(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 September 2007 (20.09.2007)

PCT

(10) International Publication Number
WO 2007/106524 A2

(51) International Patent Classification:
*C12N 1/18* (2006.01)

(21) International Application Number:
PCT/US2007/006408

(22) International Filing Date: 13 March 2007 (13.03.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/781,674 13 March 2006 (13.03.2006) US

(71) Applicant *(for all designated States except US)*: **NATURE WORKS LLC** [US/US]; 15305 Minnetonka Boulevard, Minneapolis, MN 55345 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DUNDON, Catherine, Asleson** [US/US]; 3944 45th Avenue S., Minneapolis, MN 55406 (US). **SUOMINEN, Pirkko** [FI/US]; 7801 Kingsview, Maple Grove, MN 55311 (US). **ARISTIDOU, Aristos** [CY/US]; 7809 Kingsview, Maple Grove, MN 55311 (US). **RUSH, Brian, J.** [US/US]; 3336 Emerson Avenue S, Minneapolis, MN 55408 (US). **KOIVURANTA, Kari** [FI/FI]; Makitorpantie 17 D 39, FI-00640 Helsinki (FI). **HAUSE, Benjamin, Matthew** [US/US]; 1575 250th Avenue, Currie, MN 56123 (US). **MCMULLIN, Thomas, William** [US/US]; 3509 Lilac Lane, Minnetonka, MN 55345 (US). **ROBERG-PEREZ, Kevin** [US/US]; 2250 Cleveland Street, Minneapolis, MN 55418 (US).

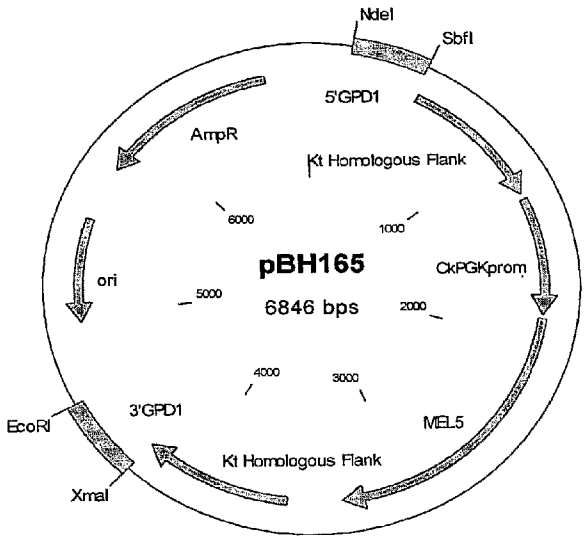
(74) Agent: **COHN, Gary, C.**; Gary C. Cohn PLLC, 1147 N. Fourth Street, Unit 6E, Philadelphia, PA 19123 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, 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, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,

*[Continued on next page]*

(54) Title: YEAST CELLS HAVING DISRUPTED PATHWAY FROM DIHYDROXYACETONE PHOSPHATE TO GLYCEROL




(57) Abstract: Yeast cells are genetically modified to disrupt a native metabolic pathway from dihydroxyacetone to glycerol. In certain aspects, the yeast cell is of the genera *Kluyveromyces, Candida or Issatchenkia*. In other aspects, the yeast cell is capable of producing at least one organic acid, such as lactate. The yeast cells produce significantly less glycerol than the wild-type strains, and usually produce greater yields of desired fermentation products. Yeast cells of the invention often grow well when cultivated, despite their curtailed glycerol production.

WO 2007/106524 A2

PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# YEAST CELLS HAVING DISRUPTED PATHWAY FROM DIHYDROXYACETONE PHOSPHATE TO GLYCEROL

This invention was made under contract no. DE-FC36-03GO13145 with the United States Department of Energy. The United States Government has certain rights to this invention.

This application claims priority from United States Provisional Application No. 60/781,674, filed 13 March 2006.

This invention relates to certain genetically modified yeast, and fermentation processes to produce lactic acid using those genetically modified yeast.

Yeast are used as biocatalysts in a number of industrial fermentations. There is an increasing interest in using yeast to ferment sugars to organic acids such as lactic acid. As more organic acid is produced in these fermentations, the fermentation medium becomes increasingly acidic. Most bacteria that produce these organic acids do not perform well in strongly acidic environments—they either do not survive under those conditions or else produce so slowly that the process becomes economically unviable. As a result, it becomes necessary to buffer the medium to maintain a higher pH. This causes difficulty in recovering the product in acid form. It is preferred to conduct the fermentation at a lower pH at which the product is partially or wholly in the acid form.

Yeast species have been considered as candidates for such low-pH fermentations. Many yeast species naturally ferment hexose sugars to ethanol, but few if any naturally produce significant yields of organic acids such as lactic acid. Accordingly, efforts have been made to genetically modify various yeast species to insert one or more genes that will enable the cell to produce lactic acid. In order to divert sugar metabolism from ethanol production to lactic acid production, these cells have also been genetically modified to disrupt or delete the native pyruvate decarboxylase (*PDC*) gene. This work is described, for example, in WO 99/14335, WO 00/71738 A1, WO 02/42471 A2, WO 03/049525 A2, WO 03/102152 A2 and WO 03/102201 A2.

Glycerol is produced in significant yield in many of these yeast fermentations. Glycerol may serve as an osmoprotectant for the cell. Glycerol formation may help regenerate redox cofactors under fermentation conditions.

Glycerol is produced in many yeast cells by metabolizing dihydroxyacetone phosphate (DHAP). In most yeast species, DHAP is reduced by a glycerol-3-

phosphate dehydrogenase (GPD, systematic name sn-glycerol-3-phosphate:NAD+ 2-oxidoreductase, EC 1.1.1.8) enzyme to form glycerol-3-phosphate (G3P). G3P serves as a precursor for lipid biosynthesis as well as a glycerol precursor. G3P is dephosphorylated to glycerol by a glycerol-3-phosphatase enzyme (GPP, systematic name glycerol-1-phosphate phosphohydrolase, EC 3.1.3.21).

There exists an alternate pathway for glycerol production, which is important for some yeast, such as *S. pombe*. In this pathway, dihydroxyacetone phosphate is dephosphorylated into dihydroxyacetone by dihydroxyacetone phosphate phosphatase. Dihydroxyacetone is then converted into glycerol in conjunction with NADH oxidation by $NADH^+$-dependent glycerol dehydrogenase (systematic name glycerol:NAD+ 2-oxidoreductase, EC 1.1.1.6).

Because glycerol production consumes carbon that could otherwise be used to produce a more desirable fermentation product, this glycerol production represents a significant source of yield loss. In addition, glycerol production comes at the expense of both ATP and NADH. This directs energy away from the production of biomass or the desired product. For both of these reasons, it would be desired to reduce or eliminate glycerol production by the cell. A further consideration is that the reduction or elimination of glycerol production could simplify recovery and purification of the desired product.

A *Saccharomyces cerevisiae* strain has been genetically engineered to delete its native GPD genes, thus depriving the cell of the GPD enzyme and preventing glycerol production. *See* Nissen *et al.*, "Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis", *Yeast*, 2000: 16:463-474. Nissen *et al.* report that the mutated cells grew very poorly under both anaerobic and aerobic conditions when both of the native GPD genes were disrupted. According to Nissen *et al.*, the mutated cells produced much less glycerol than the wild-type cells. Nissen *et al.* hypothesized that the poor growth seen in the double deletant strains was due to a depletion of the cell's $NAD^+$ pool, because glycerol production was not available to oxidize NADH in the cell.

It would be desirable to provide a yeast cell that produces a desired organic product, which produces little or no glycerol, and which also grows well under aerobic conditions, anaerobic conditions or both aerobic and anaerobic conditions.

In one aspect, this invention is a mutant yeast cell of a pre-whole genome duplication yeast species, having a deletion or disruption of a native metabolic

pathway from dihydroxyacetone phosphate to glycerol. The deletion or disruption of the native metabolic pathway may include a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase (GPD) gene. The deletion or disruption of the native metabolic pathway may include a deletion or disruption of at least one native glycerol-3-phosphatase (GPP) gene. It may include a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase (GPD) gene and at least one native glycerol-3-phosphatase (GPP) gene. The deletion or disruption of the native metabolic pathway may include a deletion or disruption of at least one native dihydroxyacetone phosphate phosphatase gene, native glycerol dehydrogenase gene, or both.

In another aspect, this invention is a mutant yeast cell of of a pre-whole genome duplication yeast species, which mutant cell produces less than 2.0 g/L of glycerol when cultivated under the following standard microaerobic conditions:

A. defined aqueous medium containing, at the start of cultivation, 5 g/L ammonium sulfate, 3 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, trace elements, vitamins, 150 g/L glucose;

B. pH at the start of cultivation of 3.5, with fermentation medium being buffered if necessary to prevent the pH from falling below 3.0 or rising above 7.0 during the cultivation;

C. Cultivation inoculated with the yeast cell to an $OD_{600}$ of 1.0;

D. Cultivation temperature 30°C;

E. Cultivation continued until glucose concentration is reduced to 10 g/L, but is not continued for more than 120 hours;

F. Aeration and agitation sufficient to produce an oxygen uptake rate of 5.0 ± 1.0 mmol/L/hr.

In another aspect, this invention is a mutant yeast cell of a pre-whole genome duplication yeast species, which lacks the ability to produce an active glycerol-3-phosphate dehydrogenase (GDP) enzyme. For purposes of this invention, a cell is considered to lack the ability to produce an active enzyme if the activity of such enzyme in the cell is reduced by at least 75%, preferably at least 90%, compared to the activity of that enzyme in the wild-type strain. Enzyme activity of any particular enzyme can be determined using appropriate assay methods. Commercial assay kits are available for determining glycerol-3-phosphate dehydrogenase activity. An

example of such a product is designated as MK426 by Takara Bio, Inc. and is available through Fisher Scientific, Pittsburgh, Pennsylvania.

In another aspect, this invention is a mutant yeast cell of a pre-whole genome duplication yeast species, which lacks the ability to produce an active glycerol-3-phosphatase enzyme.

In another aspect, this invention is a mutant yeast cell which lacks the ability to produce an active dihydroxyacetone phosphate phosphatase enzyme that is natively produced by wild type cells of the yeast species, lacks the ability to produce an active $NADH^+$-dependent glycerol dehydrogenase enzyme that is natively produced by wild type cells of the yeast species, or both

In another aspect, this invention is a mutant yeast cell that is genetically modified to produce a product organic acid, said yeast cell further having a deletion or disruption of a native metabolic pathway from dihydroxyacetone phosphate to glycerol and a deletion or disruption of a native metabolic pathway from pyruvate to ethanol. The deletion or disruption of the native metabolic pathway may include a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene. The deletion or disruption of the native metabolic pathway may include a deletion or disruption of at least one native glycerol-3-phosphatase gene. It may include a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene and at least one native glycerol-3-phosphatase gene.

Cells in accordance with the invention have been found to produce very low levels of glycerol when cultivated under fermentation conditions. Glycerol production has been found to be below 0.2 g/L under a range of fermentation conditions. Surprisingly, the cells of the invention grow well under fermentation conditions, despite the lack of glycerol production and in some embodiments despite the lack of glycerol-3-phosphate production. The cells of the invention have also been found to have improved acid tolerance in some instances. Accordingly, the invention is also a fermentation process wherein a cell of any of the foregoing aspects of the invention is cultivated under fermentation conditions to produce a fermentation product, wherein the yield of carbon source to glycerol is less than 2% by weight.

**Figure 1** is a diagram depicting the pBH158 plasmid.

**Figure 2** is a diagram depicting the pBH159 plasmid.

**Figure 3** is a diagram depicting the pBH160 plasmid.

**Figure 4** is a diagram depicting the pBH161 plasmid.

**Figure 5** is a diagram depicting the pMM28 plasmid.

**Figure 6** is a diagram depicting the pMI318 plasmid.

**Figure 7** is a diagram depicting the pMI321 plasmid.

**Figure 8** is a diagram depicting the pMI355 plasmid.

**Figure 9** is a diagram depicting the pMI357 plasmid.

**Figure 10** is a diagram depicting the pMI433 plasmid.

**Figure 11** is a diagram depicting the pMI449 plasmid.

**Figure 12** is a diagram depicting the pMI454 plasmid.

**Figure 13** is a diagram depicting the pBH165 plasmid.

**Figure 14** is a diagram depicting the pTMC61 plasmid.

The yeast cells of the invention are 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 glycerol. The native metabolic pathway may involve a metabolic pathway from dihydroxyacetone phosphate to glycerol-3-phosphate to glycerol. The native pathway may involve a metabolic pathway from dihydroxyacetone phosphate to dihydroxyacetone to glycerol. Host cells may contain both of those native metabolic pathways.

The term "native," when used herein with respect to genetic materials (e.g., a gene, promoter, terminator or other DNA sequence), refers to genetic materials that are found (apart from individual-to-individual mutations which do not affect function) within the genome of wild-type cells of that species of yeast. "Native capability" (and its variations such as "natively capable") indicates the ability of wild-type cells to perform the indicated function. For example, a cell is natively capable of metabolizing a sugar to glycerol if wild-type cells of that species possess that capability prior to any genetic modifications. A gene is considered to be "functional" within a cell if it functions within the cell to produce an active protein. A "native pathway" or "native metabolic pathway" refers to a metabolic pathway that exists and is active in wild-type cells of that species of yeast. An enzyme is "natively produced" by a yeast species if the enzyme is produced in active form by wild type cells of that species of yeast.

In this invention, "exogenous" means with respect to any genetic material that it is not native to the host cell.

Suitable host yeast cells for certain embodiments of the invention include yeast cells which are not descended from a line that underwent the ancient (~100 million years ago) whole genome duplication event described by Wolf et al., "Molecular evidence for an ancient duplication of the entire yeast genome", Nature 387, 708-713 (1997) (hereinafter "Wolf et al 1997"), Langkjaer et al., "Yeast genome duplication was followed by asynchronous differentiation of duplicated genes", Nature 421, 848-852 (2003) and Merico et al., "Fermentative lifestyle in yeasts belonging to the Saccharomyces complex", FEBS Journal 274, 967-989 (2007) (hereinafter "Merico 2007"). Such yeast cells are instead descended from one or more other lines of yeast cells that existed at the time of the whole genome duplication event, and are referred to herein as "pre-whole genome duplication yeast". The whole genome duplication event is seen as critical for the evolution of the fermentative capabilities of *Saccharomyces cerevisiae* and other species descended from the common ancestor in which the genome duplication occurred (Merico 2007). Included in the set of genes duplicated in the genome duplication are those encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, as are genes encoding fumarate reductase which is also involved in maintaining redox balance (Wolfe et al 1997).

Among the suitable pre-whole genome duplication yeast cells are hemiascomycetous yeast cells. Hemiascomycetous yeast are single-celled yeast classified within the order Saccharomycetales.

Other suitable yeast cells include those falling within any of the clades 7, 8, 9, 10, 11, 12, 13 or 14 of the Saccharomyces complex, as described in Figure 9 (p. 430) of Kurtzman and Robnett, "Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses.", FEMS Yeast Res. Vol. 4, pp. 417-432. (2003), incorporated herein by reference. Those clades are designated by the names *Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces, Eremothecium, Hanseniaspora* and *Saccharomycodes,* respectively, in Merico 2007, *supra, and in Kurtzmann, "Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other members of the Saccharomycetaceae . . ."* *FEMS Yeast. Res.* Vol. 4, pp. 233-245 (2003) (hereinafter "Kurtzman 2003").

Other suitable yeast cells include (but are not limited to) yeast cells classified under the genera *Candida, Saccharomyces, Schizosaccharomyces, Kluyveromyces, Pichia, Issatchenkia,* and *Hansenula.*

A class of host cells that are of particular interest includes any of those of a species contained within the *I. orientalis/I. terricola* clade. Members of the *I. orientalis/I. terricola* clade are identified by analysis of the variable D1/D2 domain of the 26S ribosomal DNA of yeast species, using the method described by Kurtzman and Robnett in "Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences", *Antonie van Leeuwenhoek* 73:331-371, 1998, incorporated herein by reference (hereinafter "Kurtzman and Robnett 1998"). *See especially* p. 349 and 361. Analysis of the variable D1/D2 domain of the 26S ribosomal DNA from hundreds of ascomycetes has revealed that the *I. orientalis/I. terricola* clade contains closely related species. Members of the *I. orientalis/I. terricola* clade exhibit greater similarity in the variable D1/D2 domain of the 26S ribosomal DNA to that of other members of the clade than to that of yeast species outside of the cladea. Therefore, other members of the *I. orientalis/I. terricola* clade can be identified by comparison of the D1/D2 domains of their respective ribosomal DNA and comparing to that of other members of the clade and closely related species outside of the clade, using Kurtzman and Robnett's methods. Yeast species within the *I. orientalis/I. terricola* clade are all hemiascomycetous yeast within the broader *Pichia/Issatchenkia/Saturnispora/ Dekkera* clade. Another class of host cells of interest is the *I.orientalis/P. fermentans* clade as described by Kurtzman and Robnett 1998. .That clade is the most terminal clade that contains at least the species *Issatchenkia orientalis, Pichia galeiformis, Pichia sp. YB-4149* (NRRL designation), *Candida ethanolica, P. deserticola, P. membranifaciens* and *P. fermentans.*

Other host cells of particular interest are any of those of a species contained within the *Kluyveromyces* clade of Saccharomyces complex, as described (as Clade 11) in Figure 9 (p. 430) of Kurtzman and Robnett, "Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses.". FEMS Yeast Res. Vol. 4, pp. 417-432. (2003), incorporated herein by reference, and in Figure 1 of Kurtzmann, "Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other members of the Saccharomycetaceae . . ." *FEMS Yeast. Res.,* Vol. 4, pp. 233-245 (2003) (hereinafter "Kurtzman 2003"), incorporated herein by reference. The *Kluyveromyces* clade includes at least the species *S. kluyveri, K. aestuaryii, K. nonfermentans, K. lactic, K. marxianus* and *K.*

*dobzhanskii,* and would include additional species classifiable within that clade using the multigene sequene analysis methods described in Kurtzman 2003.

Such yeast cells are of particular interest when genetically modified to produce an organic acid, especially lactate. Host cells from the *Candida, Kluyveromyces and Ittatchenkia* genera are generally preferred. Host cells from the *Kluyveromyces* and *I. orientalis/P. fermentans* clades described before are particularly preferred, in those embodiments where the mutant cell produces an organic acid, as well as in cases where the mutant cell produces another fermentation product (such as, for example, ethanol) in addition to or instead of an organic acid. Especially preferred host cells are *C. sonorensis, K. marxianus, K. thermotolerans, C. methanosorbosa, and I. orientalis.* Most preferred cells are *K. marxianus, C. sonorensis, and I. orientalis.* When first characterized, the species *I. orientalis* was assigned the name *Pichia kudriavzevii.* The anamorph (asexual form) of *I. orientalis* is known as *Candida krusei.* 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.

By "deletion or disruption" of a metabolic pathway, it means that the pathway is either rendered completely inoperative, or else its activity is reduced by at least 75%, preferably at least 90%, relative to the wild-type cell. Activity of a pathway may be reduced by reducing the amount of active enzyme that is produced, by reducing the activity of the enzyme that is produced, or some combination of both. By "deletion or disruption" of a gene it is meant that 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) so that the gene no longer produces an active enzyme, the gene produces a severely reduced quantity (at least 75% reduction, preferably at least 90% reduction) of the active enzyme, or the gene produces an enzyme with severely reduced (at least 75% reduced, preferably at least 90% reduced) activity.

In most cases, the deletion or disruption of the native metabolic pathway will involve a deletion or disruption of at least one GPD gene, at least one GPP gene, or both. In cells such as *S. pombe,* that have an alternate metabolic pathway based on dihydroxyacetone phosphate phosphatase and glycerol dehydrogenase, the deletion or disruption of the native metabolic pathway will usually include a deletion or

disruption of the dihydroxyacetone phosphate phosphatase gene, glycerol dehydrogenase gene, or both. In cells having both pathways, deletions or disruptions of both pathways can be performed.

The term "glycerol-3-phosphate dehydrogenase gene" and "GPD gene" are used herein to refer to (a) any gene that encodes for a protein with glycerol-3-phosphate dehydrogenase activity and/or (b) any chromosomal DNA sequence that encodes for an enzyme that is at least 50%, preferably at least 60% and more preferably at least 65% identical to any of the amino acid sequences identified as SEQ. ID. NO. 1, SEQ. ID. NO. 2, SEQ. ID. NO. 3, SEQ. ID. NO. 4, SEQ. ID. NO. 5, SEQ. ID. NO. 6, or SEQ. ID. NO. 7. "Glycerol-3-phosphate dehydrogenase activity" refers to the ability of a protein to catalyze the reaction of DHAP to glycerol-3-phosphate. For purposes of this invention, percent identity of amino acid sequences of DNA, RNA or proteins can conveniently computed using BLAST (NCBI Basic Local Alignment Search Tool) version 2.2.1 software with default parameters. Sequences having an identities score of at least XX%, using the BLAST version 2.2.13 algorithm with default parameters, are considered at least XX% identical. The BLAST software is available from the National Center for Biological Information, Bethesda, Maryland.

Similarly, "glycerol-3-phosphatase gene" and "GPP gene" are used herein to designate (a) any gene that encodes for a protein with glycerol-3-phosphatase activity and/or (b) any chromosomal DNA sequence that encodes for a protein that is at least 50%, preferably at least 60% and more preferably at least 65% identical to any of the amino acid sequences identified as SEQ. ID. NO.8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, SEQ. ID. NO 11 or SEQ. ID. NO 12. "Glycerol-3-phosphatase activity" refers to the ability of a protein to catalyze the dephosphorylation of glycerol-3-phosphate to form glycerol.

The term "dihydroxyacetone phosphate phosphatase" gene is used herein to denote any gene that encodes for a protein with dihydroxyacetone phosphate phosphatase activity. "Glycerol dehydrogenase" gene is used herein to denote (a) any gene coding for a protein with glycerol dehydrogenase activity and/or (b) any chromosomal DNA sequence that encodes for a protein that is at least 50%, preferably at least 60% and more preferably at least 65% identical to the amino acid sequence identified as SEQ. ID. NO. 13. "Dihydroxyacetone phosphate phosphatase activity" refers to the ability of a protein to catalyze the reaction of dihydroxyacetone

phosphate to dihydroxyacetone. "Glycerol dehydrogenase activity" refers to the ability of a protein to catalyze the reduction of dihydroxyacetone to glycerol.

The deletion or disruption of any of the foregoing genes can be accomplished by forced evolution, mutagenesis, or genetic engineering methods, followed by appropriate selection or screening to identify the desired mutants.

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. Cells having the desired mutation can be screened for on the basis of their reduced ability to produce glycerol. Disruption or deletion of any of the foregoing genes can be confirmed through PCR or Southern analysis methods.

Genetic engineering to delete or disrupt the metabolic pathway to glycerol is conveniently accomplished in one or more steps via the design and construction of appropriate deletion constructs and transformation of the host cell with those constructs. The term "construct" is used herein to denote a DNA sequence that is used to transform a cell. The construct may be, for example, in the form of a circular plasmid or vector, in the form of a linearized plasmid or vector, may be a portion of a circular plasmid or vector (such as is obtained by digesting the plasmid or vector with one or more restriction enzymes), or may be a PCR product prepared using a plasmid or vector as a template. Selection or screening follows to identify successful transformants. Electroporation and/or chemical (such as calcium chloride– or lithium acetate–based) transformation methods can be used.

The following discussion of deletion constructs is equally applicable to the deletion or disruption of any of the glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, dihydroxyacetone phosphate phosphatase or glycerol dehydrogenase genes.

A deletion construct is conveniently assembled by first cloning two DNA sequences of the target gene and/or its upstream (5') or downstream (3') flanking regions. The sequences are preferably non-contiguous, but may be contiguous if additional genetic material (such as a selection marker cassette) is to be interposed between them on the construct. In this context, "non-contiguous" means that the DNA sequences are not immediately adjacent to each other in the wild-type genome, but instead are separated from each other in the wild-type genome by an area that is

to be deleted in order to delete or disrupt the gene. "Contiguous" sequences are directly adjacent to each other in the wild-type genome. One of the 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 target gene coding region, or some combination thereof. The other 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 region. A deletion construct is then produced containing the two sequences oriented in the same direction in relation to each other as they natively appear on the chromosome of the host cell. Typically a selection marker is cloned between the sequences to allow selection of transformants, as described more fully below. This construct is used to transform the host cell. Electroporation and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods can be used.

In successful transformants, a homologous recombination event at the locus of the target gene results in the disruption or the deletion of the functional gene. All or a portion of the native target gene, its promoter and/or terminator is deleted during this recombination event. If the deletion construct contains genetic material between the two sequences taken from the target locus (such as a selection marker cassette or structural gene cassette), that genetic material is inserted into the host cell's genome at the locus of the deleted material. Analysis by PCR or Southern analysis can be performed to confirm that the desired deletion has taken place.

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" is one that encodes 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 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 selection marker cassette will further include promoter and terminator sequences, operatively linked to the selection marker gene, and which are operable in the host cell. One suitable type of promoter is at least 50%, 70%, 90%, 95% or 99% identical to a promoter that is native to a yeast gene. A more suitable type of promoter is at least 50%, 70%, 90%, 95% or 99% identical to a promoter for a gene that is native to the host cell. Particularly useful promoters include promoters for pyruvate decarboxylase (*PDC1*), phosphoglycerate kinase (*PGK*), xylose reductase (*XR*), xylitol dehydrogenase (*XDH*), L-(+)-lactate-cytochrome c oxidoreductase (*CYB2*), translation elongation factor-1 (*TEF1*) and translation elongation factor-2 (*TEF2*) genes, especially from the respective genes of the host cell. An especially useful promoter includes the functional portion of a promoter for a *PDC1, PGK, TEF1* or *TEF2* gene native to the host cell, or a sequence that is at least 80, 85, 90 or 95% identical to such a *PDC1, PGK, TEF1* or *TEF2* promoter.

One suitable type of terminator is at least 50%, 70%, 90%, 95% or 99% identical to a terminator for a gene that is native to a yeast cell. The terminator may be at least 50%, 70%, 90%, 95% or 99% identical to a terminator for a gene that is native to the host cell. Particularly useful terminators include terminators for pyruvate decarboxylase (*PDC1*), xylose reductase, (*XR*), xylitol dehydrogenase (*XDH*), L-lactate:ferricytochrome c oxidoreductase (*CYB2*) or iso-2-cytochrome c (*CYC*) genes, or a terminator from the galactose family of genes in yeast, particularly the so-called *GAL10* terminator. An especially preferred terminator includes a functional portion of a terminator for a *GAL10* gene native to the host cell, or a sequence that is at least 80, 85, 90 or 95% identical to such a terminator.

The deletion 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 structural 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 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.

The target gene deletion construct may also contain a structural gene cassette, again located downstream of the 5' flanking region and upstream of the 3' flanking region, but preferably not within any selection marker cassette as may be present. Such a construct permits the simultaneous deletion of the target gene and insertion of a structural gene. By "structural gene", it is meant any gene that encodes for a protein, other than the target gene or a selection marker gene as described above. A wide variety of structural genes can be used, but those of particular interest to this invention are a gene that confers to the cell the ability to produce an organic acid, or a gene that confers to the cell the ability to consume a particular carbon source, such as a pentose sugar.

In cases in which a selection marker is used, the transformation can be performed with pair of deletion constructs instead of a single deletion construct. One of the pair will contain the first sequence from the locus of the target gene and a non-functional part of the marker gene cassette. The other of the pair will contain the second sequence from the locus from the target gene and another non-functional part of the marker gene cassette. The two parts of the marker gene cassette are selected that that together they form a complete cassette. The ends of each of the two parts of the marker gene cassette share a common sequence, *i.e.*, a portion of the cassette is duplicated at the ends of each of the two parts. The cell is transformed with these simultaneously to perform the desired deletion or disruption, with the formation of a complete, functional marker or structural gene cassette. A proportion of the cells will

homologously integrate both deletion constructs at the target locus, and will engage in a further homologous recombination event to reconstitute a functional selection gene cassette from the two non-functional fragments. Successful transformants can be selected for on the basis of the characteristic imparted by the selection marker.

When the cell's native metabolic pathway includes the dihydroxyacetone phosphate-to-glycerol-3-phosphate-to-glycerol pathway (via GDP and GPP enzymes), either the GDP gene(s) or GPP gene(s) may be deleted or disrupted. Both the GDP and the GPP genes may be deleted. In such a case, the deletion or disruption of both the GDP and GPP genes may be done simultaneously or sequentially in either order. If the cell contains multiple GDP or GPP genes, or multiple alleles of such genes, it is preferred to delete all of those which are functional in the cell. In cases in which the cell's native metabolic pathway includes the dihydroxyacetone phosphate-to-dihydroxyacetone-to-glycerol pathway (via dihydroxyacetone phosphate phosphatase and glycerol dehydrogenase), either the dihydroxyacetone phosphate phosphatase or glycerol dehydrogenase genes may be deleted or disrupted. Both the dihydroxyacetone phosphate phosphatase or glycerol dehydrogenase genes may be deleted or disrupted, which may be done simultaneously or sequentially, in which case this can be done either order. As before, multiple functional copies or alleles of such genes are preferably all deleted.

In certain aspects of the invention, the cell is capable of producing a desired organic acid (or its salt). This capability is manifested by an ability to convert at least 5%, such at least 10%, at least 50%, at least 70%, at least 80% or at least 90%, by weight of a carbon source to the desired organic acid when cultivated under at least one set of fermentation conditions. As few yeast cells have the native ability to produce such acids, the cell of the invention will in most cases contain at least one functional, exogenous gene that enables it to produce the acid.

Cells of particular interest produce lactate, by which it is meant lactic acid or a salt thereof. In such case, the cell of the invention contains at least one functional, exogenous lactate dehydrogenase (*LDH*) gene integrated into its genome. An *LDH* gene is one that encodes for a functional lactate dehydrogenase enzyme. A functional *LDH* enzyme is one that catalyzes the reduction of pyruvate to lactate. *LDH* genes are specific to the production of either L-*LDH* or D-*LDH*, which respectively enable the cell to produce either the L- or D- lactic acid enantiomer (or their salts). It is possible that the modified cell of the invention contains both L- and D-*LDH* genes,

and thus is capable of producing both lactic acid enantiomers. However, it is preferred that only L- or only D-*LDH* genes are present, so the cell produces a more optically pure lactic acid product.

Suitable *LDH* genes include those obtained from bacterial, fungal, yeast or mammalian sources. Examples of specific L-*LDH* genes are those obtained from *L. helveticus, L. casei, B. megaterium, P. acidilactici* and bovine sources. Examples of specific D-*LDH* genes are those obtained from *L. helveticus, L. johnsonii, L. bulgaricus, L. delbrueckii, L. plantarum,* and *L. pentosus.* Functional genes that are identical or at least 80%identical to any of these L-*LDH* or D-*LDH* genes are suitable. The native genes obtained from any of these sources may be subjected to mutagenesis if necessary to provide a coding sequence starting with the usual eukaryotic starting codon (ATG), or for other purposes. A preferred L-*LDH* gene is that obtained from *L. helveticus* or one that is at least 80%, 85%, 90% or 95% identical to such gene. Another preferred L-*LDH* gene is that obtained from *B. megaterium* or one that is at least 80%, 85%, 90% or 95% identical to such gene. A preferred D-*LDH* gene is that obtained from *L. helveticus* or one that is at least 80%, 85%, 90% or 95% identical to such gene.

Particularly suitable *LDH* genes include those that encode for an enzyme with an amino acid sequence that is at least 60%, especially at least 80%, 85% or 95%, identical to SEQ. ID. NO. 45 of WO 03/049525 or compared with SEQ. ID. NO. 49 of WO 03/049525. Particularly suitable *LDH* genes also include those that encode an enzyme having a protein sequence that is at least 60%, 80%, 85% or 95% identical to SEQ ID. NO. 46 or 50 of WO 03/049525.

The transformed cell may contain a single *LDH* gene or multiple *LDH* genes, such as from 1 to 10 *LDH* genes, especially from 1 to 5 *LDH* genes. When the transformed cell contains multiple *LDH* genes, the individual genes may be copies of the same gene, or include copies of two or more different *LDH* genes. Multiple copies of the exogenous *LDH* gene may be integrated at a single locus (so they are adjacent to each other), or at several loci within the host cell's genome.

The exogenous *LDH* gene is under the transcriptional control of one or more promoters and one or more terminators, both of which are functional in the modified yeast cell. Suitable promoters and terminators are as described before with regard to the selection marker gene cassette, and are also described in WO 99/14335, WO 00/71738, WO 02/42471, WO 03/102201, WO 03/102152 and WO 03/049525. An

especially useful promoter includes the functional portion of a promoter for a *PDC1, PGK, TEF1,* or *TEF2* gene of the host cell or is at least 80%, 85%, 90% or 95% identical to such a promoter. An especially preferred terminator includes a functional portion of a terminator for a *PDC1* gene of the host cell or is at least 80%, 85%, 90% or 95% identical thereto.

When multiple exogenous *LDH* genes are introduced into the host cell, it is possible for the different *LDH* genes to be under the control of different types of promoters and/or terminators.

The exogenous *LDH* gene may be integrated randomly into the host cell's genome or inserted at one or more targeted locations. Examples of targeted locations include the locus of a gene that is desirably deleted or disrupted, such as that of a *PDC1* gene, a glycerol-3-phosphate dehydrogenase gene, a glycerol 3-phosphatase gene, a dihydroxyacetone phosphate phosphatase gene or a glycerol dehydrogenase gene. The exogenous *LDH* gene cassette may reside on a construct for the deletion or disruption of a glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase, dihydroxyacetone phosphate phosphatase or glycerol dehydrogenase gene, and in that manner be inserted into the locus of such a gene simultaneously with the deletion or disruption thereof.

Methods for transforming a yeast cell to introduce an exogenous *LDH* gene cassette are described in WO 99/14335, WO 00/71738, WO 02/42471, WO 03/102201, WO 03/102152 and WO 03/049525. Such methods are applicable to this invention.

The cell may also be modified to enable it to produce one or more other organic acids. For example, the cell may be transformed with an exogenous gene cassette that encodes for a functional beta-alanine/pyruvate aminotransferase enzyme, thus enabling the cell to produce 3-hydroxy propionic acid. Methods for accomplishing this are described in WO 2005/118719.

The genetically modified yeast cell of the invention may include additional genetic modifications that provide one or more desired attributes to the cells.

An additional modification of particular interest in some embodiments includes a deletion or disruption of pyruvate decarboxylase gene(s). This reduces the cell's ability to produce ethanol, which is particularly desirable in cases in which an organic acid such as lactate is the desired product. If the host cell contains multiple *PDC* genes, it is especially preferred to delete or disrupt all of the *PDC* genes, although it is possible to delete fewer than all such *PDC* genes. *PDC* deletion can be

accomplished using methods analogous to those described in WO 99/14335, WO 02/42471, WO 03/049525, WO 03/102152 and WO 03/102201. *PDC* deletion can also be accomplished with simultaneous insertion of an *LDH* gene cassette or other structural or selection marker gene cassette. In a method of particular interest, (1) non-contiguous sequences from the locus of the PDC gene(s) are cloned, (2) a construct containing the non-contiguous sequences is produced, and (3) the host cell is transformed with the construct. A homologous recombination event results in a deletion or disruption of the functional *PDC* gene in a portion of the transformants. This can be repeated if necessary to delete or disrupt multiple *PDC* genes or alleles. In some yeast species, such as *I. orientalis*, multiple *PDC* genes or alleles exist that are closely homologous. It has been found that in at least some such instances non-contiguous sequences taken from the locus of either gene or allele can be used in the construct to delete or disrupt both of the *PDC* genes or alleles. The construct used to disrupt the *PDC* gene(s) may include one or more functional marker or structural gene cassettes inserted downstream of the 5' flanking portion of the native *PDC* gene and upstream of the 3' flanking portions of the native *PDC* gene. This approach allows for the deletion of the *PDC* gene and insertion of the functional gene cassette in a single transformation step.

Another additional modification of particular interest is one (or more) which individually or collectively confers to the cell the ability to ferment pentose sugars to desirable fermentation products. Among the latter type of 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 modifying the cell to overexpress a functional xylulokinase are described, for example, in WO 04/099381, incorporated herein by reference.

Another additional modification of particular interest in lactate-producing cells of the invention includes a deletion or disruption of at least one L- or D-lactate:ferricytochrome c oxidoreductase gene.

In general, the cell of the invention is characterized by a reduced ability to synthesize glycerol. A useful method for evaluating a cell's ability to synthesize glycerol is by cultivating the cell under the standard microaerobic conditions described before. A defined aqueous fermentation medium is used, which contains at the start of cultivation 5 g/L ammonium sulfate, 3 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, trace elements, vitamins and 150 g/L glucose. The pH is adjusted to 3.5 at the start of cultivation. The pH is permitted to range freely during the cultivation, except that the medium is buffered if necessary to prevent the pH from falling below 3.0 or rising above 7.0 during the cultivation. The fermentation medium is inoculated with sufficient yeast cells that are the subject of the evaluation to produce an $OD_{600}$ of 1.0. The cultivation temperature is 30°C. The cultivation is continued until the glucose concentration is reduced to 5 g/L, but is not continued for more than 120 hours. During the cultivation, aeration and agitation conditions are selected to produce an oxygen uptake rate of 5.0 ± 1.0 mmol/L/hr. Under these standard conditions, the cells of the invention typically produce no more than 2.0 g/L of glycerol. More typically, they produce no more than 0.6 g/L of glycerol under these conditions and in most cases produce no more than 0.2 g/L of glycerol under these conditions. Preferred cells also produce, under these standard microaerobic conditions, at least 10 g/L of at least one desirable fermentation product, such as ethanol or an organic acid such as lactate. The cells more preferably produce at least 40 and especially at least 50 g/L of the desired fermentation produce under these conditions.

The cell of the invention can be cultivated, under the standard microaerobic conditions described before or any other useful set of fermentation conditions, to produce one or more desirable fermentation products. Ethanol is an example of a fermentation product which many yeast species produce naturally. As discussed before, the cells can be modified to enable them to produce other desirable fermentation products, including organic acids such as lactate or 3-hydroxy propionic acid. The cells may be modified to produce other fermentation products as well, including other acids or other products that are not acids.

In the fermentation process of the invention, the cell of the invention is cultivated in a fermentation medium that includes a carbon source that is fermentable by the transformed cell. The carbon source may be a hexose sugar such as glucose, or an oligomer or other polymer of glucose such as glycan, maltose, maltotriose or isomaltotriose. The carbon source may be another hexose sugar, of which panose, fructose, fructose and their respective oligomers and polymers are examples. If the cell natively has or is modified to impart an ability to ferment pentose sugars, the carbon source may include a pentose sugar such as xylose, or a xylose oligomer or polymer such as xylan. Such pentose sugars are suitably hydrolysates of a hemicellulose-containing biomass. In case of oligomeric sugars, it may be necessary to add enzymes to the fermentation broth in order to digest these to the corresponding monomeric sugar for fermentation by the cell.

The medium will typically contain 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. A so-called "complex" medium or a so-called "defined" medium can be used.

Other fermentation 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 from about 30-45°C.

During the production phase, the concentration of cells in the fermentation medium is typically in the range of from 0.1 to 20, preferably from 0.1 to 5, even more preferably from 1 to 3 g dry cells/liter of fermentation medium. The fermentation may be conducted aerobically, microaerobically, or anaerobically. If desired, oxygen uptake rate can be used as a process control, as described in WO 03/102200. Cells of the invention can perform especially well when cultivated under microaerobic conditions characterized by an oxygen uptake rate of from 4 to 12, especially from 5 to 10, mmol/L/hr.

In preferred cases in which the cell produces an organic acid such as lactate, the medium may be buffered during the production phase of the fermentation so that

the pH is maintained in a range of about 3.5 to about 9.0, or from about 4.5 to about 7.0. Suitable buffering agents are 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 general, those buffering agents that have been used in conventional fermentation processes are also suitable here.

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. The acid is then 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.

Alternatively, the pH of the fermentation medium may be permitted to drop during the cultivation from a starting pH that is above the pKa of the product acid, typically 5.5 or higher, to at or below the pKa of the acid fermentation product, such as in the range of about 1.5 to about 3.5, in the range of from about 1.5 to about 3.0, or in the range from about 1.5 to about 2.5.

It is also possible to conduct the fermentation to produce a product acid by adjusting the pH of the fermentation broth to at or below the 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 pKa of the product acid throughout the cultivation, or may be allowed to increase to above the pKa of the acid as the fermentation proceeds. In the former case, the pH is preferably maintained within the range of about 1.5 to about 3.5, in the range of about 1.5 to about 3.2, or in the range of about 2.0 to about 3.0.

The cell of the invention has a sharply reduced ability to produce glycerol under many fermentation conditions. The reduced ability of the cell to produce

glycerol is manifested by low glycerol yields. The cells of the invention typically metabolize less than 2% by weight of the carbon source that is consumed to glycerol. In most cases, the glycerol yield is less than 1% or even less than 0.1%, based on the weight of carbon source that is consumed in the cultivation. Preferably, the cell metabolizes at least 40%, such as at least 50, 60, 70, 80 or 85%, of the carbon source that is consumed to the desired fermentation product.

It has been found that the cells of the invention exhibit good ability to grow under fermentation conditions. This is surprising, because of the cell's various uses for glycerol and the role glycerol is believed to play in balancing NADH/$NAD^+$ in wild-type yeast cells. It is within the scope of the invention to add glycerol to the fermentation medium to compensate for the cell's diminished capacity to produce glycerol on its own. However, applicants have found that doing this provides little benefit, at least in some fermentation processes.

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.

**Example 1A: Mutagenesis of *K. marxianus* strain CD607 and selection of mutant strain (CD853) having resistance to glycolic acid.**

*K. marxianus* strain CD607 is described in Example 3D of WO 03/102152. This stain has a deletion of its pyruvate decarboxylase gene and an insertion of an exogenous lactate dehydrogenase gene at that locus. Cells of strain CD607 are subjected to mutagenesis via exposure to ultraviolet light.

Cells from a fresh YP (yeast extract plus peptone) + 20 g/L glucose plate are resuspended in 2 mL of yeast peptone + 50 g/L glucose to an approximate $OD_{600}$ of 6. Ten 125μl aliquots of this cell suspension are pipeted into ten wells of a 300μl 96-well microtiter plate. The microtiter plate is exposed to 12,500 μJoule/$cm^2$ of UV light to kill 90-99% of the cells. The microtiter plate is then incubated in darkness overnight at 30°C with agitation (225 rpm) to allow the cells to recover prior to plating onto selection plates.

100μl of the UV-treated cell suspensions are then plated onto a potato dextrose agar (PDA) + 15 g/L glycolic acid plate to select for glycolic acid-resistant strains. These plates are incubated at 30°C for several days until colonies appear. A single colony is isolated for further analysis.

Approximately 2 X $10^8$ of mutagenized cells are plated onto PDA plates containing 15 g/L glycolic acid and incubated at 30°C. Colonies that grow on these plates are grown overnight in baffled shake flasks at 30°C and 225 rpm agitation in YP (yeast peptone) + 100 g/L glucose without buffer. Production flasks are then inoculated with 2 g/L cell dry weight from these shake flasks. The production flasks are cultured at 30°C and 70 rpm agitation in YP + 50 g/L glucose. Samples are withdrawn periodically to measure glucose, lactate, ethanol and pyruvate by HPLC using methods such as described in Example 1M of WO 03/102201. A strain that produces about 26 g/L lactate after 88 hours is designated as strain CD635. Strain CD635 is able to grow on lactate as the sole carbon source.

Cells of strain CD635 are subjected to an additional mutagenesis step as described above. The resulting mutagenized cells are selected for colonies that are able to grow on PDA containing 25 g/L glycolic acid. Colonies that are resistant to glycolic acid are separately grown overnight in YP + 100 g/L glucose in shake flasks at 30°C and 250 rpm agitation. Biomass is collected by centrifugation and 2 g/L dry weight of cells are inoculated into 50 mL YP + 50 g/L glucose in a baffled shake flask. The flasks are cultivated at 30°C and 250 rpm agitation for approximately 92 hours. A mutant that produces significantly higher final lactate titers, compared to parent strains CD607 and CD635, is designated as strain CD853.

Strain CD853 is unable to grow on lactate as the sole carbon source, suggesting that the native L-lactate:ferricytochrome c oxidoreductase gene (*KmCYB2*) gene has become non-functional in this mutant. Therefore, the *KmCYB2* coding region plus ~500 bp up and downstream from the *KmCYB2* coding region is amplified from this strain, using PCR with high fidelity FailSafe enzyme and genomic DNA as the template. The resulting ~2.75 kbp PCR product is purified via Qiagen column purification and sequenced over the entire *KmCYB2* coding region. Strain CD853 is found to have a four-base insertion at amino acid position 62 of the *KmCYB2* gene, which causes a frame-shift mutation, resulting in a stop codon at amino acid position 76 and truncating the protein.

**Example 1B: Construction of *GPD1F* deletion vectors pBH158 (Fig. 1) and pBH159 (Fig 2).**

A plasmid designated pVR29 (described in Example 1C and Figure 4 of WO 03/102152) contains the kanamycin-resistance gene of Tn903 (G418 gene) under the control of a pyruvate decarboxylase promoter and a *GAL10* terminator. Plasmid

pVR29 is digested with *MluI* and *PstI* and a 5.1 kbp fragment containing the G418 gene cassette so obtained is gel purified and dephosphorylated. A 1.2 kbp region of DNA upstream of the *K. marxianus* GPD (*KmGPD1F*) gene is amplified by PCR using primers identified as SEQ. ID. NO. 14 and SEQ. ID. NO. 15, with *K. marxianus* genomic DNA as a template. The PCR product is gel purified, digested with *MluI* and *PstI*, and ligated to the 5.1 kbp fragment from plasmid pVR29 to produce a plasmid designated as pBH158 (Fig. 1). Plasmid pBH158 contains, in order of transcription, the 1.2 kbp upstream flank of the *KmGPD1F gene* and the G418 expression cassette.

For the second deletion vector, plasmid pVR29 is digested with *NgoMIV* and *AatII* and a 4.7 kbp fragment containing the G418 expression cassette is gel purified and dephosphorylated. A 0.7 kbp region of DNA downstream of the *KmGPD1F* gene is amplified by PCR using primers identified as SEQ. ID. NO. 16 and SEQ. ID. NO. 17, again using *K. marxianus* genomic DNA as a template. The PCR product is gel purified, digested with *NgoMIV* and *AatII*, and ligated to the 4.7 kbp fragment of pVR29 to produce a plasmid designated as pBH159 (Fig. 2). Plasmid pBH159 contains, in order of transcription, the G418 expression cassette and the 0.7 kbp downstream flank of the *KmGPD1F gene*.

**Example 1C: Transformation of strain CD853 (Ex. 1A) with plasmids pBH158 and pBH159 (Ex. 1B, Figs. 1 and 2) to produce a transformant (strain CD1606) having an exogenous *LDH* gene, a deletion of a native *PDC* gene, a disrupted native *CYB2* gene and a deleted native *GPD1F* gene.**

Plasmid pBH158 is digested with *MluI* and *HindIII*. These restriction enzymes cut the plasmid to produce a 2.6 kbp fragment that contains the 1.2 kbp upstream flank of the *KmGPD1F* gene and part of the G418 expression cassette. This fragment is isolated from an agarose gel. Plasmid pBH159 is digested with *XhoI* and *NgoMIV*. These restriction enzymes cut the plasmid to produce a 2.0 kbp fragment that contains a portion of the G418 expression cassette and the 0.7 kbp downstream flank of the *KmGPD1F* gene. This fragment is isolated from an agarose gel. The two isolated fragments together contain the entire G418 expression cassette with some duplication at the ends of the fragments.

Strain CD853 is grown overnight in YP + 60 g/L glucose + 0.2 M MES + 1% ethanol, pH 6.5, and is electroporated simultaneously with the 2.6 kbp fragment from plasmid pBH158 and the 2.0 kbp fragment from pBH159. Transformants are selected on YP + 20 g/L glucose + 300 μg/mL G418 plates at 30°C following 2 days of growth. 15 transformants are picked,, restreaked to YP + 20 g/L glucose + G418 plates and

grown overnight. Only cells which have been cotransformed with both fragments and in which both fragments have become homologously integrated at the *KmGPD1F* locus will be resistant to G418.

Deletion of the *KmGPD1F* gene is verified by PCR using primers identified as SEQ. ID. NO. 18 and SEQ. ID. NO. 19. Seven transformants exhibit a single band of 3.4 kbp by PCR, indicating that the *KmGPD1F* gene is deleted in those transformants. One of these transformants is designated as strain CD1606.

**Example 2A: Construction of *GPP* gene deletion vectors pBH160 (Fig. 3) and pBH161 (Fig. 4)**

Plasmid pVR29 is digested with *MluI* and *KpnI* and a 5.1 kbp fragment containing the G418 gene cassette so obtained is gel purified and dephosphorylated. A 0.9 kbp region of DNA immediately upstream of the native GPP gene (*KmHOR2* gene) is amplified by PCR using primers identified as SEQ. ID. NO. 20 and SEQ. ID. NO. 21, using *K. marxianus* genomic DNA as the template. The PCR product is gel purified, digested with *MluI* and *KpnI*, and ligated to the 5.1 kbp fragment from plasmid pVR29 to produce a plasmid designated as pBH160 (Fig. 3). Plasmid pBH160 contains, in order of transcription, the 0.9 kbp upstream flank of the *KmHOR2* gene and the G418 expression cassette.

Plasmid pVR29 is digested with *NgoMIV* and *SpeI* and a 4.7 kbp fragment containing the G418 expression cassette is gel purified and dephosphorylated. A 0.8 kbp region of DNA immediately downstream of the *KmHOR2* gene is amplified by PCR using primers identified as SEQ. ID. NO. 22 and SEQ. ID. NO. 23, using *K. marxianus* genomic DNA as the template. The PCR product is gel purified, digested with *NgoMIV* and *SpeI*, and ligated to the 4.7 kbp fragment of pVR29 to produce a plasmid designated as pBH161 (Fig. 4). Plasmid pBH161 contains, in order of transcription, the G418 expression cassette and the 0.8 kbp downstream flank of the *KmHOR2 gene.*

**Example 2B: Transformation of strain CD853 (Ex. 1A) with plasmids pBH160 and pBH161 (Ex. 2A, Figs. 3 and 4) to produce a transformant (strain CD1608) having an exogenous *LDH* gene, a deletion of a native *PDC* gene, a disrupted native *CYB2* gene and a deleted native *GPP* gene.**

Plasmid pBH160 is digested with *MluI* and *HindIII.* These restriction enzymes cut the plasmid to produce a 2.3 kbp fragment that contains the 0.9 kbp upstream flank of the *K. marxianus* GPP (*KmHOR2)* gene and part of the G418

expression cassette. This fragment is isolated from an agarose gel. Plasmid pBH161 is digested with *XhoI* and *NgoMIV*. These restriction enzymes cut the plasmid to produce a 2.0 kbp fragment that contains the 0.8 kbp upstream flank of the *KmHOR2* gene and part of the G418 expression cassette. This fragment is isolated from an agarose gel. The two isolated fragments together contain the entire G418 expression cassette with some duplication at the ends of the fragments.

Strain CD853 is grown overnight in YP + 60 g/L glucose + 0.2 M MES + 1% ethanol, pH 6.5, and is then electroporated with both the 2.3 kbp fragment from plasmid pBH160 and the 2.0 kbp fragment from plasmid pBH161. Transformants are selected on YP + 20 g/L glucose + 300 μg/mL G418 plates at 30°C following 2 days of growth. 15 transformants are restreaked to YP + 20 g/L glucose + 300 μg/mL G418 plates and grown overnight. All transformants grow on this medium. Only cells which have been cotransformed with both fragments and in which both fragments have become homologously integrated at the *KmHOR2* locus will be resistant to G418.

Deletion of the *KmHOR2* gene is verified by PCR using primers identified as SEQ. ID. NO. 20 and SEQ. ID. NO. 21. Three transformants yield a single 3.8 kbp band which is indicative of the deletion of the *K*mHOR2 gene. One of these transformants is designated strain CD1608.

**Example 3: Microaerobic batch culture cultivation of strains CD853 (Ex.1A), CD1606 (Ex. 1C) and CD1608 (Ex. 2B).**

Strains CD853, CD1606 and CD1608 are separately cultivated under microaerobic conditions. Duplicate fermentations are performed in the cases of strains CD1606 and CD1608. In each case, a single-stage batch-culture reactor is used. The fermentation medium is a defined medium that includes ammonium sulphate, potassium dihydrogen phosphate and magnesium sulphate, trace elements, vitamins, defoaming agent, and about 90 g/L glucose. The pH of the medium is adjusted to about 3.0 by addition of potassium hydroxide. The medium is adjusted to 30°C and inoculated with 1 mL of cells. The cells are cultured at 30°C under agitation and aeration conditions that lead to an oxygen uptake rate of 5-6 mmol/L/hr. Oxygen uptake rate is determined according to methods described in WO 03/102,200.

Samples of the fermentation broth are removed periodically and assayed for lactate, acetate, glycerol and pyruvate. Carbon dioxide production is measured by determining the carbon dioxide content of gasses vented from the reactor.

Strain CD853 (not an example of the invention) produces lactate at a rate of 0.85 g/L-hr through early stages of the fermentation, until the lactate titer is approximately 20 g/L. Lactate yield through that point is about 70%. After that, lactate production slows to about 0.76 g/L-hr and lactate yield drops slightly. Production for this strain is stopped after 86 hours, at which time the fermentation broth contains 11 g/L glucose. Lactate titer is 59 g/L. Overall lactate production rate is 0.65 g/L-hr, and overall yield to lactate is 70%. Yields to pyruvate, acetate, glycerol and carbon dioxide for strain CD853 are 0.6%, 0%, 5.1% and 14%, respectively. Yield to biomass is 6.4%.

Strain CD1606 produces lactate at a rate of 0.77-0.84 g/L-hr through early stages of the fermentation, until the lactate titer is approximately 20 g/L. Lactate yield through that point is about 72-80%. After that, lactate production slows to about 0.39 – 0.41 g/L-hr and lactate yield drops slightly. Production for this strain is stopped after 137 hours, at which time the fermentation broth contains 14-19 g/L glucose. Lactate titer is 43-45 g/L. Overall lactate production rate is 0.32 – 0.34 g/L-hr, and overall yield to lactate is 60-63%. Yields to pyruvate, acetate, glycerol and carbon dioxide for strain CD1606 are 0.1%, 0.5%, 0% and 26-29%, respectively. Yield to biomass is 7.9%. These results show that deletion of the native *KmGPD1F* gene is effective to disrupt the cell's capability to produce glycerol. Surprisingly, the deletion of this gene (and the resulting lack of glycerol production) has little or no effect on cell growth.

Strain CD1608 produces lactate at a rate of 0.66 g/L-hr through early stages of the fermentation, until the lactate titer is approximately 20 g/L. Lactate yield through that point is about 70-75%. After that, lactate production slows to about 0.37 g/L-hr and lactate yield drops slightly. Production for this strain is stopped after 137 hours, at which time the fermentation broth contains 19 g/L glucose. Lactate titer is 42 g/L. Overall lactate production rate is 0.31 g/L-hr, and overall yield to lactate is 59-60%. Yields to pyruvate, acetate, glycerol and carbon dioxide for strain CD1608 are 0.0 - 0.1%, 0.8%, 0% and 26-28%, respectively. Yield to biomass is 7.9-8.2%. These results show that deletion of the native *KmHOR2* gene also is effective to

disrupt the cell's capability to produce glycerol. Again, the deletion of this gene (and the resulting lack of glycerol production) has no effect on cell growth.

**Example 4A: Cloning of *I. orientalis* native *GPD1* gene together with upstream and downstream flanking region.**

Known glycerol-3-phosphate dehydrogenase genes from several yeast species (*S. cerevisiae, K. marxianus, Y. lipolytica, P. jadinii, D. hansenii* and *C. glabrata*) are aligned and regions which are highly conserved among the various genes are identified. Two sets of degenerate primers were designed in these regions of high homology. These sets are identified as SEQ. ID. NO. 24 and SEQ. ID. NO. 25, and SEQ. ID. NO. 26 and SEQ. ID. NO. 27, respectively. PCR is performed using the first set of primers and *I. orientalis* genomic DNA as the template, and a ~200 bp product is obtained as expected. PCR is again performed using the second set of primers and *I. orientalis* genomic DNA as the template, and a ~400 bp product is obtained as expected. The two PCR products are gel purified and sequenced using the same primers. Using the partial sequence so obtained, primers are designed for genome walking. Genome walking is performed using the BD Clontech Genome Walking Kit according to the manufacturer's instructions, using primary PCR primers identified as SEQ. ID. NO. 28 and SEQ. ID. NO. 29 and nested PCR primers identified as SEQ. ID. NO. 30 and SEQ. ID. NO. 31. Sequences obtained from both upstream and downstream genome walks are aligned and merged with the previously obtained partial sequence to construct the *I. orientalis* glycerol-3-phosphate dehydrogenase gene.

**Example 4B: Construction of a plasmid (pMM28, Fig. 5) containing the *KmCYB2* gene cassette between *K. thermotolerans* direct repeat sequences.**

The entire *K. marxianus CYB2* (*KmCYB2*) gene cassette, including promoter and terminator regions, is PCR amplified from the genomic DNA of a wild-type *K. marxianus* strain designated as CD21, with introduction of *BamHI* and *SalI* restriction sites, by PCR using primers identified as SEQ. ID. NO. 32 and SEQ. ID. NO. 33. The PCR product is ligated to a commercial vector designated as pUC18 (from Invitrogen Corp., Carlsbad, CA USA) that is digested with *BamHI* and *SalI*. The resulting plasmid is designated as pMM25.

A 705 bp sequence identified as SEQ. ID. NO. 34 is PCR-amplified from the genomic DNA of *K. thermotolerans*, with introduction of *SphI* and *SalI* restriction

sites, using primers identified as SEQ. ID. NO. 35 and SEQ. ID. NO. 36. This *K. thermotolerans* sequence does not encode for any active protein. Plasmid pMM25 is digested with *SphI* and *SalI* and the *K. thermotolerans* sequence is ligated to it upstream (5') to the *KmCYB2* cassette to form a plasmid designated as pMM27.

An identical *K. thermotolerans* sequence is PCR-amplified with addition of *BamHI* and *XmaI* restriction sites, using primers identified as SEQ. ID. NO. 37 and SEQ. ID. NO. 38. Plasmid pMM27 is digested with *BamHI* and *XmaI* and the *K. thermotolerans* sequence is ligated to it downstream (3') from the *KmCYB2* cassette to form a plasmid designated as pMM28 (Fig. 5). Plasmid pMM28 contains the *KmCYB2* cassette flanked by *K. thermotolerans* direct repeat sequences, both oriented in the same direction.

**Example 4C: Construction of a plasmid (pMI321, Fig. 7) containing a hygromycin gene cassette and a *L. helveticus LDH* gene cassette.**

A 920 bp probe fragment of the *C. sonorensis PGK1* gene is obtained from the genomic DNA of *C. sonorensis* in the same manner as described in Example 22 of WO 02/042471, using primers identified as SEQ. ID. NO. 39 and SEQ. ID. NO. 40. Genomic DNA is isolated from a growing *I. orientalis* strain and resuspended in 10 mM Tris-HCl + 1 mM EDTA (pH 8) (TE). The *I. orientalis* genomic DNA is cut with *Hind*III and a Southern blot is prepared and hybridized using standard methods with the *C. sonorensis PGK1* gene as a probe. Fragments of ~2 kb size are isolated from agarose gel and cloned into a *Hind*III-cut plasmid to generate a size-fractionated library, which is transformed into *E. coli*. Colony hybridization of the size-fractionated library with the *PGK1* probe results in isolation of a plasmid containing a *Hind*III fragment with most of the *I. orientalis PGK1 (IoPGK1)* protein coding sequences but no promoter sequence, as verified by sequencing.

Genomic fragments containing the *IoPGK1* promoter region are obtained with ligation-mediated PCR amplification (Mueller, P.R. and Wold, B. 1989, "In vivo footprinting of a muscle specific enhancer by ligation mediated PCR." *Science* 246:780-786). A mixture of a linker identified as SEQ. ID. NO. 41 and a linker identified as SEQ. ID. NO. 42 is ligated to *Hae*III-digested *I. orientalis* genomic DNA with T4 DNA ligase (New England BioLabs). Samples of the ligation mixtures are used as templates for 50 µl PCR reactions containing 0.1 µM of a primer identified as SEQ. ID. NO. 43 and 1 µM of a primer identified as SEQ. ID. NO. 44. The reaction mixture is heated at 94°C for 3 minutes after 2 U of Dynazyme EXT is added. The

reactions are cycled 30 times as follows: 1 minute at 94°C, 2 minutes at 68°C and 2 minutes at 72°C, with a final extension of 10 minutes at 72°C. A diluted sample of this first PCR-amplification is used as the template in a nested PCR reaction (50 µl) containing 0.05 µM of a primer identified as SEQ. ID. NO. 45 and 0.5 µM of a primer identified as SEQ. ID. NO. 46. The reaction mixture is heated at 94°C for 3 minutes after 2 U of Dynazyme EXT is added. The reactions are then cycled 30 times as follows: 1 minute at 94°C, 2 minutes at 67°C and 2 minutes at 72°C, with a final extension of 10 minutes at 72°C.

A ~600 bp PCR fragment is isolated and sequenced. Nested primers identified as SEQ. ID. NO. 47 and SEQ. ID. NO. 48 are designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ. ID. NO. 49 and SEQ. ID. NO. 50 similarly as above, except that *Ssp*I-digested *I. orientalis* DNA is used and the PCR is carried out using an annealing temperature of 65°C.

The *I. orientalis PGK1* promoter region is PCR amplified using primers identified as SEQ. ID. NO. 51 and SEQ. ID. NO. 52 and the *I. orientalis* genomic DNA as the template. The fragment is filled in using the Klenow enzyme and then cut with *Xba*I. A 633 bp fragment is gel isolated and ligated to a 4428 bp fragment obtained by digesting a plasmid designated as pMI270 (described in Fig. 4 of WO 03/049525) with *Xho*I, filling the fragment in using the Klenow enzyme and 0.1 mM dNTP, and digesting with *Xba*I. Plasmid pMI270 contains the *E. coli* hygromycin gene linked to a *C. sonorensis PGK1* promoter and a *S. cerevisiae GAL10* terminator. The resulting plasmid is designated pMI318 (Fig. 6). Plasmid pMI318 contains the *E. coli* hygromycin gene under the control of the *I. orientalis PGK1* promoter and the *S. cerevisiae GAL10* terminator.

The *I. orientalis PGK1* promoter is PCR amplified using primers identified as SEQ. ID. NO. 53 and SEQ. ID. NO. 54 and *I. orientalis* genomic DNA as the template. The fragment is filled in using the Klenow enzyme and 0.1 mM dNTP, and then cut with *Nco*I. A 633 bp fragment is gel isolated. Plasmid pVR1 (described in WO 03/102152 Figure 7) contains the *L. helveticus LDH* gene under the control of the *S. cerevisiae TEF1* promoter and the *S. cerevisiae CYC1* terminator. Plasmid pVR1 is digested with *Xho*I, filled in using the Klenow enzyme, and cut with *Nco*I. A 7386 bp fragment from plasmid pVR1 is ligated to the 633 bp *IoPGK1* promoter fragment. The resulting plasmid is designated pMI320. Plasmid pMI320 contains the *L.*

*helveticus LDH* gene under the control of the *IoPGK1* promoter and *S. cerevisiae CYC1* terminator.

Plasmids pMI318 and pMI320 are digested with *Apa*I and *Not*I. A 5008 bp fragment from plasmid pMI318 is ligated to a 1995 bp fragment from plasmid pMI320 to form plasmid pMI321 (Fig. 7).

The hygromycin gene (and its terminator) is positioned on plasmid pMI321 between two copies of the *IoPGK1* promoter, which serve as direct repeat sequences.

**Example 4D: Construction of a plasmid (pMI355, Fig. 8) having the *E. coli* hygromycin gene cassette, the *L. helveticus LDH* gene cassette, and the *IoPDC1A* 5' flanking region.**

A genomic library of the wild-type *I. orientalis* strain ATCC PTA-6658 is constructed into the SuperCos1 (Stratagene) cosmid vector according to instructions provided by the manufacturer. *PDC*-like sequences are amplified by PCR from the genomic DNA of the strain with primers designated as SEQ. ID. NO. 55 and SEQ. ID. NO. 56. A 700 bp fragment of a *PDC* gene is amplified. The genomic library is screened using hybridization techniques with labeled PCR fragments as the probe as described in WO 03/049525 and cosmid clones containing the *PDC* gene are isolated and sequenced. The *I. orientalis PDC1A* 5' region from 1000 bp to 167 bp upstream of the start of the open reading frame is PCR amplified using primers identified as SEQ. ID. NO. 57 and SEQ. ID. NO. 58 and the *I. orientalis PDC1A* cosmid DNA as the template. The fragment is cut with *Sal*I *and Sac*I. An 836 bp fragment is gel isolated and ligated to a 6992 bp fragment obtained by digesting plasmid pMI321 (Fig. 7, Example 4C) with *Sal*I and *Sac*I. The resulting plasmid is named pMI355 (Fig. 8).

**Example 4E: Construction of plasmids (pMI356 and pMI357 (Fig. 9)) containing the *IoPDC1A* 5' flanking region, the *E. coli* hygromycin gene cassette, the *L. helveticus LDH* gene cassette, and an *IoPDC1A* 3' flanking region.**

The *I. orientalis PDC1A* 3' region corresponding to sequences from 524 bp upstream to 217 bp downstream of the *PDC* translation stop codon is PCR amplified using primers identified as SEQ. ID. NO. 59 and SEQ. ID. NO. 60 and the *I. orientalis PDC1A* cosmid DNA (Example 4D) as the template. The fragment is cut with *Apa*I *and Sma*I. A 630 bp fragment is gel isolated and ligated to a 7809 bp fragment obtained by digesting plasmid pMI355 (Fig. 8, Ex. 4D) with *Apa*I and *Sma*I. The resulting plasmid is named pMI357 (Fig. 9). It contains the hygromycin and

LDH cassettes from plasmid pMI355 between the 5' flank and a portion of the 3' flank of the *IoPDC1A* gene.

Plasmid pMI356 is constructed in the same way, except a different section of the *I. orientalis PDC1A* 3' region is used.

**Example 4F: Construction of plasmid pMI433 (Fig. 10) containing the *IoPDC1A* 5' flanking region, a *ScMEL5* gene cassette, the *L. helveticus LDH* gene cassette and the *IoPDC1A* 3' flanking region.**

The *I. orientalis PGK1* promoter is PCR amplified using primers identified as SEQ. ID. NO. 61 and SEQ. ID. NO. 62 and the *I. orientalis* genomic DNA as the template. The fragment is filled in using the Klenow enzyme and 0.1 mm dNTP, and then cut with *Sph*I. A 669 bp fragment is gel isolated. A plasmid designated as pMI233 (described in Fig. 23C of WO 03/049525) is cut with *Xho*I. The fragment is filled in with the Klenow enzyme and cut with *Sph*I. The 4534 bp and the 669 bp fragments are ligated and the resulting plasmid is named pMI319. Plasmid pMI319 contains the S. *cerevisiae MEL5* (*ScMEL5*) gene and the *IoPGK1* promoter region.

Plasmid pMI319 plasmid is cut with *Apa*I, made blunt ended with T4 polymerase, and cut with *Not*I. A 2317 bp fragment is gel isolated. It is ligated to a 6498 bp fragment obtained by digesting plasmid pMI357 (Example 4E) with *Sal*I, making it blunt ended with the Klenow enzyme and then cutting with *Not*I. The resulting plasmid contains the *ScMEL5* gene (with its native terminator) in place of the hygromycin gene of plasmid pMI357. The resulting plasmid is designated pMI433 (Fig. 10).

**Example 4G: Construction of plasmids pMI449 (Fig. 11) and pMI454 (Fig. 12) containing *I. orientalis CYB2* 5' flanking region, *ScMEL5* gene cassette between *K. thermotolerans* direct repeat sequences and *I. orientalis CYB2* 3' flanking region.**

Plasmid pMM28 (Fig. 5, Ex. 4B) is digested with *Bam*HI, filled in with the Klenow enzyme, and digested with *Sal*I. The 4077 bp fragment so obtained is ligated to a 2317 bp *NotI* (filled in with Klenow enzyme)-*Sal*I fragment of pMI433 (Fig. 10, Ex. 4F). The resulting plasmid is designated pMI445.

*The* 3' flanking region of the *I. orientalis* L-lactate:ferricytochrome c oxidoreductase (*IoCYB2A*) gene (corresponding to sequences from 90 to 676 bp downstream of the the start of the predicted open reading frame) is amplified by PCR using primers identified as SEQ. ID. NO. 63 and SEQ. ID. NO. 64, using a *CYB2-2* cosmid clone as a template. The PCR product is digested with *Sac*I and *Sma*I and the

607 bp fragment is ligated to the 6386 bp *Sac*I - *Sma*I fragment of plasmid pMI445. The resulting plasmid is designated pMI448.

*The IoCYB2A* 5' flanking region (corresponding to sequences from 913 to 487 bp upstream of the start of the predicted open reading frame) is amplified by PCR using primers identified as SEQ. ID. NO. 65 and SEQ. ID. NO. 66, again using the *CYB2-2* cosmid clone as a template. The PCR product is digested with *Sph*I and the 454 bp fragment is ligated to the 6993 bp *Sph*I fragment obtained by partially digesting pMI448. The resulting plasmid is designated pMI449 (Fig. 11).

*The IoCYB2A* 5' flanking region (corresponding to sequences from 466 to 7 bp upstream of the predicted open reading frame) is amplified by PCR using primers identified as SEQ. ID. NO. 67 and SEQ. ID. NO. 68, once again using the *CYB2-2* cosmid clone as the template. The PCR product is digested with *Sph*I and the 493 bp fragment is ligated to the 6993 bp *Sph*I fragment obtained by partially digesting plasmid pMI448. The resulting plasmid is designated pMI453.

The *IoCYB2A* 3' flanking region (corresponding to sequences from 402 bp upstream to 77 bp downstream of the predicted stop codon) is amplified by PCR using primers identified as SEQ. ID. NO. 69 and SEQ. ID. NO. 70, using the *CYB2-2* cosmid as a template. The PCR product is digested with *Apa*I and *Sma*I and the 506 bp fragment is ligated to the 6886 bp *Apa*I – *Sma*I fragment of plasmid pMI453. The resulting plasmid is designated pMI454 (Fig. 12).

**Example 4H: Construction of a plasmid (pBH165, Fig. 13) containing an upstream fragment of the *IoGPD1* gene, a first *K. thermotolerans* direct repeat section, a *MEL5* gene cassette, a second *K. thermotolerans* direct repeat section, and a downstream fragment of the *IoGPD1* gene.**

Plasmid pMI449 is digested with *NdeI* and *SbfI* to excise the 5' *CYB2A* flanking homology. A 6.8 kbp fragment is gel purified and dephosphorylated. A 302 bp fragment of the *IoGPD1* gene from Example 4A (corresponding to base pairs 1-302 from the start codon of the gene) is amplified by PCR using primers identified as SEQ. ID. NO. 71 and SEQ. ID. NO. 72. The PCR product is gel purified, digested with *NdeI* and *SbfI*, and ligated to the 6.8 kbp fragment from plasmid pMI449 to produce plasmid pBH164. Plasmid pBH164 is then digested with *XmaI* and *EcoRI* to excise the 3' *CYB2A* flanking homology. A 6.5 kbp fragment is gel purified and dephosphorylated. A 346 bp fragment of the *IoGPD1* gene from Example 4A (corresponding to base pairs 322-668 from the start codon) is amplified by PCR using primers identified as SEQ. ID. NO. 73 and SEQ. ID. NO. 74. The PCR product is gel

purified, digested with *XmaI* and *EcoRI*, and ligated to the 6.5 kbp fragment obtained from pBH164 to produce pBH165 (Fig. 13).

Plasmid pBH165 contains, in order of transcription, the 302 bp fragment of the *IoGPD1* gene, a first *K. thermotolerans* direct repeat section, a *MEL5* gene cassette, a second *K. thermotolerans* direct repeat section, and the 346 bp fragment of the *IoGPD1* gene. It is designed for insertion at the locus of the native *IoGPD1* gene (with disruption of the gene), followed by a loop-out of the *MEL5* gene cassette.

**Example 4I: Generation of an *I. orientalis* mutant (CD1184) with deleted *IoPDC1A* and *IoPDC1B* genes and integrated *LhLDH* gene in one step by transforming wild-type *I. orientalis* strain with plasmid pMI356 (Ex. 4F).**

Wild-type *I. orientalis* strain ATCC PTA-6658 is transformed with plasmid pMI356 using standard methods. Transformed strains that grow on hygromycin plates are cultured. A transformant that does not produce ethanol is selected for Southern analysis, which confirms the deletion of both *IoPDC1A* alleles and insertion of at least one copy of the *LhLDH* gene. This strain is designated CD1184.

**Example 4J: Generation of *I. orientalis* mutant strain (CD1496) by successively transforming strain CD1184 (Ex. 4I) with plasmids pMI449 (Ex. 4G, Fig. 11) and pMI454 (Ex. 4G, Fig. 12), followed by mutagenesis.**

Strain CD1184 is transformed with plasmid pMI449 using the lithium acetate method and transformants (blue colonies) are selected based on melibiase activity on YPD X-α-gal plates. The replacement of the *IoCYB2A* gene of strain CD1184 is confirmed by colony PCR and Southern analysis in some of the transformants. The *MEL5* marker is looped out from one of those transformants via a homologous recombination event through the *K. thermotolerans* repeat sequences, as confirmed by Southern analysis. The second *CYB2A* allele is then deleted from this transformant using plasmid pMI454. Transformants are analyzed by colony PCR for the absence of a 1000 bp *CYB2A*-specific PCR product. The *MEL5* marker from plasmid pMI454 is looped out of a transformant having a deletion of the second *CYB2A* allele via recombination as before. This transformant is designated strain CD1436. Strain CD1436 has a deletion of both *PDC1* genes (with replacement by a functional L-*LDH* gene cassette), and a deletion of each of its two native *IoCYB2* genes.

Strain CD1436 is subjected to EMS mutagenesis using the conditions set forth in Example 1A, except the exposure conditions are 8 μL for 1 hour. Mutagenized cells are allowed to recover for 6 hours in 200μL of YP + 20g/L glucose media and then

plated onto PDA + 35 g/L lactic acid plates and incubated for one week at 30°C. A strain that produces more lactate and less glycerol than strain CD1436 is designated as strain CD1496.

**Example 4K: Transformation of strains CD1184 (Ex. 4I) and CD 1496 (Ex. 4J) with plasmid pBH165 (Ex. 4H, Fig. 13), followed by loop-out of the selection marker to produce transformant strains CD1667 and CD1671, respectively, which have a single *GPD1* allele deleted.**

Strain CD1184 is grown and transformed with 5 µg of the 4.4 kbp fragment obtained by digesting plasmid pBH165 with *NdeI* and *EcoRI*. Transformants are selected on yeast nitrogen base (YNB) + 2% melibiose plates overlaid with x-α-gal (--4-chloro-3-indolyl-aD-galactopyranoside). Blue-colored transformants are visible after ~4 days of growth at 30°C. Eight transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x-α-gal. A single blue colony for each transformant is picked and restreaked to YP + 20 g/L glucose plates. Genomic DNA is isolated from the transformants. Disruption of one allele of the *IoGPD1* gene is verified by PCR using primers identified as SEQ. ID. NO. 75 and SEQ. ID. NO. 76. Five transformants exhibit the expected ~2 kb product. One of those transformants is designated as strain CD1655. Disruption of one copy of the native *IoGPD1* gene is further verified by PCR using primers designated as SEQ. ID. NO. 77 and SEQ. ID. NO. 78.

Strain CD1655 is grown for several rounds in YP + 100g/L glucose at 30°C. A dilution series is plated onto YP + 20 g/L plates overlaid with x-α-gal, and grown overnight at 30°C. A white colony (indicative of the loop-out of the MEL 5 marker cassette) is selected and restreaked to YP + 20 g/L glucose + x-α-gal plates. A white colony is selected. Disruption of one allele of the native *IoGPD1* gene is verified by PCR using primers identified as SEQ. ID. NO. 69 and SEQ. ID. NO. 80. This transformant is designated as strain CD1667.

Strain CD1496 is transformed in the same manner. A transformant exhibiting the expected ~2kbp band on PCR is designated as strain CD1657. Disruption of one allele of the native *IoGPD1* gene is verified by PCR as described for strain CD1655. Strain CD1657 is further grown for several rounds, and a colony showing a deletion of the *MEL5* marker gene cassette is selected and designated as strain CD1671. Disruption of one allele of the native *IoGPD1* gene is verified by PCR as before.

**Example 4L: Transformation of strains CD1667 (Ex. 4K) and CD1671 (Ex. 4K) with plasmid pBH165 (Ex. 4H, Fig. 13) to produce transformant strains CD1688 and CD1690, respectively, with both *IoGPD1* alleles deleted.**

Strain CD1667 is transformed with 5 µg of a 4.4 kbp fragment obtained by digesting plasmid pBH165 with *NdeI* and *EcoRI*. Transformants are selected on YNB + 2% melibiose plates overlaid with x-α-gal. Blue-colored transformants are visible after ~4 days of growth at 30°C. Ten transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x-α-gal. A single blue colony for each transformant is picked and restreaked to YP + 20 g/L glucose. Genomic DNA is isolated from the transformants. Disruption of the second allele of the *IoGPD1* gene is verified in three transformants by PCR using primers identified as SEQ. ID. NO 81 and SEQ. ID. NO. 82. One of these transformants is designated as strain CD1688.

Strain CD1671 is transformed in the same manner. PCR shows that the second allele of the *IoGPD1* gene is disrupted in one transformant, which is designated strain CD1690.

**Example 5: Microaerobic batch culture cultivation of strains CD1184 (Ex. 4I) and CD1688 (Ex. 4L) at an OUR of 5.5-5.6.**

A single-stage batch-culture reactor containing a defined medium that includes ammonium sulphate, potassium dihydrogen phosphate and magnesium sulphate, trace elements, vitamins, defoaming agent, and about 50 g/L glucose is inoculated with 1 mL strain CD1688. The pH of the medium is adjusted to about 3.5 prior to adding the cells. The pH of the culture is allowed to drop to 3.0 as cells grow and begin to produce lactic acid. Afterward, pH is maintained at about 3.0 by addition of potassium hydroxide. Glucose is fed to the fermentation at about 1-2 g/L/hr until a total of 136.1 g/L glucose has been added. The cells are cultured at 30°C under aeration conditions that lead to an oxygen uptake rate of about 5.5-5.6 mmol/L/hr.

Strain CD1688 produces lactate at a rate of 1.02 g/L-hr until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 74%. Production for this strain is stopped after 77 hours, at which time the fermentation broth contains 15.3 g/L glucose. Overall lactate production rate is 1.06 g/L-hr, and overall yield to lactate is 70%. Yields to pyruvate, glycerol and carbon dioxide for strain CD1688 are 1.9%, 0% and 23.7%, respectively. Yield to biomass is 3.5%.

For comparison, strain CD1184 (not an example of the invention) is cultured under similar conditions. Strain CD1184 produces lactate at a rate of 1.24 g/L-hr

until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 74%. Production for this strain is stopped after 77 hours, at which time the fermentation broth contains 15.3 g/L glucose. Overall lactate production rate is 1.06 g/L-hr, and overall yield to lactate is 70%. Yields to pyruvate, glycerol and carbon dioxide for strain CD1184 are 2.1%, 9.3% and 15.9%, respectively. Yield to biomass is 3.2%.

These results show that under these fermentation conditions, deletion of the native *IoGPD1* genes prevents the cell from producing measurable quantities of glycerol. As before, the deletion of this gene (and the resulting lack of glycerol production) has little or no effect on cell growth.

**Example 6: Microaerobic batch culture cultivation of strains CD1184 (Ex. 4I) and CD1688 (Ex. 4L) at an OUR of 9.9-10.**

Strains CD1688 and CD1184 are separately cultivated in the general manner described in Example 5, except aeration conditions are selected to lead to an oxygen uptake rate of 9.9-10.0 mmol/L/hr, and no glucose is fed to the system during the cultivation. Yeast hulls are added to the cultivation of strain CD1688.

Under these conditions, strain CD1184 produces lactate at a rate of 1.87 g/L-hr until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 73%. Production for this strain is stopped after 67.5 hours, at which time the glucose concentration in the fermentation broth has been reduced from 60 g/L to 2.1 g/L. Overall lactate production rate is 1.43 g/L-hr, and overall yield to lactate is 70%. Yields to pyruvate, glycerol and carbon dioxide for strain CD1184 are 2.1%, 5.7% and 21.5%, respectively. Yield to biomass is 4.4%.

Strain CD1688 produces lactate at a rate of 1.68 g/L-hr until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 80%. Production for this strain is stopped after 78 hours, at which time the glucose concentration in the fermentation broth has been reduced from 53.5 g/L to 4.8 g/L. Overall lactate production rate is 1.26 g/L-hr, and overall yield to lactate is 77%. Yields to pyruvate, glycerol and carbon dioxide for strain CD1688 are 1.2%, 0% and 23.2%, respectively. Yield to biomass is 5.95%. As before, these results show that under these fermentation conditions, deletion of the native *IoGPD1* genes prevents the cell from producing measurable quantities of glycerol and that the deletion of this gene (and the resulting lack of glycerol production) has no effect on cell growth. In addition, deletion of *IoGPD1* improves overall lactate yield.

**Example 7: Microaerobic batch culture cultivations of strain CD1690 (Ex. 4L) at an OUR of 5-6.**

Strain CD1690 is cultivated in the general manner described in Example 5, except aeration conditions are selected to lead to an oxygen uptake rate of 5.75 mmol/L/hr, and the fermentation medium is YP + 70 g/L glucose.

Under these conditions, strain CD1690 produces lactate at a rate of 0.66 g/L-hr until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 78%. Production for this strain is stopped after 121 hours, at which time the glucose concentration in the fermentation broth has been reduced to 23.8 g/L (out of 127.9 g/L provided to the cultivation). Overall lactate production rate is 0.61 g/L-hr, and overall yield to lactate is 77%. Yields to pyruvate, glycerol and carbon dioxide are 0%, 0% and 31.1%, respectively. Yield to biomass is 2.4%. Once again, these results show that under these fermentation conditions, deletion of both of the native *IoGPD1* alleles prevents the cell from producing measurable quantities of glycerol, and has little or no effect on cell growth.

Strain CD1690 is cultivated twice more in the general manner described in Example 5 (using the defined medium described there), except the OUR is 5.2 mmol/L/hr and glycerol is added to the fermentation broth. In the first run, 0.1 g/L glycerol is added and 1.0 g/L glycerol is added in the second run.

When 0.1 g/L glycerol are added, strain CD 1690 produces lactate at a rate of 0.74 g/L-hr until the lactate titer is approximately 70 g/L. Lactate yield through that point is about 78%. Production for this strain is stopped after 121 hours, at which time the glucose concentration in the fermentation broth has been reduced to 10.2 g/ (out of 117.8 g/L provided to the cultivation). Overall lactate production rate is 0.68 g/L-hr, and overall yield to lactate is 76%. Yields to pyruvate, glycerol and carbon dioxide are 0.2%, 0% and 25.3%, respectively. Yield to biomass is 4.1%.

Very similar results are obtained when 1.0 g/L glycerol are added.

These results unexpectedly show that the addition of glycerol to the fermentation medium has little or no effect on the ability of these transformants to grow and produce lactate, despite the disruption of the cells' native ability to produce glycerol.

**Example 8A: Construction of a plasmid (pTMC61 (Fig. 14)) containing the IoGPD1 5' flanking region, the E. coli hygromycin gene cassette between direct repeats, and the IoGPD1 3' flanking region.**

The hygromycin gene cassette is PCR amplified using primers identified as SEQ. ID. NO. 83 and SEQ. ID. NO. 84, with plasmid pMI356 (Ex. 4E, *see* Fig. 9) as the template. PCR conditions are 95°C for 5 minutes (once), 30 cycles of 95°C (30 seconds), 56°C (30 seconds) and 72°C (2 minutes), followed by one cycle of 72°C for 10 minutes. The resulting PCR product is digested with *SpeI* and *SalI*, and ligated onto plasmid pBH165 (Ex. 4H, Fig. 13), which has been similarly digested, to produce plasmid pTMC61 (Fig. 14).

**Example 8B: Transformation of selected wild-type *I. orientalis* strain with plasmid pBH165 (Ex. 4H, Fig. 13), followed by loop-out of the selection marker to produce transformant strain CD2624, which has a single *GPD1* allele deleted.**

Wild-type *I. orientalis* strain ATCC PTA-6658 is grown for many generations in continuous culture in a medium containing a low concentration of glucose and a high concentration of lactic acid. A cell that grows well under these conditions is isolated and designated as strain CD1822. Strain CD1822 produces ethanol and glycerol when cultivated in a medium containing glucose as the carbon source. Strain CD1822 is grown and transformed with plasmid pBH165 in the same manner as described in Example 4K. Transformants are selected on yeast nitrogen base (YNB) + 2% melibiose plates overlaid with x-α-gal (5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside), as described in Example 4K, with a blue colony being picked and restreaked to YP + 20 g/L glucose plates. Genomic DNA is isolated from the transformant, and analyzed for integration of the deletion construct by two sets of PCR reactions. The first of these used primers designated as SEQ. ID. NO. 85 and SEQ. ID. NO. 86, and the second of these was performed with primers designated as SEQ. ID. NO. 87 and SEQ. ID. NO. 88. These produced PCR products of 2.0 kbp and 1.4 kbp, respectively, indicating that one of the *GPD1* alleles has been disrupted. A third PCR reaction is performed, using primers designated as SEQ. ID. NO. 85 and SEQ. ID. NO. 88; this produces a 0.8 kbp product indicating that an undisrupted *GPD1* allele is still present in the transformant. The transformant is designated as strain CD2624.

**Example 8C: Transformation of strain CD2624 (Ex. 8B) with plasmid pTMC61 (Ex. 8A, Fig. 14) to produce transformant strains CD2627, having both *IoGPD1* alleles deleted.**

PCR is performed using primers identified as SEQ. ID. NO. 89 and SEQ. ID. NO. 90, with plasmid pTMC61 as the template. A 4.1 kbp fragment is obtained, and is used to transform strain CD2624. Transformants are selected on YPD + 300µg/ml hygromycin. Genomic DNA is isolated from 100 of the transformants, and used as a template in three sets of PCR reactions. The first uses primers identified as SEQ. ID. NO. 91 and SEQ. ID. NO. 88, and produces a 1.5 kbp product in 30 of the transformants. A second PCR reaction is conducted on genomic DNA from those 30 transformants, using primers identified as SEQ. ID. NO. 85 and SEQ. ID. NO. 92. Ten strains exhibited the expected 2.5 kbp product. Genomic DNA from those ten strains are then analysed using primers identified as SEQ. ID. NO. 85 and SEQ. ID. NO. 88. Two strains that do not product a 0.8 kbp fragment have both *GPD1* alleles disrupted. These are tested for grown on YNB + 2.0% melibiose plates. One strain is able to grow, and is designated as strain CD2627.

**Example 8D: Microaerobic cultivation of strain CD1822, strain CD2624 (Example 8B) and strain CD2627 (Example 8C).**

Strains CD1822, CD2624 and CD2627 are cultivated in duplicate microaerobic shake flask fermentations. The strains are grown overnight in 25 mL a defined medium containing ~100g/mL glucose, at 30°C and 250 rpm stirring in 250 mL baffled flasks. The defined medium is as described in Peter M. Bruinenberg, Johannes P. Van Dijken and W. Alexander Schefferes, 1983, An Enzymatic Analysis of NADPH Production and Consumption in *Candida utilis*, *J. General Microbiology* vol.129, pp.965-971, except for the presence of additional glucose as indicated and an increase in nicotinic acid to 5 mg/L.

The resulting cultures are used to inoculate 50 mL of the defined medium containing 100 g/L glucose in 250 mL baffled flasks to an $OD_{600}$ of 0.2. These flasks are then incubated at 100 rpm for 22 hours at 30°C. The medium is then analyzed by HPLC for glucose, glycerol and ethanol. Yield to biomass is also determined.

Strain CD1822 consumes all of the glucose during the 22 hour cultiviation, producing 6.0 g/kg of glycerol, 34.54 g/kg of ethanol and biomass to an $OD_{600}$ of 14.8.

Strain CD2624, which has a disruption of one *GPD1* allele, consumes all of the glucose, producing 5.88 g/kg of glycerol and 35.25 g/kg of ethanol. Biomass is produced to an $OD_{600}$ of 14.5.

Strain CD2627, which has a disruption of both GPD1 alleles, consumes all but 12.39 g/kg of the glucose during 22 hours. Glycerol production is 0.34 g/kg. Ethanol production is 23.06 g/kg and biomass is produced to an $OD_{600}$ of 11.5. These results indicate that disruption of the *GPD1* alleles in *I. orientalis* results in a small reduction in glucose consumption rates, and a small reduction in ethanol production and biomass production, under these conditions. However, strain CD2627 grows well and produces ethanol well, with minimal glycerol production. The results further indicate that the ability of the *GPD1* deletants to grow and produce is not dependent on the disruption of PDC activity or the addition of a pathway from pyruvate to lactate.

Claims

1. A cell of a pre-whole genome duplication yeast, which is genetically modified to delete or disrupt a native metabolic pathway from dihydroxyacetone phosphate to glycerol.

2. The cell of claim 1, which is hemiascomycetous.

3. The cell of claim 2 which, when cultivated in the presence of a carbon source that can be metabolized by the cell, metabolizes less than 2% of the weight of carbon source that is consumed by the cell to glycerol.

4. The cell of claim 3 which is genetically modified to produce an organic acid.

5. The cell of claim 4 which contains a functional *LDH* gene cassette, and which produces lactate.

6. The cell of claim 5 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene.

7. The cell of claim 5 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphatase gene.

8. The cell of claim 5 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene and of at least one native glycerol-3-phosphatase gene.

9. The cell of claim 5 wherein the deletion of disruption of the native metabolic pathway may include a deletion or disruption of at least one native dihydroxyacetone phosphatase gene, a deletion or disruption of a native glycerol dehydrogenase gene, or a deletion or disruption of both a native dihydroxyacetone phosphatase gene and a native glycerol dehydrogenase gene.

10. The cell of any of claims 4-9 which contains a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

11. A cell within the *Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces, Eremothecium* or *Hanseniaspora* clades of the Saccharomyces complex (Kurtzman 2003) which is genetically modified to delete or disrupt a native metabolic pathway from dihydroxyacetone phosphate to glycerol.

12. The cell of claim 12 which, when cultivated in the presence of a carbon source that can be metabolized by the cell, metabolizes less than 2% of the weight of carbon source that is consumed by the cell to glycerol.

13. The cell of claim 12 which is genetically modified to produce an organic acid.

14. The cell of claim 13 which contains a functional *LDH* gene cassette, and which produces lactate.

15. The cell of claim 14 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene.

16. The cell of claim 14 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphatase gene.

17. The cell of claim 14 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene and of at least one native glycerol-3-phosphatase gene.

18. The cell of claim 14 wherein the deletion of disruption of the native metabolic pathway may include a deletion or disruption of at least one native dihydroxyacetone phosphatase gene, a deletion or disruption of a native glycerol dehydrogenase gene, or a deletion or disruption of both a native dihydroxyacetone phosphatase gene and a native glycerol dehydrogenase gene.

19. The cell of any of claims 13-18 which contains a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

20. The cell of the genera *Kluyveromyces, Candida* or *Issatchenkia,* which is genetically modified to delete or disrupt a native metabolic pathway from dihydroxyacetone phosphate to glycerol.

21. The cell of claim 20 which, when cultivated in the presence of a carbon source that can be metabolized by the cell, metabolizes less than 2% of the weight of carbon source that is consumed by the cell to glycerol.

22. The cell of claim 21 which is genetically modified to produce an organic acid.

23. The cell of claim 22 which contains a functional *LDH* gene cassette, and which produces lactate.

24. The cell of claim 23 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene.

25. The cell of claim 23 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphatase gene.

26. The cell of claim 23 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene and of at least one native glycerol-3-phosphatase gene.

27. The cell of any of claims 21-26 which contains a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

28. A cell within the *I. orientalis/P. fermentans* clade (Kurtzman and Robnett 1998) or within the *Kluyveromyces* clade of the Saccharomyces complex (Kurtzman

2003), which is genetically modified to delete or disrupt a native metabolic pathway from dihydroxyacetone phosphate to glycerol.

29. The cell of claim 28 which, when cultivated in the presence of a carbon source that can be metabolized by the cell, metabolizes less than 2% of the weight of carbon source that is consumed by the cell to glycerol.

30. The cell of claim 29 which is genetically modified to produce an organic acid.

31. The cell of claim 30 which contains a functional *LDH* gene cassette, and which produces lactate.

32. The cell of claim 31 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene.

33. The cell of claim 31 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphatase gene.

34. The cell of claim 31 wherein the deletion or disruption of the native metabolic pathway includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene and of at least one native glycerol-3-phosphatase gene.

35. The cell of claim 31, which is a *K. marxianus* cell.

36. The cell of claim 35, which contains a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

37. The cell of claim 31, which is an *I. orientalis* cell.

38. The cell of claim 37, which contains a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

39. A genetically modified cell of a pre-whole genome duplication yeast species, which produces less than 2.0 g/L of glycerol when cultivated under the following standard microaerobic conditions:

A. defined aqueous medium containing, at the start of cultivation, 5 g/L ammonium sulfate, 3 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, trace elements, vitamins, 150 g/L glucose;

B. pH of 3.5 at the start of cultivation, with the fermentation medium being buffered if necessary to prevent the pH from falling below 3.0 or rising above 7.0 during the cultivation;

C. Cultivation inoculated with the yeast cell to an $OD_{600}$ of 1.0;

D. Cultivation temperature 30°C;

E. Cultivation is continued until the glucose concentration is reduced to 10 g/L, but is not continued for longer than 120 hours;

F. Aeration and agitation sufficient to produce an oxygen uptake rate of 5.0 ± 1.0 mmol/L/hr.

40. The cell of claim 39 which is hemiascomycetous.

41. The cell of claim 39 which is within the *Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces, Eremothecium* or *Hanseniaspora* clades of the Saccharomyces complex (Kurtzman 2003).

42. The cell of claim 40, which is of the genera *Kluyveromyces, Candida* or *Issatchenkia.*

43. The cell of any of claims 39-42 which produces no more than 0.6 g/L of glycerol when cultivated under the standard microaerobic conditions.

44. The cell of claim 43 which produces at least 10 g/L of a desired fermentation product when cultivated under the standard microaerobic conditions.

45. A genetically modified cell of a pre-whole genome duplication yeast species, which genetically modified cell lacks the ability to produce an active glycerol-3-

phosphate dehydrogenase enzyme that is natively produced by cells of the wild-type yeast species.

46. The cell of claim 45 which is hemiascomycetous.

47. The cell of claim 45 which is within the *Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces, Eremothecium* or *Hanseniaspora* clades of the Saccharomyces complex (Kurtzman 2003).

48. The cell of claim 45, which is of the genera *Kluyveromyces, Candida* or *Issatchenkia.*

49. A cell of any of claims 44-48 which further lacks the ability to produce an active glycerol-3-phosphatase enzyme that is natively produced by cells of the yeast species.

50. A cell of a pre-whole genome duplication yeast species, which lacks the ability to produce an active glycerol-3-phosphatase enzyme that is natively produced by wild-type cells of the yeast species.

51. The cell of claim 50 which is hemiascomycetous.

52. The cell of claim 50 which is within the *Zygosaccharomyces, Zygotorulaspora, Torulaspora, Lachancea, Kluyveromyces, Eremothecium* or *Hanseniaspora* clades of the Saccharomyces complex (Kurtzman 2003).

53. The cell of claim 50 which is of the genera *Kluyveromyces, Candida* or *Issatchenkia.*

54. A genetically modified cell of a yeast species which lacks the ability to produce an active dihydroxyacetone phosphate phosphatase enzyme that is natively produced by wild type cells of the yeast species, lacks the ability to produce an active $NADH^+$-dependent glycerol dehydrogenase enzyme that is natively produced by wild type cells of the yeast species, or both.

55. A yeast cell that is genetically modified to produce a product organic acid, said yeast cell further having a deletion or disruption of a native metabolic pathway from dihydroxyacetone phosphate to glycerol and a deletion or disruption of a native metabolic pathway from pyruvate to ethanol.

56. The yeast cell of claim 55 wherein the deletion or disruption of the native metabolic pathway from dihydroxyacetone phosphate to glycerol includes a deletion or disruption of at least one native glycerol-3-phosphate dehydrogenase gene.

57. The yeast cell of claim 55 wherein the deletion or disruption of the native metabolic pathway from dihydroxyacetone phosphate to glycerol includes a deletion or disruption of at least one native glycerol-3-phosphatase gene.

58. The yeast cell of claim 57 wherein the deletion or disruption of the native metabolic pathway from dihydroxyacetone phosphate to glycerol includes a deletion or disruption of at least one native glycerol-3-phosphase dehydrogenase gene and of at least one native glycerol-3-phosphatase gene.

59. The yeast cell of claim 57 wherein the deletion of disruption of the native metabolic pathway from dihydroxyacetone phosphate to glycerol includes a deletion or disruption of at least one native dihydroxyacetone phosphate phosphatase gene, a deletion or disruption of a native glycerol dehydrogenase gene, or a deletion or disruption of both a native dihydroxyacetone phosphate phosphatase gene and a native glycerol dehydrogenase gene.

60. The yeast cell of any of claims 55-59 wherein the organic acid is lactate.

61. A fermentation process wherein a cell of any of claims 1-3, 11-12, 20-21 or 28-29 is cultivated under fermentation conditions and in the presence of a carbon source to produce a desired fermentation product, wherein the glycerol yield is less than 2% based on the weight of the carbon source that is consumed by the cell.

62. The fermentation process of claim 61 wherein the desired fermentation product is produced in a yield of at least 40% based on the weight of the carbon source that is consumed by the cell.

63. The process of claim 62, wherein the glycerol yield is less than 0.5% based on the weight of the carbon source that is consumed by the cell.

64. The process of claim 63, wherein the desired fermentation product is ethanol.

65. A fermentation process wherein a cell of any of claims 4, 13, 22 or 30 is cultivated under fermentation conditions and in the presence of a carbon source to produce at least one organic acid, wherein the glycerol yield is less than 2% based on the weight of the carbon source that is consumed by the cell.

66. The fermentation process of claim 66 wherein the organic acid is produced in a yield of at least 40% based on the weight of the carbon source that is consumed by the cell and the glycerol yield is less than 0.5% based on the weight of the carbon source that is consumed by the cel.

67. A fermentation process wherein a cell of any of claims 5-9, 14-18, 23-26 or 31-38 is cultivated under fermentation conditions and in the presence of a carbon source to produce lactate, wherein the glycerol yield is less than 2% based on the weight of the carbon source that is consumed by the cell.

68. The fermentation process of claim 67 wherein lactate is produced in a yield of at least 40% based on the weight of the carbon source that is consumed by the cell and the glycerol yield is less than 0.5% based on the weight of the carbon source that is consumed by the cell.

69. A fermentation process wherein a cell of any of claims 1-3, 11-12, 20-21 or 28-29 is cultivated under fermentation conditions and in the presence of a carbon source to produce ethanol, wherein the glycerol yield is less than 2% based on the weight of the carbon source that is consumed by the cell.

FIG. 1

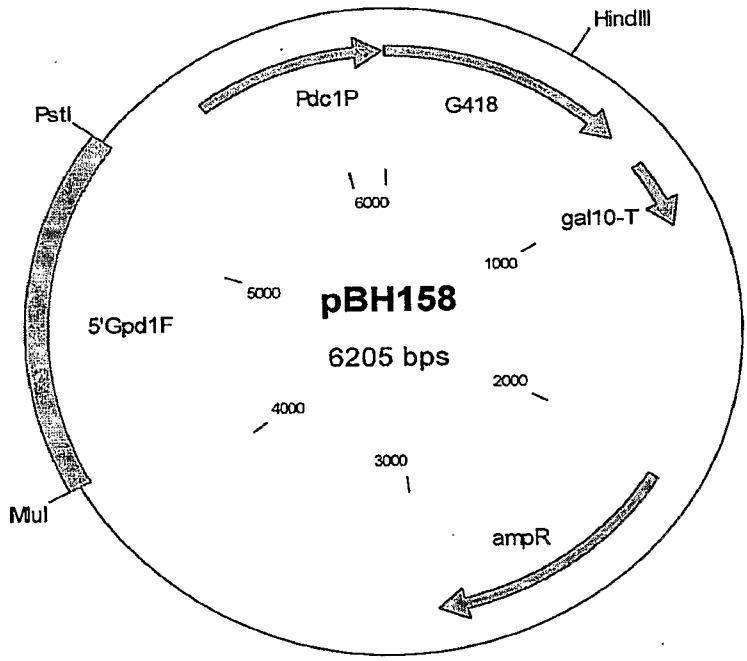

HindIII
Pdc1P
G418
PstI
6000
gal10-T
1000
5000
pBH158
5'Gpd1F
6205 bps
2000
4000
3000
MluI
ampR

FIG. 2

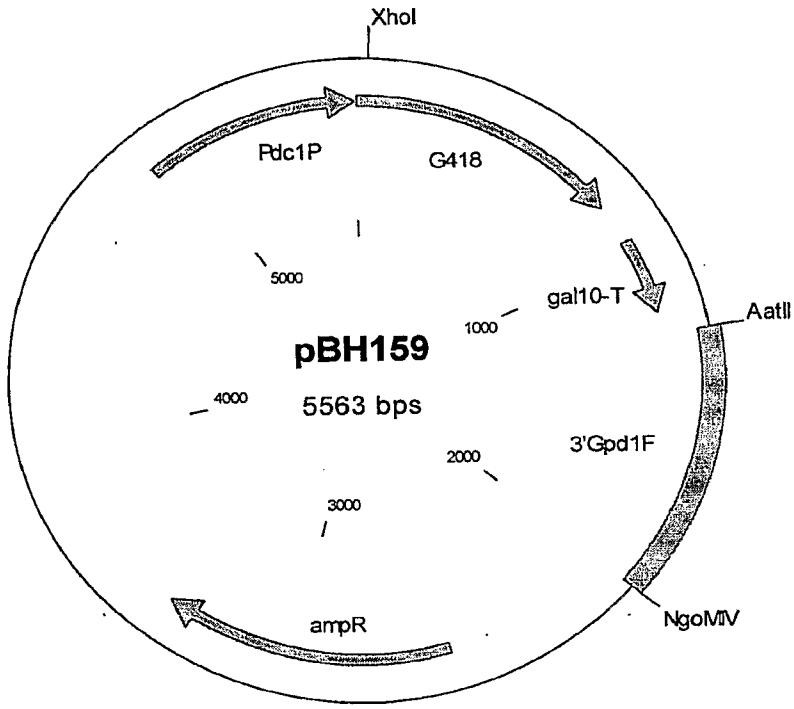

XhoI
Pdc1P
G418
5000
gal10-T
1000
AatII
pBH159
5563 bps
4000
2000
3'Gpd1F
3000
ampR
NgoMIV

FIG. 3

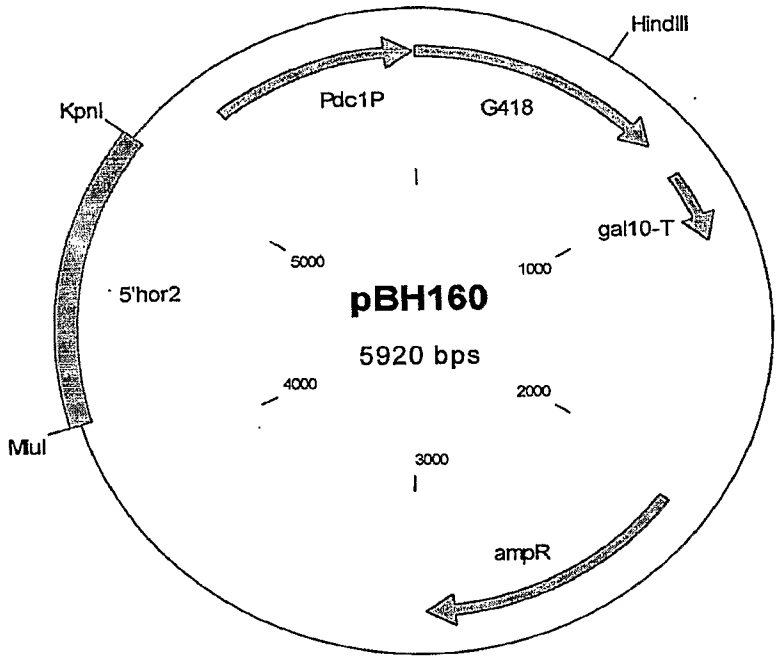

HindIII
Pdc1P
G418
KpnI
gal10-T
5000
1000
5'hor2
pBH160
5920 bps
4000
2000
MluI
3000
ampR

FIG. 4

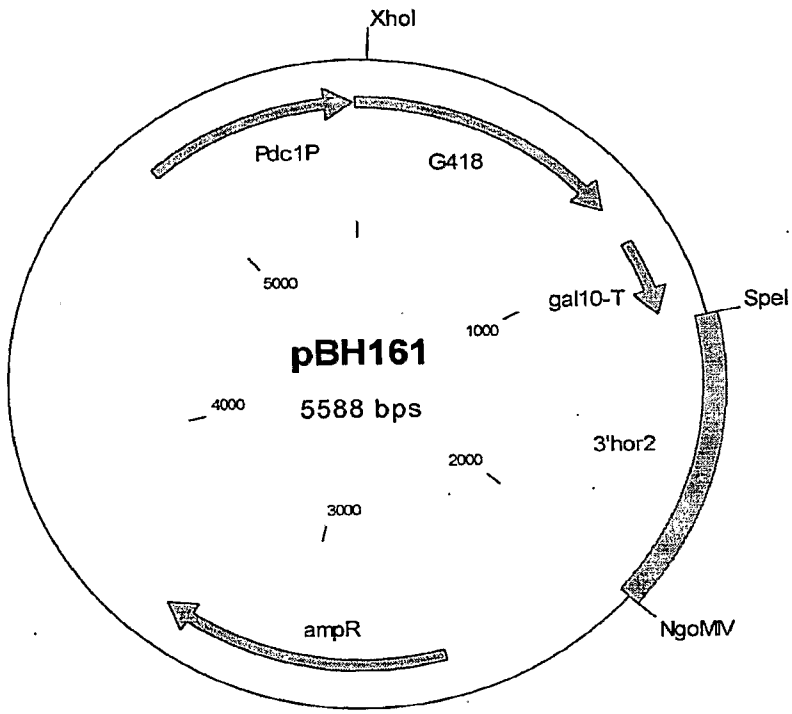

XhoI
Pdc1P
G418
5000
gal10-T
SpeI
1000
pBH161
4000
5588 bps
3'hor2
2000
3000
ampR
NgoMIV

FIG. 5

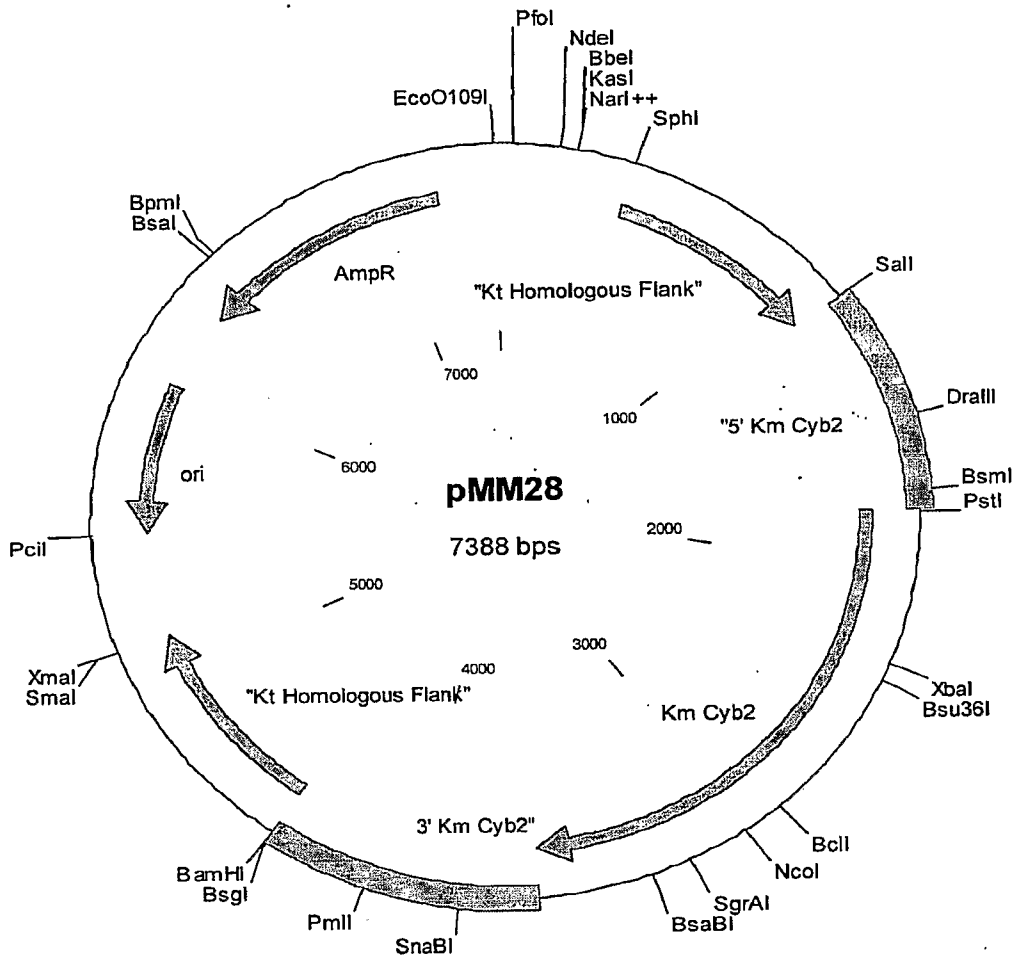

PfoI
NdeI
BbeI
KasI
NarI++
SphI
EcoO109I
BpmI
BsaI
AmpR
"Kt Homologous Flank"
SalI
DraIII
BsmI
PstI
"5' Km Cyb2
pMM28
7388 bps
ori
PciI
XmaI
SmaI
"Kt Homologous Flank"
Km Cyb2
XbaI
Bsu36I
3' Km Cyb2"
BclI
NcoI
SgrAI
BsaBI
BamHI
BsgI
PmlI
SnaBI
1000
2000
3000
4000
5000
6000
7000

FIG. 6

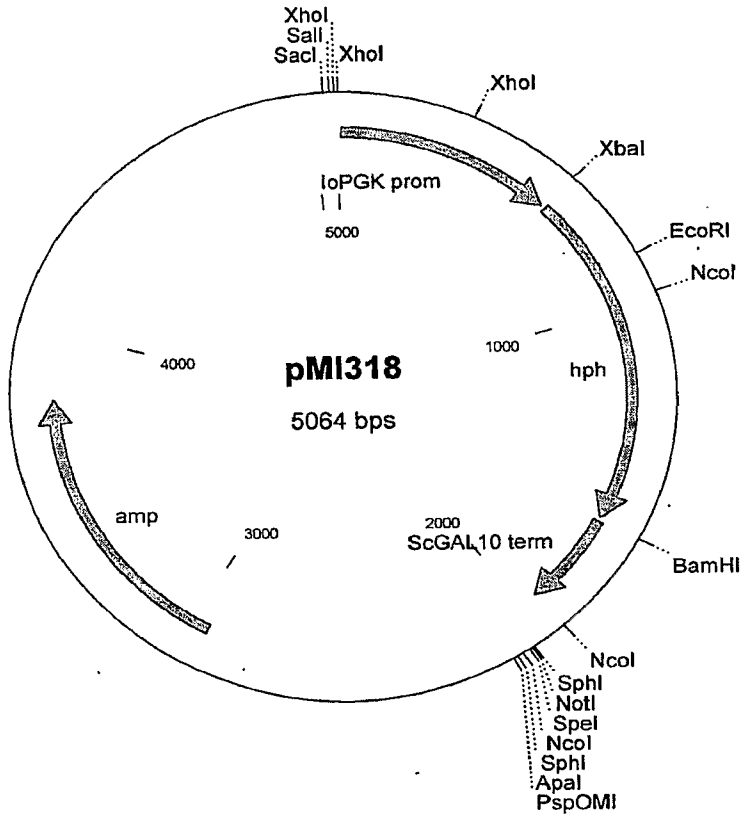

XhoI
SalI
SacI
XhoI
XhoI
XbaI
loPGK prom
5000
EcoRI
NcoI
1000
hph
4000
pMI318
5064 bps
amp
3000
2000
ScGAL10 term
BamHI
NcoI
SphI
NotI
SpeI
NcoI
SphI
ApaI
PspOMI

FIG. 7

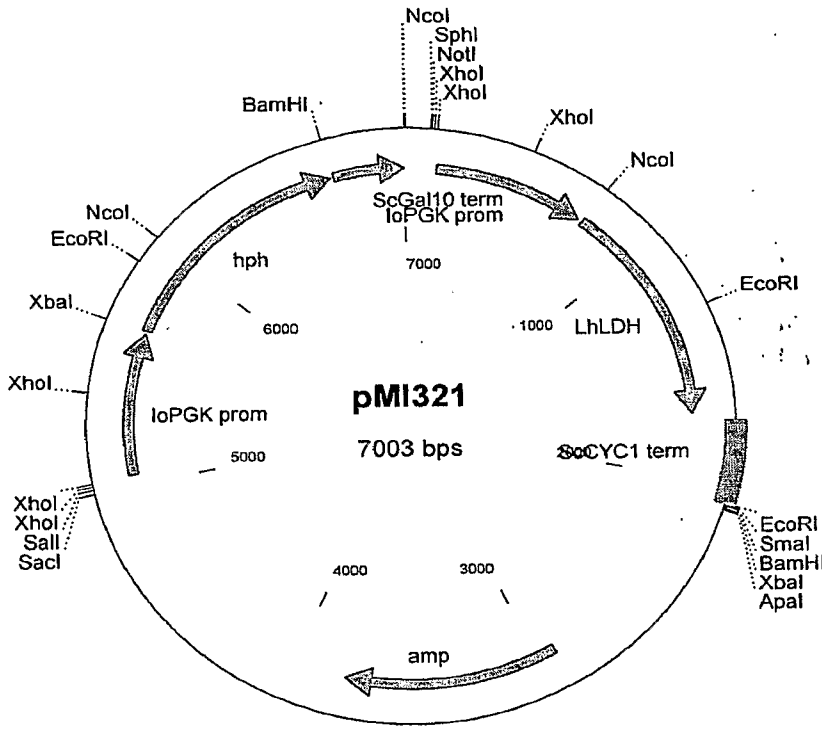

NcoI
SphI
NotI
XhoI
XhoI
BamHI
XhoI
NcoI
ScGal10 term
IoPGK prom
7000
NcoI
EcoRI
hph
6000
XbaI
1000
LhLDH
EcoRI
XhoI
pMI321
IoPGK prom
7003 bps
5000
ScCYC1 term
XhoI
XhoI
SalI
SacI
4000
3000
EcoRI
SmaI
BamHI
XbaI
ApaI
amp

FIG. 8

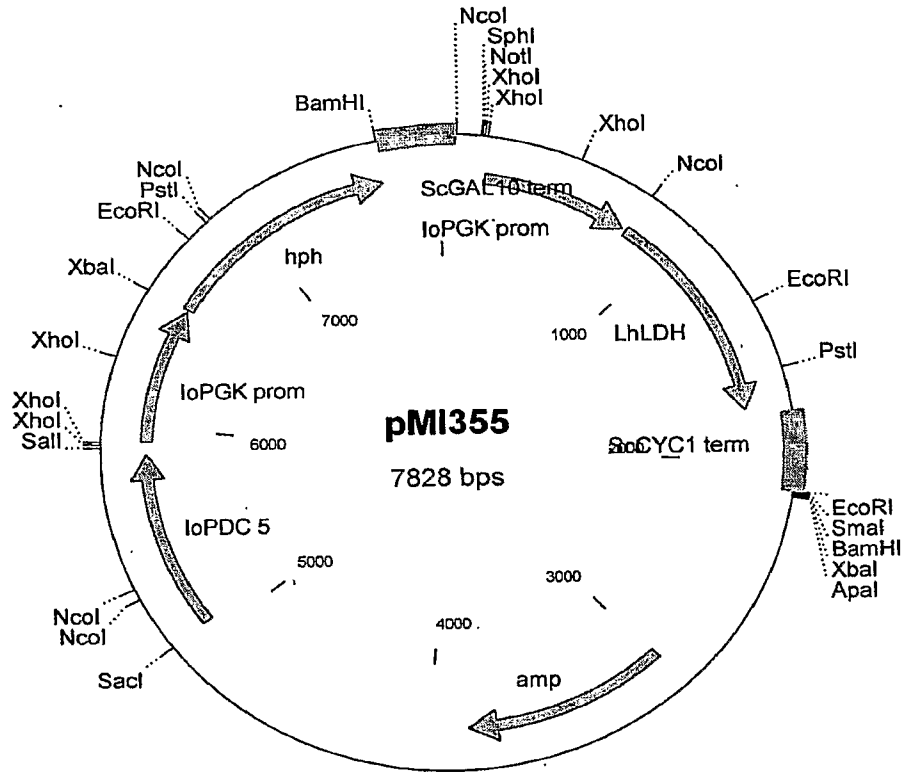

NcoI
SphI
NotI
XhoI
XhoI
BamHI
XhoI
NcoI
EcoRI
PstI
NcoI
ScGAL10 term
IoPGK prom
hph
XbaI
7000
1000
LhLDH
EcoRI
XhoI
PstI
IoPGK prom
XhoI
XhoI
SalI
6000
pMI355
7828 bps
ScCYC1 term
IoPDC 5
EcoRI
SmaI
BamHI
XbaI
ApaI
5000
3000
NcoI
NcoI
4000
SacI
amp

FIG. 9

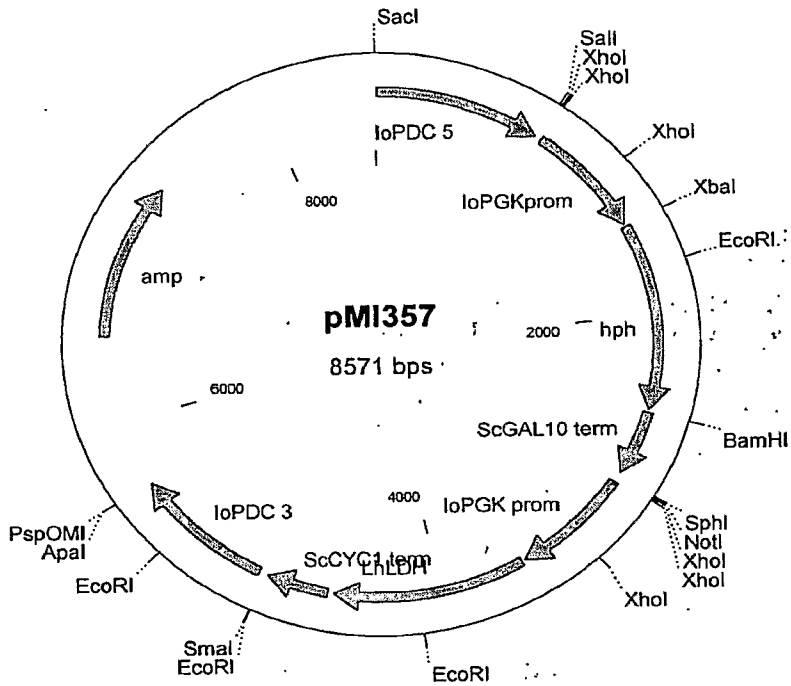

SacI
SalI
XhoI
XhoI
XhoI
XbaI
EcoRI
BamHI
SphI
NotI
XhoI
XhoI
EcoRI
SmaI
EcoRI
PspOMI
ApaI
EcoRI
loPDC 5
loPGKprom
hph
ScGAL10 term
loPGK prom
ScCYC1 term
LnLDH
loPDC 3
amp
pMI357
8571 bps
2000
4000
6000
8000

FIG. 10

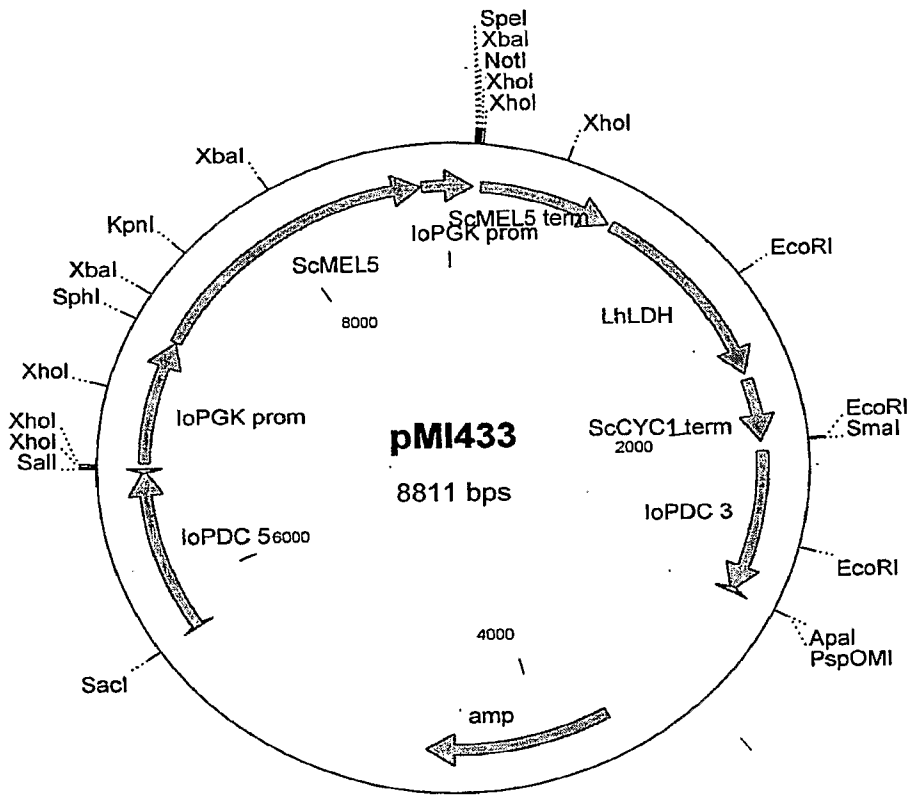

SpeI
XbaI
NotI
XhoI
XhoI
XhoI
XbaI
KpnI
XbaI
SphI
XhoI
XhoI
XhoI
SalI
SacI
ScMEL5 term
IoPGK prom
ScMEL5
8000
LhLDH
EcoRI
EcoRI
SmaI
ScCYC1 term
2000
IoPDC 3
EcoRI
ApaI
PspOMI
4000
amp
IoPGK prom
pMI433
8811 bps
IoPDC 5
6000

FIG. 11

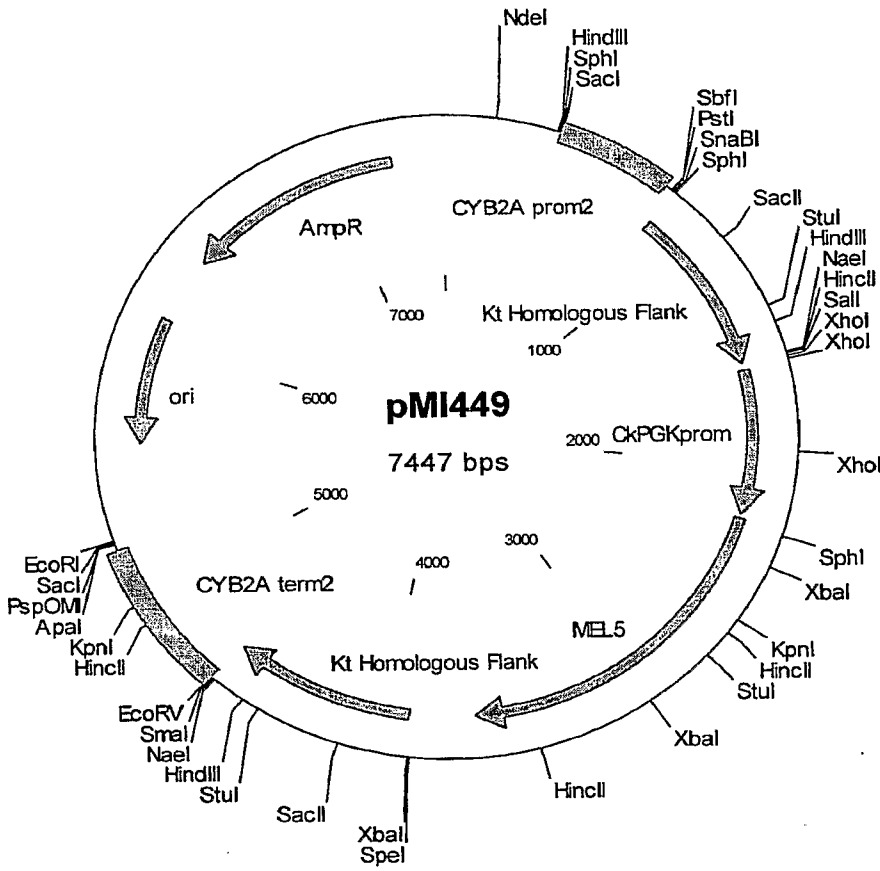

NdeI
HindIII
SphI
SacI
SbfI
PstI
SnaBI
SphI
SacII
StuI
HindIII
NaeI
HincII
SalI
XhoI
XhoI
XhoI
SphI
XbaI
KpnI
HincII
StuI
XbaI
HincII
XbaI
SpeI
SacII
StuI
HindIII
NaeI
SmaI
EcoRV
KpnI
ApaI
PspOMI
SacI
EcoRI
AmpR
CYB2A prom2
Kt Homologous Flank
CkPGKprom
MEL5
Kt Homologous Flank
CYB2A term2
ori
pMI449
7447 bps
1000
2000
3000
4000
5000
6000
7000

FIG. 12

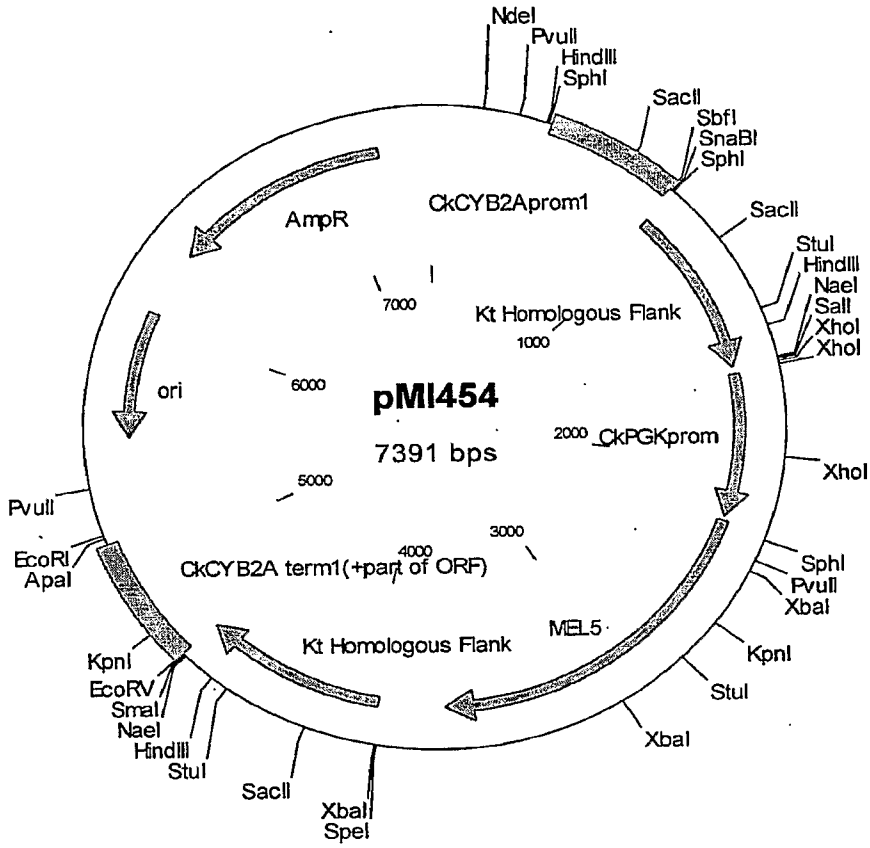

NdeI
PvuII
HindIII
SphI
SacII
SbfI
SnaBI
SphI
SacII
StuI
HindIII
NaeI
SalI
XhoI
XhoI
XhoI
SphI
PvuII
XbaI
KpnI
StuI
XbaI
SacII
XbaI
SpeI
StuI
HindIII
NaeI
SmaI
EcoRV
KpnI
PvuII
EcoRI
ApaI
AmpR
CkCYB2Aprom1
Kt Homologous Flank
ori
pMI454
7391 bps
CkPGKprom
CkCYB2A term1(+part of ORF)
MEL5
Kt Homologous Flank
1000
2000
3000
4000
5000
6000
7000

FIG. 13

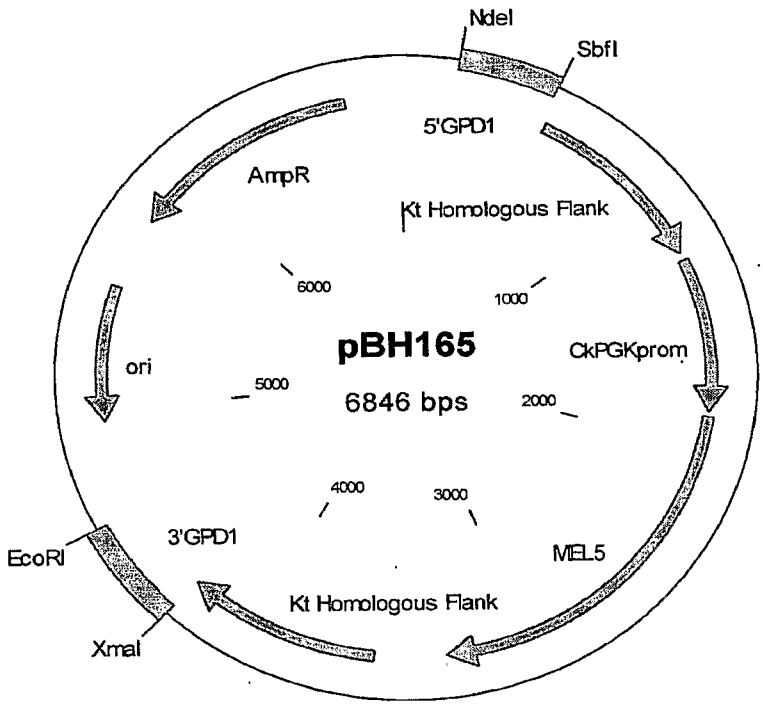
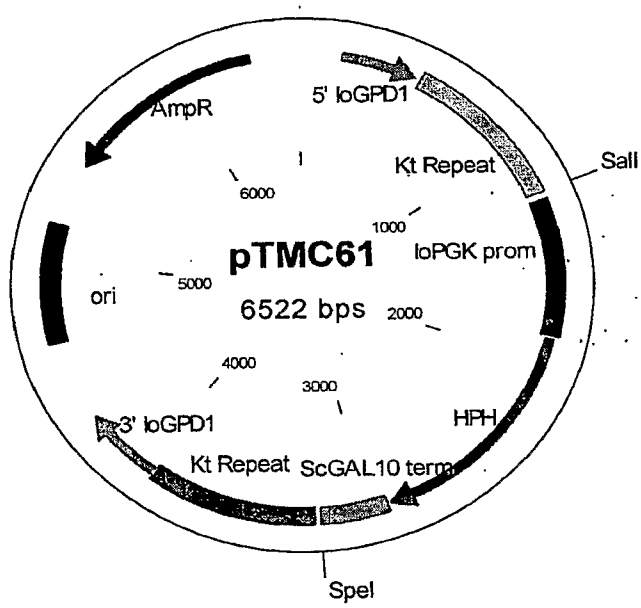

NdeI
SbfI
5'GPD1
AmpR
Kt Homologous Flank
6000
1000
pBH165
6846 bps
ori
CkPGKprom
5000
2000
4000
3000
3'GPD1
MEL5
EcoRI
Kt Homologous Flank
XmaI

FIG. 14


AmpR
5' loGPD1
Kt Repeat
SalI
6000
1000
pTMC61
loPGK prom
ori
5000
6522 bps
2000
4000
3000
3' loGPD1
HPH
Kt Repeat
ScGAL10 term
SpeI