



(51) International Patent Classification:

C12N 9/04 (2006.01) *C12N 9/92* (2006.01)
C12N 9/02 (2006.01) *C12P 7/46* (2006.01)
C12N 9/88 (2006.01)

(21) International Application Number:

PCT/US2013/052066

(22) International Filing Date:

25 July 2013 (25.07.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/675,785 25 July 2012 (25.07.2012) US

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(81) Designated States (unless otherwise indicated, for every
 kind of national protection available): AE, AG, AL, AM,
 AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
 BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
 DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
 HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR,
 KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
 MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
 OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC,
 SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
 TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
 kind of regional protection available): ARIPO (BW, GH,
 GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
 UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
 TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
 EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
 MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
 TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
 KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
 claims and to be republished in the event of receipt of
 amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: YEAST CELLS HAVING NADP(H)-DEPENDENT REDUCTIVE TCA PATHWAY FROM PYRUVATE TO SUC-
 CINATE

(57) Abstract: Recombinant yeast cells contain a reductive TCA pathway from phosphoenolpyruvate or pyruvate to succinate. At
 least one metabolic step in the pathway includes a reaction of NADPH to produce NADP⁺. The yeast cell contains at least one exo-
 genous NADPH-dependent gene in the pathway from phosphoenolpyruvate or pyruvate to succinate, preferably an NADPH-depend-
 ent malate dehydrogenase or fumarate reductase gene (or both).



WO 2014/018755 A1

YEAST CELLS HAVING NADP(H)-DEPENDENT REDUCTIVE TCA PATHWAY FROM PYRUVATE TO SUCCINATE

This invention relates to recombinant yeast having an active reductive TCA pathway from pyruvate to succinate. The inventions disclosed and claimed herein were made pursuant to a joint research agreement between Cargill Incorporated, Wayzata, Minnesota, US, and BioAmber S.A.S, Bazancourt, France.

Succinic acid is a chemical intermediate useful as a precursor for making compounds such as 1,4-butanediol, tetrahydrofuran and gamma-butyrolactone. It is also a useful diacid that can be polymerized with a polyol to make polyester resins. Succinic acid can be produced industrially from butane. However, butane is a petrochemical, and there is a strong desire to develop processes for making many chemical compounds from annually renewable resources such as plant or animal feedstocks.

Some microorganisms have evolved the ability to produce succinate from carbohydrate feedstocks. In some cases, these strains have been engineered to improve yield and/or productivity. WO 2007/061590 describes recombinant yeast cells that produce succinate. Some yeast species are of interest as candidates for succinic acid-producing fermentations because they are resistant to low pH conditions, and so can produce acidic fermentation products at a low pH at which the product acid exists mainly in the acid form rather than in the salt form. Producing the acid directly in the acid form simplifies recovery and purification, as salt splitting, with its attendant requirements for raw materials, capital, operating and disposal costs, can be reduced if not eliminated.

There are three primary fermentation pathways by which a microorganism can produce succinate: oxidative tricarboxylic acid (TCA), glyoxylate shunt, and reductive TCA. The oxidative TCA pathway begins with the conversion of oxaloacetate (OAA) and acetyl-CoA to citrate. OAA can be generated from carboxylation of phosphoenolpyruvate (PEP) or pyruvate, while acetyl-CoA is generated from the decarboxylation of pyruvate by pyruvate dehydrogenase (PDH) or pyruvate formate lyase (PFL). Citrate is converted to isocitrate, isocitrate is converted to α -ketoglutarate, α -ketoglutarate is converted to succinyl-CoA, and succinyl-CoA is converted to succinate.

Like the oxidative TCA pathway, the glyoxylate shunt pathway begins with the generation of citrate from OAA and acetyl-CoA and the conversion of citrate to

isocitrate. Isocitrate is converted to glyoxylate and succinate. Glyoxylate is condensed with acetyl-CoA to form malate, and the resultant malate is converted to succinate via a fumarate intermediate.

The reductive TCA pathway begins with carboxylation of phosphoenolpyruvate (PEP) or pyruvate to oxaloacetate (OAA) (by PEP carboxylase (PPC) and pyruvate carboxylase (PYC), respectively). OAA is converted to malate by malate dehydrogenase (MDH), malate is converted to fumarate by fumarase (FUM, also known as fumarate hydratase), and fumarate is converted to succinate by fumarate reductase (FRD). The reductive TCA pathway provides the highest succinate yield of the three succinate fermentation pathways, per mole of glucose consumed, and for that reason offers the best economic potential.

A problem with the reductive TCA pathway is that the MDH enzyme consumes NADH as a cofactor. In addition, certain efficient FRD enzymes also consume NADH. Examples of such NADH-dependent FRD enzymes are described, for example, in WO 2009/065778 and PCT/US2011/022612. Thus, certain efficient metabolic pathways from pyruvate to succinate consume two molecules of NADH. One molecule of NADH is produced when sugars such as glucose are metabolized to pyruvate via the glycolytic pathway, but this still leaves a net deficit of one NADH, which results in a redox imbalance. A living cell must correct this redox balance if it is to remain healthy and continue to metabolize through the reductive TCA pathway. This typically means that the cell must balance the net NADH consumption by replacing the consumed NADH from other metabolic processes that produce NADH. For example, the reductive TCA pathway can be combined with one or both of the oxidative TCA or glyoxylate shunt pathways to help with the redox balance, but the oxidative TCA and glyoxylate shunt pathways produce less succinic acid per mole of starting sugar, and taking this approach therefore results in a loss of yield. It is possible for the cell to use one or more unrelated pathways to produce the needed NADH, but this can have adverse consequences for cell health and productivity, and may create other imbalances within the cell.

Therefore, there remains a desire to provide a yeast strain that efficiently produces succinic acid (or its salts).

This invention is in one aspect a recombinant cell having an active reductive TCA pathway from pyruvate to succinate, which reductive TCA pathway includes at

least one reaction that oxidizes NADPH to NADP⁺. In specific embodiments, this recombinant cell has one or more of the following features:

In any of the foregoing recombinant cells, the active reductive TCA pathway from pyruvate to succinate may include a step of converting pyruvate or phosphoenolpyruvate to oxaloacetate, a step of converting oxaloacetate to malate, a step of converting malate to fumarate, and a step of converting fumarate to succinate.

In another aspect, this invention is a recombinant yeast cell that overexpresses an NADPH-dependent malate dehydrogenase enzyme. Such a yeast cell in some embodiments has at least one exogenous NADPH-dependent malate dehydrogenase gene integrated into its genome. The recombinant cell of this aspect of the invention may express a NADPH-dependent fumarate reductase enzyme.

In another aspect this invention is a recombinant yeast cell that overexpresses an NADPH-dependent fumarate reductase enzyme. Such a yeast cell in some embodiments has at least one exogenous NADPH-dependent fumarate reductase gene integrated into its genome. The recombinant cells of this aspect of the invention may also express a NADPH-dependent malate dehydrogenase enzyme.

Yeast cells of the foregoing aspects of the invention avoid the net consumption of NADH in the production of succinate through the reductive TCA pathway, and in that manner mitigates if not eliminates the NAD(H) redox imbalance. This permits better cell health and productivity.

Because the metabolic pathway to succinate in the cells of the invention is a net consumer of NADPH, the NADPH must be provided by other metabolic processes in the cell. One potential source of NADPH is certain NADPH-producing reactions in the pentose phosphate pathway. Therefore, in some embodiments of the invention, NADPH is produced in one or more pentose phosphate pathways. Thus, in certain additional aspects, the invention is recombinant cell of any of the foregoing aspects, which exhibits an increased flux through the pentose phosphate pathway and/or overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH. By increasing flux through the pentose phosphate pathway and/or overexpressing one or more of such enzymes, the cell can produce NADPH for consumption in the reductive TCA pathway to succinate, and maintain redox imbalance in the cell. Another advantage of this approach is that the pentose phosphate pathway leads to pyruvate and/or phosphoenolpyruvate production, which are the starting materials for the reductive TCA to succinate. Thus, carbon directed

through the pentose phosphate pathway is not diverted to other products, and yields to succinate can remain high.

In still other aspects, the invention is a recombinant cell of any of the foregoing aspects, which has a deletion or disruption of a native phosphoglucose isomerase gene. This modification has the effect of increasing carbon flux through the pentose phosphate pathway, producing NADPH which can be consumed in the reductive TCA pathway to succinate.

In still another aspect, the invention is a recombinant yeast of any of the foregoing aspects which overexpresses at least one *Stb5p* protein. Such a yeast cell in some embodiments has at least one exogenous *Stb5p* gene (*i.e.* a gene that encodes for the *Stb5p* protein) integrated into its genome. The *Stb5p* gene activates genes that produce enzymes which catalyze certain NADPH-producing enzymes in the pentose phosphate pathway. Again, the cell can consume NADPH in the reductive TCA pathway to succinate.

In still other aspects, the invention is a recombinant cell of any of the foregoing aspects, which further overexpresses a NAD(P)⁺ transhydrogenase enzyme. Such a yeast cell in some embodiments has at least one exogenous NAD(P)⁺-transhydrogenase gene integrated into its genome. By overexpressing a NAD(P)⁺ transhydrogenase enzyme, the cell can better balance redox factors by converting NADP(H) to NAD(H) as needed.

In yet another aspect, the invention is a recombinant yeast having an active reductive TCA pathway from pyruvate to succinate and a deletion or disruption of a native phosphoglucose isomerase gene.

In still another aspect, the invention is a recombinant yeast having an active reductive TCA pathway from pyruvate to succinate which overexpresses at least one *Stb5p* protein. Such a yeast cell in some embodiments has at least one exogenous *Stb5p* gene integrated into its genome.

The cell of any of the foregoing aspects of the invention may produce succinate and transport it from the cell. In some embodiments, the cell may further metabolize some or all of the succinate into one or more other succinate metabolization products, and transport one or more of such succinate metabolization product from the cell. In such embodiments, the cell contains native or non-native metabolic pathways which perform the further metabolization of succinate into such succinate metabolization product(s).

In yet other aspects, the invention is a method of producing succinate or a succinate metabolization product, comprising culturing a cell of any of the foregoing aspects in a fermentation medium that includes at least one carbon source.

The term "NADPH-dependent" refers to the property of an enzyme to preferentially use NADPH, rather than NADH, as the redox cofactor. An NADPH-dependent enzyme has a higher specificity constant (k_{cat}/K_M) with the cofactor NADPH than with the cofactor NADH as determined by *in vitro* enzyme activity assays such as are described in Examples 1C and 2T below. The term "NADH-dependent" as used herein refers to the property of an enzyme to preferentially use NADH rather than NADPH as the redox cofactor. An NADH-dependent enzyme has a higher specificity constant (k_{cat}/K_M) with the cofactor NADH than with the cofactor NADPH as determined by *in vitro* enzyme activity assays, such as described in Example 1C below.

For purposes of this application, "native" as used herein with regard to a metabolic pathway refers to a metabolic pathway that exists and is active in the wild-type host strain. Genetic material such as genes, promoters and terminators is "native" for purposes of this application if the genetic material has a sequence identical to (apart from individual-to-individual mutations which do not affect function) a genetic component that is present in the genome of the wild-type host cell (*i.e.*, the exogenous genetic component is identical to an endogenous genetic component).

For purposes of this application, genetic material such as a gene, a promoter and a terminator is "endogenous" to a cell if it is (i) native to the cell, (ii) present at the same location as that genetic material is present in the wild-type cell and (iii) under the regulatory control of its native promoter and its native terminator.

For purposes of this application, genetic material such as genes, promoters and terminators is "exogenous" to a cell if it is (i) non-native to the cell and/or (ii) is native to the cell, but is present at a location different than where that genetic material is present in the wild-type cell and/or (iii) is under the regulatory control of a non-native promoter and/or non-native terminator. Extra copies of native genetic material are considered as "exogenous" for purposes of this invention, even if such extra copies are present at the same locus as that genetic material is present in the wild-type host strain.

As used herein, the term "promoter" refers to an untranslated sequence located upstream (*i.e.*, 5') to the translation start codon of a gene (generally a sequence of about 1 to 1500 base pairs (bp), preferably about 100 to 1000 bp and especially of about 200 to 1000 bp) which controls the start of transcription of the gene. The term "terminator" as used herein refers to an untranslated sequence located downstream (*i.e.*, 3') to the translation finish codon of a gene (generally a sequence of about 1 to 1500 bp, preferably of about 100 to 1000 bp, and especially of about 200 to 500 bp) which controls the end of transcription of the gene. A promoter or terminator is "operatively linked" to a gene if its position in the genome relative to that of the gene is such that the promoter or terminator, as the case may be, performs its transcriptional control function.

"Identity" for nucleotide or amino acid sequences are for purposes of this invention calculated using BLAST (National Center for Biological Information (NCBI) Basic Local Alignment Search Tool) version 2.2.13 software with default parameters. A sequence having an identity score of XX% with regard to a reference sequence using the BLAST version 2.2.13 algorithm with default parameters is considered to be at least XX% identical or, equivalently, have XX% sequence identity to the reference sequence.

"Deletion or disruption" with regard to a gene means that either the entire coding region of the gene is eliminated (deletion) or the coding region of the gene, its promoter, and/or its terminator region is modified (such as by deletion, insertion, or mutation) such that the gene no longer produces an active enzyme, produces a severely reduced quantity (at least 75% reduction, preferably at least 85% reduction, more preferably at least 95% reduction) of the enzyme, or produces an enzyme with severely reduced (at least 75% reduced, preferably at least 85% reduced, more preferably at least 95% reduced) activity. A deletion or disruption of a gene can be accomplished by, for example, forced evolution, mutagenesis or genetic engineering methods, followed by appropriate selection or screening to identify the desired mutants.

"Overexpress" means the artificial expression of an enzyme in increased quantity by a gene. Overexpression of an enzyme may result from the presence of one or more exogenous gene(s), or from other conditions. For purposes of this invention, a yeast cell containing at least one exogenous gene is considered to overexpress the enzyme(s) encoded by such exogenous gene(s).

The recombinant yeast of the invention is made by performing certain genetic modifications to a host yeast cell. The host yeast cell is one which as a wild-type strain is natively capable of metabolizing at least one sugar to pyruvate. Suitable host yeast cells include (but are not limited to) yeast cells classified under the genera

5 *Candida*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Issatchenkia*, *Yarrowia* and *Hansenula*. Examples of specific host yeast cells include *C. sonorensis*, *K. marxianus*, *K. thermotolerans*, *C. methanesorbosa*, *Saccharomyces bulderi* (*S. bulderi*), *I. orientalis*, *C. lambica*, *C. sorboxylosa*, *C. zemplinina*, *C. geochares*, *P. membranifaciens*, *Z.*

10 *kombuchaensis*, *C. sorbosivorans*, *C. vanderwaltii*, *C. sorbophila*, *Z. bisporus*, *Z. lentus*, *Saccharomyces bayanus* (*S. bayanus*), *D. castellii*, *C. boidinii*, *C. etchellsii*, *K. lactis*, *P. jadinii*, *P. anomala*, *Saccharomyces cerevisiae* (*S. cerevisiae*), *Pichia galeiformis*, *Pichia* sp. YB-4149 (NRRL designation), *Candida ethanolica*, *P. deserticola*, *P. membranifaciens*, *P. fermentans* and *Saccharomycopsis crataegensis*

15 (*S. crataegensis*). Suitable strains of *K. marxianus* and *C. sonorensis* include those described in WO 00/71738 A1, WO 02/42471 A2, WO 03/049525 A2, WO 03/102152 A2 and WO 03/102201A2. Suitable strains of *I. orientalis* are ATCC strain 32196 and ATCC strain PTA-6648.

In some embodiments of the invention the host cell is Crabtree negative as a

20 wild-type strain. The Crabtree effect is defined as the occurrence of fermentative metabolism under aerobic conditions due to the inhibition of oxygen consumption by a microorganism when cultured at high specific growth rates (long-term effect) or in the presence of high concentrations of glucose (short-term effect). Crabtree negative phenotypes do not exhibit this effect, and are thus able to consume oxygen even in

25 the presence of high concentrations of glucose or at high growth rates.

In some embodiments, the host cell is succinate-resistant as a wild-type strain. A cell is considered to be "succinate-resistant" if the cell exhibits a growth rate in media containing 75 g/L or greater succinate at pH 2.8 that is at least 50% as high as its growth rate in the same media containing 0 g/L succinate, according to the

30 test method described in Example 1A of WO 2012/103261.

In some embodiments, the host cell exhibits a volumetric glucose consumption rate of at least 3, at least 5 or at least 8 grams of glucose per liter of broth per hour, as a wild-type strain.

In some embodiments, the host cell exhibits a specific glucose consumption rate of at least 0.5, at least 1.0 or at least 1.5 gram of glucose per gram dry weight of cells per hour, as a wild-type strain.

Volumetric and specific glucose consumption can be measured by cultivating the cells in shake flasks yeast extract peptone dextrose (YPD) media containing 0 g/l succinate at pH 3.0 as described in Example 1 of WO 2012/103261. The flasks are inoculated with biomass harvested from seed flasks grown overnight to an OD₆₀₀ of 6 to 10. 250 mL baffled glycolytic assay flasks (50 mL working volume) are inoculated to an OD₆₀₀ of 0.1 and grown at 250 RPM and 30°C. Samples are taken throughout the time course for the assay and analyzed for glucose consumption by electrophoretic methods (such as by using a 2700 Biochemistry Analyzer from Yellow Springs Instruments or equivalent device). The data is plotted and volumetric glucose consumption rate calculated. Specific glucose consumption rate is calculated by dividing the volumetric glucose consumption by the cell dry weight at the end of fermentation.

In certain embodiments, the genetically modified yeast cells provided herein have an active reductive TCA active pathway from pyruvate to succinate. Such an active reductive TCA pathway includes a step of converting pyruvate or phosphoenolpyruvate (PEP) (or each) to oxaloacetate (OAA), a step of converting oxaloacetate to malate, a step of converting malate to fumarate, and a step of converting fumarate to succinate.

The step of converting pyruvate to OAA is catalyzed by a *PYC* (pyruvate carboxylase) enzyme, *i.e.*, an enzyme having the ability to catalyze the conversion of pyruvate to OAA. A *PYC* enzyme is encoded by a *PYC* (pyruvate carboxylase) gene integrated into the genome of the recombinant yeast cell. The *PYC* gene may be native or non-native to the host cell, and may be endogenous (if native) or exogenous (if non-native or if additional copies of a native gene are present). In certain embodiments, a *PYC* gene may be a yeast gene. For example, the *PYC* gene may be an *I. orientalis* *PYC* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 116, an *S. cerevisiae* *PYC1* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 117, or a *K. marxianus* *PYC1* gene encoding for an enzyme having amino acid SEQ ID NO: 118. In other embodiments, the gene may encode for an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least

99% sequence identity to any of SEQ ID NOs: 116, 117 or 118. In certain embodiments, the gene may have the nucleotide sequence set forth in SEQ ID NOs: 4, 59 or 60, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 4, 59 or 60. In other embodiments, the *PYC* gene may be fungal.

The step of converting PEP to OAA is catalyzed by a *PPC* (phosphoenolpyruvate carboxylase) enzyme, *i.e.*, an enzyme having the ability to catalyze the conversion of PEP to OAA. A *PPC* enzyme is encoded by a *PPC* (phosphoenolpyruvate carboxylase) gene integrated into the genome of the recombinant yeast cell. The *PPC* gene may be native or non-native to the host cell, and may be endogenous (if native) or exogenous (if non-native or if additional copies of a native gene are present). The *PPC* gene may encode for an enzyme having either of amino acid sequences SEQ ID NO: 119 or 120, or for an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 119 or 120. In certain embodiments, the *PPC* gene may have the nucleotide sequence set forth in either of SEQ ID NOs: 63 or 64, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 63 or 64.

The step of converting OAA to malate is catalyzed by a *MDH* (malate dehydrogenase) enzyme, *i.e.*, an enzyme having the ability to catalyze the conversion of OAA to malate. A *MDH* (malate dehydrogenase) enzyme is encoded by a *MDH* gene integrated into the genome of the recombinant yeast cell. The *MDH* gene may be native or non-native to the host cell, and may be endogenous (if native) or exogenous (if non-native or if additional copies of a native gene are present).

In certain embodiments, the *MDH* gene may be a yeast gene. For example, the *MDH* gene may be an *I. orientalis* *MDH1*, *MDH2*, or *MDH3* gene which encode, respectively, for enzymes having any of amino acid sequences SEQ ID NOs: 121, 122 or 123, a *Z. rouxii* *MDH* gene encoding the amino acid sequence set forth in SEQ ID NO: 124, or a *K. marxianus* *MDH1*, *MDH2*, or *MDH3* gene, which encode, respectively for enzymes having amino acid sequences SEQ ID NOs: 125, 126 and 127. In other embodiments, the gene may encode for an enzyme having an amino

acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 121, 122, 123, 124, 125, 126 or 127. In certain embodiments, the *MDH* gene may have the nucleotide sequence of any of SEQ ID NOs: 72, 73, 74, 27, 75, 76 or 25 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 72, 73, 74, 27, 75, 76 or 25.

In certain embodiments, the *MDH* gene is bacterial. For example, the *MDH* gene is in some embodiments an *Escherichia coli* (*E. coli*) *MDH* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 128. In other embodiments, the gene encodes for an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 128. In certain embodiments, the *MDH* gene may have the nucleotide sequence set forth in SEQ ID NO: 78 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 78.

In certain embodiments, the *MDH* gene is fungal. For example, the *MDH* gene in some embodiments is a *Rhizopus oryzae* (*R. oryzae*) or *Rhizopus delemar* (*R. delemar*) *MDH* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 129 or 149. In other embodiments, the gene encodes for an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NO: 129 or 149. In certain embodiments, the *MDH* gene may have the nucleotide sequence SEQ ID NO: 80 or 23 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NO: 80 or 23.

The step of converting malate to fumarate is catalyzed by a *FUM* (fumarase) enzyme, *i.e.*, an enzyme having the ability to catalyze the conversion of malate to fumarate. A *FUM* (fumarase) enzyme is encoded by a *FUM* (fumarase) gene integrated into the genome of the recombinant yeast cell. The *FUM* gene may be native or non-native to the host cell, and may be endogenous (if native) or exogenous (if non-native or if additional copies of a native gene are present). In certain embodiments, a *FUM* gene is a yeast gene. The *FUM* gene is in some embodiments

an *I. orientalis FUM* gene encoding an enzyme having amino acid sequence SEQ ID NO: 130, or an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 130. In certain embodiments, the *FUM* gene may have nucleotide sequence SEQ ID NO: 35 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 35. In other embodiments, a *FUM* gene may be a bacterial gene.

The step of converting fumarate to succinate is catalyzed by a *FRD* (fumarate reductase) enzyme, *i.e.*, an enzyme having the ability to catalyze the conversion of fumarate to succinate. A *FRD* enzyme is encoded by a *FRD* (fumarate reductase) gene integrated into the genome of the recombinant yeast cell. The *FRD* gene may be native or non-native to the host cell, and may be endogenous (if native) or exogenous (if non-native or if additional copies of a native gene are present). In certain embodiments, the *FRD* gene is a yeast gene. For example, the *FRD* gene may be an *S. cerevisiae FRD1* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 131, a *Saccharomyce mikatae* (*S. mikatae*) *FRD1* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 132, a *K. polyspora FRD1* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 133, or a *K. marxianus FRD1* gene encoding amino acid sequence SEQ ID NO: 134. In other embodiments, the gene may encode for an enzyme having an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of amino acid sequences SEQ ID NOs: 131, 132, 133 or 134. In certain embodiments, a yeast-derived *FRD* gene may have a nucleotide sequence as set forth in any of SEQ ID NOs: 86, 87, 88, or 89, or have a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of the nucleotide sequences set forth in SEQ ID NOs: 86, 87, 88 or 89.

In certain embodiments, the *FRD* gene is protozoan. For example, the *FRD* gene may be a *Trypanosoma brucei* (*T. brucei*) *FRD* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 135, a *Trypanosoma cruzi* (*T. cruzi*) *FRD* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 136, a *Leishmania braziliensis* (*L. braziliensis*) *FRD* gene encoding for an enzyme having amino acid sequence SEQ ID NO: 137, a *Leishmania mexicana* (*L. mexicana*) *FRD*

gene encoding for an enzyme having amino acid sequence SEQ ID NO: 138, or an *FRD* gene encoding for an enzyme having amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 135, 136, 137 or 138. In certain embodiments, the *FRD* gene may have any of nucleotide sequences SEQ ID NOs: 13, 94, 95 or 10, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 13, 94, 95 or 10.

In this invention, at least one of the reactions in the reductive TCA pathway oxidizes NADPH to NADP⁺. This may be a reaction of oxaloacetate to malate or fumarate to succinate. The oxidation of NADPH to NADP⁺ typically occurs in cases in which the reaction in any one or more of these steps is catalyzed by an NADPH-dependent enzyme. The NADPH-dependent enzyme may be an NADPH-dependent *MDH* gene, an NADPH-dependent *FRD* gene, or both of these.

In some embodiments, the reductive TCA pathway includes an NADPH-dependent *MDH* gene, which encodes for an NADPH-dependent *MDH* enzyme. The NADPH-dependent *MDH* enzyme catalyzes the conversion of oxaloacetate to malate using NADPH as a cofactor, oxidizing NADPH to NADP⁺. The NADPH-dependent *MDH* gene may be naturally-occurring, *i.e.*, present in a wild-type organism (such as, for example, a *Sorghum bicolor* (*S. bicolor*) *MDH* gene or a *Chlamydomonas reinhardtii* (*C. reinhardtii*) *MDH* gene). Alternatively, the NADPH-dependent *MDH* gene may be a mutated or otherwise modified *MDH* gene. The NADPH-dependent *MDH* gene in some embodiments encodes for an enzyme having an amino acid sequence of either of SEQ ID NOs: 143 or 144, or for an enzyme with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 143 or 144. The NADPH-dependent *MDH* gene may have the nucleotide sequence of either of SEQ. ID. NOs. 29 or 31 or may have a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 29 or 31.

Other suitable NADPH-dependent *MDH* genes can be produced through site-directed mutagenesis at the portion of the coding sequence of any of SEQ. ID. NOs: 72, 73, 74, 27, 25, 75, 76, 78 or 80 that encodes for the putative NADH binding domain of the enzyme, in the general manner described in Example 2F below. An

example of such a mutated NADPH-dependent *MDH* gene is SEQ ID NO: 32. In addition, genes that encode for NADPH-dependent *MDH* enzymes can be produced from genes that encode for NADH-dependent *MDH* enzymes by techniques such as enzyme evolution, gene shuffling, site saturation mutagenesis or mutagenesis of another portion of the coding sequence of the gene.

In some embodiments, the reductive TCA pathway includes an NADPH-dependent *FRD* gene, which encodes for an NADPH-dependent *FRD* enzyme. The NADPH-dependent *FRD* enzyme catalyzes the conversion of fumarate to succinate using NADPH as a cofactor, oxidizing the NADPH to NADP⁺. The NADPH-dependent *FRD* gene in some embodiments encodes for an enzyme having any of amino acid sequences SEQ ID NOs: 110, 111, 112, 113, 114 or 115, or for an enzyme with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 110, 111, 112, 113, 114 or 115. The NADPH-dependent *FRD* gene may have any of nucleotide sequences SEQ. ID. NOs: 15, 16, 17, 18, 19 or 20 or may have a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 15, 16, 17, 18, 19 or 20.

Other suitable NADPH-dependent *FRD* genes can be produced through site-directed mutagenesis at the portion of the coding sequence of any of SEQ. ID. NOs 13, 94, 95 or 10 that encode for the putative NADH binding domain of the enzyme, in the general manner described in Examples 1A and 1B below. In addition, genes that encode for NADPH-dependent *FRD* enzymes can be produced from genes that encode for NADH-dependent *FRD* enzymes by techniques such as enzyme evolution, gene shuffling, site saturation mutagenesis or mutagenesis of another portion of the coding sequence of the gene.

In some embodiments, the reductive TCA pathway includes one reaction that oxidizes NADPH to NADP⁺, and one reaction that oxidizes NADH to NAD⁺. In certain such embodiments, the reaction that oxidizes NADPH to NADP⁺ is the conversion of oxaloacetate to malate, catalyzed by an NADPH-dependent *MDH* enzyme, and the reaction that oxidizes NADH to NAD⁺ is the conversion of fumarate to succinate, catalyzed by an NADH-dependent *FRD* enzyme. In other such embodiments, the reaction that oxidizes NADPH to NADP⁺ is the conversion of fumarate to succinate, catalyzed by an NADPH-dependent *FRD* enzyme, and the

reaction that oxidizes NADH to NAD⁺ is the conversion of oxaloacetate to malate, catalyzed by an NADH-dependent *MDH* enzyme.

In some embodiments, the recombinant cell exhibits increased flux, relative to the wild-type host strain, through the pentose phosphate pathway and/or overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH. Doing so produces NADPH which can be consumed by the NADPH-dependent step in the reductive TCA pathway from pyruvate to succinate, helping to maintain cofactor balance in the cell.

One way of increasing flux through the pentose phosphate pathway is to disrupt the glycolytic pathway from glucose to pyruvate. This can be done, for example, by disrupting or removing the step of isomerising glucose-6-phosphate to fructose-6-phosphate, which is catalyzed by a phosphoglucose (*PGI*) enzyme. Therefore, in certain embodiments, the recombinant cell of the invention produces a severely reduced quantity (at least 75% reduction, preferably at least 85% reduction, more preferably at least 95% reduction) of an active phosphoglucose isomerase (*PGI*) enzyme, or produces a *PGI* enzyme with severely reduced (at least 75% reduced, preferably at least 85% reduced, more preferably at least 95% reduced) activity. In some embodiments, the recombinant cell includes a deletion or disruption of at least one native phosphoglucose isomerase (*PGI*) gene. If the host cell contains multiple alleles of the *PGI* gene, all such alleles may be deleted or disrupted.

The overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH may be an enzyme that catalyzes a reaction in the pentose phosphate pathway. The pentose phosphate pathway metabolizes glucose-6-phosphate to glyceraldehyde-3-phosphate through 6-phosphogluconolactone, 6-phosphogluconate and ribulose 5-phosphate intermediates. The conversion of glucose-6-phosphate to 6-phosphogluconolactone is catalyzed by a glucose-6-phosphate dehydrogenase (*G6PDH*) enzyme that uses NADP⁺ as a cofactor, thereby reducing NADP⁺ to NADPH. Similarly, the conversion of 6-phosphogluconate to ribulose-5-phosphate is catalyzed by a 6-phosphogluconate dehydrogenase (*6PGDH*) enzyme that uses NADP⁺ as a cofactor, thereby reducing NADP⁺ to NADPH.

Therefore, in certain embodiments, the yeast cell of the invention overexpresses a *G6PDH* enzyme. Such a yeast cell in some embodiments includes one or more exogenous *G6PDH* genes, which may be native or non-native to the strain, integrated into its genome. In certain of these embodiments, the exogenous

G6PDH gene may be an *I. orientalis* *G6PDH* gene (*ZWF1*) that encodes for an enzyme having amino acid sequence SEQ ID NO: 139 or which encodes for an enzyme having with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 139. In certain embodiments, the *G6PDH* gene may have nucleotide sequence SEQ ID NO: 97 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to nucleotide sequence SEQ ID NO: 97.

Similarly, in other embodiments, the recombinant yeast cells provided herein contains one or more exogenous *6PGDH* genes, which may be native or non-native to the host strain, integrated into its genome. In certain embodiments, a *6PGDH* gene may be a yeast *6PGDH* gene such as an *I. orientalis* *6PGDH* gene. In certain embodiments, the exogenous *6PGDH* gene encodes for an enzyme having amino acid sequence SEQ ID NO: 140, or an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 140. In certain embodiments, the exogenous *6PGDH* gene has the nucleotide sequence of SEQ ID NO: 99, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to nucleotide sequence SEQ ID NO: 99.

In certain embodiments, the recombinant cell of the invention overexpresses an oxidative stress-activated zinc cluster protein *Stb5p*. This zinc cluster protein regulates genes involved in certain NADPH-producing reactions, including the *G6PDH* and *6PGDH* genes. In certain embodiments, the recombinant cell includes one or more exogenous *Stb5p* genes, which may be native or non-native to the host cell, integrated into its genome. In certain embodiments, a *Stb5p* gene may be a yeast *Stb5p* gene such as an *S. cerevisiae* *Stb5p* gene. In certain embodiments, the exogenous *Stb5p* gene encodes for an enzyme having amino acid sequence SEQ ID NO: 148, or an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to SEQ ID NO: 148. In certain embodiments, the exogenous *Stb5p* gene has the nucleotide sequence of SEQ ID NO: 51, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least

95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence of SEQ ID NO: 51.

The recombinant cell of the invention may further include one or more exogenous succinate exporter genes, which may be native or non-native to the host cell. A "succinate exporter gene" as used herein refers to any gene that encodes a polypeptide with succinate export activity, meaning the ability to transport succinate out of a cell and into the extracellular environment. The exogenous succinate exporter gene may be a fungal succinate exporter gene such as a *Schizosaccharomyces pombe* (*S. pombe*) succinate exporter gene or *Aspergillus oryzae* (*A. oryzae*) source succinate exporter gene. The exogenous succinate exporter gene in some embodiments encodes for an enzyme having either of amino acid sequence SEQ ID NOs: 141 or 142, or at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 141 or 142. In certain embodiments, the exogenous succinate exporter gene has either of nucleotide sequence SEQ ID NOs: 102 or 103, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to either of SEQ ID NOs: 102 or 103.

In still other embodiments, the recombinant cell of the invention may overexpress an active transhydrogenase enzyme and/or include an exogenous transhydrogenase gene, which may be native or non-native to the host cell. A "transhydrogenase" (*SthA*) gene refers to any gene that encodes a polypeptide that catalyzes the reaction of NADP(H) to form NAD(H). The transhydrogenase (*SthA*) enzyme preferably is soluble in the cytosol of the recombinant cell. The exogenous *SthA* gene may be of bacterial, fungal, yeast or other origin. The exogenous *SthA* gene in some embodiments is an *E. coli*, *Azotobacter vinelandii* (*A. vinelandii*) or *Pseudomonas fluorescens* gene. The exogenous *SthA* gene in some embodiments encodes for an enzyme having any of amino acid sequences SEQ ID NOs: 145, 146 or 147 or at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 145, 146, 147 or 155. In certain embodiments, the exogenous *SthA* gene has any of nucleotide sequences SEQ ID NOs: 45, 47 or 49 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least

95%, at least 97%, or at least 99% sequence identity to any of SEQ ID NOs: 45, 47, 49 or 154.

In certain embodiments, the recombinant yeast cells provided herein may have a deletion or disruption of one or more other endogenous genes. The deleted or
5 disrupted genes may include genes which produce enzymes that catalyze the reaction of pyruvate or phosphoenolpyruvate (or their metabolizes) to downstream products other than succinate. Among such genes are, for example, native pyruvate decarboxylase (*PDC1*, EC 4.1.1.1), alcohol dehydrogenase 1 (*ADH1*, catalyzes the conversion of acetaldehyde to ethanol) and/ or alcohol dehydrogenase 2 (*ADH2*,
10 catalyzes the conversion of ethanol to acetaldehyde), glycerol-3-phosphate dehydrogenase (*GPD*, systematic name sn-glycerol-3-phosphate:NAD⁺ 2-oxidoreductase, EC 1.1.1.8), and glycerol-3-phosphatase enzyme (*GPP*, systematic name glycerol-1-phosphate phosphohydrolase, EC 3.1.3.21) and NADH⁺-dependent glycerol dehydrogenase (systematic name glycerol:NAD⁺ 2-oxidoreductase, EC
15 1.1.1.6) genes.

Other endogenous genes that may be deleted in certain embodiments of the invention include genes which encode for enzymes that catalyze a reverse reaction that consumes PEP, pyruvate, succinate or any intermediates produced in the reductive TCA pathway (other than the TCA pathway reactions leading to succinate).
20 Examples of such genes include a native pyruvate carboxylase gene (which encodes an enzyme that converts OAA to pyruvate), PEP carboxykinase (*PCK*) gene (which encodes an enzyme that converts OAA to PEP) and/or malic enzyme (*MAE*) gene (which encodes an enzyme that converts malate to pyruvate), and a native succinate dehydrogenase (*SDH*) gene (which encodes for an enzyme that catalyzes the back-
25 reaction of succinate to fumarate).

In some embodiments, the modified yeast cells provided herein have a deletion or disruption of a native succinate importer gene, which as used herein refers to any gene that encodes a polypeptide that allows for growth on and consumption of succinate.

30 In certain embodiments, the cells may comprise all or part of an active oxidative TCA or glyoxylate shunt succinate fermentation pathway. In these embodiments, the cells comprise one or more genes encoding enzymes selected from the group consisting of citrate synthase, *PDH* (pyruvate dehydrogenase), *PFL* (pyruvate formate lyase), aconitase, *IDH* (isocitrate dehydrogenase), α -*KGDH* (α -

ketoglutarate dehydrogenase), succinate thiokinase, isocitrate lyase, and malate synthase.

The recombinant cell of the invention may further include one (or more) modifications which individually or collectively confers to the cell the ability to ferment pentose sugars to for xylulose 5-phosphate. Among such modifications are (1) insertion of a functional xylose isomerase gene, (2) a deletion or disruption of a native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol, (3) a deletion or disruption of a functional xylitol dehydrogenase gene and/or (4) modifications that cause the cell to overexpress a functional xylulokinase. Methods for introducing those modifications into yeast cells are described, for example, in WO 04/099381, incorporated herein by reference. Suitable methods for inserting a functional xylose isomerase gene, deleting or disrupting a native gene that produces an enzyme that catalyzes the conversion of xylose to xylitol, deleting or disrupting a functional xylitol dehydrogenase gene and modifying the cell to overexpress a functional xylulokinase are described, for example, in WO 04/099381, incorporated herein by reference.

In this invention, any exogenous gene, including without limitation any of the exogeneous genes in the reductive TCA pathway from pyruvate to succinate, any succinate exporter gene, any *G6PDH* gene, any *6PGDH* gene, any *SthA* gene, or any other exogenous gene introduced into the host cell, is operatively linked to one or more regulatory elements, and in particular to a promoter sequence and a terminator sequence that each are functional in the host cell. Such regulatory elements may be native or non-native to the host cell.

Examples of promoters that may be linked to one or more exogenous genes in the yeast cells provided herein include, but are not limited to, promoters for pyruvate decarboxylase (*PDC1*), phosphoglycerate kinase (*PGK*), xylose reductase (*XR*), xylitol dehydrogenase (*XDH*), L-(+)-lactate-cytochrome c oxidoreductase (*CYB2*), translation elongation factor-1 or -2 (*TEF1*, *TEF2*), enolase (*ENO1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), orotidine 5'-phosphate decarboxylase (*URA3*) genes, as well as any of those described in the various Examples that follow. Where the promoters are non-native, they may be identical to or share a high degree of sequence identity (*i.e.*, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%) with one or more native promoters. Other suitable

promoters and terminators include those described, for example, in WO99/14335, WO00/71738, WO02/42471, WO03/102201, WO03/102152 and WO03/049525.

Examples of terminators that may be linked to one or more exogenous genes in the yeast cells provided herein include, but are not limited to, terminators for *PDC1*, *XR*, *XDH*, transaldolase (*TAL*), transketolase (*TKL*), ribose 5-phosphate ketol-isomerase (*RKI*), *CYB2*, or iso-2-cytochrome c (*CYC*) genes or the galactose family of genes (especially the *GAL10* terminator), as well as any of those described in the various Examples that follow. Where the terminators are non-native, they may be identical to or share a high degree of sequence identity (*i.e.*, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%) with one or more native terminators.

Modifications (insertion, deletions and/or disruptions) to the genome of the host cell described herein can be performed using methods known in the art. Exogenous genes may be integrated into the genome in a targeted or a random manner using, for example, well known electroporation and chemical methods (including calcium chloride and/or lithium acetate methods). In those embodiments where an exogenous gene is integrated in a targeted manner, it may be integrated into the locus for a particular native gene, such that integration of the exogenous gene is coupled with deletion or disruption of a native gene. Alternatively, the exogenous gene may be integrated into a portion of the native genome that does not correspond to a gene. Methods for transforming a yeast cell with an exogenous construct are described in, for example, WO99/14335, WO00/71738, WO02/42471, WO03/102201, WO03/102152, WO03/049525, WO2007/061590, WO 2009/065778 and PCT/US2011/022612.

Insertion of exogenous genes is generally performed by transforming the cell with one or more integration constructs or fragments. The terms "construct" and "fragment" are used interchangeably herein to refer to a DNA sequence that is used to transform a cell. The construct or fragment may be, for example, a circular plasmid or vector, a portion of a circular plasmid or vector (such as a restriction enzyme digestion product), a linearized plasmid or vector, or a PCR product prepared using a plasmid or genomic DNA as a template. An integration construct can be assembled using two cloned target DNA sequences from an insertion site target. The two target DNA sequences may be contiguous or non-contiguous in the native host genome. In this context, "non-contiguous" means that the DNA sequences are not

immediately adjacent to one another in the native genome, but are instead are separated by a region that is to be deleted. "Contiguous" sequences as used herein are directly adjacent to one another in the native genome. Where targeted integration is to be coupled with deletion or disruption of a target gene, the integration construct also functions as a deletion construct. In such an integration/deletion construct, one of the target sequences may include a region 5' to the promoter of the target gene, all or a portion of the promoter region, all or a portion of the target gene coding sequence, or some combination thereof. The other target sequence may include a region 3' to the terminator of the target gene, all or a portion of the terminator region, and/or all or a portion of the target gene coding sequence. Where targeted integration is not to be coupled to deletion or disruption of a native gene, the target sequences are selected such that insertion of an intervening sequence will not disrupt native gene expression. An integration or deletion construct is prepared such that the two target sequences are oriented in the same direction in relation to one another as they natively appear in the genome of the host cell. The gene expression cassette is cloned into the construct between the two target gene sequences to allow for expression of the exogenous gene. The gene expression cassette contains the exogenous gene, and may further include one or more regulatory sequences such as promoters or terminators operatively linked to the exogenous gene.

It is usually desirable that the deletion construct may also include a functional selection marker cassette. When a single deletion construct is used, the marker cassette resides on the vector downstream (*i.e.*, in the 3' direction) of the 5' sequence from the target locus and upstream (*i.e.*, in the 5' direction) of the 3' sequence from the target locus. Successful transformants will contain the selection marker cassette, which imparts to the successfully transformed cell some characteristic that provides a basis for selection.

A "selection marker gene" may encode a protein needed for the survival and/or growth of the transformed cell in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, (such as, for example, zeocin (*Streptoalloteichus hindustanus ble* bleomycin resistance gene), *G418* (kanamycin-resistance gene of Tn903) or hygromycin (aminoglycoside antibiotic resistance gene from *E. coli*)), (b) complement auxotrophic deficiencies of the cell (such as, for example, amino acid leucine deficiency (*K. marxianus LEU2*

gene) or uracil deficiency (e.g., *K. marxianus* or *S. cerevisiae* *URA3* gene)); (c) enable the cell to synthesize critical nutrients not available from simple media, or (d) confer ability for the cell to grow on a particular carbon source, (such as a *MEL5* gene from *S. cerevisiae*, which encodes the alpha-galactosidase (melibiase) enzyme and confers the ability to grow on melibiose as the sole carbon source). Preferred selection markers include the zeocin resistance gene, G418 resistance gene, a *MEL5* gene, a *URA3* gene and hygromycin resistance gene. Another preferred selection marker is an L-lactate:ferricytochrome c oxidoreductase (*CYB2*) gene cassette, provided that the host cell either natively lacks such a gene or that its native *CYB2* gene(s) are first deleted or disrupted.

The construct may be designed so that the selection marker cassette can become spontaneously deleted as a result of a subsequent homologous recombination event. A convenient way of accomplishing this is to design the vector such that the selection marker gene cassette is flanked by direct repeat sequences. Direct repeat sequences are identical DNA sequences, native or not native to the host cell, and oriented on the construct in the same direction with respect to each other. The direct repeat sequences are advantageously about 50-1500 bp in length. It is not necessary that the direct repeat sequences encode for anything. This construct permits a homologous recombination event to occur. This event occurs with some low frequency, resulting in cells containing a deletion of the selection marker gene and one of the direct repeat sequences. It may be necessary to grow transformants for several rounds on nonselective or selective media to allow for the spontaneous homologous recombination to occur in some of the cells. Cells in which the selection marker gene has become spontaneously deleted can be selected or screened on the basis of their loss of the selection characteristic imparted by the selection marker gene, or by using PCR or Southern Analysis methods to confirm the loss of the selection marker.

In some embodiments, an exogenous gene may be inserted using DNA from two or more integration fragments, rather than a single fragment. In these embodiments, the 3' end of one integration fragment contains a region of homology with the 5' end of another integration fragment. One of the fragments will contain a first region of homology to the target locus and the other fragment will contain a second region of homology to the target locus. The gene cassette to be inserted can reside on either fragment, or be divided among the fragments, with a region of

homology at the 3' and 5' ends of the respective fragments, so the entire, functional gene cassette is produced upon a crossover event. The cell is transformed with these fragments simultaneously. A selection marker may reside on any one of the fragments or may be divided between the fragments with a region of homology as described. In other embodiments, transformation from three or more constructs can be used in an analogous way to integrate exogenous genetic material.

Deletions and/or disruptions of native genes can be performed by transformation methods, by mutagenesis and/or by forced evolution methods. In mutagenesis methods cells are exposed to ultraviolet radiation or a mutagenic substance, under conditions sufficient to achieve a high kill rate (60-99.9%, preferably 90-99.9%) of the cells. Surviving cells are then plated and selected or screened for cells having the deleted or disrupted metabolic activity. Disruption or deletion of the desired native gene(s) can be confirmed through PCR or Southern analysis methods.

Cells of the invention can be cultivated to produce succinic acid, either in the free acid form or in salt form (or both). The recombinant cell is cultured in a medium that includes at least one carbon source that can be fermented by the cell. Examples include, but are not limited to, twelve carbon sugars such as sucrose, hexose sugars such as glucose or fructose, glycan, starch, or other polymer of glucose, glucose oligomers such as maltose, maltotriose and isomaltotriose, panose, and fructose oligomers, and pentose sugars such as xylose, xylan, other oligomers of xylose, or arabinose.

The medium will typically contain, in addition to the carbon source, nutrients as required by the particular cell, including a source of nitrogen (such as amino acids, proteins, inorganic nitrogen sources such as ammonia or ammonium salts, and the like), and various vitamins, minerals and the like. In some embodiments, the cells of the invention can be cultured in a chemically defined medium.

Other cultivation conditions, such as temperature, cell density, selection of substrate(s), selection of nutrients, and the like are not considered to be critical to the invention and are generally selected to provide an economical process. Temperatures during each of the growth phase and the production phase may range from above the freezing temperature of the medium to about 50°C, although this depends to some extent on the ability of the strain to tolerate elevated temperatures. A preferred temperature, particularly during the production phase, is about 30 to 45°C.

During cultivation, aeration and agitation conditions may be selected to produce a desired oxygen uptake rate. The cultivation may be conducted aerobically, microaerobically, or anaerobically, depending on pathway requirements. In some embodiments, the cultivation conditions are selected to produce an oxygen uptake rate of around 2-25 mmol/L/hr, preferably from around 5-20 mmol/L/hr, and more preferably from around 8-15 mmol/L/hr. "Oxygen uptake rate" or "OUR" as used herein refers to the volumetric rate at which oxygen is consumed during the fermentation. Inlet and outlet oxygen concentrations can be measured with exhaust gas analysis, for example by mass spectrometers. OUR can be calculated using the Direct Method described in Bioreaction Engineering Principles 2nd Edition, 2003, Kluwer Academic/Plenum Publishers, p. 449, equation 1.

The culturing process may be divided up into phases. For example, the cell culture process may be divided into a cultivation phase, a production phase, and a recovery phase.

The pH may be allowed to range freely during cultivation, or may be buffered if necessary to prevent the pH from falling below or rising above predetermined levels. For example, the medium may be buffered to prevent the pH of the solution from falling below around 2.0 or above about 8.0 during cultivation. In certain of these embodiments, the medium may be buffered to prevent the pH of the solution from falling below around 3.0 or rising above around 7.0, and in certain of these embodiments the medium may be buffered to prevent the pH of the solution from falling below around 3.0 or rising above around 4.5. Suitable buffering agents include basic materials that neutralize the acid as it is formed, and include, for example, calcium hydroxide, calcium carbonate, sodium hydroxide, potassium hydroxide, potassium carbonate, sodium carbonate, ammonium carbonate, ammonia, ammonium hydroxide and the like.

In a buffered fermentation, acidic fermentation products are neutralized to the corresponding salt as they are formed. Recovery of the acid therefore involves regenerating the free acid. This is typically done by removing the cells and acidulating the fermentation broth with a strong acid such as sulfuric acid. A salt by-product is formed (gypsum in the case where a calcium salt is the neutralizing agent and sulfuric acid is the acidulating agent), which is separated from the broth.

In other embodiments, the pH of the fermentation medium may be permitted to drop during cultivation from a starting pH that is at or above the lower pKa

(4.207) of succinate, typically 6 or higher, to at or below the lower pKa of the acid fermentation product, such as in the range of about 2.0 to about 4.2, in the range of from about 3.0 to about 4.2, or in the range from about 3.8 to about 4.2.

In still other embodiments, fermentation may be carried out to produce a product acid by adjusting the pH of the fermentation broth to at or below the lower pKa of the product acid prior to or at the start of the fermentation process. The pH may thereafter be maintained at or below the lower pKa of the product acid throughout the cultivation. In certain embodiments, the pH may be maintained at a range of about 2.0 to about 4.2, in the range of from about 3.0 to about 4.2, or in the range from about 3.8 to about 4.2.

When the pH of the fermentation broth is low enough that the succinate is present in acid form, the acid can be recovered from the broth through techniques such as liquid-liquid extraction, distillation, absorption, etc., such as are described in T.B. Vickroy, Vol. 3, Chapter 38 of *Comprehensive Biotechnology*, (ed. M. Moo-Young), Pergamon, Oxford, 1985; R. Datta, et al., *FEMS Microbiol. Rev.*, 1995, 16:221-231; U.S. Patent Nos. 4,275,234, 4,771,001, 5,132,456, 5,420,304, 5,510,526, 5,641,406, and 5,831,122, and WO 93/00440.

The cultivation may be continued until a yield of succinate on the carbon source is, for example, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, or greater than 50% of the theoretical yield. The yield to succinate may at least 80% or at least 90% of the theoretical yield. The concentration, or titer, of succinate produced in the cultivation will be a function of the yield as well as the starting concentration of the carbon source. In certain embodiments, the titer may reach at least 1, at least 3, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or greater than 50 g/L at some point during the fermentation, and preferably at the end of the fermentation.

In certain embodiments, the genetically modified yeast cells produce ethanol in a yield of 10% or less, preferably in a yield of 2% or less of the theoretical yield. In certain of these embodiments, ethanol is not detectably produced. In other embodiments, however, succinate and ethanol may be co-produced. In these embodiments, ethanol may be produced at a yield of greater than 10%, greater than 25%, or greater than 50% of the theoretical yield.

The recombinant cell of the invention may exhibit a volumetric glucose consumption rate of at least 0.5 gram, at least 0.75 gram, or at least 0.9 gram of

glucose per liter of broth per hour, when cultivated in the manner described in Example 17 below. The data is plotted and volumetric glucose consumption rate calculated. Specific glucose consumption rate can also be calculated by dividing the glucose consumption by the cell dry weight at the end of fermentation.

5 The cell of the invention may produce succinate as an end-product of the fermentation process. In such a case, the cell preferably transports succinate out of the cell and into the surrounding culture medium.

In some embodiments, the cell may further metabolize some or all of the succinate into one or more succinate metabolization products, i.e., a compound
10 formed in the further metabolization of succinate by the cell. Examples of such succinate metabolization products include, for example, 1,4-butanediol, 1,3-butadiene, propionic acid, and 3-hydroxyisobutyric acid. In such embodiments, the cell contains native or non-native metabolic pathways which perform such a further metabolization of succinate into such succinate metabolization product(s). The cell
15 may then transport such succinate metabolization products out of the cell and into the surrounding medium. In some embodiments, the cell may transport one or more succinate metabolization products, but not succinate, out of the cell. In other embodiments, the cell may transport both succinate itself and one or more succinate metabolization products out of the cell. For example, the cell may transport less than
20 10% by weight of succinate from the cell, based on the combined weight of succinate and succinate metabolization products exported from the cell.

The following examples are provided to illustrate the invention, but are not intended to limit the scope thereof. All parts and percentages are by weight unless otherwise indicated.

25 Construction of Preparatory Strains P1-P6

P-1. An *I. orientalis* strain host strain is generated by evolving *I. orientalis* strain ATCC PTA-6658 for 91 days in a glucose-limited chemostat. The system is fed 15 g/L glucose in a defined medium and operated at a dilution rate of 0.06 h^{-1} at pH=3
30 with added lactic acid in the feed medium. The conditions are maintained with an oxygen transfer rate of approximately $2 \text{ mmol L}^{-1}\text{h}^{-1}$, and dissolved oxygen concentration remains constant at 0% of air saturation. Single colony isolates from the final time point are characterized in two shake flask assays. In the first assay, the isolates are characterized for their ability to ferment glucose to ethanol in the

presence of 25 g/L total lactic acid with no pH adjustment in the defined medium. In the second assay, the growth rate of the isolates is measured in the presence of 45 g/L of total lactic acid, with no pH adjustment in the defined medium. Strain P-1 is a single isolate exhibiting the highest glucose consumption rate in the first assay and the highest growth rate in the second assay.

P-2. Strain P-1 is transformed with linearized integration fragment P2 (having nucleotide sequence SEQ ID NO: 1) designed to disrupt the *URA3* gene, using the LiOAc transformation method as described by Gietz et al., in *Met. Enzymol.* 350:87 (2002). Integration fragment P2 includes a *MEL5* selection marker gene. Transformants are selected on YNB-melibiose plates and screened by PCR to confirm the integration of the integration piece and deletion of a copy of the *URA3* gene. A *URA3*-deletant strain is grown for several rounds until PCR screening identifies an isolate in which the *MEL5* selection marker gene has looped out. The PCR screening is performed using primers having nucleotide sequences SEQ ID NOs: 56 and 57 to confirm the 5'-crossover and primers having nucleotide sequences SEQ ID NOs: 58 and 61 to confirm the 3' crossover. That isolate is again grown for several rounds on 5-fluoroorotic acid (FOA) plates to identify a strain in which the *URA3* marker has looped out. PCR screening is performed on this strain using primers having nucleotide sequences SEQ ID NOs: 56 and 61, identifies an isolate in which both *URA3* alleles have been deleted. In a preferred aspect, the strain is selected on 5-fluoroorotic acid (FOA) plates prior to the PCR screening described in the previous sentence. This isolate is named strain P-2.

P-3. Strain P-2 is transformed with integration fragment P3 (having the nucleotide sequence SEQ ID NO: 2), which is designed to disrupt the *PDC* gene. Integration fragment P3 contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis PDC* open reading frame, a *PDC* transcriptional terminator, the *URA3* promoter, the *I. orientalis URA3* gene, an additional *URA3* promoter direct repeat for marker recycling and a DNA fragment with homology for integration corresponding to the region directly downstream of the *I. orientalis PDC* open reading frame. A successful integrant (and single-copy *PDC* deletant) is identified on selection plates lacking uracil and confirmed by PCR using primers having nucleotide sequences SEQ ID NOS: 62 and 65 to confirm the 5'-crossover and primers having nucleotide sequences SEQ ID NOs: 66 and 67 to confirm the 3'-crossover. That

integrant is grown for several rounds and plated on 5-fluoroorotic acid (FOA) plates to identify a strain in which the *URA3* marker has looped out. Loopout of the *URA3* marker is confirmed by PCR. That strain is again transformed with integration fragment P3 to delete the second copy of the native *PDC* gene. A successful transformant is again identified by selection on selection plates lacking uracil, and further confirmed by culturing the strain over two days and measuring ethanol production. Lack of ethanol production further demonstrates a successful deletion of both copies of the *PDC* gene in a transformant. That transformant is grown for several rounds and plated on FOA plates until PCR identifies a strain in which the *URA3* marker has looped out. The PCR screening is performed using primers having nucleotide sequences SEQ ID NOs: 62 and 65 to confirm the 5'-crossover and SEQ ID NOs: 66 and 67 to confirm the 3'-crossover. That strain is plated on selection plates lacking uracil to confirm the loss of the *URA3* marker, and is designated strain P-3.

P-4. Integration fragment P4-1, having nucleotide sequence SEQ ID NO: 3, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis* ADH9091 open reading frame, an *I. orientalis PDC1* promoter, the *I. orientalis PYC* gene (having the nucleotide sequence SEQ ID NO: 4), the *I. orientalis TAL* terminator, the *I. orientalis URA3* promoter, and the first 530 bp of the *I. orientalis URA3* open reading frame.

Integration fragment P4-2, having nucleotide sequence SEQ ID NO: 5, contains the following elements, 5' to 3': a DNA fragment corresponding to the last 568 bp of the *I. orientalis URA3* open reading frame, the *I. orientalis URA3* terminator, the *I. orientalis URA3* promoter, the *I. orientalis TDH3* promoter, the *S. pombe MAE* gene (having the nucleotide sequence SEQ ID NO: 6), the *I. orientalis TKL* terminator, and a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis* ADH9091 open reading frame.

Strain P-3 is transformed simultaneously with integration fragments P4-1 and P4-2, using lithium acetate methods, to insert the *I. orientalis PYC* gene and the *S. pombe MAE* gene at the ADH9091 locus. Integration occurs via three cross-over events: in the regions of the ADH9091 upstream homology, in the regions of the ADH9091 downstream homology and in the region of URA3 homology between SEQ ID NO: 3 and SEQ ID NO: 5. Transformants are streaked to isolates and the correct integration of the cassette at the AHD9091 locus is confirmed in a strain by PCR. The

PCR screening is performed using primers having nucleotide sequences SEQ ID NOs: 69 and 70 to confirm the 5'-crossover and SEQ ID NOs: 71 and 77 to confirm the 3'-crossover. That strain is grown and plated on FOA as before until the loopout of the *URA3* marker from an isolate is confirmed by PCR.

5 That isolate is then transformed simultaneously with integration fragments P4-3 and P4-4 using LiOAc transformation methods, to insert a second copy of each of the *I. orientalis* *PYC* gene and the *S. pombe* *MAE* gene at the ADH9091 locus.

Integration fragment P4-3, having the nucleotide sequence SEQ ID NO: 7, contains the following elements, 5' to 3': a DNA fragment with homology for
10 integration corresponding to the region immediately downstream of the *I. orientalis* ADH9091 open reading frame, an *I. orientalis* *PDC1* promoter, the *I. orientalis* *PYC* gene as found in SEQ ID NO: 4, the *I. orientalis* *TAL* terminator, the *I. orientalis* *URA3* promoter, and the first 530 bp of the *I. orientalis* *URA3* open reading frame.

Integration fragment P4-4, having the nucleotide sequence SEQ ID NO: 8,
15 contains the following elements, 5' to 3': a DNA fragment corresponding to the last 568 bp of the *I. orientalis* *URA3* open reading frame, the *I. orientalis* *URA3* terminator, the *I. orientalis* *URA3* promoter, the *I. orientalis* *TDH3* promoter, the *S. pombe* *MAE* gene (having nucleotide sequence SEQ ID NO: 6), the *I. orientalis* *TKL* terminator, and a DNA fragment with homology for integration corresponding to the
20 region immediately upstream of the *I. orientalis* ADH9091 open reading frame.

Integration again occurs via three crossover events. Transformants are streaked to isolates and screened by PCR to identify a strain containing both copies of the *I. orientalis* *PYC* and *S. pombe* *MAE* genes at the ADH9091 locus. The PCR screening to confirm the first copy is performed using primers having nucleotide
25 sequences SEQ ID NOs: 69 and 70 to confirm the 5'-crossover and SEQ ID NOs: 71 and 77 to confirm the 3'-crossover. The PCR screening to confirm the second copy is performed using primers having nucleotide sequences SEQ ID NOs: 69 and 71 to confirm the 5'-crossover and SEQ ID NOs: 70 and 77 to confirm the 3'-crossover. That strain is grown and replated on FOA until a strain in which the *URA3* marker
30 has looped out is identified. That strain is designated strain P-4.

P-5. Strain P-4 is transformed with integration fragment P5-1 (having the nucleotide sequence SEQ ID NO: 9) using LiOAc transformation methods as described in previous examples, to integrate the *L. mexicana* *FRD* gene at the locus of the native *CYB2b* open reading frame. The integration fragment P5-1 contains the

following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis* *CYB2b* open reading frame, an *I. orientalis* *PDC1* promoter, the *L. mexicana* *FRD* gene (having the DNA sequence SEQ ID NO: 10, the *I. orientalis* *PDC1* terminator, the *I. orientalis* *URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat for marker recycling, and a region immediately upstream of the *I. orientalis* *CYB2b* open reading frame.

Successful integration of a single copy of the *L. mexicana* *FRD* gene in one isolate is identified by selection on selection plates lacking uracil and confirmed by PCR. The PCR screening is performed using primers having nucleotide sequences SEQ ID NOs: 79 and 81 to confirm the 5'-crossover and SEQ ID NOs: 82 and 83 to confirm the 3'-crossover. That isolate is grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified by PCR. That isolate is transformed with the integration fragment P5-2 in the same manner as before, to integrate a second copy of the *L. mexicana* *FRD* gene at the native locus of the *CYB2b* open reading frame.

Integration fragment P5-2 (having nucleotide sequence SEQ ID NO: 11), contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis* *CYB2b* open reading frame, an *I. orientalis* *PDC1* promoter, the *L. mexicana* *FRD* gene (having the nucleotide sequence SEQ ID NO: 10), the *I. orientalis* *PDC1* terminator, the *I. orientalis* *URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat for marker recycling, and a region immediately downstream of the *I. orientalis* *CYB2b* open reading frame.

Correct integration of the second copy of the *L. mexicana* *FRD* gene in one isolate is confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 82 and 81 to confirm the 5'-crossover and SEQ ID NOs: 79 and 83 to confirm the 3'-crossover. Retention of the first integration is reconfirmed by repeating the PCR reactions used to verify proper integration of fragment P5-1 above. The confirmed isolate is grown and plated on FOA as before until the loop out of the *URA3* marker is confirmed by PCR in one isolate. That isolate is designated strain P-5.

P-6. Strain P-4 is transformed with the integration fragment P6-1 (having SEQ ID NO: 12) using LiOAc transformation methods as described before to

integrate a copy of the *T. brucei FRD* gene at the native *CYB2b* open reading frame. Integration fragment P6-1 contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis CYB2b* open reading frame, an *I. orientalis PDC1* promoter, the
5 *T.brucei FRD* gene (having the nucleotide sequence SEQ ID NO: 13), the *I. orientalis PDC1* terminator, the *I. orientalis URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat for marker recycling, and a region immediately upstream of the *I. orientalis CYB2b* open reading frame.

10 Successful integration of a single copy of the *T. brucei FRD* gene in one isolate is identified by selection on selection plates lacking uracil and confirmed by PCR. That isolate is grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified by PCR. That isolate is transformed with integration piece P6-2 (having SEQ ID NO: 14) in the same manner as before, to
15 integrate a second copy of the *T. brucei FRD* gene at the *CYB2b* open reading frame. Integration piece P6-2 contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis CYB2b* open reading frame, an *I. orientalis PDC1* promoter, the *T.brucei FRD* gene (having the nucleotide sequence SEQ ID NO: 13), the *I. orientalis PDC1*
20 terminator, the *I. orientalis URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat for marker recycling, and a region immediately downstream of the *I. orientalis CYB2b* open reading frame.

PCR confirms correct integration of both copies of the *T. brucei FRD* gene in
25 an isolate. That isolate is grown and plated on FOA until the loop out of the *URA3* marker is confirmed by PCR in an isolate. That isolate is designated strain P-6.

Table 1: *I. orientalis* URA and PDC Deletion Strains

Strain name	Description	Parent strain
P-1	Organic acid tolerant isolate	Wild-type
P-2	URA3 deletion (2)	P-1
P-3	URA3 deletion (2) PDC deletion (2)	P-2
P-4	URA deletion (2) PDC deletion (2) <i>I. orientalis</i> PYC1 insertion at ADHa (2) <i>S. pombe</i> MAE insertion at ADHa (2)	P-3
P-5	URA deletion (2) PDC deletion (2) <i>I. orientalis</i> PYC1 insertion at ADHa (2) <i>S. pombe</i> MAE insertion at ADHa (2) <i>L.mexicana</i> FRD insertion at CYB2b (2)	P-4
P-6	URA deletion (2) PDC deletion (2) <i>I. orientalis</i> PYC1 insertion at ADHa (2) <i>S. pombe</i> MAE insertion at ADHa (2) <i>T.brucei</i> FRD insertion at CYB2b (2)	P-4

5 Example 1

Example 1A: Changing the co-factor preference of the *L.mexicana* FRD to a NADPH-dependent enzyme

A codon-optimized *L. mexicana* FRD gene having nucleotide sequence SEQ ID NO: 10 is used as a template to modify the coding sequence in five separate reactions to introduce substitutions to the amino acid residues of the putative NADH binding domain of the enzyme.

A mutated *L. mexicana* FRD gene having nucleotide sequence SEQ ID NO. 15 is prepared by performing site-directed substitutions at amino acids 219 (glutamic acid) and 220 (tryptophan).

A mutated *L. mexicana* FRD gene having nucleotide sequence SEQ ID NO. 16 is prepared by performing site-directed substitution at amino acid 417 (glutamic acid).

A mutated *L. mexicana* FRD gene having nucleotide sequence SEQ ID NO. 17 is prepared by performing site-directed substitutions at amino acid 641 (aspartic acid).

A mutated *L. mexicana FRD* gene having nucleotide sequence SEQ ID NO. 18 is prepared by performing site-directed substitutions at amino acids 861 (glutamic acid) and 862 (cysteine).

5 A mutated *L. mexicana FRD* gene having nucleotide sequence SEQ ID NO. 19 is prepared by performing site-directed substitutions at amino acids 1035 (aspartic acid) and 1036 (serine).

Example 1B: Changing the co-factor preference of the *T.brucei FRD* to a NADPH-dependent enzyme.

10 A codon-optimized *T. brucei FRD* gene having nucleotide sequence SEQ ID NO: 13 is used as a template to modify the coding sequence to introduce an amino acid substitution in the putative NADH binding domain of the enzyme. The site-directed mutagenesis is performed to target amino acid 411 (glutamic acid). The resulting mutated gene encodes for a mutated *T. brucei FRD* enzyme having amino
15 acid sequence SEQ ID NO: 20.

Example 1C: FRD Enzyme Assay method

The *FRD* genes having SEQ ID NOs: 10 and 15-20 are each modified at 5' end by the addition of a short DNA sequence having SEQ ID NO: 21, immediately
20 downstream of the initiation codon. This short nucleotide sequence encodes a peptide consisting of six histidine residues, followed by a single methionine, four aspartate residues and a single lysine residue, and effectively adds a 6His affinity tag and an enterokinase cleavage site to the *FRD* sequence. The expression of the *FRD* gene is driven by the IPTG inducible T5 promoter. The construct in each case also contains
25 an optimized Shine-Dalgarno sequence followed by 8 bp of AT rich spacer sequence upstream of the initiation codon as shown in SEQ ID NO: 42. Each of these constructs is separately transformed into *E. coli* Top10 (Invitrogen) according to manufacturer's instructions. Successful transformants are cultured and lysed, and clarified lysate is separated from insoluble material. The *FRD* enzymes in each case,
30 with attached 6His affinity tag and enterokinase cleavage site, are purified using standard methods. Enterokinase (NEB) is added to protein to cleave the 6His affinity tag, and the resulting *FRD* enzyme is purified and collected.

The activities (v_0) of the purified enzymes are measured in an assay with and without 2 mM fumarate in 100mM NaPO₄ buffer (pH7.5) and either 250 μ M NADH

or 250 μ M NADPH as the co-factor. The reaction is carried out in a cuvette containing a total volume of 0.8 mL assay solution. The initial velocity of the enzyme is determined by monitoring the change in $A_{340\text{nm}}$ over the course of 10 minutes; assays are run using a Beckman DU-800 spectrophotometer and cuvettes with a 1cm pathway length. V_{max} and K_M of the enzymes to NADH and NADPH is determined from the v_o using a Lineweaver-Burk plot (Lineweaver, H and Burk, D. (1934), "The Determination of Enzyme Dissociation Constants". *Journal of the American Chemical Society* **56** (3): 658–666) where the concentration of NADH or NADPH is varied from 25 to 400 μ M.

Protein concentration ($[E]_T$) is determined against a BSA standard curve by measuring the absorbance at $OD_{595\text{nm}}$ using the Advanced Protein Assay (Cytoskeleton, Inc.). k_{cat} for both cofactors is calculated $V_{\text{max}}/[E]_T$, and the specificity constant is given as k_{cat}/K_M . The enzyme is NADPH-dependent when k_{cat}/K_M for NADPH is greater than k_{cat}/K_M for NADH.

Example 1D—Integration of NADPH-dependent *FRD* producing strains

Two integration fragments are made from each of the mutated *L. mexicana* *FRD* genes (SEQ ID NOs: 15, 16, 17, 18 and 19). The first fragment in each case is made by digesting the gene with *Xba*I and *Pac*I and subcloning it into SEQ ID NO: 9, replacing the wild-type *FRD* gene with the mutated gene. The second fragment in each case is made by digesting the gene with *Xba*I and *Pac*I and subcloning it into SEQ ID NO: 11, again replacing the wild-type *FRD* gene with the mutated gene.

Two integration fragments are made from the mutated *T. brucei* *FRD* gene (SEQ ID NO: 20). The first fragment is made by digesting the gene with *Xba*I and *Pac*I and subcloning it into SEQ ID NO: 12, replacing the wild-type *FRD* gene with the mutated gene. The second fragment in each case is made by digesting the gene with *Xba*I and *Pac*I and subcloning it into SEQ ID NO: 14, again replacing the wild-type *FRD* gene with the mutated gene.

Separate samples of strain P-4 are separately transformed with the first of the integration constructs for each *FRD* gene, using the LiOAc transformation method described in previous examples, to integrate the respective NADPH-dependent *FRD* genes (as shown in Table 2) at the native *CYB2b* locus. After correct integration is confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 79 and 81 to confirm the 5'-crossover and SEQ ID NOs: 82 and 83 to confirm the 3'-crossover, an isolate from each transformation is grown and plated until the *URA3* marker has

looped out, and in each case the resulting isolate is transformed again with the the second of the integration fragments for each *FRD* gene to introduce a second copy of the same NADPH-dependent *FRD* gene at the *CYB2b* locus. Loopout of the second *URA3* marker is confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 82 and 81 to confirm the 5'-crossover and SEQ ID NOs: 79 and 83 to confirm the 3'-crossover. Retention of the first integration for each *FRD* gene is also reconfirmed by repeating the PCR reactions used to verify proper integration of the first of the integration constructs for each *FRD* gene as above. The resulting strains are designated Examples 1-1, 1-2, 1-3, 1-4, 1-5 and 1-6 respectively.

Table 2 - FRD Site Directed Mutants

Example Designation	Description (in addition to transformations as indicated for strain C-4)	Parent strain
1-1	<i>Mutated L.mexicana FRD</i> SEQ ID NO. 15 insertion at CYB2b (2)	P-4
1-2	<i>Mutated L.mexicana FRD</i> SEQ ID NO. 16 insertion at CYB2b (2)	P-4
1-3	<i>Mutated L.mexicana FRD</i> SEQ ID NO. 17 insertion at CYB2b (2)	P-4
1-4	<i>Mutated L.mexicana FRD</i> SEQ ID NO. 18 insertion at CYB2b (2)	P-4
1-5	<i>Mutated L.mexicana FRD</i> SEQ ID NO. 19 insertion at CYB2b (2)	P-4
1-6	<i>Mutated T.brucei FRD</i> SEQ ID NO. 20 insertion at CYB2b (2)	P-4

Example 2: Integration of malate dehydrogenase (MDH) and fumarase (FUM) genes

Example 2A: RdMDH integration fragment

Integration fragment 2A (SEQ ID NO: 22) contains the *Rhizopus delemar* *MDH* gene (having nucleotide sequence SEQ ID NO: 153, (which is similar to SEQ ID NO: 23, except for a T to A substitution at bp 438, both encoding for the amino acid of SEQ ID NO: 149), *ADHb* upstream integration arm, *ENO* promoter, *RKI* terminator, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2B: Cytosolic *Kluyveromyces marxianus* MDH (*KmMDH3*) integration fragment

Integration fragment 2B (SEQ ID NO: 24) contains the cytosolic *Kluyveromyces marxianus* *MDH* (*KmMDH3*) gene (having nucleotide sequence SEQ ID NO: 25), *ADHb* upstream integration arm, *ENO* promoter, *RKI* terminator, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2C: Cytosolic *Zygosaccharomyces rouxii* MDH (ZrMDH) integration fragment

Integration fragment 2C (SEQ ID NO: 26) contains the cytosolic *Zygosaccharomyces rouxii* MDH (ZrMDH) gene (having nucleotide sequence SEQ ID NO: 27), *ADHb* upstream integration arm, *ENO* promoter, *RKI* terminator, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2D: *Sorghum bicolor* NADPH-dependent MDH (SbMDH) integration fragment

Integration fragment 2D (SEQ ID NO: 28) contains the *Sorghum bicolor* NADPH-dependent MDH (SbMDH) gene (having nucleotide sequence SEQ ID NO: 29), *ADHb* upstream integration arm, *ENO* promoter, *RKI* terminator, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2E: *Chlamydomonas reinhardtii* NADPH-dependent MDH (CrMDH) integration fragment

Integration fragment 2E (SEQ ID NO: 30) contains the *Chlamydomonas reinhardtii* NADPH-dependent MDH (CrMDH) gene (having nucleotide sequence SEQ ID NO: 31), *ADHb* upstream integration arm, *ENO* promoter, *RKI* terminator, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2F: Engineered NADPH-dependent *Rhizopus delemar* MDH (RdPMDH) integration fragment

A *R. delemar* MDH gene having the nucleotide sequence SEQ ID NO: 153 (which is similar to SEQ ID NO: 23, except for T to A substitution at bp 438, both encoding for the amino acid of SEQ ID NO: 149) is used as a template to modify the coding sequence to introduce substitutions of amino acid residues of the putative NADH binding domain of the enzyme. A mutated *R. delemar* MDH gene having the nucleotide sequence SEQ ID NO. 32 is prepared by performing site-directed substitutions.

Integration fragment 2F (SEQ ID NO: 33) contains the NADPH-dependent engineered variant of the *Rhizopus delemar* MDH (RdPMDH) gene, *ADHb* upstream integration arm, *ENO* promoter, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2G: *I. orientalis* FUM (IoFUM) integration fragment

Integration fragment 2G (having SEQ ID NO: 34) contains the *I. orientalis* FUM (IoFUM) gene (having nucleotide sequence, SEQ ID NO: 35), the last 568 base

pairs of the *URA3* marker, *URA3* promoter, *PGK* promoter, *PDC* terminator and *ADHb* downstream integration arm.

Example 2H: *Actinobacillus succinogenes FUM (AsFUM)* integration fragment

Integration fragment 2H (having SEQ ID NO: 36) contains the *Actinobacillus succinogenes FUM (AsFUM)* gene (having nucleotide sequence SEQ ID NO: 37), the last 568 base pairs of the *URA3* marker, *URA3* promoter, *PGK* promoter, *PDC* terminator and *ADHb* downstream integration arm.

Example 2I: *Rhizopus delemar MDH (RdMDH)* reverse integration fragment

Second *R. delemar MDH (RdMDH)* integration fragment 2I (having SEQ ID NO: 38) contains the *R. delemar MDH (RdMDH)* gene (having nucleotide sequence SEQ ID NO: 23), *ADHb* downstream integration arm, *ENO* promoter, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2J: Engineered *Rhizopus delemar MDH (RdPMDH)* reverse integration fragment

Reverse *Rhizopus delemar MDH (RdPMDH)* integration fragment 2J (having SEQ ID NO: 39) contains the engineered NADPH-dependent *RdMDH* gene (having nucleotide sequence SEQ ID NO: 32), *ADHb* downstream integration arm, *ENO* promoter, *URA3* promoter and first 583 base pairs of the *URA3* marker.

Example 2K: *I. orientalis FUM (IoFUM)* reverse integration fragment

Second *I. orientalis FUM (IoFUM)* integration fragment 2K (having SEQ ID NO: 40) contains the *IoFUM* gene (having SEQ ID NO: 35), the last 568 base pairs of the *URA3* marker, *URA3* promoter, *PGK* promoter, *PDC* terminator and *ADHb* upstream integration arm.

Example 2L: *A. succinogenes FUM (AsFUM)* reverse integration fragment

A. succinogenes FUM (AsFUM) reverse integration fragment (having SEQ ID NO: 41) contains the truncated *AsFUM* gene (having nucleotide sequence SEQ ID NO: 37), the last 568 base pairs of the *URA3* marker, *URA3* promoter, *PGK* promoter, *PDC* terminator and *ADHb* upstream integration arm.

Example 2M: Preparation of Examples 2-1 through 2-12.

To produce Example 2-1, Example 1-4 is simultaneously transformed with each of integration fragments 2A and 2G using the standard lithium acetate process described before. Successful transformants are selected on selection plates lacking uracil and confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 84 and 85 to confirm the 5'-crossover, SEQ ID NOs: 107 and 108 to confirm the

junction of integration fragments 2A and 2G, and SEQ ID NOs: 91 and 93 to confirm the 3'-crossover. These transformants are grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified. This strain, which contains the NADPH-dependent *L. Mexicana FRD* gene having SEQ. ID. NO: 18, is designated as Example 2-1.

Examples 2-2 and 2-12 are made in the same general manner. The integration fragments used to make those strains, and their respective genotypes, are identified in Table 3.

Table 3 - *I. orientalis* MDH/FUM Insertion Strains

Ex. No.	Integration Fragments	Description (in addition to transformations as indicated for Example 1-4)
2-1	2A/2G	<i>R. delemar MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-2	2B/2G	<i>K. marxianus MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-3	2C/2K	<i>Z. rouxii MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-4	2D/2K	<i>S. bicolor MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-5	2E/2K	<i>C. reinhardtii MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-6	2F/2K	Engineered <i>R. delemar MDH</i> insertion at ADHb (1) <i>I. orientalis FUM</i> insertion at ADHb (1)
2-7	2A/2H	<i>R. delemar MDH</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)
2-8	2B/2H	<i>K. marxianus MDH3</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)
2-9	2C/2H	<i>Z. rouxii MDH</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)
2-10	2D/2H	<i>S. bicolor MDH</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)
2-11	2E/2H	<i>C. reinhardtii MDH</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)
2-12	2F/2H	Engineered <i>R. delemar MDH</i> insertion at ADHb (1) <i>A. succinogenes FUM</i> insertion at ADHb (1)

Example 2N: Preparation of Examples 2-13 through 2-15

To produce Example 2-13, Example 2-2 is simultaneously transformed with each of fragments 2I and 2K using the lithium acetate process described before. Successful transformants are identified by PCR using primers having nucleotide sequences SEQ ID NOs: 84 and 85 to confirm the 5'-crossover, SEQ ID NOs: 107 and 108 to confirm the junction between the integration fragments and SEQ ID NOs: 91

and 93 to confirm the 3'-crossover. Retention of the first integration at ADHb is also reconfirmed by repeating the PCR reactions used to verify the 5'- and 3'-crossovers as in Example 2M above. The successful transformants are grown and plated until a strain in which the *URA3* marker has looped out is identified as before as confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 84 and 91 to confirm the 5'-crossover and SEQ ID NOs: 85 and 93 to confirm the 3'-crossover. This strain is designated as Example 2-13.

Examples 2-14 and 2-15 are made in the same general manner by integrating Examples 2-6 and 2-7, respectively. The integration fragments used to make those strains, and their respective genotypes, are identified in Table 4.

Table 4

Ex. No.	Parent Strain	Integration Fragments	Description (in addition to transformations as indicated for strain 1-4)
2-13	2-2	2I/2K	<i>R. delemar MDH</i> insertion at ADHb (2) <i>I. orientalis FUM</i> insertion at ADHb (2)
2-14	2-6	2J/2K	Engineered <i>R. delemar MDH</i> insertion at ADHb (2) <i>I. orientalis FUM</i> insertion at ADHb (2)
2-15	2-7	2K/2L	<i>R. delemar MDH</i> insertion at ADHb (2) <i>A. succinogenes FUM</i> insertion at ADHb (2)

Example 2O: Preparation of Examples 2-16 through 2-30.

Examples 2-16 through 2-30 are produced in the same manner as Examples 2-1 through 2-15, respectively, except that the host strain is Example 1-1 rather than Example 1-4. The genotypes of Examples 2-16 through 2-30 are the same as described in Tables 3 and 4 above for Examples 2-1 through 2-15, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having SEQ ID NO: 15.

Example 2P: Preparation of Examples 2-31 through 2-45.

Examples 2-31 through 2-45 are produced in the same manner as Examples 2-1 through 2-15, respectively, except that the host strain is Example 1-2 rather than Example 1-4. The genotypes of Examples 2-31 through 2-45 are the same as described in Tables 3 and 4 above for Examples 2-31 through 2-45, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having SEQ ID NO: 16.

Example 2Q: Preparation of Examples 2-46 through 2-60.

Examples 2-46 through 2-60 are produced in the same manner as Examples 2-1 through 2-15, respectively, except that the host strain is Example 1-3 rather than Example 1-4. The genotypes of Examples 2-46 through 2-60 are the same as

described in Tables 3 and 4 above for Examples 2-46 through 2-60, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having SEQ ID NO: 17.

Example 2R: Preparation of Examples 2-61 through 2-75.

Examples 2-61 through 2-75 are produced in the same manner as Examples 2-1 through 2-15, respectively, except that the host strain is Example 1-5 rather than Example 1-4. The genotypes of Examples 2-61 through 2-75 are the same as described in Tables 3 and 4 above for stains 2-61 through 2-75, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having SEQ ID NO: 19.

Example 2S: Preparation of Examples 2-76 through 2-90.

Examples 2-76 through 2-90 are produced in the same manner as Examples 2-1 through 2-15, respectively, except that the host strain is Example 1-6 rather than Example 1-4. The genotypes of Examples 2-76 through 2-90 are the same as described in Tables 3 and 4 above for Examples 2-76 through 2-90, respectively, except the *FRD* gene is the mutated *T. brucei* gene having SEQ ID NO: 20.

Example 2T:

The *MDH* genes having SEQ ID NOs 29, 31 and 32 are each modified at 5' end by the addition of a short DNA sequence having SEQ ID NO: 21, immediately downstream of the initiation codon. This short nucleotide sequence encodes a peptide consisting of six histidine residues, followed by a single methionine, four aspartate residues and a single lysine residue, and effectively adds a 6His affinity tag and an enterokinase cleavage site to the *MDH* sequence. The expression of the *MDH* gene is driven by the IPTG inducible T5 promoter. The construct in each case also contains an optimized Shine-Dalgarno sequence followed by 8 bp of AT rich spacer sequence upstream of the initiation codon as shown in SEQ ID NO: 42. Each of these constructs is separately transformed into *E. coli* Top10 (Invitrogen) according to manufacturer's instructions. Successful transformants are cultured and lysed, and clarified lysate is separated from insoluble material. The *MDH* enzymes in each case, with attached 6His affinity tag and enterokinase cleavage, are purified using standard methods. Enterokinase (NEB) is added to protein to cleave the 6His affinity tag, and the resulting *MDH* enzyme is purified and collected.

The activities (v_0) of the purified enzymes are measured in an assay with and without 100 micromoles M OAA in Tris-HCl (pH 8.0) and either 400 μ M NADH or 400 μ M NADPH as the co-factor. The reaction is carried out in a cuvette containing a total volume of 0.8 mL assay solution. The initial velocity of the enzyme is

determined by monitoring the change in $A_{340\text{nm}}$ over the course of 10 minutes; assays are run using a Beckman DU-800 spectrophotometer and cuvettes with a 1cm pathway length. V_{max} and K_M of the enzymes to NADH and NADPH is determined from the v_o using a Lineweaver-Burk plot (Lineweaver, H and Burk, D. (1934), "The
 5 Determination of Enzyme Dissociation Constants". *Journal of the American Chemical Society* **56** (3): 658–666) where the concentration of NADH or NADPH is varied from 25 to 400 μM .

Protein concentration ($[E]_T$) is determined against a BSA standard curve by measuring the absorbance at $OD_{595\text{nm}}$ using the Advanced Protein Assay
 10 (Cytoskeleton, Inc.). k_{cat} for both cofactors is calculated $V_{\text{max}}/[E]_T$, and the specificity constant is given as k_{cat}/K_M . The enzyme is NADPH-dependent when k_{cat}/K_M for NADPH is greater than k_{cat}/K_M for NADH.

Example 3: Integration of soluble transhydrogenase

Integration fragment 3A: Right Hand integration fragment – marker only

Integration fragment 3A having nucleotide sequence SEQ ID NO: 43, contains the following elements, 5' to 3': a DNA fragment corresponding to the last 567 bp of the *I. orientalis URA3* open reading frame, the *I. orientalis URA3* terminator, the *I. orientalis URA3* promoter, the *I. orientalis TDH3* promoter, the *I. orientalis TKL*
 20 terminator, the *I. orientalis PGK* promoter, the *I. orientalis PDC* terminator and a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis MAE1* open reading frame.

Integration fragment 3B: Left Hand integration fragment with the *E. coli SthA* gene

Integration fragment 3B, having nucleotide sequence SEQ ID NO: 44, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis MAE1* open reading frame, an *I. orientalis PDC1* promoter, the *I. orientalis TAL* terminator, the *I. orientalis ENO* promoter, the *E. coli SthA* gene (having the
 25 nucleotide sequence SEQ ID NO: 45), *I. orientalis RKI* terminator, *URA3* promoter, and the first 582 bp of the *I. orientalis URA3* open reading frame.

Integration fragment 3C: Left Hand integration fragment with a codon optimized *E. coli SthA* gene

Integration fragment 3C, having nucleotide sequence SEQ ID NO: 46, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis* MAE1 open reading frame, an *I. orientalis* PDC1 promoter, the *I. orientalis* TAL terminator, the *I. orientalis* ENO promoter, the codon-optimized *E. coli SthA* gene (having the nucleotide sequence SEQ ID NO: 47), *I. orientalis* RKI terminator, *URA3* promoter, and the first 582 bp of the *I. orientalis* *URA3* open reading frame.

Integration fragment 3D: Left Hand integration fragment with the *A. vinelandii SthA* gene

Integration fragment 3D, having nucleotide sequence SEQ ID NO: 48, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis* MAE1 open reading frame, an *I. orientalis* PDC1 promoter, the *I. orientalis* TAL terminator, the *I. orientalis* ENO promoter, the *A. vinelandii SthA* gene (having the nucleotide sequence, SEQ ID NO: 49), *I. orientalis* RKI terminator, *URA3* promoter, and the first 582 bp of the *I. orientalis* *URA3* open reading frame.

To produce Example 3-1, Example 1-4 is simultaneously transformed with each of integration fragments 3B and 3A using the lithium acetate process described before. Successful transformants are selected on selection plates lacking uracil and confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 96 and 98 to confirm the 5'-crossover and SEQ ID NOs: 100 and 101 to confirm the 3'-crossover. Successful transformants are grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified. This strain is designated as Example 3-1. Examples 3-2 and 3-3 are made in the same general manner. The integration fragments used to make those strains, and their respective genotypes, are identified in Table 4.

To produce Example 3-4, Example 2-13 is simultaneously transformed with each of integration fragments 3B and 3A using the standard lithium acetate process described before. Successful transformants are selected on selection plates lacking uracil, confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 96 and 98 to confirm the 5'-crossover and SEQ ID NOs: 100 and 101 to confirm the 3'-crossover. Successful transformants are grown and plated on FOA as before until a

strain in which the *URA3* marker has looped out is identified. This strain is designated as Example 3-4. Examples 3-5 and 3-6 are made in the same general manner. The integration fragments used to make those strains, and their respective genotypes, are identified in Table 5.

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Table 5 - *I. orientalis* Insertion Strains

Ex. No.	Integration Fragments	Description (in addition to transformations as indicated for parent strain)	Parent strain
3-1	3A, 3B	<i>E. coli SthA</i> insertion at MAE1 (1)	Ex. 1-4
3-2	3A, 3C	Codon optimized <i>E.coli SthA</i> insertion at MAE1 (1)	Ex. 1-4
3-3	3A, 3D	<i>A. vinelandii SthA</i> insertion at MAE1 (1)	Ex. 1-4
3-4	3A, 3B	<i>E. coli SthA</i> insertion at MAE1 (1)	Ex. 2-13
3-5	3A, 3C	Codon optimized <i>E.coli SthA</i> insertion at MAE1 (1)	Ex. 2-13
3-6	3A, 3D	<i>A. vinelandii SthA</i> insertion at MAE1 (1)	Ex. 2-13

Examples 3-7 through 3-100 are produced by separately transforming Examples 1-1, 1-2, 1-3, 1-5, 1-6 and 2-1 through 2-12 and 2-14 through 2-90 with integration fragments 3A and 3B in the same manner described with regard to Example 3-1. All of Examples 3-9 through 3-100 have the *E. coli SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as the modifications indicated in Tables 1-4, as applicable.

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Examples 3-101 through 3-194 are produced by separately transforming Examples 1-1, 1-2, 1-3, 1-5, 1-6 and 2-1 through 2-12 and 2-14 through 2-90 with integration fragments 3A and 3C in the same manner described with regard to Example 3-1. All of Examples 3-101 through 3-194 have the codon optimized *E. coli SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as the modifications indicated in Tables 1-4, as applicable.

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Examples 3-195 through 3-288 are produced by separately transforming Examples 1-1, 1-2, 1-3, 1-5, 1-6 and 2-1 through 2-11 and 2-13 through 2-90 with integration fragments 3A and 3D in the same manner described with regard to Example 3-1. All of Examples 3-195 through 3-288 have the *A. vinelandii SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as the modifications indicated in Tables 1-4, as applicable.

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Example 4 – Integration of *Stb5p* geneIntegration fragment 4A: Left Hand integration fragment – marker only

Integration fragment 4A, having the nucleotide sequence SEQ ID NO: 109, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis* MAE1 open reading frame, an *I. orientalis* PDC1 promoter, the *I. orientalis* TAL terminator, the *I. orientalis* ENO promoter, *I. orientalis* RKI terminator, URA3 promoter, and the first 582 bp of the *I. orientalis* URA3 open reading frame.

Integration fragment 4B: Right Hand integration fragment with the *S. cerevisiae* *Stb5p* gene

Integration fragment 4B, having nucleotide sequence SEQ ID NO: 50, contains the following elements, 5' to 3': a DNA fragment corresponding to the last 567 bp of the *I. orientalis* URA3 open reading frame, the *I. orientalis* URA3 terminator, the *I. orientalis* URA3 promoter, the *I. orientalis* TDH3 promoter, the *S. cerevisiae* *Stb5p* gene (having nucleotide sequence SEQ ID NO: 51), the *I. orientalis* TKL terminator, the *I. orientalis* PGK promoter, the *I. orientalis* PDC terminator and a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis* MAE1 open reading frame.

Example 4-1 is produced by simultaneously transforming Example 1-1 with integration fragments 4A and 4B using the lithium acetate methods described before. Successful transformants having the *S. cerevisiae* *Stb5p* gene integrated at the MAE1 locus are selected.

Examples 4-2 through 4-6 are produced in the same manner by transforming Examples 1-2 through 1-6, respectively, with integration fragments 4A and 4B. Examples 4-7 through 4-96 are produced in the same manner by transforming Examples 2-1 through 2-90, respectively, with integration fragments 4A and 4B. All of Examples 4-2 through 4-96 have the *S. cerevisiae* *Stb5p* gene integrated at the MAE1 locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 4-1 through 4-96 each are transformed with integration fragments 3B and 4B in the manner described in Example 3 to produce Examples 4-97 through 4-192, respectively. Examples 4-97 through 4-192 contain the *E. coli* *SthA* gene and the *S. cerevisiae* *Stb5p* gene integrated at the MAE1 locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 4-1 through 4-96 each are transformed with integration fragments 3C and 4B in the manner described in Example 3 to produce Examples 4-193 through 4-288, respectively. Examples 4-193 through 4-288 contain the codon optimized *E. coli SthA* gene and the *S. cerevisiae Stb5p* gene integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 4-1 through 4-96 each are transformed with integration fragments 3D and 4B in the manner described in Example 3 to produce Examples 4-289 through 4-384, respectively. Examples 4-289 through 4-384 contain the *A. vinelandii SthA* gene and the *S. cerevisiae Stb5p* gene integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Example 5 – Deletion of Native *GPD* gene

Example 1-1 is transformed with integration fragment 5 (having nucleotide sequence SEQ ID NO: 52) using lithium acetate methods as described before. This integration fragment contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis GPD1* open reading frame, a *PDC* transcriptional terminator, the *URA3* promoter, the *I. orientalis URA3* gene, an additional *URA3* promoter direct repeat for marker recycling and a DNA fragment with homology for integration corresponding to the region directly downstream of the *I. orientalis GPD1* open reading frame. Successful transformants are selected on selection plates lacking uracil, confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 150 and 151 to confirm the 5'-crossover and SEQ ID NOs: 152 and 101 to confirm the 3'-crossover, and grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified. This strain is then transformed with an integration fragment having nucleotide sequence SEQ ID NO: 53. This integration fragment contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis GPD1* open reading frame, the *URA3* promoter, the *I. orientalis URA3* gene, an additional *URA3* promoter direct repeat for marker recycling a *PDC* transcriptional terminator, and a DNA fragment with homology for integration corresponding to the region directly downstream of the *I. orientalis GPD1* open reading frame. Successful transformants are again selected on selection plates lacking uracil, and integration of the second *GPD1* deletion construct confirmed by PCR using primers having nucleotide

sequences SEQ ID NOs: 151 and 101 to confirm the 5'-crossover and SEQ ID NOs: 150 and 152 to confirm the 3'-crossover. Retention of the first *GPD1* deletion construct is also reconfirmed by repeating the PCR reactions used to verify proper integration of integration fragment 5 above. Confirmed isolates are grown and plated until a strain in which the *URA3* marker has looped out is identified as before. One such transformant which has a deletion of both native *GPD* genes, is designated Example 5-1.

Examples 5-2 through 5-6 are made in the same manner by transforming Examples 1-1 through 1-6, respectively.

Examples 5-7 through 5-96 are made in the same manner by transforming Examples 2-1 through 2-12, respectively.

Examples 5-97 through 5-384 are made in the same manner by transforming Examples 3-1 through 3-288, respectively.

Examples 5-385 through 5-768 are made in the same manner by transforming Examples 4-1 through 4-384, respectively.

All of Examples 5-1 through 5-768 have a deletion of a native *GPD* gene in addition to the modification indicated before in relation to the respective parent strains.

Example 6 - Deletion of Phosphoglucosomerase (*PGI*) gene

Integration fragment 6-1 (having SEQ ID NO: 54) for the deletion of the first copy of the *I. orientalis PGI* gene, contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately upstream of the *I. orientalis PGI* open reading frame, a *PDC1* transcriptional terminator, , the *I. orientalis URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat for marker recycling, and a region immediately downstream of the *I. orientalis PGI* open reading frame.

Integration fragment 6-2 (having SEQ ID NO: 55) for the deletion of the second copy of the *I. orientalis PGI* gene contains the following elements, 5' to 3': a DNA fragment with homology for integration corresponding to the region immediately downstream of the *I. orientalis PGI* open reading frame, a *PDC1* transcriptional terminator, the *I. orientalis URA3* promoter, gene, and terminator in succession, followed by an additional *URA3* promoter which serves as a direct repeat

for marker recycling, and a region immediately upstream of the *I. orientalis* PGI open reading frame.

Example 1-1 is transformed with integration fragment 6-1 using the lithium acetate process described before. Successful transformants are selected on PGI deletion selection plates lacking uracil (SC –ura, +20 g/L fructose, +0.5 g/L glucose) incubated 3-5 days and confirmed by PCR using primers having nucleotide sequences SEQ ID NOs: 104 and 105 to confirm the 5'-crossover and SEQ ID NOs: 79 and 106 to confirm the 3'-crossover. Successful transformants are grown and plated on FOA as before until a strain in which the *URA3* marker has looped out is identified. That strain is then transformed with integration fragment 6-2 in the same manner, and a successful deletant is identified using primers having nucleotide sequences SEQ ID NOs: 79 and 104 to confirm the 5'-crossover and SEQ ID NOs: 104 and 106 to confirm the 3'-crossover. A strain in which the *URA3* marker has looped out as before is designated Example 6-1-1.

Examples 6-1-2 through 6-1-6 are prepared in the same manner as strain 6-1-1, by transforming each of strains 1-2 through 1-6 sequentially with integration fragments 6-1 and 6-2.

Strains 6-2-1 through 6-2-90 are prepared in the same manner as strain 6-1-1, by transforming each of strains 2-1 through 2-90 sequentially with integration fragments 6-1 and 6-2.

Strains 6-3-1 through 6-3-288 are prepared in the same manner as strain 6-1-1, by transforming each of strains 3-1 through 3-288 sequentially with integration fragments 6-1 and 6-2.

Strains 6-4-1 through 6-4-384 are prepared in the same manner as strain 6-1-1, by transforming each of strains 4-1 through 4-384 sequentially with integration fragments 6-1 and 6-2.

Strains 6-5-1 through 6-5-768 are prepared in the same manner as strain 6-1-1, by transforming each of strains 5-1 through 5-768 sequentially with integration fragments 6-1 and 6-2.

Example 7

7A: Construction of Preparatory Strains

Preparatory Strains P-2sc through P-4sc are engineered in a manner analogous to Strains P-2 through P-4 above, starting from a wild-type *Saccharomyces*

cerevisiae strain (CEN.PK 113-7D, on deposit in the CBS culture collection as CBS 8340). The wild-type strain is transformed with an integration fragment designed to disrupt the *URA3* gene. A successful deletent strain is then transformed with integration fragment P3 as described in P-3 above, modified to target the *S. cerevisiae* YLR044C gene by replacing first 855bp of the fragment with 855bp of DNA from immediately downstream of the target YLR044C gene and replacing the final 803bp of the fragment with 803bp from immediately upstream of the target YLR044C gene. The resultant *PDC* deletant strain is designated strain P-3sc.

One copy of each of the *PYC* and *MAE* genes are inserted into Strain P-3sc in a manner analogous to that described in P-4 above. The integration is performed using the integration fragments P4-1 and P4-2 described in P-4 above, modified in each case to target the *S. cerevisiae* YOL086C locus. Integration fragment P4-1 is modified by replacing the first 855bp with 855bp from immediately upstream of the ATG start codon for the YOL086C locus and integration fragment P4-2 is modified by replacing the last 1003p with the 1003bp from immediately downstream of the TAA stop codon for the YOL086C locus. This strain, after loopout of the *URA3* marker, is designated strain P-4sc.

Table 6: *S. cerevisiae* URA and PDC Deletion Strains

Strain name	Description	Parent strain
P-1sc	Wild-type strain	Wild-type
P-2sc	URA3 deletion (1)	P-1sc
P-3sc	URA3 deletion (1) PDC deletion (1)	P-2sc
P-4sc	URA deletion (1) PDC deletion (1) <i>I. orientalis</i> PYC1 insertion at ADH1 (1) <i>S. pombe</i> MAE insertion at ADH1 (1)	P-3sc

6B. Insertion of NADPH-dependent FRD gene

Six separate transformations of strain P-4sc are performed to insert one copy of the mutated NADPH-dependent *FRD* genes, analogously to Example 1D. The integration constructs in each case are the same as described in Examples 1-1 through 1-6, respectively, except in each case the 5' and 3' flanks of the fragments are replaced with a 300-1200 bp upstream (5') sequence or downstream (3') sequence of the target *CYB2b* gene. Successful transformants, after loopout of the *URA3* marker, are designated Examples 7-1, 7-2, 7-3, 7-4, 7-5 and 7-6 respectively.

Table 7

Ex. No.	Description (in addition to transformations as indicated for strain P-1sc)	Parent strain
7-1	<i>L.mexicana</i> FRD SEQ ID NO: 15 insertion at <i>CYB2</i> (1)	P-4sc
7-2	<i>L.mexicana</i> FRD SEQ ID NO: 16 insertion at <i>CYB2</i> (1)	P-4sc
7-3	<i>L.mexicana</i> FRD SEQ ID NO: 17 insertion at <i>CYB2</i> (1)	P-4sc
7-4	<i>L.mexicana</i> FRD SEQ ID NO: 18 insertion at <i>CYB2</i> (1)	P-4sc
7-5	<i>L.mexicana</i> SEQ ID NO: 19 insertion at <i>CYB2</i> (1)	P-4sc
7-6	<i>T.brucei</i> FRD SEQ ID NO: 20 insertion at <i>CYB2</i> (1)	P-4sc

Example 8

5 Integration fragments analogous to those described in Examples 2A through 2E above are prepared by replacing the first 769 bp of each of integration fragments 2A through 2E with the 769bp from immediately upstream of the ATG start codon for the *S. cerevisiae* YMR303C gene.

10 Integration fragments analogous to those described in Examples 2G, 2H, 2I and 2K above are prepared by replacing the last 615 bp of each of integration fragments 2G, 2H, 2I and 2K above with the 615 bp from immediately downstream of the stop codon for the *S. cerevisiae* YMR303C gene.

15 Examples 8-1 through 8-12 are prepared in the same manner as Examples 2-1 through 2-12, respectively, using the corresponding modified integration fragments and Example 7-1 as the parent strain. Successful transformants are selected as before and grown and plated on FOA as before until the *URA3* marker has looped out. The genotypes of those respective Examples are identified in Table 8.

Table 8. *S. cerevisiae* MDH/FUM Insertion Strains

Ex. No.	Description (in addition to transformations as indicated for the indicated parent strain)	Parent Strain
8-1	<i>R. delemar</i> MDH insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-2	<i>K. marxianus</i> MDH3 insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-3	<i>Z. rouxii</i> MDH insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-4	<i>S. bicolor</i> MDH insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-5	<i>C. reinhardtii</i> MDH insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-6	Engineered <i>R. delemar</i> MDH insertion at YMR303C (1) <i>I. orientalis</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-7	<i>R. delemar</i> MDH insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-8	<i>K. marxianus</i> MDH3 insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-9	<i>Z. rouxii</i> MDH insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-10	<i>S. bicolor</i> MDH insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-11	<i>C. reinhardtii</i> MDH insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1
8-12	Engineered <i>R. delemar</i> MDH insertion at YMR303C (1) <i>A. succinogenes</i> FUM insertion at YMR303C (1)	Ex. 7-1

5 Examples 8-13 through 8-24 are produced in the same manner as Examples 8-1 through 8-12, respectively, except that the host strain is Example 7-2 rather than Example 7-1. The genotypes of Examples 8-13 through 8-24 are the same as described in Table 8 above for Examples 8-1 through 8-12, respectively, except the *FRD* gene is the mutated *L. mexicana* *FRD* gene having nucleotide sequence no. 16.

10 Examples 8-25 through 8-36 are produced in the same manner as Examples 8-1 through 8-12, respectively, except that the host strain is Example 7-3 rather than Example 7-1. The genotypes of Examples 8-25 through 8-36 are the same as described in Table 8 above for Examples 8-1 through 8-12, respectively, except the *FRD* gene is the mutated *L. mexicana* *FRD* gene having nucleotide sequence no. 17.

15 Examples 8-37 through 8-48 are produced in the same manner as Examples 8-1 through 8-12, respectively, except that the host strain is Example 7-4 rather than

Example 7-1. The genotypes of Examples 8-37 through 8-48 are the same as described in Table 8 above for Examples 8-1 through 8-12, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having nucleotide sequence no. 18.

Examples 8-49 through 8-60 are produced in the same manner as Examples 8-1 through 8-12, respectively, except that the host strain is Example 7-5 rather than Example 7-1. The genotypes of Examples 8-49 through 8-60 are the same as described in Table 8 above for Examples 8-1 through 8-12, respectively, except the *FRD* gene is the mutated *L. mexicana FRD* gene having nucleotide sequence no. 19.

Examples 8-61 through 8-72 are produced in the same manner as Examples 8-1 through 8-12, respectively, except that the host strain is Example 7-6 rather than Example 7-1. The genotypes of Examples 8-61 through 8-72 are the same as described in Table 8 above for Examples 8-1 through 8-12, respectively, except the *FRD* gene is the mutated *T. brucei FRD* gene having nucleotide sequence no. 20.

Example 9

Integration fragments 3A through 3D are modified as follows:

Integration fragment 3A is modified for insertion at the *S. cerevisiae* YKL029C locus by replacing the last 377 bp with the 377 bp from immediately upstream of the ATG start codon of the native *S. cerevisiae* YKL029C gene.

Integration fragments 3B, 3C and 3D each are separately modified for insertion at the *S. cerevisiae* YKL029C locus by replacing the first 361 bp with the 361 bp from immediately upstream of the ATG start codon of the native *S. cerevisiae* YKL029C gene.

To produce Example 9-1, Example 7-1 is simultaneously transformed with each of the modified integration fragments 3B and 3A using the standard lithium acetate process described before. Successful transformants are selected on selection plates lacking uracil, confirmed by PCR, and grown and plated until a strain in which the *URA3* marker has looped out is identified as before. This strain is designated as Example 9-1. Examples 9-2 and 9-3 are made in the same general manner. The integration fragments used to make those strains, and their respective genotypes, are identified in Table 9.

Table 9 *S. cerevisiae* Insertion Strains

Example No.	Integration Fragments (modified)	Description (in addition to transformations as indicated for parent strain)	Parent strain
9-1	3A, 3B	<i>E. coli SthA</i> insertion at MAE1 (1)	Ex. 1-4
9-2	3A, 3C	Codon optimized <i>E.coli SthA</i> insertion at MAE1 (1)	Ex. 1-4
9-3	3A, 3D	<i>A. vinelandii SthA</i> insertion at MAE1 (1)	Ex. 1-4

Examples 9-4 through 9-80 are produced by separately transforming Examples 7-2 through 7-6 and 8-1 through 8-72, respectively, with modified integration fragments 3A and 3B in the same manner described with regard to Example 9-1. Examples 9-4 through 9-80 all contain the *E. coli SthA* gene at the native YKL029C locus, with deletion of the native gene.

Examples 9-81 through 9-157 are produced by separately transforming Examples 7-2 through 7-6 and 8-1 through 8-72 with modified integration fragments 3A and 3C in the same manner described with regard to Example 9-2. Examples 9-81 through 9-157 all contain the codon-optimized *E. coli SthA* gene at the native YKL029C locus, with deletion of the native gene.

Examples 9-158 through 9-234 are produced by separately transforming Examples 7-2 through 7-6 and 8-1 through 8-72 with modified integration fragments 3A and 3D in the same manner described with regard to Example 9-3. Examples 9-158 through 9-234 all contain the *A. vinelandii SthA* gene at the native YKL029C locus, with deletion of the native gene.

Example 10

Integration fragment 5 is modified for insertion at the native *S. cerevisiae* YDL022W gene by replacing the first 853 bp with 853 bp of DNA from immediately upstream of the YDL022W gene and the final 1004 bp with 1004 bp of DNA from immediately downstream of the YDL022W gene. Example 7-1 is transformed with the modified integration fragment using methods as described before. A successful transformant in which the native *GPD* gene is deleted and the *URA3* marker has looped out is designated Example 10-7-1.

Examples 10-7-2 through 10-7-6 are made in the same manner by transforming Examples 7-2 through 7-6, respectively. Examples 10-7-2 through 10-7-6 have a deletion of a native *GPD* gene in addition to the genetic modifications indicated earlier with respect to the respective parent strains.

5 Examples 10-8-1 through 10-8-72 are made in the same manner by transforming strains 8-1 through 8-72, respectively. Examples 10-8-1 through 10-8-72 have a deletion of a native *GPD* gene in addition to the genetic modifications indicated earlier with respect to the respective parent strains.

10 Examples 10-9-1 through 10-9-234 are made in the same manner by transforming strains 9-1 through 9-234, respectively. Examples 10-9-1 through 10-9-234 have a deletion of a native *GPD* gene in addition to the genetic modifications indicated earlier with respect to the respective parent strains.

Example 11-Construction of Preparatory strains P1wtIo through P6wtIo

15 Preparatory Strains P-2wtIo through P-6wtIo are made in the same manner as Preparatory Strains P-2 through P6, except the starting strain is *I. orientalis* strain ATCC PTA-6658.

 Examples 11-1 through 11-6 are made in the same way as Examples 1-1 through 1-6, respectively, by transforming Preparatory Strain P-4wtIo.

Example 12

 Examples 12-1 through 12-15 are made in the same way as Examples 2-1 through 2-15, respectively, by transforming strain 11-4 instead of strain 1-4.

25 Examples 12-16 through 12-30 are made in the same way as Examples 2-16 through 2-30, respectively, by transforming strain 11-1 instead of strain 1-1.

 Examples 12-31 through 12-45 are made in the same way as Examples 2-31 through 2-45, respectively, by transforming strain 11-2 instead of strain 1-2.

 Examples 12-46 through 12-60 are made in the same way as Examples 2-46 through 2-60, respectively, by transforming strain 11-3 instead of strain 1-3.

30 Examples 12-61 through 12-75 are made in the same way as Examples 2-61 through 2-75, respectively, by transforming strain 11-5 instead of strain 1-5.

 Examples 12-76 through 12-90 are made in the same way as Examples 2-76 through 2-90, respectively, by transforming strain 11-6 instead of strain 1-6.

Example 13

Examples 13-1 through 12-3 are made in the same way as Examples 3-1 through 3-3, respectively, by transforming strain 11-4 instead of Strain 1-4.

Examples 13-4 through 13-6 are made in the same way as Examples 3-4 through 3-6, respectively, by transforming strain 12-13 instead of Strain 2-13.

Examples 13-7 through 13-100 are produced by separately transforming Examples 11-1, 11-2, 11-3, 11-5, 11-6, 12-1 through 12-12 and 12-14 through 12-90 with integration fragments 3A and 3B in the same manner described with regard to Example 3-1. All of Examples 13-7 through 13-100 have the *E. coli SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as modifications analogous to those indicated in Tables 1-4, as applicable.

Examples 13-101 through 13-194 are produced by separately transforming Examples 11-1, 11-2, 11-3, 11-5, 11-6, 12-1 through 12-12 and 12-14 through 12-90 with integration fragments 3A and 3C in the same manner described with regard to Example 3-1. All of Examples 13-101 through 13-194 have the codon optimized *E. coli SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as modifications analogous to those indicated in Tables 1-4, as applicable.

Examples 13-195 through 13-288 are produced by separately transforming Examples 11-1, 11-2, 11-3, 11-5, 11-6, 12-1 through 12-12 and 12-14 through 12-90 with integration fragments 3A and 3D in the same manner described with regard to Example 3-1. All of Examples 13-195 through 13-288 have the *A. vinelandii SthA* gene inserted at the locus of the *MAE1* gene, which is deleted, as well as modifications analogous to those indicated in Tables 1-4, as applicable.

Example 14 – Integration of *Stb5p* gene

Example 14-1 is produced by simultaneously transforming Example 11-1 with integration fragments 4A and 4B using the lithium acetate methods described before. Successful transformants having the *S. cerevisiae Stb5p* gene integrated at the *MAE1* locus are selected.

Examples 14-2 through 14-6 are produced in the same manner by transforming Examples 11-2 through 11-6, respectively, with integration fragments 4A and 4B. Examples 14-7 through 14-96 are produced in the same manner by transforming Examples 12-1 through 12-90, respectively, with integration fragments 4A and 4B. All of Examples 14-2 through 14-96 have the *S. cerevisiae Stb5p* gene

integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 14-1 through 14-96 each are transformed with integration fragments 3B and 4B in the manner described in Example 3 to produce Examples 14-97 through 14-192, respectively. Examples 14-97 through 14-192 contain the *E. coli* *SthA* gene and the *S. cerevisiae* *Stb5p* gene integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 14-1 through 14-96 each are transformed with integration fragments 3C and 4B in the manner described in Example 3 to produce Examples 14-193 through 14-288, respectively. Examples 14-193 through 14-288 contain the codon optimized *E. coli* *SthA* gene and the *S. cerevisiae* *Stb5p* gene integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Examples 14-1 through 14-96 each are transformed with integration fragments 3D and 4B in the manner described in Example 3 to produce Examples 14-289 through 14-384, respectively. Examples 14-289 through 14-384 contain the *A. vinelandii* *SthA* gene and the *S. cerevisiae* *Stb5p* gene integrated at the *MAE1* locus, as well as the modifications indicated in Tables 1-4, as applicable.

Example 15

Example 11-1 is transformed with integration fragment 5 in the manner described in Example 5 to delete the native *GPD* gene. A successful transformant having the looped out marker is designated Example 15-1. Examples 15-2 through 15-6 are made in the same manner by transforming Examples 11-2 through 11-6, respectively.

Examples 15-7 through 15-96 are made in the same manner by transforming Examples 12-1 through 12-90, respectively.

Examples 15-97 through 15-384 are made in the same manner by transforming Examples 13-1 through 13-288, respectively.

Examples 15-385 through 15-768 are made in the same manner by transforming Examples 14-1 through 14-384, respectively.

All of Examples 15-1 through 15-768 have a deletion of a native *GPD* gene in addition to the modification indicated before in relation to the respective parent strains.

Example 16

Both alleles of the phosphoglucosomerase (*PGI*) gene are deleted in each of Examples 11-1 through 11-6, 12-1 through 12-90, 13-1 through 13-288, 14-1 through 14-384 and 15-1 through 15-768 by transforming the cells sequentially with integration fragments 6-1 and 6-2 as described in Example 6. The resulting cells are designated Examples 16-11-1 through 16-11-6, 16-12-1 through 16-12-90, 16-13-1 through 16-13-288, 16-14-1 through 16-14-384 and 16-15-1 through 16-15-768, respectively.

10 Example 17 – Shake flask evaluation for succinate production

Example 1-1 is streaked out for single colonies on URA selection plates and incubated at 30°C until colonies are visible (1-2 days). Cells from plates are scraped into sterile growth medium and the optical density (OD₆₀₀) is measured. Optical density is measured at wavelength of 600 nm with a 1 cm pathlength using a model Genesys20 spectrophotometer (Thermo Scientific). Dry cell mass is calculated from the measured OD₆₀₀ value using an experimentally derived conversion factor of 1.7 OD₆₀₀ units per 1 g dry cell mass.

A shake flask is inoculated with the cell slurry to reach an initial OD₆₀₀ of 0.1 – 0.3. Prior to inoculation, the 250 mL baffled shake flasks containing 1.75 g/L dry CaCO₃ are sterilized by autoclave at 121° C for 15 minutes. Immediately prior to inoculating, 50 mL of shake flask medium is added to the dry calcium carbonate. The shake flask medium is a sterilized, 5.5 pH aqueous solution of urea (2.3 g/L), magnesium sulfate heptahydrate (0.5 g/L), potassium phosphate monobasic (3.0 g/L), trace element solution (1 mL/L) and vitamin solution (1 mL/L), glucose (120.0 g/L), glycerol (0.1 g/L), 2-(N-Morpholino) ethanesulfonic acid (MES) (4.0 g/L). For strains lacking the *URA3* gene (URA-) 20 mg/L uracil is added to the media. The trace element solution is an aqueous solution of EDTA (15.0 g/L), zinc sulfate heptahydrate (4.5 g/L), manganese chloride dehydrate (1.0 g/L), cobalt(II) chloride hexahydrate (0.3 g/L), copper(II)sulfate pentahydrate (0.3 g/L), disodium molybdenum dehydrate (0.4 g/L), calcium chloride dehydrate (4.5 g/L), iron sulphate heptahydrate (3 g/L), boric acid (1.0 g/L), and potassium iodide (0.1 g/L). The vitamin solution is an aqueous solution of biotin (D-; 0.05 g/L), calcium pantothenate (D+; 1 g/L), nicotinic acid (5 g/L), myo-inositol (25 g/L), pyridoxine hydrochloride (1 g/L), p-aminobenzoic acid (0.2 g/L).

The inoculated flask is incubated at 30°C with shaking at 150 rpm for 72 hours and taken to analysis. Succinate concentration in the broth at the end of 72 hours fermentation is determined by gas chromatography with flame ionization detector and glucose by high performance liquid chromatography with refractive index detector.

Examples 1-2 through 1-6, 2-1 through 2-90, 3-1 through 3-288, 4-1 through 4-384, 5-1 through 5-768, 6-1-1 through 6-1-6, 6-2-1 through 6-290, 6-3-1 through 6-3-288, 6-4-1 through 6-4-384, 6-5-1 through 6-5-768, are made in the same manner by transforming Examples 7-1 through 7-6, 8-1 through 8-72, 9-1 through 9-234, 10-7-1 through 10-7-6, 10-8-1 through 10-8-72, 10-9-1 through 10-9-234, 11-1 through 11-6, 12-1 through 12-90, 13-1 through 13-288, 14-1 through 14-384 and 15-1 through 15-768, 16-11-1 through 16-11-6, 16-12-1 through 16-12-90, 16-13-1 through 16-13-288, 16-14-1 through 16-14-384 and 16-15-1 through 16-15-768, are separately cultured in shake flasks in similar manner and found to produce succinate. The succinate concentration in the broth is measured and yield and titer are calculated.

Further specific embodiments

The invention includes but is not limited to the following specific embodiments:

1. A recombinant cell having an active reductive TCA pathway from pyruvate to succinate which reductive TCA pathway includes at least one reaction that oxidizes NADPH to NADP⁺.

2. The recombinant cell of embodiment 1, wherein the reaction that oxidizes NADPH to NADP⁺ is a conversion of oxaloacetate to malate catalyzed by an NADPH-dependent malate dehydrogenase enzyme.

3. The recombinant cell of embodiment 2, which overexpresses the NADPH-dependent malate dehydrogenase enzyme.

4. The recombinant cell of embodiment 2 or 3, which has integrated into its genome at least one exogenous malate dehydrogenase gene that encodes for the overexpressed NADPH-dependent malate dehydrogenase enzyme.

5. The recombinant cell of embodiment 4, wherein the malate dehydrogenase gene is non-native to the yeast cell.

6. The recombinant cell of any of embodiments 3-5, wherein the NADPH-dependent malate dehydrogenase enzyme has an amino acid sequence at least 80% identical to either of SEQ. ID. NOs: 143 or 144.

7. The recombinant cell of any of embodiments 3-5, wherein the NADPH-dependent malate dehydrogenase enzyme has either of amino acid sequences SEQ. ID. NOs: 143 or 144.

8. The recombinant cell of any of embodiments 4-7, wherein the malate dehydrogenase gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NO: 29, 31 or 32.

9. The recombinant cell of any of embodiments 4-7, wherein the malate dehydrogenase gene has nucleotide sequence SEQ. ID. NO: 29, 31 or 32.

10. The recombinant cell of embodiment 1, wherein the reaction that oxidizes NADPH to NADP⁺ is a conversion of fumarate to succinate catalyzed by an NADPH-dependent fumarate reductase enzyme.

11. The recombinant cell of embodiment 10, which overexpresses the NADPH-dependent fumarate reductase enzyme.

12. The recombinant cell of embodiment 11 or 12, which has integrated into its genome at least one exogenous fumarate reductase gene that encodes for the overexpressed NADPH-dependent fumarate reductase enzyme.

13. The recombinant cell of embodiment 12, wherein the fumarate reductase gene is non-native to the yeast cell.

14. The recombinant cell of any of embodiments 10-13, wherein the NADPH-dependent fumarate reductase enzyme has an amino acid sequence at least 80% identical to any of SEQ. ID. NOs: 110, 111, 112, 113, 114 or 115.

5 15. The recombinant cell of any of embodiments 10-13, wherein the NADPH-dependent fumarate reductase enzyme has any of amino acid sequences SEQ. ID. NOs: 110, 111, 112, 113, 114 or 115.

10 16. The recombinant cell of any of embodiments 10-15, wherein the fumarate reductase gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NOs: 15, 16, 17, 18, 19 or 20.

15 17. The recombinant cell of any of embodiments 10-15, wherein the fumarate reductase gene has any of nucleotide sequences SEQ. ID. NOs: 15, 16, 17, 18, 19 or 20.

20 18. The recombinant cell of any of embodiments 1-17 wherein the active reductive TCA pathway from pyruvate to succinate includes a step of converting pyruvate or phosphoenolpyruvate to oxaloacetate, a step of converting oxaloacetate to malate, a step of converting malate to fumarate, and a step of converting fumarate to succinate.

19. A recombinant yeast cell that overexpresses an NADPH-dependent malate dehydrogenase enzyme.

25 20. The recombinant cell of embodiment 19 having integrated into its genome an exogenous malate dehydrogenase gene that encodes for the NADPH-dependent malate dehydrogenase enzyme.

30 21. The recombinant cell of embodiment 19 or 20, wherein the NADPH-dependent malate dehydrogenase enzyme has an amino acid sequence at least 80% identical to either of SEQ. ID. NOs: 143 or 144.

22. The recombinant cell of embodiment 19 or 20, wherein the NADPH-dependent malate dehydrogenase enzyme has either of amino acid sequences SEQ. ID. NOs: 143 or 144.

23. The recombinant cell of any of embodiments 19-22, wherein the malate dehydrogenase gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NO: 29, 31 or 32.

24. The recombinant cell of any of embodiments 19-22, wherein the malate dehydrogenase gene has any of nucleotide sequences SEQ. ID. NO: 29, 31 or 32.

25. The recombinant cell of any of embodiments 19-24 which expresses a NADPH-dependent fumarate reductase enzyme.

26. A recombinant yeast cell that overexpresses an NADPH-dependent fumarate reductase enzyme.

27. The recombinant cell of embodiment 26 having integrated into its genome an exogenous fumarate reductase gene that encodes for the NADPH-dependent fumarate reductase enzyme.

28. The recombinant cell of embodiment 26 or 27, wherein the NADPH-dependent fumarate reductase enzyme has an amino acid sequence at least 80% identical to any of SEQ. ID. NOs: 110, 111, 112, 113, 114 or 115.

29. The recombinant cell embodiment 26 or 27, wherein the NADPH-dependent fumarate reductase enzyme has any of amino acid sequences SEQ. ID. NOs: 110, 111, 112, 113, 114 or 115.

30. The recombinant cell of any of embodiments 26-29, wherein the fumarate reductase gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NO.: 15, 16, 17, 18, 19 or 20.

31. The recombinant cell of any of embodiments 26-29, wherein the fumarate reductase gene has any of nucleotide sequences SEQ. ID. NO.: 15, 16, 17, 18, 19 or 20.

32. The recombinant cell of any of embodiments 26-31 which expresses a NADPH-
5 dependent malate dehydrogenase enzyme.

33. The recombinant cell of any preceding embodiment which has integrated into its genome one or more of (i) an exogenous pyruvate carboxylase gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an
10 exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate, (iii) an exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

15

34. The recombinant cell of any preceding embodiment which has integrated into its genome one or more of (i) a non-native pyruvate carboxylase gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) a non-native malate dehydrogenase gene which encodes for an enzyme that catalyzes the
20 conversion of oxaloacetate to malate, (iii) a non-native exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) a non-native exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

25 35. The recombinant cell of any preceding embodiment which overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH.

36. The recombinant cell of embodiment 35, wherein the overexpressed enzyme
30 which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is in the pentose phosphate pathway.

37. The recombinant cell of embodiment 35 or 36, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a 6-phosphogluconate dehydrogenase enzyme.

5 38. The recombinant cell of embodiment 37, which has integrated into its genome at least one exogenous 6-phosphogluconate dehydrogenase gene that encodes for the overexpressed 6-phosphogluconate dehydrogenase enzyme.

10 39. The recombinant cell of embodiment 38, wherein the exogenous 6-phosphogluconate dehydrogenase gene is native to the cell.

40. The recombinant cell of embodiment 38, wherein the 6-phosphogluconate dehydrogenase enzyme has an amino acid sequence at least 80% identical to SEQ. ID. NO: 140.

15

41. The recombinant cell of embodiment 19 or 20, wherein the 6-phosphogluconate dehydrogenase enzyme has amino acid sequence SEQ. ID. NO: 140.

20 42. The recombinant cell of embodiment 38, wherein the exogenous 6-phosphogluconate dehydrogenase gene has a nucleotide sequence at least 80% identical to SEQ. ID. NO: 99.

43. The recombinant cell of embodiment 38, wherein the exogenous 6-phosphogluconate dehydrogenase gene has the nucleotide sequence SEQ. ID. NO: 99.

25

44. The recombinant cell of embodiment 35 or 36, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a glucose 6-phosphate dehydrogenase enzyme.

30 45. The recombinant cell of embodiment 44, which has integrated into its genome at least one exogenous glucose 6-phosphate dehydrogenase gene that encodes for the overexpressed glucose 6-phosphate dehydrogenase enzyme.

46. The recombinant cell of embodiment 45, wherein the exogenous glucose 6-phosphate dehydrogenase gene is native to the cell.

47. The recombinant cell of embodiment 44 or 45, wherein the glucose 6-phosphate dehydrogenase enzyme has an amino acid sequence at least 80% identical to SEQ ID NO: 139.

48. The recombinant cell of embodiment 44 or 45, wherein the glucose 6-phosphate dehydrogenase enzyme has amino acid sequence SEQ ID NO: 139.

49. The recombinant cell of embodiment 45, wherein the exogenous glucose 6-phosphate dehydrogenase gene has a nucleotide sequence at least 80% identical to SEQ. ID. NO: 97.

50. The recombinant cell of any of embodiments 19-22, wherein the exogenous glucose 6-phosphate dehydrogenase gene has the nucleotide sequence SEQ. ID. NO: 97.

51. The recombinant cell of any preceding embodiment, which overexpresses at least one *Stb5p* enzyme.

52. The recombinant cell of embodiment 51, which has integrated into its genome at least one exogenous *Stb5p* gene that encodes for the overexpressed *Stb5p* enzyme.

53. The recombinant cell of embodiment 52, wherein the exogenous *Stb5p* gene is non-native to the cell.

54. The recombinant cell of any of embodiments 51-54, wherein the *Stb5p* enzyme has an amino acid sequence at least 80% identical to SEQ. ID. NO: 148.

55. The recombinant cell of embodiment 52, wherein the *Stb5p* enzyme has amino acid sequence SEQ. ID. NO: 148.

56. The recombinant cell of embodiment 52, wherein the exogenous *Stb5p* gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NO: 51.

57. The recombinant cell of embodiment 52, wherein the exogenous *Stb5p* gene has nucleotide sequence SEQ. ID. NO: 51.

58. The recombinant cell of any preceding embodiment, which further overexpresses a NAD(P)⁺ transhydrogenase enzyme.

59. The recombinant yeast cell of embodiment 58 which has integrated into its genome an exogenous NAD(P)⁺ transhydrogenase gene that encodes for the NAD(P)⁺ transhydrogenase enzyme.

60. The recombinant yeast cell of embodiment 58 or 59 wherein the exogenous NAD(P)⁺ transhydrogenase enzyme has an amino acid sequence at least 80% identical to any of SEQ. ID. NOs: 145, 146 or 147.

61. The recombinant cell of embodiment 58 or 59, wherein the NAD(P)⁺ transhydrogenase enzyme has any of amino acid sequences SEQ. ID. NOs: 145, 146 or 147.

62. The recombinant cell of embodiment 58, wherein the exogenous NAD(P)⁺ transhydrogenase gene has a nucleotide sequence at least 80% identical to any of SEQ. ID. NO: 45, 47 or 49.

63. The recombinant cell of embodiment 59, wherein the exogenous NAD(P)⁺ transhydrogenase gene has nucleotide sequence SEQ. ID. NO: 45, 47 or 49.

64. The recombinant cell of any preceding embodiment, which has a deletion or disruption of a native phosphoglucose isomerase gene.

65. The recombinant cell of any preceding embodiment, which has a deletion or disruption of a native pyruvate decarboxylase gene.

66. A malate dehydrogenase gene having nucleotide sequence SEQ. ID. NO: 32.

67. The malate dehydrogenase gene of embodiment 66 which is produced by converting an NADH- dependent malate dehydrogenase gene to a NADPH-dependent malate dehydrogenase gene.

68. A fumarate reductase gene having any of nucleotide sequences SEQ. ID. NOs: 15, 16, 17, 18, 19 or 20.

69. The fumarate reductase gene of embodiment 68 which is produced by converting an NADH- dependent fumarate reductase gene to a NADPH-dependent fumarate reductase gene.

70. A recombinant yeast having a deletion or disruption of a native phosphoglucose isomerase gene, has an active reductive TCA pathway from pyruvate to succinate and has integrated into its genome one or more of (i) an exogenous pyruvate carboxylate gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate (iii) an exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

71. A recombinant yeast having an active reductive TCA pathway from pyruvate to succinate and which has integrated into its genome at least one exogenous *Stb5p* gene and one or more of (i) an exogenous pyruvate carboxylate gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate (iii) an exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate

72. The recombinant cell of embodiment 71, wherein the exogenous *Stb5p* gene is non-native to the cell.

73. The recombinant cell of any of embodiments 70-72 which overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH.

74. The recombinant cell of embodiment 73, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is in the pentose phosphate pathway.

75. The recombinant cell of embodiment 73 or 74, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a 6-phosphogluconate dehydrogenase enzyme.

76. The recombinant cell of embodiment 75, which has integrated into its genome at least one exogenous 6-phosphogluconate dehydrogenase gene that encodes for the overexpressed 6-phosphogluconate dehydrogenase enzyme.

77. The recombinant cell of embodiment 73 or 74, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a glucose 6-phosphate dehydrogenase enzyme.

78. The recombinant cell of embodiment 77, which has integrated into its genome at least one exogenous glucose 6-phosphate dehydrogenase gene that encodes for the overexpressed glucose 6-phosphate dehydrogenase enzyme.

79. The recombinant cell of any of embodiments 70-78, which has a deletion or disruption of a native phosphoglucose isomerase gene.

80. The recombinant cell of any of embodiments 70-79, which further overexpresses a NAD(P)⁺ transhydrogenase enzyme.

81. The recombinant yeast cell of embodiment 80 which has integrated into its genome an exogenous NAD(P)⁺ transhydrogenase gene that encodes for the NAD(P)⁺ transhydrogenase enzyme.

5 82. The recombinant cell of any of embodiments 70-81, which has a deletion or disruption of a native pyruvate decarboxylase gene.

83. The recombinant cell of any of embodiments 1-65 and 70-82, wherein the host cell is a yeast cells classified under one or more of the genera *Candida*, *Pichia*,
10 *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Issatchenkia*, and *Hansenula*.

84. The recombinant cell of any of embodiments 1-65 and 70-82, wherein the host cell is selected from *C. sonorensis*, *K. marxianus*, *K. thermotolerans*, *C.*
15 *methanesorbosa*, *S. bulderi*, *I. orientalis*, *C. lambica*, *C. sorboxylosa*, *C. zemplanina*, *C. geochares*, *P. membranifaciens*, *Z. kombuchaensis*, *C. sorbosivorans*, *C. vanderwaltii*, *C. sorbophila*, *Z. bisporus*, *Z. lentus*, *S. bayanus*, *D. castellii*, *C. boidinii*, *C. etchellsii*, *K. lactis*, *P. jadinii*, *P. anomala*, *Saccharomyces cerevisiae* and *Saccharomycopsis crataegensis*.

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85. The recombinant cell of any of embodiments 1-65 and 70-82, wherein the host cell is selected from *Issatchenkia orientalis*, *Pichia galeiformis*, *Pichia sp. YB-4149* (NRRL designation), *Candida ethanolica*, *P. deserticola*, *P. membranifaciens* and *P. fermentans*.

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86. The recombinant cell of any of embodiments 1-65 and 70-82, wherein the host cell is *I. orientalis*.

87. The recombinant cell of any of embodiments 1-65 and 70-82, wherein the host
30 cell is *S. cerevisiae*.

88. The recombinant cell of any of embodiments 1-65 and 70-86, wherein the host cell is Crabtree negative as a wild-type strain.

89. The recombinant cell of any of embodiments 1-65 and 70-88, wherein the host cell is succinate-resistant as a wild-type strain.

90. The recombinant cell of any any of embodiments 1-65 and 70-89, wherein the host cell exhibits a specific glucose consumption rate of at least 0.5 gram of glucose per gram dry weight of cells per hour, as a wild-type strain.

91. The recombinant cell of embodiment 90, wherein the host cell exhibits a specific glucose consumption rate of at least 1.0 gram of glucose per gram dry weight of cells per hour, as a wild-type strain.

92. The recombinant cell of embodiment 91, wherein the host cell exhibits a specific glucose consumption rate of at least 1.5 gram of glucose per gram dry weight of cells per hour, as a wild-type strain.

93. The recombinant cell of any any of embodiments 1-65 and 70-89, wherein the host cell exhibits a volumetric glucose consumption rate of at least 3 gram of glucose per liter per hour, as a wild-type strain.

94. The recombinant cell of embodiment 93, wherein the host cell exhibits a volumetric glucose consumption rate of at least 5 gram of glucose per liter per hour, as a wild-type strain.

95. The recombinant cell of embodiment 91, wherein the host cell exhibits a volumetric glucose consumption rate of at least 8 gram of glucose per liter per hour, as a wild-type strain.

96. The recombinant cell of any of embodiments 1-65 and 70-95, which exhibits a volumetric glucose consumption rate of at least 0.5 gram of glucose per liter per hour.

97. The recombinant cell of any embodiment 96, which exhibits a glucose consumption rate of at least 0.75 gram of glucose per liter per hour.

98. The recombinant cell of any embodiment 97, which exhibits a glucose consumption rate of at least 0.9 gram of glucose per minute per liter per hour.

99. The recombinant cell of any embodiments 1-65 and 70-98 which produces succinate and transports succinate out of the cell.

100. The recombinant cell of any embodiments 1-65 and 70-99, which further metabolizes succinate to one or more succinate metabolization products.

101. The recombinant cell of embodiment 100, which transports at least one said succinate metabolization product out of the cell.

102. The recombinant cell of embodiment 100 or 101, wherein the succinate metabolization product is one or more of 1,4-butanediol, 1,3-butadiene, propionic acid, and 3-hydroxyisobutyric acid.

103. A process for producing succinate or a succinate metabolization product of succinate, comprising culturing the recombinant cell of any of embodiments 1-65 and 70-102 under fermentation conditions in a fermentation broth that includes a sugar that is fermentable by the cell.

104. The process of embodiment 103, wherein the recombinant cell produces succinate and transports succinate out of the cell.

105. The process of embodiment 103, wherein the recombinant cell further metabolizes succinate to one or more succinate metabolization products, and the recombinant cell transports at least one of said succinate metabolization product out of the cell.

106. The process of embodiment 105, wherein the succinate metabolization product is one or more of 1,4-butanediol, 1,3-butadiene, propionic acid, and 3-hydroxyisobutyric acid.

Claims

1. A recombinant cell having an active reductive TCA pathway from pyruvate to succinate which reductive TCA pathway includes at least one reaction that oxidizes NADPH to NADP⁺.

2. The recombinant cell of claim 1, wherein the reaction that oxidizes NADPH to NADP⁺ is a conversion of oxaloacetate to malate catalyzed by an NADPH-dependent malate dehydrogenase enzyme.

3. The recombinant cell of claim 2, which overexpresses the NADPH-dependent malate dehydrogenase enzyme.

4. The recombinant cell of claim 2 or 3, which has integrated into its genome at least one exogenous malate dehydrogenase gene that encodes for the overexpressed NADPH-dependent malate dehydrogenase enzyme.

10. The recombinant cell of claim 1, wherein the reaction that oxidizes NADPH to NADP⁺ is a conversion of fumarate to succinate catalyzed by an NADPH-dependent fumarate reductase enzyme.

6. The recombinant cell of claim 5, which overexpresses the NADPH-dependent fumarate reductase enzyme.

7. The recombinant cell of claim 5 or 6, which has integrated into its genome at least one exogenous fumarate reductase gene that encodes for the overexpressed NADPH-dependent fumarate reductase enzyme.

8. The recombinant cell of any of claims 1-7 wherein the active reductive TCA pathway from pyruvate to succinate includes a step of converting pyruvate or phosphoenolpyruvate to oxaloacetate, a step of converting oxaloacetate to malate, a step of converting malate to fumarate, and a step of converting fumarate to succinate.

9. A recombinant yeast cell that overexpresses an NADPH-dependent malate dehydrogenase enzyme.

10. The recombinant cell of claim 9 which expresses a NADPH-dependent fumarate reductase enzyme.

11. A recombinant yeast cell that overexpresses an NADPH-dependent fumarate reductase enzyme.

12. The recombinant cell of claim 11 having integrated into its genome an exogenous fumarate reductase gene that encodes for the NADPH-dependent fumarate reductase enzyme.

13. The recombinant cell of claim 11 or 12 which expresses a NADPH-dependent malate dehydrogenase enzyme.

14. The recombinant cell of any preceding claim which has integrated into its genome one or more of (i) an exogenous pyruvate carboxylase gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate, (iii) an exogenous fumarate gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

15. The recombinant cell of any preceding claim which has integrated into its genome one or more of (i) a non-native pyruvate carboxylase gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) a non-native malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate, (iii) a non-native exogenous fumarate gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) a non-native exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

16. The recombinant cell of any preceding claim which overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH.

17. The recombinant cell of claim 16, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is in the pentose phosphate pathway.

18. The recombinant cell of claim 16 or 17, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a 6-phosphogluconate dehydrogenase enzyme.

19. The recombinant cell of claim 18, which has integrated into its genome at least one exogenous 6-phosphogluconate dehydrogenase gene that encodes for the overexpressed 6-phosphogluconate dehydrogenase enzyme.

20. The recombinant cell of claim 19, wherein the exogenous 6-phosphogluconate dehydrogenase gene is native to the cell.

21. The recombinant cell of claim 16 or 17, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a glucose 6-phosphate dehydrogenase enzyme.

22. The recombinant cell of claim 21, which has integrated into its genome at least one exogenous glucose 6-phosphate dehydrogenase gene that encodes for the overexpressed glucose 6-phosphate dehydrogenase enzyme.

23. The recombinant cell of claim 22, wherein the exogenous glucose 6-phosphate dehydrogenase gene is native to the cell.

24. The recombinant cell of any preceding claim, which overexpresses at least one *Stb5p* enzyme.

25. The recombinant cell of claim 24, which has integrated into its genome at least one exogenous *Stb5p* gene that encodes for the overexpressed *Stb5p* enzyme.

26. The recombinant cell of claim 25, wherein the exogenous *Stb5p* gene is non-native to the cell.

27. The recombinant cell of any preceding claim, which further overexpresses a NAD(P)⁺ transhydrogenase enzyme.

28. The recombinant yeast cell of claim 27 which has integrated into its genome an exogenous NAD(P)⁺ transhydrogenase gene that encodes for the NAD(P)⁺ transhydrogenase enzyme.

29. The recombinant cell of any preceding claim, which has a deletion or disruption of a native phosphoglucose isomerase gene.

30. The recombinant cell of any preceding claim, which has a deletion or disruption of a native pyruvate decarboxylase gene.

31. A recombinant yeast having a deletion or disruption of a native phosphoglucose isomerase gene, has an active reductive TCA pathway from pyruvate to succinate and has integrated into its genome one or more of (i) an exogenous pyruvate carboxylate gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes the conversion of oxaloacetate to malate (iii) an exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

32. A recombinant yeast having an active reductive TCA pathway from pyruvate to succinate and which has integrated into its genome at least one exogenous *Stb5p* gene and one or more of (i) an exogenous pyruvate carboxylate gene that encodes for an enzyme which catalyzes the conversion of pyruvate to oxaloacetate, (ii) an exogenous malate dehydrogenase gene which encodes for an enzyme that catalyzes

the conversion of oxaloacetate to malate (iii) an exogenous fumarase gene that encodes for an enzyme which catalyzes the conversion of malate to fumarate and (iv) an exogenous fumarate reductase gene which encodes for an enzyme which catalyzes the conversion of fumarate to succinate.

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33. The recombinant cell of claim 32, wherein the exogenous *Stb5p* gene is non-native to the cell.

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34. The recombinant cell of any of claims 31-33 which overexpresses at least one enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH.

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35. The recombinant cell of claim 34, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is in the pentose phosphate pathway.

36. The recombinant cell of claim 34 or 35, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a 6-phosphogluconate dehydrogenase enzyme.

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37. The recombinant cell of claim 36, which has integrated into its genome at least one exogenous 6-phosphogluconate dehydrogenase gene that encodes for the overexpressed 6-phosphogluconate dehydrogenase enzyme.

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38. The recombinant cell of claim 34 or 35, wherein the overexpressed enzyme which catalyzes a reaction that includes the reduction of NADP⁺ to NADPH is a glucose 6-phosphate dehydrogenase enzyme.

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39. The recombinant cell of claim 38, which has integrated into its genome at least one exogenous glucose 6-phosphate dehydrogenase gene that encodes for the overexpressed glucose 6-phosphate dehydrogenase enzyme.

40. The recombinant cell of any of claims 31-39, which has a deletion or disruption of a native phosphoglucose isomerase gene.

41. The recombinant cell of any of claims 31-40, which further overexpresses a NAD(P)⁺ transhydrogenase enzyme.

42. The recombinant yeast cell of claim 41 which has integrated into its genome an exogenous NAD(P)⁺ transhydrogenase gene that encodes for the NAD(P)⁺ transhydrogenase enzyme.

43. The recombinant cell of any of claims 31-42, which has a deletion or disruption of a native pyruvate decarboxylase gene.

44. The recombinant cell of any preceding claim, wherein the host cell is a yeast cells classified under one or more of the genera *Candida*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Issatchenkia*, and *Hansenula*.

45. The recombinant cell of any of claims 1-43, wherein the host cell is selected from *C. sonorensis*, *K. marxianus*, *K. thermotolerans*, *C. methanesorbosa*, *S. bulderi*, *I. orientalis*, *C. lambica*, *C. sorboxylosa*, *C. zemplinina*, *C. geochares*, *P. membranifaciens*, *Z. kombuchaensis*, *C. sorbosivorans*, *C. vanderwaltii*, *C. sorbophila*, *Z. bisporus*, *Z. lentus*, *S. bayanus*, *D. castellii*, *C. boidinii*, *C. etchellsii*, *K. lactis*, *P. jadinii*, *P. anomala*, *Saccharomyces cerevisiae* and *Saccharomycopsis crataegensis*.

46. The recombinant cell of any of claims 1-43, wherein the host cell is selected from *Issatchenkia orientalis*, *Pichia galeiformis*, *Pichia sp. YB-4149* (NRRL designation), *Candida ethanolica*, *P. deserticola*, *P. membranifaciens* and *P. fermentans*.

47. The recombinant cell of any of claims 1-43, wherein the host cell is *I. orientalis*.

48. The recombinant cell of any of claims 1-43, wherein the host cell is *S. cerevisiae*.

49. The recombinant cell of any preceding claim which produces succinate and transports succinate out of the cell.

50. The recombinant cell of any preceding claim, which further metabolizes succinate to one or more succinate metabolization products.

51. The recombinant cell of claim 50, which transports at least one said downstream metabolization product out of the cell.

52. The recombinant cell of claim 50 or 51, wherein the succinate metabolization product is one or more of 1,4-butanediol, 1,3-butadiene, propionic acid, and 3-hydroxyisobutyric acid.

53. A process for producing succinate or a succinate metabolization product, comprising culturing the recombinant cell of any of claims 1-52 under fermentation conditions in a fermentation broth that includes a sugar that is fermentable by the cell.

54. The process of claim 53, wherein the recombinant cell produces succinate and transports succinate out of the cell.

55. The process of claim 53, wherein the recombinant cell further metabolizes succinate to one or more succinate metabolization products, and the recombinant cell transports at least one of said succinate metabolization product out of the cell.

56. The process of claim 55, wherein the recombinant cell transports less than 10% by weight of succinate from the cell, based on the combined weight of succinate and succinate metabolization products exported from the cell.

57. The process of claim 55 or 56, wherein the downstream metabolization product is one or more of 1,4-butanediol, 1,3-butadiene, propionic acid, and 3-hydroxyisobutyric acid.

58. A malate dehydrogenase gene having nucleotide sequence SEQ. ID. NO: 32.

59. The malate dehydrogenase gene of claim 58 which is produced by converting an NADH- dependent malate dehydrogenase gene to a NADPH-dependent malate dehydrogenase gene.

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60. A fumarate reductase gene having any of nucleotide sequences SEQ. ID. NOs: 15, 16, 17, 18, 19 or 20.

61. The fumarate reductase gene of claim 60 which is produced by converting an
10 NADH- dependent fumarate reductase gene to a NADPH-dependent fumarate reductase gene.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/052066

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/04 C12N9/02 C12N9/88 C12N9/92 C12P7/46 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011/201089 A1 (BURGARD ANTHONY P [US] ET AL) 18 August 2011 (2011-08-18) the whole document	1-61
A	WO 00/03021 A2 (NIELSEN JENS [DK]; NISSEN TORBEN LAUESGAARD [DK]; KIELLAND BRANDT MORT) 20 January 2000 (2000-01-20) the whole document	1-61
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----- -/-		
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
12 November 2013		29/11/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Roscoe, Richard

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/052066

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANDREAS M. RAAB ET AL: "Oxidative versus reductive succinic acid production in the yeast <i>saccharomyces cerevisiae</i>", BIOENGINEERED BUGS, vol. 2, no. 2, 1 March 2011 (2011-03-01), pages 120-123, XP55087538, ISSN: 1949-1018, DOI: 10.4161/bbug.2.2.14549 the whole document -----	1-61
A	MD. M. KABIR ET AL: "Fermentation characteristics and protein expression patterns in a recombinant <i>Escherichia coli</i> mutant lacking phosphoglucose isomerase for poly(3-hydroxybutyrate) production", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 62, no. 2-3, 1 August 2003 (2003-08-01), pages 244-255, XP55087572, ISSN: 0175-7598, DOI: 10.1007/s00253-003-1257-z the whole document -----	17-23, 29,31, 35-40
A	CADIERE AXELLE ET AL: "The <i>Saccharomyces cerevisiae</i> zinc factor protein Stb5p is required as a basal regulator of the pentose phosphate pathway", FEMS YEAST RESEARCH, WILEY-BLACKWELL PUBLISHING LTD, GB, NL, vol. 10, no. 7, 1 November 2010 (2010-11-01), pages 819-827, XP002620924, ISSN: 1567-1356, DOI: 10.1111/J.1567-1364.2010.00672.X [retrieved on 2010-07-23] the whole document -----	24-26, 32,33
A	HUA QIANG ET AL: "Responses of the central metabolism in <i>Escherichia coli</i> to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase knockouts.", JOURNAL OF BACTERIOLOGY DEC 2003, vol. 185, no. 24, December 2003 (2003-12), pages 7053-7067, XP002716219, ISSN: 0021-9193 the whole document ----- -/--	21,22

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/052066

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HALL N ET AL: "Structure-function analysis of NADPH:nitrate reductase from <i>Aspergillus nidulans</i> : analysis of altered pyridine nucleotide specificity in vivo.", MICROBIOLOGY (READING, ENGLAND) JUN 2000, vol. 146 (Pt 6), June 2000 (2000-06), pages 1399-1406, XP002716220, ISSN: 1350-0872 the whole document -----	58-61
A	DÖHR O ET AL: "Engineering of a functional human NADH-dependent cytochrome P450 system.", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 2 JAN 2001, vol. 98, no. 1, 2 January 2001 (2001-01-02), pages 81-86, XP002716221, ISSN: 0027-8424 the whole document -----	58-61

INTERNATIONAL SEARCH REPORT

International application No.

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. (means)
- ☐ on paper
- ☒ in electronic form
- b. (time)
- ☒ in the international application as filed
- ☐ together with the international application in electronic form
- ☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2013/052066

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011201089 A1	18-08-2011	US 2011201089 A1	18-08-2011
		WO 2012099621 A1	26-07-2012

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