* gene is amplified using the AFT1UPfullF (SEQ ID NO: 67) and pENO2R primers. This product is submitted for sequencing using the AFT1UPsequence1 (SEQ ID NO: 68) and AFT1UPsequence2 (SEQ ID NO: 69) primers to ensure that the proper mutation is in the genome.

[00321] For AFT2: Primer AFT2Upforward (SEQ ID NO: 70) is used with primer pENO2R to yield an approximately 350 base pair product that will not be present in the parental strain. Primer AFT2UP forward is used with primer AFT2termR (SEQ ID NO: 71) to ensure that the parental *Sc_AFT2* does not remain in the strain. If integrated correctly these primers give an approximately 1819 base pair product. If the parental *Sc_AFT2* remains in the strain the product size is 195 base pairs. Finally, the *Sc_AFT2-1^{UP}* gene is amplified using the AFT2UPfullF (SEQ ID NO: 72) and pENO2R primers. This product is submitted for sequencing using the AFT2UPsequence1 (SEQ ID NO: 73) and AFT2UPsequence2 (SEQ ID NO: 74)

primers to ensure that the proper mutation is in the genome.

[00322] Preparation of Yeast Cells: Yeast strains are grown in 50 mL YPD with 0.2 g/L G418 (if carrying the AFT^{UP} allele) to mid-log phase (1-3 OD_{600}). A volume of cells so that 20 OD_{600} of cells are acquired are spun down at 4°C, 3000 rcf for 5 min. The medium is decanted and the cells are resuspended in 10 mL of cold MilliQ water. The cells are centrifuged a second time at 4°C, 3000 rcf for 5 min. The medium is again decanted and the cells are centrifuged at 4°C, 3000 rcf for 5 min. The remaining medium is removed and the cell pellet is frozen at -80°C.

[00323] DHAD Assays are performed as described in the general methods section. Yeast lysate protein concentration was determined as described in the general methods section.

[00324] Gas Chromatography, Liquid chromatography method 1 and liquid chromatography method 2 are performed as described in the general methods section.

[00325] Shake-Flask Fermentation: Fermentations for the *AFT1-1*^{UP} and *AFT2-1*^{UP} transformant strains are performed. Starter cultures with each transformed strain are inoculated into 3 mL YPD with 0.2 g/L G418 and 1% v/v EtOH and incubated shaking at 250 rpm at 30°C. Pre-cultures for the fermentations are inoculated to 0.05 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20μM $CuSO_4$ at pH 6.5 in 250 mL baffled flasks, shaking at 250 rpm at 30°C. Fermentation cultures are inoculated to 5.0 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20μM $CuSO_4$ at pH 6.5 in 250 mL unbaffled flasks, shaking at 75 rpm at 30°C. All cultures are done in biological triplicate. Samples are collected at 24, 48 and 72 h and analyzed using the liquid chromatography, method 1, and gas chromatography protocols.

[00326] Results for DHAD activity: Data is presented as specific DHAD activity (U/mg total cell lysate protein) averages from biological and technical triplicates with standard deviations. DHAD activity in GEVO2843 transformed with pGV2227 is generally expected to be lower than that of GEVO2843 + pGV2227 transformed with either the *Sc_AFT1-1*^{UP} or *Sc_AFT2-1*^{UP} allele.

[00327] Results for Isobutanol Fermentation: Data is presented as specific isobutanol titer (g/L/O_{D600}); averages from biological and technical triplicates with standard deviations. Isobutanol titers in GEVO2843 transformed with pGV2227 is

generally expected to be lower than that of GEVO2843 + pGV2227 transformed with either the *Sc_AFT1-1^{UP}* or *Sc_AFT2-1^{UP}* allele.

Example 7: Overexpression of *AFT1* in *S. cerevisiae* Carrying an Isobutanol Producing Metabolic Pathway Increases AFT Regulon Genes as Measured by mRNA

[00328] The purpose of this example is to demonstrate that overexpression of *AFT1* in strains expressing an isobutanol producing metabolic pathway increases the expression of genes in the AFT regulon in fermentation vessels. This in turn increases DHAD activity and isobutanol titer, productivity, and yield.

[00329] Media: Medium used was standard yeast medium (for example Sambrook, J., Russel, D.W. Molecular Cloning, A Laboratory Manual. 3rd ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press and Guthrie, C. and Fink, G.R. eds. Methods in Enzymology Part B: Guide to Yeast Genetics and Molecular and Cell Biology **350**:3-623 (2002)). YP medium contains 1% (w/v) yeast extract, 2% (w/v) peptone. YPD is YP containing 2% (w/v) glucose. Medium used for the fermentation was YP with 80 g/L glucose, 0.2 g/L G418, 0.1 g/L hygromycin, 100μM $CuSO_4.5H_2O$ and 1% v/v ethanol. The medium was filter sterilized using a 1L bottle top Corning PES 0.22μm filter (431174). Medium was pH adjusted to 6.0 in the fermenter vessels using 6N KOH.

[00330] Fermentation vessel preparation and operating conditions: Batch fermentations were conducted using six 2 L top drive motor DasGip vessels with a working volume of 0.9 L per vessel. Vessels were sterilized, along with the appropriate dissolved oxygen probes and pH probes, for 60 min at 121°C. pH probes were calibrated prior to sterilization, however, dissolved oxygen probes were calibrated post sterilization in order to allow for polarization.

[00331] Process control parameters: Initial volume, 900 mL. Temperature, 30°C. pH 6.0, pH was controlled using 6N KOH and 2N H_2SO_4 (Table 20).

Table 18. Process Control Parameters.

Growth phase	**Oxygen transfer rate**	**10 mM/h**
	Air overlay	**5.0slph**
	Agitation	**700 rpm**
	Dissolved oxygen	**Not controlled**
Fermentation phase	**Oxygen transfer rate**	**0.5 mM/h to 1.8mM/h***

	Air overlay	**5.0slph**
	Agitation	**300 rpm/400 rpm***
	Dissolved oxygen	**Not controlled**

*Oxygen transfer rate increased from 0.5 mM/h to 1.8 mM/h by increase in agitation from 300 rpm to 400 rpm 56 h post inoculation.

[00332] Fermentation: The fermentation was run for 119 h. Vessels were sampled 3 times daily. Sterile 5 mL syringes were used to collect 3 mL of fermenter culture via a sterile sample port. The sample was placed in a 2 mL microfuge tube and a portion was used to measure cell density (OD_{600}) on a Genesys 10 spectrophotometer (Thermo Scientific). An additional 2 mL portion was taken in the same manner as described above, for use in qRT-PCR analysis. This sample was spun in a microcentrifuge for 1 min at 14,000 rpm.

[00333] Yeast Transformations: Co-transformations with the CEN and 2μ plasmids are described below. Briefly, the *S. cerevisiae* strain GEVO2843 (Table 5) was grown on YPD medium. The strain was re-suspended in 100 mM lithium acetate. Once the cells were re-suspended, a mixture of DNA (final volume of 15 μL with sterile water), 72 μL 50% w/v PEG, 10 μL 1 M lithium acetate, and 3 μL of denatured salmon sperm DNA (10 mg/mL) was prepared for each transformation. In a 1.5 mL tube, 15 μL of the cell suspension was added to the DNA mixture (100 μL), and the transformation suspension was vortexed for 5 short pulses. The transformation was incubated for 30 min at 30°C, followed by incubation for 22 min at 42°C. The cells were collected by centrifugation (18,000 rcf, 10 sec, 25°C). The cells were resuspended in 1 mL YPD and after an overnight recovery shaking at 30°C and 250 rpm, the cells were spread over YPD supplemented with 0.2 g/L G418 and 0.1 g/L hygromycin selective plates. Transformants were then single colony purified onto G418 and hygromycin selective plates.

[00334] RNA preparation: RNA was isolated using the YeaStar RNAKit™ (Zymo Research Corp. Orange, CA). Cells were resuspended in 80 μl of YR Digestion Buffer, 1 μl RNAsin (Promega, Madison, WI) and 5 μl of Zymolyase™ (provided with YeaStar RNAKit). The pellet was completely resuspended by repeated pipetting. The suspension was incubated at 37°C for 60 min. Following the incubation, 160 μl of YR Lysis Buffer was added to the suspension, which was then mixed thoroughly by vortexing. The mixture was centrifuged at 7,000 g for 2 min in a microcentrifuge, and the supernatant was transferred to a Zymo-Spin Column in a collection tube.

The column was centrifuged at 10,000 g for 1 min in a microcentrifuge. To the column, 200 µl RNA Wash Buffer was added, and the column was centrifuged for 1 min at full speed in a microcentrifuge. The flow-through was discarded and 200 µl RNA Wash Buffer was added to the column. The column was centrifuged for 1 min at 14,000g in a microcentrifuge. The Zymo-Spin Column was transferred to a new RNase-free 1.5 mL centrifuge tube, and 60 µl of DNase/RNase-free water was added directly to the column membrane and let stand for 1 min at room temperature. The RNA was eluted by centrifugation for 1 min at full speed in the microcentrifuge. Concentrations were determined by measuring the OD_{260} with the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA 02454). RNA was stored at -80°C until use.

[00335] qRT-PCR analysis: RNA prepared from the fermentation samples (at a dilution of 5 ng/µl) was used as a template for one-step quantitative RT-PCR using the qScript One-Step SYBR Green qRT-PCR kit (Quanta Biosciences™ Gaithersburg, MD). Each PCR reaction contained 10 ng of RNA, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 6.1 µL of sterile water, and 10 µL of the One-Step SYBR Green Master Mix, 0.5 µL RNAsin, and 0.4 µL of qScript One-Step Reverse Transcriptase. qRT-PCR was done in triplicate for each sample. For the purpose of normalizing the experimental samples, qRT-PCR was also done for the TFC1 housekeeping gene. Primers used to target the AFT regulon genes and for the TFC1 gene are presented in Table 19. The reactions were incubated in an Eppendorf Mastercycler ep thermocycler (Eppendorf, Hamburg, Germany) using the following conditions: 50°C for 10 min, 95°C for 5 min, 40 cycles of 95°C for 15 sec and 60°C for 45 sec (amplification), then 95°C for 15 sec, 60°C for 15 sec, and a 20 min slow ramping up of the temperature until it reaches 95°C (melting curve analysis). The fluorescence emitted by the SYBR dye was measured at the 60°C incubation step during each of the 40 cycles, as well as during the ramping up to 95°C for melting curve analysis of the PCR product.

Table 19. Primers used for qRT-PCR analysis to target the *AFT* regulon.

Target	Primer	Sequence
TFC1	**2649**	TCCAGGCGGTATTGACAGCAGG (SEQ ID NO: 75)
	2650	CAATCTGCAACATCAGGTACCACGG (SEQ ID NO: 76)
AFT1	**2962**	ACGCCAACATCTTCGCAACACTC (SEQ ID NO: 77)
	2963	TGCCGGCAGTGGCAAGATTTC (SEQ ID NO: 78)
AFT2	**2966**	CCTCTTCAAGATCCCATGCATGTCC (SEQ ID NO: 79)

	2967	TGTAACCGCACAGAGTAGGCTGC (SEQ ID NO: 80)
FET3	**2972**	TGGCCACTGAAGGTAACGCCG (SEQ ID NO: 81)
	2973	CCGGTAGGAATGAAGGCATGCTG (SEQ ID NO: 82)
ENB1	**2976**	TGGCGCTGAGATTGTGGTCGG (SEQ ID NO: 83)
	2977	TGAAGCGTGCACTAGCGTCC (SEQ ID NO: 84)
SMF3	**2978**	TGCCGGGCAAATCGTTTCTGAG (SEQ ID NO: 85)
	2979	CTTGTGGCCCAAGGTGGTAAAGACC (SEQ ID NO: 86)

[00336] Standard molecular biology methods for cloning and plasmid construction were generally used, unless otherwise noted (Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

[00337] Cloning techniques included gel purification of DNA fragments (using the Zymoclean Gel DNA Recovery Kit, Cat# D4002, Zymo Research Corp, Orange, CA).

[00338] GEVO2843 (Table 5) was co-transformed with two plasmids. GEVO3342 (Table 8) has plasmids pGV2227 (Table 6) and pGV2196 (empty vector, Table 6); GEVO3343 (Table 8) has plasmids pGV2227 (Table 6) and pGV2472 (Table 6 – contains *Sc_AFT1*).

[00339] In Table 20, the fold change data was normalized to the strain without *Sc_AFT1* overexpression at 24 h. Thus, all data points for the strain without *Sc_AFT1* overexpression at 24 h have been set to one. The overexpression of *Sc_AFT1* in *S. cerevisiae* strains increased predicted *Sc_AFT1* target genes, *ENB1* (SEQ ID NO: 123) and *FET3* (SEQ ID NO: 91). SMF3 (SEQ ID NO: 159) is predicted to be more dependent on *Sc_AFT2* for expression and SMF3 had a much weaker response to the overexpression of *Sc_AFT1*, as can be seen in Table 20.

Table 20. Fold change in mRNA expression between strains with and without *Sc_AFT1* overexpressed.

	Expression at 24h		**Expression at 119h**	
qRT-PCR target	Without overexpression of *Sc_AFT1*	With overexpression of *Sc_AFT1*	Without overexpression of *Sc_AFT1*	With overexpression of *Sc_AFT1*
AFT1	1.00	16.17	0.83	7.29
AFT2	1.00	1.02	0.86	0.79
ENB1	1.00	18.00	0.83	7.59
FET3	1.00	31.89	0.92	10.16
SMF3	1.00	5.37	1.23	3.23

[00340] Overexpression of *Sc_AFT1* increased gene expression of targeted genes in the *AFT* regulon. As shown in Example 1, the increased expression of *Sc_AFT1* in

these strains also caused increased isobutanol titers, production rates and yields and DHAD activity in fermentations. Thus, it is likely that one or more genes in the *AFT* regulon impacts DHAD activity and isobutanol production.

Example 8: Overexpression of Specific Genes in the *AFT1* and *AFT2* Regulons

[00341] The purpose of this example is to demonstrate that a specific gene or genes from the *AFT1* or *AFT2* regulon are important for an increase in DHAD activity and isobutanol production.

[00342] Standard molecular biology methods for cloning and plasmid construction are generally used, unless otherwise noted (Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

[00343] Media: Medium used is described in the general methods section. Cloning techniques include gel purification of DNA fragments (using the Zymoclean Gel DNA Recovery Kit, Cat# D4002, Zymo Research Corp, Orange, CA).

[00344] *AFT1* and *AFT2* regulon genes presented in Table 21 are synthesized by DNA 2.0 (Menlo Park, CA, USA) removing any *Hpa*I or *Sac*I restriction sites within the genes. The synthesized *AFT* regulon genes are cloned behind the PGK1 promoter in pGV2196 (empty vector – Table 6) creating a series of 50 plasmids that are co-transformed with pGV2227 (Table 6) into *S. cerevisiae* strain GEVO2843 (Table 5). Isobutanol production from strain GEVO2843 containing pGV2227 has been shown to be limited by DHAD activity. Thus, this provides a suitable background for detecting increases in DHAD activity and subsequent increases in the production of a metabolite from a DHAD-requiring biosynthetic pathway, such as an isobutanol producing metabolic pathway.

Table 21. Genes in the *AFT1* and *AFT2* Regulon For Screening DHAD Activity

Gene name	Gene (SEQ ID NO)	Protein (SEQ ID NO)
FIT3	SEQ ID NO: 87	SEQ ID NO: 88
FIT1	SEQ ID NO: 89	SEQ ID NO: 90
FET3	SEQ ID NO: 91	SEQ ID NO: 92
FRE1	SEQ ID NO: 93	SEQ ID NO: 94
FTR1	SEQ ID NO: 95	SEQ ID NO: 96
FIT2	SEQ ID NO: 97	SEQ ID NO: 98
COT1	SEQ ID NO: 99	SEQ ID NO: 100
OYE3	SEQ ID NO: 101	SEQ ID NO: 102
TIS11/CTH2	SEQ ID NO: 103	SEQ ID NO: 104

VMR1	SEQ ID NO: 105	SEQ ID NO: 106
AKR1	SEQ ID NO: 107	SEQ ID NO: 108
BIO5	SEQ ID NO: 109	SEQ ID NO: 110
YOR387C	SEQ ID NO: 111	SEQ ID NO: 112
YDR271C	SEQ ID NO: 113	SEQ ID NO: 114
YMR034C	SEQ ID NO: 115	SEQ ID NO: 116
FRE2	SEQ ID NO: 117	SEQ ID NO: 118
ARN1	SEQ ID NO: 119	SEQ ID NO: 120
ATX1	SEQ ID NO: 121	SEQ ID NO: 122
ENB1/ARN4	SEQ ID NO: 123	SEQ ID NO: 124
SIT1/ARN3	SEQ ID NO: 125	SEQ ID NO: 126
ARN2	SEQ ID NO: 127	SEQ ID NO: 128
TAF1/TAF130/TAF145	SEQ ID NO: 129	SEQ ID NO: 130
FRE5	SEQ ID NO: 131	SEQ ID NO: 132
FRE6	SEQ ID NO: 133	SEQ ID NO: 134
FRE3	SEQ ID NO: 135	SEQ ID NO: 136
BNA2	SEQ ID NO: 137	SEQ ID NO: 138
ECM4/GTO2	SEQ ID NO: 139	SEQ ID NO: 140
HSP26	SEQ ID NO: 141	SEQ ID NO: 142
YAP2/CAD1	SEQ ID NO: 143	SEQ ID NO: 144
LAP4/APE1/YSC1/API	SEQ ID NO: 145	SEQ ID NO: 146
ECL1	SEQ ID NO: 147	SEQ ID NO: 148
OSW1	SEQ ID NO: 149	SEQ ID NO: 150
NFT1	SEQ ID NO: 151	SEQ ID NO: 152
YBR012C	SEQ ID NO: 153	SEQ ID NO: 154
YOL083W	SEQ ID NO: 155	SEQ ID NO: 156
ARA2	SEQ ID NO: 157	SEQ ID NO: 158
SMF3	SEQ ID NO: 159	SEQ ID NO: 160
MRS4	SEQ ID NO: 161	SEQ ID NO: 162
ISU1/NUA1	SEQ ID NO: 163	SEQ ID NO: 164
FET4	SEQ ID NO: 165	SEQ ID NO: 166
FET5	SEQ ID NO: 167	SEQ ID NO: 168
FTH1	SEQ ID NO: 169	SEQ ID NO: 170
CCC2	SEQ ID NO: 171	SEQ ID NO: 172
FRE4	SEQ ID NO: 173	SEQ ID NO: 174
ISU2	SEQ ID NO: 175	SEQ ID NO: 176
HMX1	SEQ ID NO: 177	SEQ ID NO: 178
PCL5	SEQ ID NO: 179	SEQ ID NO: 180
ICY2	SEQ ID NO: 181	SEQ ID NO: 182
PRY1	SEQ ID NO: 183	SEQ ID NO: 184
YDL124w	SEQ ID NO: 185	SEQ ID NO: 186

[00345] Yeast Transformations are performed as described in the general methods section.

[00346] Preparation of Yeast Cells for Enzyme Assays: Yeast strains are grown in

50 mL YPD with 0.2 g/L G418 and 0.1 g/L hygromycin to mid-log phase (1-3 OD_{600}). A volume of cells so that 20 OD_{600} of cells are acquired are spun down at 4°C, 3000 rcf for 5 min. The medium is decanted and the cells are resuspend in 10 mL of cold MilliQ water. The cells are centrifuged a second time at 4°C, 3000 rcf for 5 min. The medium is again decanted and the cells are centrifuged at 4°C, 3000 rcf for 5 min. The remaining media is removed and the cell pellet is frozen at -80°C.

[00347] Preparation of Yeast Lysate for Enzyme Assays: Cell pellets are thawed on ice. Y-PER Plus reagent (Thermo Scientific #78999) is added to each pellet at a ratio of 12.5 µL of reagent per one OD of cells and the cells resuspended by vortexing. The suspension is gently agitated for 20 min at room temperature. After 20 min, a volume equal to the Y-PER Plus volume of universal lysis buffer (0.1 M Sodium Phosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT) is added. The suspension is shaken for another 40 min. Samples are centrifuged at 5300 g for 10 min at room temperature. The clarified lysates are transferred to a fresh tube and kept on ice until assayed.

[00348] DHAD Assays are performed as described in the general methods section.

[00349] Yeast lysate protein concentration was determined as described in the general methods section.

[00350] Gas Chromatography, liquid chromatography method 1 and liquid chromatography method 2 are performed as described in the general methods section.

[00351] Shake-Flask Fermentation: Fermentations with the *AFT* regulon gene transformant strains are performed. Starter cultures with each transformed strain are inoculated into 3 mL YPD supplemented with 0.2 g/L G418 and 1% v/v EtOH and incubated shaking at 250 rpm at 30°C. Pre-cultures for the fermentations are inoculated to 0.05 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20µM $CuSO_4$ at pH 6.5 in 250 mL baffled flasks, shaking at 250 rpm at 30°C. Fermentation cultures are inoculated to 5.0 OD_{600} into 50 mL YPD (8% w/v glucose) with 200 mM MES, 0.2 g/L G418, 1% v/v stock solution of 3 g/L ergosterol and 132 g/L Tween 80 dissolved in ethanol, and 20µM $CuSO_4$ at pH 6.5 in 250 mL unbaffled flasks, shaking at 75 rpm at 30°C. All cultures are done in biological triplicate. Samples are collected at 24, 48 and 72 h and analyzed using the liquid chromatography, method 1, and gas chromatography protocols.

[00352] Results for DHAD activity: Data is presented as specific DHAD activity

(U/mg total cell lysate protein) averages from biological and technical triplicates with standard deviations. DHAD activity in GEVO2843 transformed with pGV2227 + pGV2196 (empty vector) is generally expected to be lower than that of GEVO2843 transformed with either *AFT1* or *AFT2* genes. In addition, GEVO2843 transformed with pGV2227 and clones containing *AFT* regulon genes that are important for increasing DHAD activity will generally have similar or higher DHAD activity to GEVO2843 transformed with pGV2227 and the *AFT1* or *AFT2* genes.

[00353] Results for Isobutanol Fermentation: Data is presented as specific isobutanol titer (g/L/OD_{600}); averages from biological and technical triplicates with standard deviations. Isobutanol titers in GEVO2843 transformed with pGV2227 + pGV2196 (empty vector) are generally expected to be lower than that of GEVO2843 transformed with either *AFT1* or *AFT2* genes. In addition, GEVO2843 transformed with pGV2227 and clones containing *AFT* regulon genes that are important for increasing DHAD activity will generally have similar or higher isobutanol titers to GEVO2843 transformed with pGV2227 and *AFT1* or *AFT2*.

Example 9: Overexpression of the *Kluyveromyces lactis AFT* Increases DHAD Activity in *K. lactis*

[00354] The purpose of this example is to demonstrate that overexpression of *AFT* from *K. lactis* increases DHAD activity in *K. lactis*.

[00355] Standard molecular biology methods for cloning and plasmid construction were generally used, unless otherwise noted (Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

[00356] Cloning techniques included gel purification of DNA fragments (using the Zymoclean Gel DNA Recovery Kit, Cat# D4002, Zymo Research Corp, Orange, CA).

[00357] Strains and plasmids used in Example 9 are described in Tables 22 and 23, respectively.

Table 22. Genotype of strains disclosed in Example 9.

GEVO Number	Genotype
***K. lactis* GEVO1287**	*MATalpha uraA1 trp1 leu2 lysA1 ade1 lac4-8* [pKD1]
***K. lactis* GEVO4378**	*MATalpha uraA1 trp1 leu2 lysA1 ade1 lac4-8* [pKD1] + pGV2273
K. lactis *GEVO6169*	*MATalpha uraA1 trp1 leu2 lysA1 ade1 lac4-8* [pKD1] + pGV2273 Random integrant of KL_AFT and G418. Linear fragment from plasmid pGV2962 - cut: SalI, BglII, PfoI

Table 23. Plasmids disclosed in Example 9.

Plasmid Name	Relevant Genes/Usage	Genotype
pGV2273	Plasmid pGV2273 is a 1.6micron vector that expresses KARI, KIVD, DHAD and ADH, encodes hygromycin resistance.	P_{TDH3}: *Ec_ilvC_coSc*$^{P2D1-A1}$ P_{TEF1}: *Ll_ilvD_coSc* P_{PGK1}: *Ll_kivD2_coEc* P_{ENO2}: *Ll_adhA* *1.6μ ori, bla,* HygroR
pGV2796	A CEN plasmid carrying used as a backbone for creating pGV2962 and pGV2963.	P_{TEF1}: *Ll_ilvD_coSc* P_{TPI1}: *G418* P_{ENO2}: *Ll_adhA*^{REI} *CEN ori, bla*
pGV2962	A CEN plasmid carrying *Ll_ilvD*, *Kl_AFT* genes, and G418 resistance. The plasmid was used to create linearization fragments for integration into *K. lactis*.	P_{TEF1}: *Ll_ilvD_coSc* P_{TPI1}: *G418* P_{ENO2}: *KL_AFT* *CEN ori, bla*

[00358] *K. lactis* strains: *K. lactis* strain GEVO1287 was transformed with pGV2273 to form GEVO4378. *KL_AFT* was PCR amplified from template DNA from strain GEVO4378 using primers oGV3432 (SEQ ID NO: 189) (contains *Kpn*I) and oGV3433 (SEQ ID NO: 190) (contains *Avr*II). Plasmid pGV2796 and the *KL_AFT* PCR product were cut with *Kpn*I and *Avr*II and ligated together to form plasmid pGV2962. The linear fragment containing *Kl_AFT*:G418 was obtained by the restriction digest of pGV2962 with restriction enzymes, *Sal*I, *Bgl*II and *Pfo*I. The linear *Kl_AFT:G418* (SEQ ID NO: 201) fragment was randomly integrated by transformation into GEVO4378 to make GEVO6169.

[00359] Yeast transformations – *K. lactis*: *K. lactis* strain GEVO1287 or GEVO4378 was inoculated into a 3 mL YPD culture and incubated overnight at 250 rpm and 30°C. A 50 mL YPD culture in a baffled 250 mL shake flask was inoculated and shaken at 30°C until the *K. lactis* strain GEVO1287 reached an OD_{600} of 0.83 and K. lactis strain GEVO4378 reached an OD_{600} of 0.79. Cells were made chemically competent by the following procedure. Cells were collected by centrifugation at 2700 rcf for 2 min. To wash, cells were re-suspended with 50 mL of sterile milliQ water and again centrifuged at 2700 rcf for 2 min. The wash was repeated by re-suspending cells with 25 mL sterile milliQ water, cells were collected by centrifugation at 2700 rcf for 2 min. Finally the cells were resuspend with 1 mL 100 mM lithium acetate (LiOAc) and transferred to sterile 1.5 mL microcentrifuge tube. Cells were then collected by centrifugation in microfuge (set to max speed) for 10 sec. The supernatant was removed and the cells were re-suspended with 4 times the pellet volume of 100 mM LiOAc. Once the cells were prepared, a mixture of DNA (approximately 1ug for linear DNA fragment and about 500ng of plasmid DNA, wasbrought to 15 μL with sterile water), 72 μL 50% w/v PEG, 10 μL 1 M lithium

acetate, and 3 µL of denatured salmon sperm DNA (10 mg/mL) was prepared for each transformation. In a 1.5 mL tube, 15 µL of the cell suspension was added to the DNA mixture (100 µL), and the transformation suspension was vortexed for 5 short pulses. The transformation was incubated for 30 min at 30°C, followed by incubation for 22 min at 42°C. The cells were collected by centrifugation (18,000 rcf, 10 sec, 25°C). The cells were resuspended in 1 mL YPD and, after an overnight recovery shaking at 30°C and 250 rpm, 200 µL of the GEVO1287 transformation wasspread over YPD supplemented with 0.1 g/L hygromycin. 200 µL of the GEVO4378 transformation was spread over YPD supplemented with 0.1 g/L hygromycin and 0.2 g/L G418. Transformants were selected at 30°C. Transformants were then single colony purified onto either hygromycin and G418 or hygromycin selective plates.

[00360] Preparation of Yeast Lysate: *K. lactis* strains GEVO4378 and GEVO6169 were inoculated into 3 mL of YPD with 0.1 g/L hygromycin and incubated at 30°C at 250 rpm overnight culture. After approximately 18 h a 50 mL YPD or YPD + 0.1 g/L hygromycin culture in a baffled 250 mL shake flask was inoculated and shaken at 250 rpm until the culture reached approximately 2-3 OD_{600}. 20 OD_{600} of cells were harvested in 15 mL Falcon tubes and centrifuged at 4°C, 3000 rcf for 5 min. The medium was decanted and the cells were re-suspended in 2 mL of ice-cold MilliQ water. The cells were centrifuged a second time at 4°C, 3000 rcf for 5 min. The supernatant was again decanted, and the cells were centrifuged at 4°C, 3000 rcf for 5 min. The remaining medium was removed. The cell pellet was frozen at -80°C. The cell pellets were thawed on ice and 750 µL of lysis buffer (0.1 M Sodium Phosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT) was used to re-suspend each pellet. 800 µL of re-suspended cell pellet was added to a 1.5 mL centrifuge tube with 1 mL of 0.5 mm glass beads. The tubes containing the glass beads and cell suspension were put into the two bead beater blocks chilled to -20°C. The Retsch MM301 bead beater was set to 1 min and 300 1/sec frequency. To lyse the cells, the cell suspensions were beat 6 times for 1 min each, with 2 min of cooling the tubes and the bead beater blocks on ice in between beatings. After bead beating, the tubes were centrifuged at 4°C at 21,500g for 10 min in a tabletop centrifuge. The supernatant was transferred into 1.5 mL tubes and placed on ice for use in the DHAD assay. Yeast lysate protein concentration was determined as described under General Methods.

[00361] DHAD Assay: The assay was performed in triplicate for each sample. In addition, a no lysate control with lysis buffer was included. To assay each sample,

10 µL of a 1:10 dilution of lysate in lysis buffer (0.1 M Sodium Phosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT) was mixed with 90 µL of assay buffer (5 µL of 0.1 M $MgSO_4$, 10 µL of 0.1 M DHIV, and 75 µL 50 mM Tris pH 7.5), and incubated in a thermocycler for 30 min at 30°C, then at 95°C for 5 min. Insoluble material was removed from the samples by centrifugation at 3000 rcf for 5 min. The supernatants are transferred to fresh PCR tubes and submitted to analytics for analysis by liquid chromatography, method 2.

[00362] Liquid Chromatography, Method 2: DNPH reagent (4:1 of 15 mM 2,4 - Dinitrophenyl Hydrazine:100 mM Citric Acid pH 3.0) was added to each sample in a 1:1 ratio. Samples were incubated for 30 min at 70°C in a thermo-cycler (Eppendorf, Mastercycler). Analysis of keto-isovalerate was performed on an Agilent 1200 High Performance Liquid Chromatography system equipped with an Eclipse XDB C-18 reverse phase column (Agilent) and a C-18 reverse phase column guard (Phenomenex). Ketoisovalerate were detected using an Agilent 1100 UV detector (360 nm). The column temperature was 50°C. This method was isocratic with 70% acetonitrile 2.5% phosphoric acid (0.4%), 27.5% water as mobile phase. Flow was set to 3 mL/min. Injection size was 10 µL and run time was 2 min.

[00363] DHAD Assay Results: The *in vitro* DHAD enzymatic activity of lysates from the microaerobic fermentation of *K. lactis* strains was determined as described above. All values are the specific DHAD activity (U/mg total cell lysate protein) as averages from technical triplicates. In *K. lactis*, overexpression of the *Kl_AFT* gene resulted in an increase in DHAD activity (U/mg total cell lysate protein). GEVO4378 without Kl_AFT overexpression had an activity of 0.053 ± 0.009 U/mg while GEVO6169, overexpressing Kl_AFT had a specific activity of 0.131 ± 0.012 U/mg.

Example 10: Overexpression of the *Kluyveromyces marxianus AFT*

[00364] The purpose of this example is to demonstrate that overexpression of *K. marxianus AFT* (*Km_AFT*) is generally expected to increase DHAD activity in *K. marxianus.*

[00365] Standard molecular biology methods for cloning and plasmid construction are generally used, unless otherwise noted (Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press). Cloning techniques include gel purification of DNA fragments (using the Zymoclean Gel DNA Recovery Kit, Cat# D4002, Zymo Research Corp, Orange, CA).

[00366] Strains used in Example 10 are described in Table 24.

Table 24. Genotype of strains disclosed in Example 10.

GEVO Number	Genotype
***K. marxianus* GEVO1068**	*K. marxianus*, NRRL-Y7571
***K. marxianus* GEVO1947**	*ura3*Δ
***K. marxianus* GEVO6222**	*ura3*Δ Random integration of: P_{KmPDC}:*LI_ilvD*:P_{TPI}:*G418*:P_{PGK1}:*Km_AFT*:*T*:$_{ScAFT}$
***K. marxianus* GEVO6223**	Δ*ura3* Random integration of: P_{KmPDC}:*LI_ilvD*:P_{TPI}:*G418*:P_{PGK1}

[00367] In this example, the *K. marxianus URA3* gene was deleted by transformation of GEVO1068 with a PCR fragment (SEQ ID NO: 191) of *K. marxianus URA3* carrying a deletion of 348 base pairs that was amplified from pGV1799 (SEQ ID NO: 192) using primers oGV394 (SEQ ID NO: 193) and oGV395 (SEQ ID NO: 194). The *K. marxianus ura3* deletion strain transformants were selected by plating on 5-FOA (5-fluoroorotic acid) plates (For 500 mL: 10 g agar, 400 mL dH_2O, 0.5 g 5-FOA (in 5 mL DMSO), 50 mL 10Xa.a (14g yeast synthetic drop-out supplement (US Biological) dissolved in 1L water), 3.35 g YNB, 10 g glucose, 10 mL 50X HIS (0.95g histidine/250 mL H_2O), 10 mL 50X TRP (1.9 g in 500 mL H_2O), 10 mL 10X LEU (4.75 g Leucine/250 mL H_2O), 3.15 mL 25X URA(0.475 g uracil/250 mL H_2O). The 5-FOA resistant colonies were confirmed for the correct phenotype (auxotrophic for uracil). PCR demonstrated a partial deletion of approximately 200 bp in the *ura3* gene and this strain was named GEVO1947.

[00368] A linear DNA fragment containing *Km_AFT, LI_ilvD,* and a G418 resistance marker (SEQ ID NO: 195, Figure 5) is synthesized by DNA2.0. The fragment is randomly integrated by transformation into *K. marxianus* strain GEVO1947 to obtain GEVO6222. A linear fragment containing *LI_ilvD* and a G418 marker is also synthesized by DNA2.0 (SEQ ID NO: 196, Figure 6) and is randomly integrated by transforming *K. marxianus* strain GEVO1947 to obtain GEVO6223.

[00369] Transformations are carried out as follows: *K. marxianus* strain GEVO1947 is incubated in 50 mL of YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) shaking at 250 RPM at 30°C until the culture is at an OD_{600} of approximately 5. The cells are collected in a sterile 50 mL conical tube by centrifugation (1600 rcf, 5 min at room temperature). The cells are then resuspended in 10 mL of electroporation buffer (10 mM Tris-HCl, 270 mM sucrose, 1 mM $MgCl_2$, pH 7.5), and collected at 1600 rcf for 5 min at room temperature. The cells are then

resuspended in 10 mL IB (YPD medium, 25 mM DTT, 20 mM HEPES, pH 8.0; prepared fresh by diluting 100 µL of 2.5M DTT and 200 µL of 1 M HEPES, pH 8.0 into 10 mL of YPD) and are incubated for 30 min, 250 RPM, 30°C (tube standing vertical). The cells are collected at 1600 rcf for 5 min at room temperature and resuspended in 10 mL of chilled electroporation buffer. The cells are then pelleted at 1600 rcf for 5 min at 4°C. The cells are then resuspended in 1 mL of chilled electroporation buffer and transferred to a microfuge tube. The cells are collected by centrifugation at >10,000 rcf for 20 sec at 4°C. The cells are then resuspended in an appropriate amount of chilled electroporation buffer for a final biomass concentration of 30 OD_{600}/mL. 400 µL of cell suspension is added to a chilled electroporation cuvette (0.4cm gap) and 50 µL of DNA (SEQ ID NO: 195 or SEQ ID NO: 196 or water control) is added and mixed by pipetting up and down, and the cuvette is incubated on ice for 15-30 min. The samples are then electroporated at 1.8 kV, 1000 Ohm, 25 µF. The samples are transferred to a 50 mL tube with 1 mL YPD medium, and the samples are incubated for 4 h at 250 rpm at 30°C. 200 µL of each transformation culture are spread onto YPD plates containing 0.2 g/L G418 and the plates are incubated at 30°C until individual colonies develop.

[00370] *K. marxianus* strain GEVO6222 is verified by colony PCR for the integration of *Km_AFT* using primers PGK1F (SEQ ID NO: 197) and KmAFTR (SEQ ID NO: 198) (yielding an approximately 325 base pair product) and integration of *LI_ilvD* using primers oGV2107 (SEQ ID NO: 199) and oGV2108 (SEQ ID NO: 200) (yielding an approximately 104 base pair product). *K. marxianus* strain GEVO6223 is verified by colony PCR for the integration of *LI_ilvD* using primers oGV2107 and oGV2108.

[00371] Next, *K. marxianus* strains GEVO1947, GEVO6222 and GEVO6223 are inoculated into 3 mL of YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) and incubated at 30°C at 250 rpm. After approximately 18 h, a 50 mL YPD culture in a baffled 250 mL shake flask is inoculated and shaken at 250 rpm until the culture reaches approximately 2-3 OD_{600}. Cell pellets are prepared by taking 20 OD units of culture [OD_{600nm} × volume (mL) = 20] and centrifuging the appropriate volume at 3000 rpm and 4°C for 5 min. The medium is decanted and the cells are resuspended in 2 mL of ice-cold MilliQ water. The cells are centrifuged a second time at 4°C, 3000 rcf for 5 min. The supernatant is again decanted, and the cells are centrifuged at 4°C, 3000 rcf for 5 min. The remaining medium is removed. The cell pellet is frozen at -80°C. To prepare lysate, the cell pellets are thawed on ice and

750 µL of lysis buffer (0.1 M Sodium Phosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT) is used to re-suspend each pellet. 800 µLof re-suspended cell pellet is added to a 1.5 mL centrifuge tube with 1 mL of 0.5 mm glass beads. The tubes containing the glass beads and cell suspension are put into the two bead beater blocks chilled to -20°C. A Retsch MM301 bead beater is set to 1 min and 300 1/sec frequency. To lyse the cells, the cell suspensions are beat 6 times for 1 min each, with 2 min of cooling the tubes and the bead beater blocks on ice in between beatings. After bead beating, the tubes are centrifuged at 4°C at 21,500g for 10 min in a tabletop centrifuge. The supernatant is transferred into 1.5 mL tubes and placed on ice for use in the DHAD activity assay. Yeast lysate protein concentration is determined as described under General Methods.

[00372] DHAD assays are performed as described in the general methods sectionLiquid chromatography method 2 is performed as described in the general methods section.

[00373] Results for DHAD activity: Data is presented as specific DHAD activity (U/mg total cell lysate protein) averages from biological and technical triplicates with standard deviations. DHAD activity in GEVO6223, containing DHAD is generally expected to be lower than that of GEVO6222 containing both Km_AFT and DHAD.

Example 11: Construction of *Issatchenkia orientalis* Strain with Isobutanol Pathway Genes Integrated into the Genome

[00374] The purpose of this example is to demonstrate that overexpression of *Issatchenkia orientalis AFT1-2* (herein referred to as *Io_AFT1-2)* increases DHAD activity in *I. orientalis.*

[00375] An *I. orientalis* strain derived from PTA-6658 (US 2009/0226989) was grown overnight and transformed using the lithium acetate method as described in Gietz, *et al* (1992, *Nucleic Acids Research* 20: 1524). The strain was transformed with homologous integration constructs using native *I. orientalis* promoters to drive protein expression. *Issatchenkia orientalis* strains used are described in Table 25.

Table 25. Genotype of strains disclosed in Example 11.

Strain Number	Genotype
GEVO6155	*ura3/ura3* *gpd1Δ::P_{Io_PDC}: Ll_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Io_URA3: loxP: P_{Io_ENO1}: Ll_ilvD-1/* *gpd1Δ:: P_{Io_PDC}:Ll_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Sc_MEL5: loxP: P_{Io_ENO1}:Ll_ilvD-1*

	TMA29/tma29Δ::P_{Io_PDC1}:LI_adhA^{RE1}:P_{Io_TDH3}:Ec_ilvC$^{P2D1-A1}$: loxP: Io_URA3: loxP: P_{Io_ENO1}: LI_ilvD-4
GEVO6162	*ura3/ura3* *gpd1Δ::P_{Io_PDC}: LI_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Io_URA3: loxP: P_{Io_ENO1}: LI_ilvD-1/* *gpd1Δ:: P_{Io_PDC}:LI_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Sc_MEL5: loxP: P_{Io_ENO1}:LI_ilvD-1 (SEQ ID NO: 204)* *TMA29/tma29Δ:: P_{Io_PDC1}: LI_adhA^{RE1}: P_{Io_TDH3}:Ec_ilvC$^{P2D1-A1}$: loxP: Io_URA3: loxP: P_{ENO1}:LI_ilvD-4 (SEQ ID NO: 206): P_{PYK1}:Io_AFT1-2*
GEVO6203	*ura3/ura3* *gpd1Δ::P_{Io_PDC}: LI_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Io_URA3: loxP: P_{Io_ENO1}: LI_ilvD/* *gpd1Δ:: P_{Io_PDC}:LI_adhA^{RE1}: T_{ScCYC1}: P_{Io_TDH3}: Ec_ilvC$^{P2D1-A1}$: $T_{ScGAL10}$: loxP: Sc_MEL5: loxP: P_{Io_ENO1}:LI_ilvD* *TMA29/tma29Δ:: P_{Io_PDC1}: LI_adhA^{RE1}: P_{Io_TDH3}:Ec_ilvC$^{P2D1-A1}$: loxP: Io_URA3: loxP: P_{ENO1}:LI_ilvD: P_{PYK1}:Io_AFT1-2*

[00376] Three strains were used to demonstrate that the overexpression of *I. orientalis AFT1-2* increases DHAD activity in *I. orientalis*. GEVO6155 does not contain the heterologous *AFT1-2* expression construct, while both GEVO6162 and GEVO6203 have the heterologous *AFT1-2* construct integrated into the genome. All three strains were cultured in two different conditions and then tested for DHAD activity.

[00377] In the first condition, cultures were started for each strain (GEVO6155, GEVO6162, and GEVO6203) in 12 mL YP medium (1% (w/v) yeast extract, 2% (w/v) peptone) containing 5% (w/v) glucose and incubated at 30°C and 250 RPM for 9 h. The OD_{600} of the 12 mL cultures was determined and the appropriate volume of each culture was used to inoculate 50 mL of YP medium containing 8% glucose in separate 250 mL baffled flasks to an OD_{600} of 0.01. The flasks were incubated at 30°C and 250 RPM for 18 h. A total of 80 OD_{600} of cells were harvested and the cell suspension was transferred to 50 mL Falcon tubes. Cells were pelleted at 3000 rcf for 5 min at 4°C, and washed twice in 2 mL cold, sterile water. The cell pellets were stored at -80°C until analysis by DHAD assay.

[00378] In the second condition, cultures were inoculated at a starting OD_{600} of 0.1 and were incubated at 30°C with 250 rpm shaker speed for 20 h and then the shaker speed was reduced to 75 rpm for an additional 28 h prior to sampling. Cells were washed twice with cold sterile water and stored at -80°C until analysis.

[00379] To determine DHAD activity in whole cell lysates, the frozen cell pellets were thawed on ice and resuspended in 750 µL lysis buffer (100 mM $NaPO_4$ pH 7.0, 5 mM $MgCl_2$ and 1 mM DTT). One mL of glass beads (0.5 mm diameter) were added to a 1.5 mL microcentrifuge tube and the entire cell suspension for each strain was added to seperate tubes containing glass beads. Yeast cells were lysed using a

Retsch MM301 bead beater (Retsch Inc. Newtown, PA), bead beating six times for 1 min each at full speed with 1 min icing in between each bead beating step. The tubes were centrifuged for 10 min at 23,500 x*g* at 4°C and the supernatant was removed. Supernatants were held on ice until assayed. Yeast lysate protein concentration was determined as described under General Methods.

[00380] DHAD assays were performed in triplicate for each sample. In addition, an assay on a no lysate control with lysis buffer was performed. To assay each sample, 10 µL of lysate in assay buffer was mixed with 90 µL of assay buffer (5 µL of 0.1 M $MgSO_4$, 10 µL of 0.1 M DHIV, and 75 µL 50 mM Tris pH 7.5), and incubated in a thermocycler (Eppendorf, Mastercycler) for 30 min at 30°C, then at 95°C for 5 min. Insoluble material was removed from the samples by centrifugation at 3000 rcf for 5 min. The supernatants were transferred to fresh PCR tubes. 100 µL DNPH reagent (12 mM 2,4 - dinitrophenyl hydrazine, 10 mM citric acid, pH 3.0, in 80% acetonitrile, 20% MilliQ H_2O) was added to 50 µL of each sample and 50 µL of MilliQ H_2O. Samples were incubated for 30 min at 70°C in a thermocycler.

[00381] Analysis of keto-isovalerate (KIV) was performed on an Agilent 1200 High Performance Liquid Chromatography system equipped with an Eclipse XDB C-18 reverse phase column (Agilent) and a C-18 reverse phase column guard (Phenomenex). Ketoisovalerate was detected using an Agilent 1100 UV detector (360 nm). The column temperature was 50°C. This method was isocratic with 70% acetonitrile 2.5% phosphoric acid (0.4%), 27.5% water as mobile phase. Flow was set to 3 mL/min. Injection size was 10 µL and run time was 2 min. KIV was quantified on a 3-point linear calibration curve.

[00382] The *in vitro* DHAD enzymatic activity of lysates from the samples of *I. orientalis* strains were carried out as described above. DHAD activity (U/mg total cell lysate protein) is reported as averages from biological triplicate samples. In *I. orientalis*, overexpression of the *I. orientalis AFT1-2* gene resulted in an increase in DHAD activity (U/mg total cell lysate protein). The cultures harvested at 18 h (samples inoculated at 0.01) had DHAD activity values as follows: GEVO6155 had an activity of 0.039 ± 0.004 U/mg while GEVO6162 had an activity of 0.082 ± 0.005 U/mg and GEVO6203 had an activity of 0.060 ± 0.011 U/mg. The cultures harvested at 48 h (cultures inoculated at 0.1) had DHAD activity values as follows: GEVO6155 had an activity of 0.085 ± 0.014 U/mg while GEVO6162 had an activity of 0.155 ± 0.020 U/mg and GEVO6203 had an activity of 0.140 ± 0.033 U/mg. Therefore, this example demonstrates that overexpression of *Io_AFT1-2* increases DHAD activity in

I. orientalis.

Example 12: Overexpression of Fe-S Assembly Machinery

[00383] To ascertain the effects of overexpressing a cytosolic 2Fe-2S or 4Fe-4S cluster-containing DHAD with candidate assembly machinery, the following steps, or equivalent steps can be carried out. First, the coding sequence for the open reading frame of the DHAD from spinach or other 2Fe-2S or 4Fe-4S cluster-containing DHAD is cloned into the high-copy (2micron origin) *S.cerevisiae* expression vector pGV2074, such that expression of the coding sequence is directed by the PGK1 promoter sequence, yielding plasmid pGV2074-1. Next, the NifU and NifS genes from *Entamoeba histolytica* or the homologous NIF genes from *Lactococcus lactis* are successively introduced into the aforementioned vector, eventually yielding a single plasmid (pGV2074-2) where the expression of all 3 genes is directed by strong constitutive *S.cerevisiae* promoter sequences. Plasmids pGV2074-1 and pGV2074-2 are transformed into *S. cerevisiae* strain GEVO2244 (relevant genotype, *ilv3Δ*) and transformants selected by resistance to Hygromycin B (0.1 g/L). At least 3 individual colonies arising from each transformation are cultured, a cell lysate produced, and the DHAD activity present therein measured, all according to previously-described methods.

[00384] The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood there from as modifications will be obvious to those skilled in the art.

[00385] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

[00386] The disclosures, including the claims, figures and/or drawings, of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A recombinant yeast microorganism comprising a recombinantly overexpressed polynucleotide encoding a dihydroxy acid dehydratase (DHAD), wherein said recombinant yeast microorganism is engineered to delete or attenuate the expression or activity of one or more endogenous glutathione-dependent oxidoreductases selected from the group consisting of Grx3 and Grx4.

2. The recombinant yeast microorganism of claim 1, wherein said recombinant microorganism comprises an isobutanol producing metabolic pathway, said isobutanol producing metabolic pathway comprising the following substrate to product conversions:

(a) pyruvate to acetolactate;

(b) acetolactate to 2,3-dihydroxyisovalerate;

(c) 2,3-dihydroxyisovalerate to α-ketoisovalerate;

(d) α-ketoisovalerate to isobutyraldehyde; and

(e) isobutyraldehyde to isobutanol;

and wherein said DHAD catalyzes the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate.

3. The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of pyruvate to acetolactate is an acetolactate synthase.

4. The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of acetolactate to 2,3-dihydroxyisovalerate is a ketol-acid reductoisomerase.

5. The recombinant yeast microorganism of claim 4, wherein said ketol-acid reductoisomerase is an NADH-dependent ketol-acid reductoisomerase.

6. The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the cytosol.

7. The recombinant yeast microorganism of claim 1, wherein said DHAD is localized in the mitochondria.

8. The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Lactococcus lactis.*

9. The recombinant yeast microorganism of claim 1, wherein said DHAD is derived from *Streptococcus mutans*.

10. The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of α-ketoisovalerate to isobutyraldehyde is a 2-keto acid decarboxylase.

11. The recombinant yeast microorganism of claim 2, wherein the enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol is an alcohol dehydrogenase.

12. The recombinant yeast microorganism of claim 11, wherein said alcohol dehydrogenase is an NADH-dependent alcohol dehydrogenase.

13. The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is engineered to inactivate one or more endogenous pyruvate decarboxylase (PDC) genes.

14. The recombinant yeast microorganism of claim 2, wherein said recombinant yeast microorganism is engineered to inactivate one or more endogenous glycerol-3-phosphate dehydrogenase (GPD) genes.

15. The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is engineered to overexpress one or more polynucleotides encoding one or more activator of ferrous transport (Aft) proteins.

16. The recombinant yeast microorganism of claim 1, wherein said recombinant yeast microorganism is engineered to express one or more polynucleotides encoding one or more constitutively active activator of ferrous transport (Aft) proteins.

17. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following genera: *Saccharomyces*, *Kluyveromyces*, *Pachysolen*, *Zygosaccharomyces*, *Debaryomyces*, *Pichia*, *Schizosaccharomyces*, *Candida*, *Issatchenkia*, *Hansenula*, *Yarrowia*, *Tricosporon*, *Rhodotorula,* and *Myxozyma.*

18. The recombinant yeast microorganism of claim 1, wherein the recombinant yeast microorganism is a yeast microorganism selected from one of the following species: *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces bayanus*, *Saccharomyces paradoxus*, *Saccharomyces castelli*, *Saccharomyces kluyveri*, *Kluyveromyces thermotolerans*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces waltii*, *Pachysolen tannophilis*, *Zygosaccharomyces bailli*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Debaromyces carsonii*, *Pichia pastorius*, *Pichia anomala*, *Pichia stipitis*, *Pichia castillae*, *Schizosaccharomyces pombe*, *Candida utilis*, *Candida glabrata*, *Candida tropicalis*, *Candida xestobii*, *Issatchenkia orientalis*, *Issatchenkia occidentalis*, *Issatchenkia scutulata*, *Hansenula anomala*, and *Yarrowia lipolytica*.

19. A method of producing isobutanol comprising: (a) providing the recombinant yeast microorganism of claim 2; and (b) cultivating the recombinant yeast microorganism of claim 2 in a culture medium containing a feedstock providing a carbon source, until a recoverable quantity of the isobutanol is produced.

ABSTRACT

The present invention is directed to recombinant microorganisms comprising one or more dihydroxyacid dehydratase (DHAD)-requiring biosynthetic pathways and methods of using said recombinant microorganisms to produce beneficial metabolites derived from said DHAD-requiring biosynthetic pathways. In various aspects of the invention, the recombinant microorganisms may be engineered to overexpress one or more polynucleotides encoding one or more Aft proteins or homologs thereof. In some embodiments, the recombinant microorganisms may comprise a cytosolically localized DHAD enzyme. In additional embodiments, the recombinant microorganisms may comprise a mitochondrially localized DHAD enzyme. In various embodiments described herein, the recombinant microorganisms may be microorganisms of the Saccharomyces clade, Crabtree-negative yeast microorganisms, Crabtree-positive yeast microorganisms, post-WGD (whole genome duplication) yeast microorganisms, pre-WGD (whole genome duplication) yeast microorganisms, and non-fermenting yeast microorganisms.

FIGURES

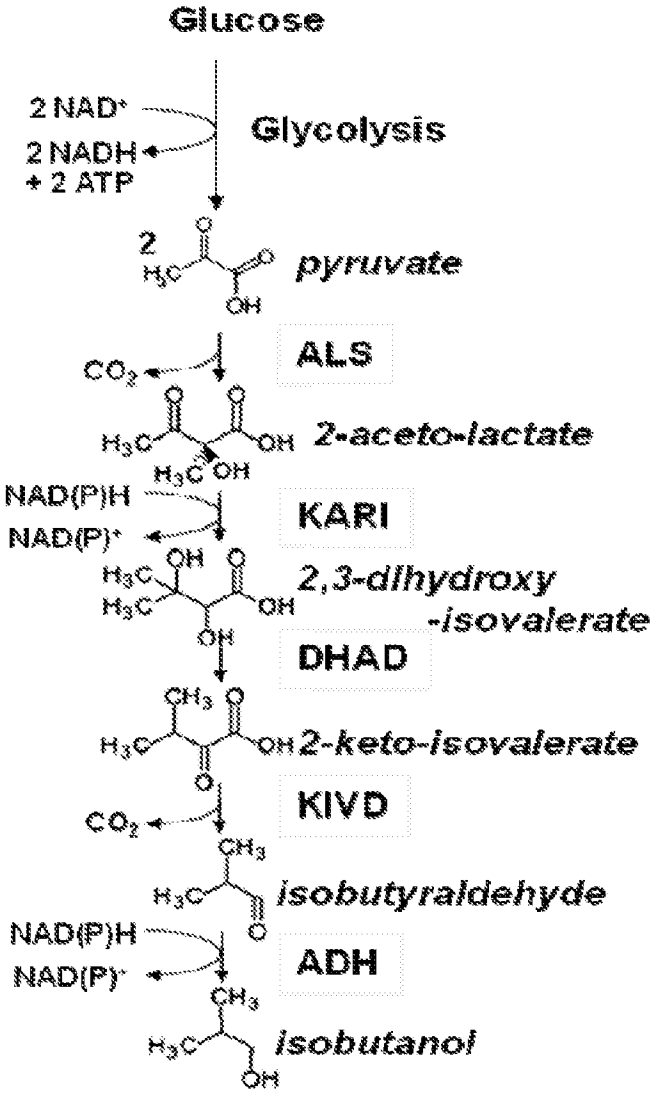
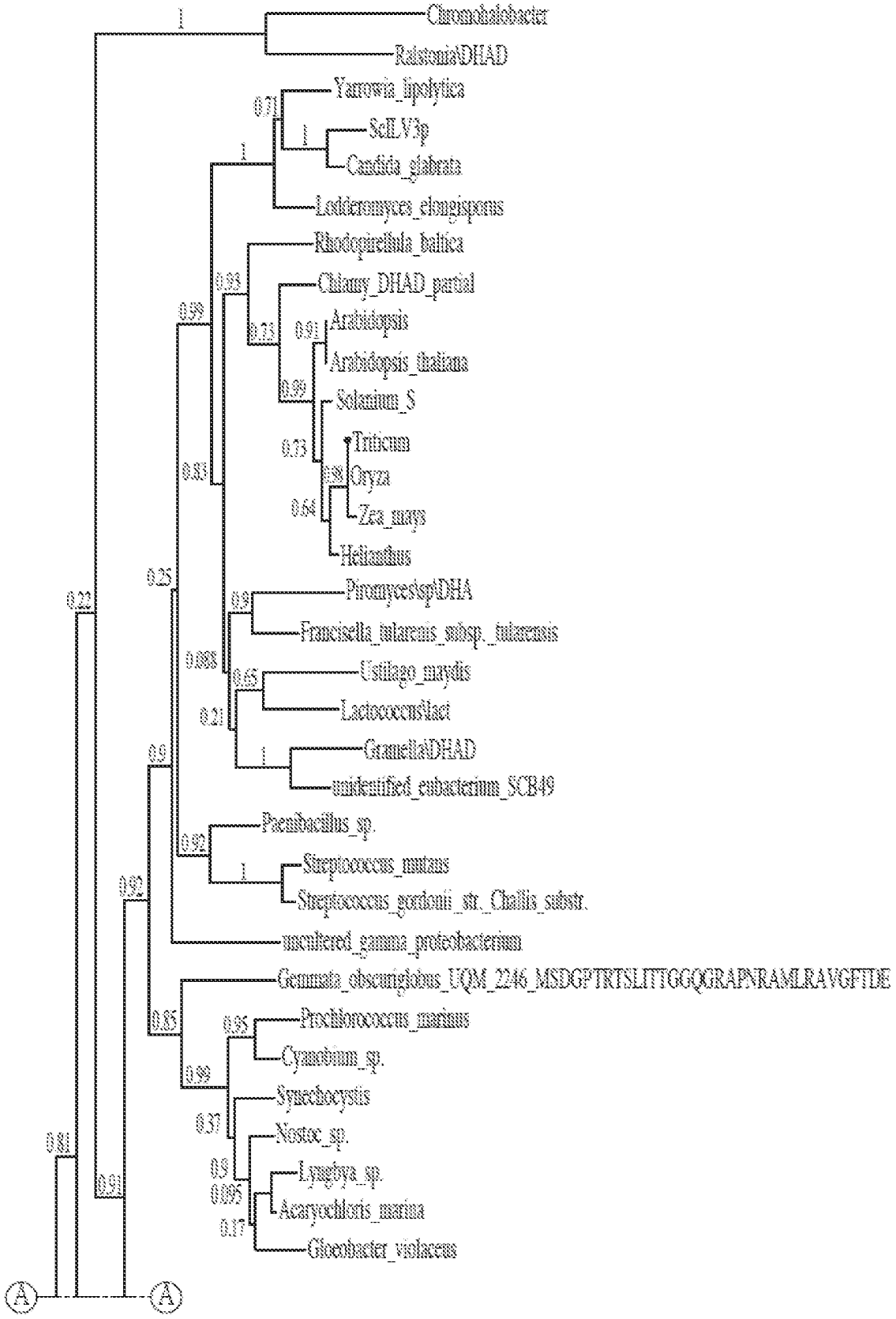


FIGURE 1


Citrobacter
Ralstonia_DHAD
Yarrowia_lipolytica
ScILV3p
Candida_glabrata
Lodderomyces_elongisporus
Rhodopirellula_baltica
Chlamy_DHAD_partial
Arabidopsis
Arabidopsis_thaliana
Solanum_S
Triticum
Oryza
Zea_mays
Helianthus
Pirellula_sp_DHA
Francisella_tularensis_subsp._tularensis
Ustilago_maydis
Lactococcus_lactis
Granulo_DHAD
unidentified_eubacterium_SCB49
Paenibacillus_sp.
Streptococcus_mutans
Streptococcus_gordonii_str._Challis_substr.
uncultured_gamma_proteobacterium
Gemmata_obscuriglobus_UQM_2246_MSDGPTRTSLITTGQQGRAPNRAMLRAVGFTDE
Prochlorococcus_marinus
Cyanothece_sp.
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Lyngbya_sp.
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Gloeobacter_violaceus
A
A

FIGURE 2

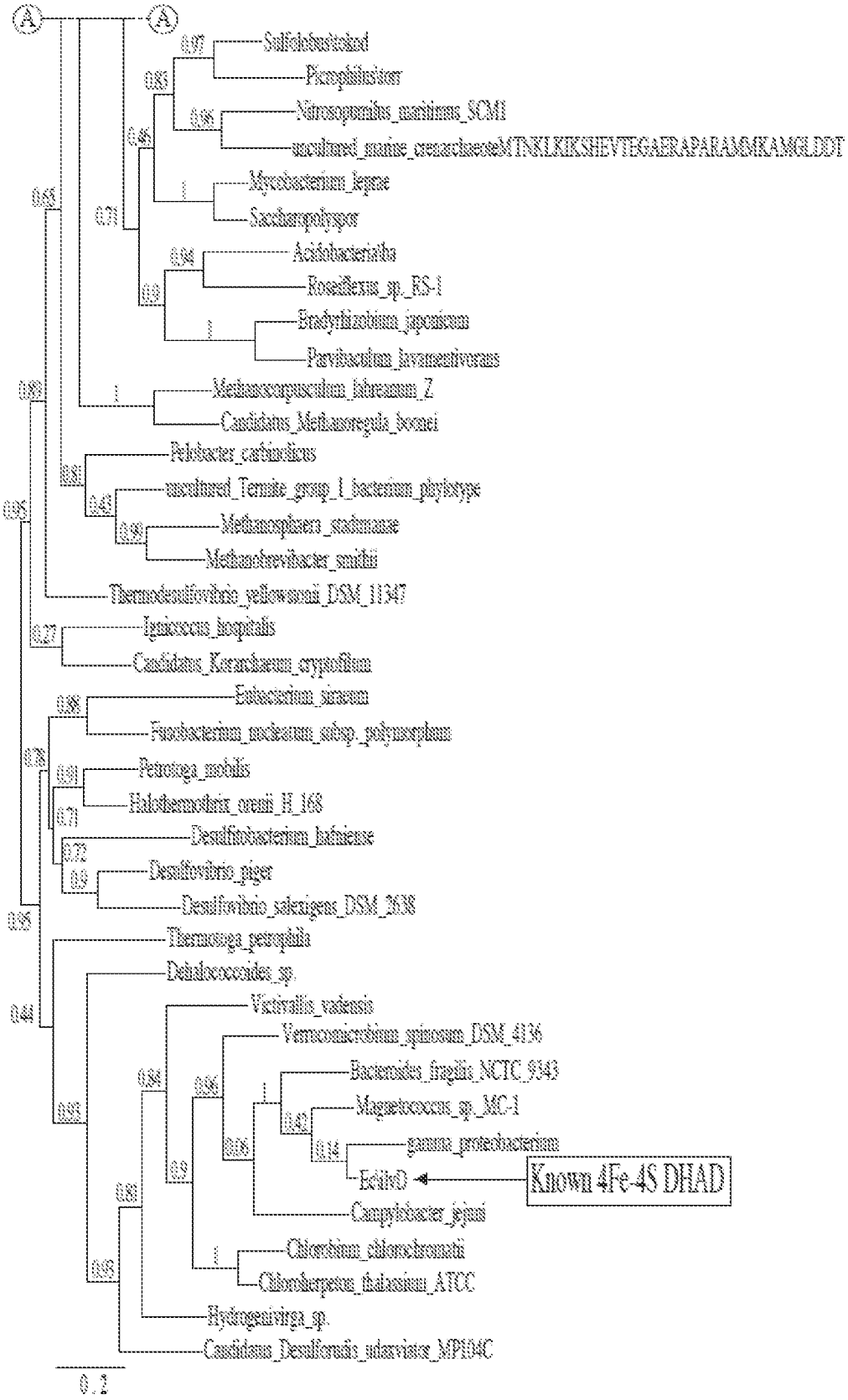
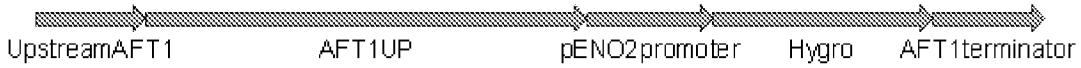


FIGURE 2 (CONT.)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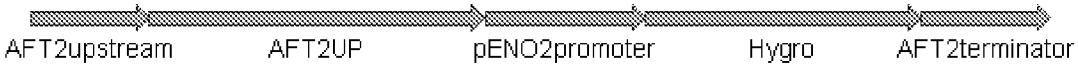
AFT1UP
pENO2promoter
Hygro
AFT1terminator

FIGURE 3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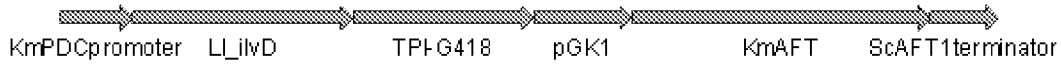
AFT2UP
pENO2promoter
Hygro
AFT2terminator

FIGURE 4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Ll_ilvD
TPI-G418
pGK1
KmAFT
ScAFT1terminator

FIGURE 5

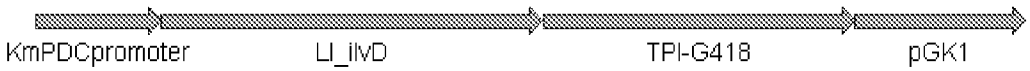

FIGURE 6

PTO/SB/14 (07-07)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	GEVO-041/13US 310142-000
	Application Number	
Title of Invention	METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Applicant Information:

Applicant 1

Applicant Authority ☑ Inventor	☐ Legal Representative under 35 U.S.C. 117	☐ Party of Interest under 35 U.S.C. 118

Prefix	**Given Name**	**Middle Name**	**Family Name**	**Suffix**
	Catherine	Asleson	DUNDON	

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City	Englewood	**State/Province**	CO	**Country of Residence** [i]	US
Citizenship under 37 CFR 1.41(b) [i]	US				

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Postal Code	80112	**Country** [i]	US

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Applicant Authority ☑ Inventor	☐ Legal Representative under 35 U.S.C. 117	☐ Party of Interest under 35 U.S.C. 118

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Citizenship under 37 CFR 1.41(b) [i]	CY				

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City	Englewood	**State/Province**	CO
Postal Code	80112	**Country** [i]	US

Applicant 3

Applicant Authority ☑ Inventor	☐ Legal Representative under 35 U.S.C. 117	☐ Party of Interest under 35 U.S.C. 118

Prefix	**Given Name**	**Middle Name**	**Family Name**	**Suffix**

	Andrew		HAWKINS	

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
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Citizenship under 37 CFR 1.41(b) [i]	US				
Mailing Address of Applicant:					
Address 1	c/o Gevo, Inc., 345 Inverness Drive South				
Address 2	Building C, Suite 310				
City	Englewood			**State/Province**	CO
Postal Code	80112			**Country** [i]	US

Applicant 4			
Applicant Authority ☑ Inventor	☐ Legal Representative under 35 U.S.C. 117	☐ Party of Interest under 35 U.S.C. 118	

Prefix	**Given Name**	**Middle Name**	**Family Name**	**Suffix**
	Doug		LIES	

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Parker	**State/Province**	CO	**Country of Residence** [i]	US
Citizenship under 37 CFR 1.41(b) [i]	US				
Mailing Address of Applicant:					
Address 1	c/o Gevo, Inc., 345 Inverness Drive South				
Address 2	Building C, Suite 310				
City	Englewood			**State/Province**	CO
Postal Code	80112			**Country** [i]	US

Applicant 5			
Applicant Authority ☑ Inventor	☐ Legal Representative under 35 U.S.C. 117	☐ Party of Interest under 35 U.S.C. 118	

Prefix	**Given Name**	**Middle Name**	**Family Name**	**Suffix**
	Lynne		ALBERT	

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Golden	**State/Province**	CO	**Country of Residence** [i]	US
Citizenship under 37 CFR 1.41(b) [i]	US				
Mailing Address of Applicant:					
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Address 2	Building C, Suite 310				
City	Englewood			**State/Province**	CO
Postal Code	80112			**Country** [i]	US

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).	
☐ **An Address is being provided for the correspondence Information of this application.**	
Customer Number	58249
Email Address	

Application Information:

Title of the Invention	**METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**		
Attorney Docket Number	GEVO-041/13US 310142-000	**Small Entity Status Claimed** ☒	
Application Type	Nonprovisional		
Subject Matter	UTILITY		
Suggested Class (if any)		**Sub Class (if any)**	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any) 7 [FIGS 1-6]		**Suggested Figure for Publication (if any)**	

Publication Information:

☒	Request Early Publication (Fee required at time of Request 37 CFR 1.219)
☐	Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Enter either Customer Number or complete the Representative Name section below. If both sections are completed the Customer Number will be used for the Representative Information during processing.

Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ US Representative (37 CFR 11.9)
Customer Number	58249		

Domestic Benefit/National Stage Information:

This section allows for the applicant to claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78(a)(2) or CFR 1.78(a)(4), and need not otherwise be made part of the specification.

Prior Application Status	Pending		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
This application	divisional	13/228,342	2011-09-08
Prior Application Status	Pending		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
This application	divisional	12/953,884	2010-11-24
Prior Application Status	Pending		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
12/953,884	nonprovisional	61/263,952	2009-11-24
Prior Application Status	Pending		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
12/953,884	nonprovisional	61/350,209	2010-06-01

Foreign Priority Information:

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

Application Number	Country [1]	Parent Filing Date (YYYY-MM-DD)	Priority Claimed
			☐ Yes ☒ No

Assignee Information:

Providing this information in the application data sheet does not substitute for compliance with any requirement of part 3 of Title 37 of the CFR to have an assignment recorded in the Office. .

Assignee 1			
If the Assignee is an Organization check here. ☑			
Organization Name	GEVO, INC.		
Mailing Address Information:			
Address 1	345 Inverness Drive South		
Address 2	Building C, Suite 310		
City	Englewood	**State/Province**	CO
Country [i]	US	Postal Code	80112
Phone Number		Fax Number	
Email Address			

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.					
Signature	**/Paul Wickman/**			Date (YYYY-MM-DD)	2011-09-27
First Name	Paul	Last Name	Wickman	Registration Number	61242

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require completing this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Attorney Docket No. GEVO-041/13US 310142-000 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Catherine Asleson DUNDON *et al.*		Confirmation No.: *To Be Assigned*
Serial No.:	*To Be Assigned* (DIV of U.S. Ser. No. 13/228,342)	Group Art Unit: *To Be Assigned*
Filed:	September 27, 2011	Examiner: *To Be Assigned*

FOR: **METHODS OF INCREASING DIHYDROXY ACID DEHYDRATASE ACTIVITY TO IMPROVE PRODUCTION OF FUELS, CHEMICALS, AND AMINO ACIDS**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DELETION OF INVENTORS UNDER 37 C.F.R. § 1.63(d)(iv)(2)

In accordance with the requirement for an oath or declaration under 37 C.F.R. § 1.63, a copy of the executed declaration filed in a parent application, U.S. Application Serial No. 12/953,884, is submitted herewith for the divisional application.

For the purposes of the present application, please delete the following individuals, who are not inventors of the present application:

Jun Urano

Peter Meinhold

Reid M. Renny Feldman

Thomas Buelter

Matthew Peters

Stephanie Porter-Scheinman

Christopher Smith

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. **50-1283**.

Dated: September 27, 2011

Respectfully submitted,
COOLEY LLP

COOLEY LLP
ATTN: Patent Group
777 6th Street NW, Suite 1100
Washington, DC 20001

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By: /Paul A. Wickman/
Paul A. Wickman
Reg. No. 61,242