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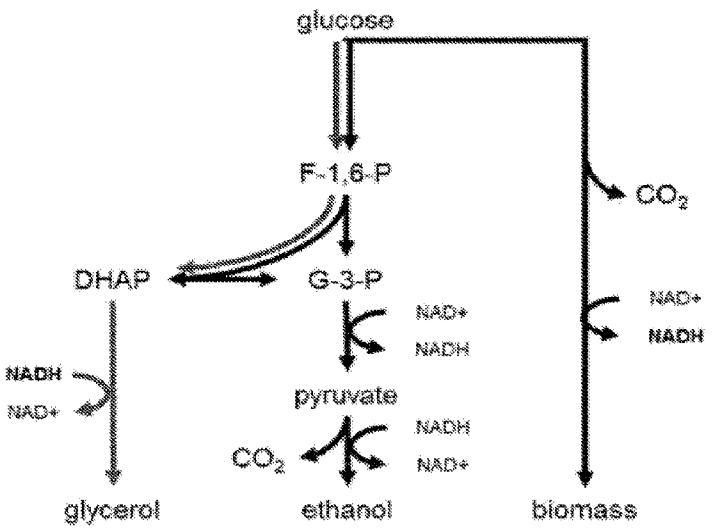

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The present invention provides for a mechanism to reduce glycerol production and increase nitrogen utilization and ethanol production of recombinant microorganisms. One aspect of this invention relates to strains of S. cerevisiae with reduced glycerol productivity that get a kinetic benefit from higher nitrogen concentration without sacrificing ethanol yield. A second aspect of the invention relates to -metabolic modifications resulting in altered transport and/or intracellular metabolism of nitrogen sources present in com mash.

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(54) Title: METHODS FOR REGULATING NITROGEN METABOLISM DURING THE PRODUCTION OF ETHANOL FROM CORN BY METABOLICALLY ENGINEERED YEAST STRAINS

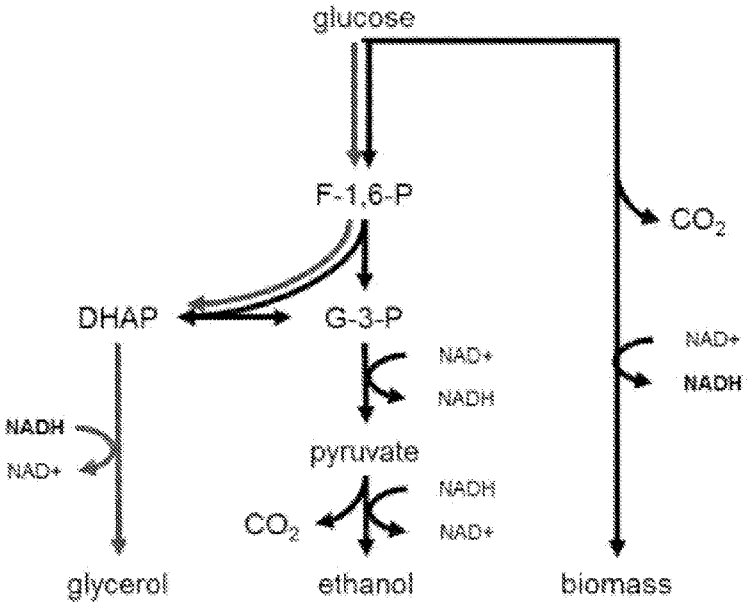

FIGURE 1

(57) Abstract: The present invention provides for a mechanism to reduce glycerol production and increase nitrogen utilization and ethanol production of recombinant microorganisms. One aspect of this invention relates to strains of *S. cerevisiae* with reduced glycerol productivity that get a kinetic benefit from higher nitrogen concentration without sacrificing ethanol yield. A second aspect of the invention relates to -metabolic modifications resulting in altered transport and/or intracellular metabolism of nitrogen sources present in com mash.

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METHODS FOR REGULATING NITROGEN METABOLISM DURING THE PRODUCTION OF ETHANOL FROM CORN BY METABOLICALLY ENGINEERED YEAST STRAINS

BACKGROUND OF THE INVENTION

[0001] Energy conversion, utilization and access underlie many of the great challenges of our time, including those associated with sustainability, environmental quality, security, and poverty. New applications of emerging technologies are required to respond to these challenges. Biotechnology, one of the most powerful of the emerging technologies, can give rise to important new energy conversion processes. Plant biomass and derivatives thereof are a resource for the biological conversion of energy to forms useful to humanity.

[0002] Among forms of plant biomass, both grain-based biomass and lignocellulosic biomass (collectively "biomass") are well-suited for energy applications. Each feedstock has advantages and disadvantages. For example, because of its large-scale availability, low cost, and environmentally benign production lignocellulosic biomass has gained attention as a viable feed source for biofuel production. In particular, many energy production and utilization cycles based on cellulosic biomass have near-zero greenhouse gas emissions on a life-cycle basis.

[0003] However, grain-based feed stocks are more readily converted to fuels by existing microorganisms, although grain-based feed stock is more expensive than lignocellulosic feed stock and conversion to fuel competes with alternative uses for the grain.

[0004] Biomass processing schemes involving enzymatic or microbial hydrolysis commonly involve four biologically mediated transformations: (1) the production of saccharolytic enzymes (cellulases and hemicellulases); (2) the hydrolysis of carbohydrate components present in pretreated biomass to sugars; (3) the fermentation of hexose sugars (*e.g.*, glucose, mannose, and galactose); and (4) the fermentation of pentose sugars (*e.g.*, xylose and arabinose). These four transformations can occur in a single step in a process configuration called consolidated bioprocessing ("CBP"), which is distinguished from other less highly integrated configurations in that it does not involve a dedicated process step for cellulase and/or hemicellulase production.

[0005] CBP offers the potential for lower cost and higher efficiency than processes featuring dedicated cellulase production. The benefits result in part from avoided capital

costs, substrate and other raw materials, and utilities associated with cellulase production. In addition, several factors support the realization of higher rates of hydrolysis, and hence reduced reactor volume and capital investment using CBP, including enzyme-microbe synergy and the use of thermophilic organisms and/or complexed cellulase systems. Moreover, cellulose-adherent cellulolytic microorganisms are likely to compete successfully for products of cellulose hydrolysis with non-adhered microbes, *e.g.*, contaminants. Successful competition of desirable microbes increases the stability of industrial processes based on microbial cellulose utilization. Progress in developing CBP-enabling microorganisms is being made through two strategies: engineering naturally occurring cellulolytic microorganisms to improve product-related properties, such as yield and titer; and engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase and hemicellulase system enabling cellulose and hemicellulose utilization.

[0006] One way to meet the demand for ethanol production is to convert sugars found in biomass, *i.e.*, materials such as agricultural wastes, corn hulls, corncobs, cellulosic materials, and the like to produce ethanol. Efficient biomass conversion in large-scale industrial applications requires a microorganism that is able to tolerate high concentrations of sugar and ethanol, and which is able to ferment more than one sugar simultaneously.

[0007] Bakers' yeast (*Saccharomyces cerevisiae*) is the preferred microorganism for the production of ethanol (Hahn-Hägerdal, B., *et al.*, *Adv. Biochem. Eng. Biotechnol. 73*, 53–84 (2001)). Attributes in favor of this microbe are (i) high productivity at close to theoretical yields (0.51 g ethanol produced/g glucose used), (ii) high osmo- and ethanol tolerance, (iii) natural robustness in industrial processes, also (iv) being generally regarded as safe (GRAS) due to its long association with wine and bread making, and beer brewing. Furthermore, *S. cerevisiae* exhibits tolerance to inhibitors commonly found in hydrolysates resulting from biomass pretreatment. Exemplary metabolic pathways for the production of ethanol are depicted in Figure 1. However, *S. cerevisiae* does not naturally break down components of cellulose, nor does it efficiently use pentose sugars.

[0008] Glycerol is a metabolic end-product of native yeast ethanolic fermentation (Figure 1). During anaerobic growth on carbohydrates, production of ethanol and carbon dioxide is redox neutral, while the reactions that create cell biomass and associated carbon

dioxide are more oxidized relative to carbohydrates. The production of glycerol, which is more reduced relative to carbohydrates, functions as an electron sink to off-set cell biomass formation, so that overall redox neutrality is conserved. This is essential from a theoretical consideration of conservation of mass, and in practice strains unable to produce glycerol are unable (or only very poorly able) to grow under anaerobic conditions.

[0009] There is a strong commercial incentive not to produce glycerol as a byproduct because it represents lost ethanol yield. In industrial corn ethanol fermentations, this yield loss can be up to 6% of theoretical, for a market of ~14 billion gallons/yr. At selling price of $2.50/gal, this is a total market value of $2 B/yr.

[0010] Strategies from the literature to address this problem include decreasing glycerol formation by engineering ammonia fixation to function with NADH instead of NADPH via up-regulation of GLN1, encoding glutamine synthetase, or GLT1, encoding glutamate synthase with deletion of GDH1, encoding the NADPH-dependent glutamate dehydrogenase. (Nissen, T.L., *et al.*, *Metabolic Engineering 2*: 69-77 (2000)). Another strategy engineering cells to produce excess NADPH during glycolysis via expression of a NADPH linked glyceraldehyde-3-phosphate dehydrogenase. (Bro, C., *et al.*, *Metabolic Engineering 8*: 102-111 (2006)). Another strategy contained a deletion of GDH1, and over-expression of glutamate synthase (GLT1) and glutamine synthase (GLN1), which also resulted in reduced glycerol formation. However, growth rates and biomass formation were well below the control strain and improvements on the initial performance have not been demonstrated. Additionally, industrially relevant yields, titers and fermentation rates were never demonstrated. (U.S. Patent No. 7,018,829). Another strategy describes deletion of only GDH1 without overexpression of GDH2 or GLT1/GLN1. However, the strategy was dependent on the use of an industrial polyploid yeast strain capable of tolerating high ethanol concentrations. It is noted in the patent that GDH1 was the only deletion, and that there are no heterologous DNA sequences in the genome. Additionally, the maximum reduction in glycerol production seen was 12.04%, and the technology was not demonstrated on an industrially relevant substrate (U.S. Patent No. 7,935,514). Most glycerol reduction strategies either only partially reduce the requirement for glycerol formation, or create a by-product other than ethanol. The present invention overcomes the shortcomings of these other strategies.

[0011] Corn mash contains free amino nitrogen. However the amount is too low to enable yeast biomass formation sufficient to meet the needs of the process. Nitrogen is added to industrial corn ethanol fermentations to promote yeast growth, most commonly in the form of urea and ammonia. Excess nitrogen can improve the fermentation kinetics of conventional yeasts; however ethanol yields are often lower due to excess biomass and glycerol formation. Typically, urea is added to industrial corn fermentations in concentrations that range from 500ppm to 1000ppm.

[0012] Yeast take up and assimilate ammonium as its preferred nitrogen source, followed by amino acids, and finally urea (Figures 2-4) (extensively reviewed by Lungdhal *et al.*, *Genetics 190*: 885-929 (2012)). The mechanism of nitrogen catabolite repression (NCR) control is established by transcription factors which control the expression of ammonium, amino acid and urea transporters. These transcription factors also control expression of proteins responsible for degradation and assimilation of nitrogen containing molecules. It has been shown that de-repression of non-preferred nitrogen source assimilation pathways can improve fermentation kinetics (Salmon, J. M., and Barre, P., *Appl. Environ. Microbiol. 64*:3831–3837 (1998)); however, effects on e thanol productivity were not measured.

[0013] *S. cerevisiae* contains three known ammonium transporters, MEP1, MEP2 and MEP3. MEP1 and MEP2 are high affinity transporters while MEP3 is a low affinity transporter. *S. cerevisiae* breaks down urea through the enzymatic action of a urea-amido lyase (EC 6.3.4.6). This activity is encoded by the enzyme DUR1/2 in *S. cerevisiae* (Figures 2-4). Overexpression of DUR1/2 in wine yeasts has been shown to enhance urea degradation rates during fermentation of grape must (Coulon, J., *et al.*, *Am. J. Enol. Vitic. 57*:2 (2006)). There are two known urea transporters in *S. cerevisiae*, DUR3 and DUR4 (Figures 2-4). It has been shown that overexpression of DUR3 resulted in improved urea degradation rates during wine fermentation (Dahabieh, M.S., *et al.*, *Am. J. Enol. Vitic. 60*:4 (2009)). U.S. Patent Publ. No. 2011/0129566 describes the expression of DUR1/2 and DUR3 in wine yeasts.

[0014] Industrial corn mash substrates contain as much as 3% protein (w/v); however, much of the amino acid content contained in these proteins is unavailable to *S. cerevisiae*. Expression of one or more proteases would release amino acids that could serve as a nitrogen source for yeast. Additionally, the use of amino acids as a nitrogen source for *S.*

cerevisiae in corn ethanol fermentations would improve yield through a reduction in the surplus NADH generated from *in vivo* amino acid synthesis during anaerobic growth.

[0015] Guo *et al.* engineered *S. cerevisiae* to express a heterologous protease for the purpose of improving ethanol yield (Guo, Z-p, *et al.*, *Enzyme and Microbial Technology 48*: 148-154 (2011)). However, the work was conducted in a wild type yeast background that had not been previously engineered for reduced glycerol formation, and the activity of the expressed endoprotease primarily breaks protein into short polypeptides which are not transported by *S. cerevisiae*.

[0016] One aspect of the present invention relates to improved fermentation performance through co-expression of an exoprotease to release single amino acids. Additionally, corn kernel protein is primarily a class of storage proteins known as zeins. Zeins have been shown to be recalcitrant to hydrolysis by many proteases and it is possible that expression of zein specific proteases would result in improved proteolysis. Thus, another aspect of the present invention relates to expressing zein-specific proteases to improve corn protein hydrolysis and amino acid utilization by the yeast.

[0017] Amino acids are transported by a large family of amino acid permeases. One aspect of this invention relates to deregulation or over-expression of a specific or general amino acid permease to complement protease expression or metabolic engineering by improving the uptake rate of free amino acids released during proteolysis. For example, expression of the general amino acid permease GAP1 is negatively regulated by AUA1. One aspect of this invention relates to the deletion of AUA1 or over expression of GAP1 that could result in improved amino acid uptake rates.

[0018] PCT/US2012/032443 teaches a method of eliminating glycerol formation through the production of formate. The formate production pathway can also be combined with strains engineered for reduced activity of the native glycerol production pathway. These combinations can be designed such that strains are built with different degrees of glycerol reduction as shown in Figure 5. Several embodiments of the current invention relate to a combination of those or related genetic modifications described in PCT/US2012/032443, with additional genetic modifications that are designed to alter nitrogen transport and assimilation.

[0019] One aspect of this invention relates to strains of *S. cerevisiae* with reduced glycerol production that get a kinetic benefit from higher nitrogen concentration without

sacrificing ethanol yield. A second aspect of the invention relates to metabolic modifications resulting in altered transport and/or intracellular metabolism of nitrogen sources present in corn mash.

BRIEF SUMMARY OF THE INVENTION

[0020] Some embodiments are direct to a recombinant microorganism comprising: at least one engineered genetic modification that leads to the up-regulation or down-regulation of one or more native and/or heterologous enzymes that function in one or more ethanol production pathways; at least one engineered genetic modification that leads to the down-regulation of an enzyme in a glycerol-production pathway: and at least one engineered genetic modification that leads to the up-regulation or down-regulation of an enzyme in a nitrogen-assimilation pathway.

[0020a] In one aspect of the invention, there is provided a recombinant yeast including: a. at least one engineered genetic modification that leads to the up-regulation of one or more native and/or heterologous enzymes that function in one or more ethanol production pathways; b. at least one engineered genetic modification that leads to the down-regulation of an enzyme in a glycerol-production pathway; and, c. at least one engineered genetic modification that leads to the up-regulation or down-regulation of an enzyme in a nitrogen-assimilation pathway; wherein the up-regulated enzyme that acts in an ethanol production pathway is pyruvate formate lyase (EC 2.3.1.54), pyruvate formate lyase activating enzyme (EC 1.91.1.4), and/or bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having both of the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.1; wherein the down-regulated enzyme that acts in the glycerol production pathway is at least one enzyme selected from the group consisting of: a glycerol-3-phosphate dehydrogenase 1 polynucleotide (GPD1) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 1 polypeptide (Gpd1) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 2 polynucleotide (GPD2) (EC 1.1 1.8), a glycerol-3-phosphate dehydrogenase 2 polypeptide (Gpd2) (EC 1.1 1 8), a glycerol-3-phosphate phosphatase I polynucleotide (GPP1) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase polypeptide 1 (Gpp1) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase 2 polynucleotide (GPP2) (EC 3.1.3.21), and a glycerol-3-phosphate phosphatase polypeptide 2 (Gpp2) (EC 3.1.3.21); and wherein the down-regulated enzyme that acts in the nitrogen-assimilation pathway is glutamate dehydrogenase (Gdh) (EC 1.4.1.4) and wherein the up-regulated enzyme in the

nitrogen-assimilation pathway is at least one enzyme selected from the group consisting of glutamate dehydrogenase (Gdh) (EC 1.4.1.2), glutamate synthase (Glt) (EC 1.4.1.14), and glutamine synthase (Gln) (EC 6.3.1.2), an ammonium transporter, a urea amido lyase (EC 6.3.4.6), and a urea transporter.

[0021] In some embodiments of the invention, the down-regulated enzyme in the nitrogen-assimilation pathway is glutamate dehydrogenase (Gdh) (EC 1.4.1.4).

[0022] In some embodiments of the invention, the microorganism further comprises least one genetic modification that leads to the up-regulation of an enzyme in a nitrogen-assimilation pathway.

[0023] In some embodiments of the invention, the up-regulated enzyme in the nitrogen-assimilation pathway is at least one enzyme selected from the group consisting of glutamate dehydrogenase (Gdh) (EC 1.4.1.2), glutamate synthase (Glt) (EC 1.4.1.14), and glutamine synthase (Gln) (EC 6.3.1.2). In some embodiments of the invention, the up-regulated enzyme in the nitrogen-assimilation pathway is a native ammonium transporter. In some embodiments of the invention, the up-regulated enzyme in the nitrogen-assimilation pathway is a MEP protein from the genus *Saccharomyces*. In some embodiments of the invention, the up-regulated enzyme in the nitrogen assimilation pathway is a urea-amido lyase (EC 6.3.4.6). In some embodiments of the invention, the up-regulated enzyme in the nitrogen assimilation pathway is a urea transporter. In some embodiments of the invention, the up-regulated enzyme in the nitrogen assimilation pathway is G1n3.

[0024] In some embodiments of the invention, the enzyme in the glycerol-production pathway is encoded by at least one enzyme selected from the group consisting of: a glycerol-3-phosphate dehydrogenase 1 polynucleotide (*GPD1*) (EC 1.1.1.8), a glycerol-3-

phosphate dehydrogenase 1 polypeptide (Gpd1) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 2 polynucleotide (*GPD2*) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 2 polypeptide (Gpd2) (EC 1.1.1.8), a glycerol-3-phosphate phosphatase 1 polynucleotide (*GPP1*) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase polypeptide 1 (Gpp1) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase 2 polynucleotide (*GPP2*) (EC 3.1.3.21), and a glycerol-3-phosphate phosphatase polypeptide 2 (Gpp2) (EC 3.1.3.21).

[0025] In some embodiments of the invention, up-regulated enzyme that acts in an ethanol production pathway is pyruvate formate lyase (EC 2.3.1.54). In some embodiments of the invention, the up-regulated enzyme that acts in the ethanol production pathway is pyruvate formate lyase activating enzyme (EC 1.91.1.4).

[0026] In some embodiments of the invention, the up-regulated enzyme that acts in the ethanol production pathway is bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having both of the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.1.

[0027] In some embodiments of the invention, the up-regulated enzyme that acts in the ethanol production pathway is an NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having both of the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.2.

[0028] In some embodiments, the microorganism further comprises a down-regulation in one or more native enzymes encoded by a formate dehydrogenase enzyme selected from the group consisting of: EC 1.2.1.43 and EC 1.2.1.2.

[0029] In some embodiments of the invention, the recombinant microorganism further comprises a heterologous GPD1 polynucleotide operably linked to a native GPD2 promoter. In some embodiments of the invention, the recombinant microorganism further comprises a heterologous GPD2 polynucleotide operably linked to a native GPD1 promoter.

[0030] In some embodiments of the invention, the microorganism further comprises an up-regulation or down-regulation of a regulatory element. In some embodiments the regulatory element is selected from the group consisting of: Ure2 and Aua1.

[0031] In some embodiments of the invention, the microorganism further comprises at least one additional up-regulated enzyme. In some embodiments of the invention, the additional up-regulated enzyme is a glucoamylase enzyme with EC number 3.2.1.3. In

some embodiments of the invention, the additional up-regulated enzyme is a permease. In some embodiments of the invention, the additional up-regulated enzyme is a protease with EC number: 3.4.23.41.

[0032] In some embodiments of the invention, the up-regulated or down-regulated enzymes are under the control of a heterologous promoter. In some embodiments of the invention, the heterologous promoter is selected from a group consisting of: *TEF2* (SEQ ID NO: 58), *HXT7* (SEQ ID NO: 59), *ADH1* (SEQ ID NO: 60), and *TPI* (SEQ ID NO: 61).

[0033] In some embodiments, the microorganism is a yeast. In some embodiments, the yeast is from the genus *Saccharomyces*. In some embodiments, the yeast is *Saccharomyces cerevisiae*. In some embodiments, the microorganism produces ethanol at a higher yield than an otherwise identical microorganism lacking the genetic modifications. In some embodiments, the microorganism produces an ethanol titer about 1% to about 10% more than an otherwise identical microorganism lacking the genetic modifications. In some embodiments, the microorganism produces an ethanol titer of at least about 125 g/L.

[0034] In some embodiments, the microorganism produces glycerol at a lower yield than an otherwise identical microorganism lacking the genetic modifications. In some embodiments, the microorganism produces a glycerol titer of about 10 to about 100% less than an otherwise identical microorganism lacking the genetic modifications.

[0035] In some embodiments, the invention relates to a composition comprising any recombinant microorganism herein, and a carbon-containing feedstock.

[0036] Some embodiments of the invention are directed to a method of producing a fermentation product using any composition herein, wherein the recombinant microorganism is capable of fermenting the carbon containing feedstock to yield the fermentation product.

[0037] Some embodiments of the invention are directed to a method of producing a fermentation product comprising: any composition provided herein; contacting the composition with a carbon containing feedstock, wherein the recombinant microorganism is capable of fermenting the carbon containing feedstock to yield the fermentation product; and, optionally recovering the fermentation production

[0038] Some embodiments of the invention are directed to a method of producing ethanol comprising: providing any recombinant microorganism herein; culturing the recombinant microorganism in the presence of a carbon containing feedstock for sufficient time to produce ethanol; and, optionally, extracting the ethanol.

[0039] Some embodiments of the invention are directed to a co-culture comprising at least two host cells, wherein one of the host cells comprises any recombinant microorganism herein; and another host cell that is genetically distinct from the recombinant microorganism.

[0040] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, *GPD1* under the control of the *GPD2* promoter, *GPD2* under the control of the *GPD1* promoter, and up-regulated Gdh2.

[0041] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, *GPD1* under the control of the *GPD2* promoter, *GPD2* under the control of the *GPD1* promoter, up-regulated Glt1 and up-regulated Gln1.

[0042] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activated enzyme, *GPD1* under the control of the *GPD2* promoter, *GPD2* under the control of the *GPD1* promoter, up-regulated Glt1 and up-regulated Gln1.

[0043] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0044] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-

regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and *GPD2* under the control of the *GPD1* promoter.

[0045] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Fdhl, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0046] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, upregulated-DUR/12, and *GPD2* under the control of the *GPD1* promoter.

[0047] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and up-regulated-DUR/12.

[0048] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Ure2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, *GPD1* under the control of the *GPD2* promoter, and *GPD2* under the control of the *GPD1* promoter.

[0049] Some embodiments of the invention are directed to a recombinant microorganism comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdhl, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, up-regulated *GDH2*, *GPD1* under the control of the *GPD2* promoter, and *GPD2* under the control of the *GPD1* promoter.

[0049a] In one aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0049b] In another aspect of the invention, there is provided a recombinant yeast including down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE,

up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0049c] In a further aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0049d] In yet another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, and up-regulated Gdh2.

[0049e] In another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, up-regulated Glt1 and up-regulated Gln1.

[0049f] In a further aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, up-regulated Glt1 and up-regulated Gln1.

[0049g] In yet another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0049h] In another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and GPD2 under the control of the GPD1 promoter.

[0049i] In a further aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated

Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

[0049j] In another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, upregulated-DUR/12, and GPD2 under the control of the GPD1 promoter.

[0049k] In yet another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and up-regulated-DUR/12.

[0049l] In another aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Ure2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, and GPD2 under the control of the GPD1 promoter.

[0049m] In a further aspect of the invention, there is provided a recombinant yeast including: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, up-regulated GDH2, GPD1 under the control of the GPD2 promoter, and GPD2 under the control of the GPD1 promoter.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0050] Figure 1 depicts simplified carbon and redox pathways utilized by wildtype *S. cerevisiae* during anaerobic growth. Ethanol formation is redox neutral while cell biomass formation generates net NADH, which is balanced by glycerol formation.

[0051] Figure 2 depicts urea transport and intracellular catabolism. The enzymes Dur3 and Dur4 are known transporters of urea. Once inside the cell, urea is broken down into 2 ammonia molecules and 2 carbon dioxide molecules in a reaction catalyzed by Dur1,2.

[0052] Figure 3 depicts the process by which an unmodified *S. cerevisiae* assimilates urea.

[0053] Figure 4 depicts process by which a genetically modified glycerol reduction strain which contains a deletion of Gdh1 assimilates urea.

[0054] Figure 5 depicts the glycerol titers of the wild type and glycerol reduction strains containing the formate pathway (M2390 (wildtype), M3465, M3467, M3469, and M3624 are depicted). This data shows total glycerol present in corn mash which contained 7 g/l glycerol prior to fermentation.

[0055] Figure 6 depicts a schematic diagram of the MA0631 insertion cassette.

[0056] Figure 7 depicts a schematic diagram of the MA0425 insertion cassette.

[0057] Figure 8 depicts a schematic diagram of the MA0426 insertion cassette.

[0058] Figure 9 depicts a schematic diagram of the MA0888 insertion cassette.

[0059] Figure 10 depicts a schematic diagram of the MA0837 insertion cassette.

[0060] Figure 11 depicts a schematic diagram of the MA0616 insertion cassette.

[0061] Figure 12 depicts a schematic diagram of the MA0616.1 insertion cassette.

[0062] Figure 13 depicts a schematic diagram of the MA0615 insertion cassette.

[0063] Figure 14 depicts a schematic diagram of the MA0615.1 insertion cassette.

[0064] Figure 15 depicts a schematic diagram of the MA0622 insertion cassette.

[0065] Figure 16 depicts a schematic diagram of the MA0622.1 insertion cassette.

[0066] Figure 17 depicts a schematic diagram of the MA0580 insertion cassette.

[0067] Figure 18 depicts a schematic diagram of the MA0581 insertion cassette.

[0068] Figure 19 depicts a schematic diagram of the MA0582 insertion cassette.

[0069] Figure 20 depicts a schematic diagram of the MA0583 insertion cassette.

[0070] Figure 21 depicts a schematic diagram of the MA0584 insertion cassette.

[0071] Figure 22 depicts a schematic diagram of the MA0585 insertion cassette.

[0072] Figure 23 depicts a schematic diagram of the MA0617 insertion cassette.

[0073] Figure 24 depicts a schematic diagram of the MA0617.1 insertion cassette.

[0074] Figure 25 depicts a schematic diagram of the MA0434 insertion cassette.

[0075] Figure 26 depicts a schematic diagram of the MA0434.2 insertion cassette.

[0076] Figure 27 depicts a schematic diagram of the MA0434.3 insertion cassette.

[0077] Figure 28 depicts a schematic diagram of the MA0434.4 insertion cassette.

[0078] Figure 29 depicts a schematic diagram of the MA0434.5 insertion cassette.

[0079] Figure 30 depicts a schematic diagram of the MA0454.14 insertion cassette.

[0080] Figure 31 depicts a schematic diagram of the MA0464 insertion cassette.

[0081] Figure 32 depicts a schematic diagram of the MA0464.1 insertion cassette.

[0082] Figure 33 depicts a schematic diagram of the MA0464.2 insertion cassette.

[0083] Figure 34 depicts a schematic diagram of the MA0464.3 insertion cassette.

[0084] Figure 35 depicts a schematic diagram of the MA0464.4 insertion cassette.

[0085] Figure 36 depicts a schematic diagram of the MA0464.5 insertion cassette.

[0086] Figure 37 depicts a schematic diagram of the MA0465.1 insertion cassette.

[0087] Figure 38 depicts a schematic diagram of the MA0467 insertion cassette.

[0088] Figure 39 depicts a schematic diagram of the MA0467.1 insertion cassette.

[0089] Figure 40 depicts a schematic diagram of the MA0467.2 insertion cassette.

[0090] Figure 41 depicts a schematic diagram of the MA0467.3 insertion cassette.

[0091] Figure 42 depicts a schematic diagram of the MA0467.4 insertion cassette.

[0092] Figure 43 depicts a schematic diagram of the MA0881insertion cassette.

[0093] Figure 44 depicts a schematic diagram of the MA0881.1 insertion cassette.

[0094] Figure 45 depicts a plasmid map for pMU2873.

[0095] Figure 46 depicts a plasmid map for pMU2879.

[0096] Figure 47 depicts a plasmid map for pMU2908.

[0097] Figure 48 depicts a plasmid map for pMU2909.

[0098] Figure 49 depicts a plasmid map for pMU2911.

[0099] Figure 50 depicts a plasmid map for pMU2913.

[0100] Figure 51 depicts a plasmid map for pMU3409.

[0101] Figure 52 depicts a plasmid map for pMU3410.

[0102] Figure 53 depicts a plasmid map for pMU3411.

[0103] Figure 54 depicts a plasmid map for pMU3459.

[0104] Figure 55 depicts a plasmid map for pMU3460.

[0105] Figure 56 depicts a plasmid map for pMU3461.

[0106] Figure 57 depicts a plasmid map for pMU3463.

[0107] Figure 58 depicts a plasmid map for pMU3464.

[0108] Figure 59 depicts a plasmid map for pMU3465.

[0109] Figure 60 depicts a plasmid map for pMU3466.

[0110] Figure 61 depicts a plasmid map for pMU3468.

[0111] Figure 62 depicts a plasmid map for pMU3471.

[0112] Figure 63 depicts a plasmid map for pMU3472.

[0113] Figure 64 depicts a plasmid map for pMU3473.

[0114] Figure 65 depicts a plasmid map for pMU3475.

[0115] Figure 66 depicts a plasmid map for pMU3605.

[0116] Figure 67 depicts a plasmid map for pMU3606.

[0117] Figure 68 depicts a plasmid map for pMU3607.

[0118] Figure 69 depicts the final ethanol titers measured following fermentation of 31% solids corn mash in wildtype cells (M2390), a glycerol reduction strain containing the formate pathway (M3624), and 2 strains with modification of the ammonium assimilation pathway (M4117, which contains a deletion of *gdh1* and an over-expression of *Gdh2*, and M4118, which contains a deletion of gdh1 and an over-expression of *Glt1* and *Glu1*).

[0119] Figure 70 depicts the ethanol titers measured following fermentation of 31% solids corn mash for glycerol reduction strains containing the formate pathway (M3465, M3467, M3469) that additionally have a deletion of *gdh1* (M4400, M4401, M4402). M2390 was a parental control. Figure 71 depicts the ethanol titers measured following fermentation of 31% solids corn mash for M2390, M3467, M3469, M4427 (M3467 parent strain: expression of DUR1/2 driven by the TEF2 promoter), M4428 (M3467 parent strain: expression of DUR1/2 driven by the HXT7 promoter), M4429 (M3467 parent strain: expression of DUR1/2 driven by the ADH1 promoter), M4430 (M3467 parent strain: expression of DUR1/2 driven by the HXT7/TEF2 promoters), M4431 (M3469 parent strain: expression of DUR1/2 driven by the TEF2 promoter), M4432 (M3469 parent strain: expression of DUR1/2 driven by the HXT7 promoter), M4433 (M3469 parent strain: expression of DUR1/2 driven by the ADH1 promoter), and M4434 (M3469 parent strain: expression of DUR1/2 driven by the HXT7/TEF2 promoters)

[0120] Figure 72 depicts the ethanol titers measured following fermentation of 31% solids corn mash for M2390, M3624, M4406, and M4407.

[0121] Figure 73 depicts the ethanol titers produced after 68hrs fermentation in mini-vials for strains M2390, M3624, M4117, M5841, M5842, M5843, and M5844.

[0122] Figure 74 depicts the glycerol titers produced after 68hrs fermentation in mini-vials for strains M2390, M3624, M4117, M5841, M5842, M5843, and M5844.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0123] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art of microbial metabolic engineering. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, exemplary methods, devices and materials are described herein.

[0124] The embodiments described, and references in the specification to "one embodiment", "an embodiment", "an example embodiment", etc., indicate that the embodiments described can include a particular feature, structure, or characteristic, but every embodiment does not necessarily include the particular feature, structure, or characteristic. M oreover, such phrases are not necessarily referring to the same embodiment. Further, when a particular feature, structure, or characteristic is described in connection with an embodiment, it is understood that it is within the knowledge of one skilled in the art to effect such feature, structure, or characteristic in connection with other embodiments whether or not explicitly described.

[0125] The description of "a" or "an" item herein may refer to a single item or multiple items. It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided. Thus, for example, reference to "a polynucleotide" includes a plurality of such polynucleotides and reference to "the microorganism" includes reference to one or more microorganisms, and so forth.

[0126] The term "heterologous" is used in reference to a polynucleotide or a gene not normally found in the host organism. "Heterologous" includes up-regulated or down-regulated endogenous genes. "Heterologous" also includes a native coding region, or portion thereof, that is reintroduced into the source organism in a form that is different from the corresponding native gene, *e.g.*, not in its natural location in the organism's genome. "Heterologous" also includes any gene that has been modified and placed into an organism. A heterologous gene may include a native coding region that is a portion of

a chimeric gene including a non-native regulatory region that is reintroduced into the native host or modifications to the native regulatory sequences that affect the expression level of the gene. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A heterologous polynucleotide, gene, polypeptide, or an enzyme may be derived or isolated from any source, *e.g.*, eukaryotes, prokaryotes, viruses, or synthetic polynucleotide fragments, and includes up-regulated endogenous genes.

[0127] The terms "gene(s)" or "polynucleotide" or "nucleic acid" or "polynucleotide sequence(s)" are intended to include nucleic acid molecules, *e.g.*, polynucleotides which include an open reading frame encoding a polypeptide, and can further include non-coding regulatory sequences, and introns. In addition, the terms are intended to include one or more genes that map to a functional locus. Also, the terms are intended to include a specific gene for a selected purpose. The gene may be endogenous to the host cell or may be recombinantly introduced into the host cell, *e.g.*, as a plasmid maintained episomally or a plasmid (or fragment thereof) that is stably integrated into the genome. In addition to the plasmid form, a gene may, for example, be in the form of linear DNA or RNA. The term "gene" is also intended to cover multiple copies of a particular gene, *e.g.*, all of the DNA sequences in a cell encoding a particular gene product. A "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. "Gene" also refers to a nucleic acid fragment that expresses a specific protein, including intervening sequences (introns) between individual coding segments (exons), as well as regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native" or "endogenous" refers to a gene as found in nature with its own regulatory sequences.

[0128] A "nucleic acid," "polynucleotide," or "nucleic acid molecule" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semi-synthetic DNA.

[0129] An "isolated nucleic acid molecule" or "isolated nucleic acid fragment" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine,

deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

[0130] The term "expression" is intended to include the expression of a gene at least at the level of mRNA production, generally subsequently translated into a protein product. The term "expression," refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

[0131] As used herein, an "expression vector" is a vector capable of directing the expression of genes to which it is operably linked.

[0132] A "vector," *e.g.*, a "plasmid" or "YAC" (yeast artificial chromosome) refers to an extrachromosomal element often carrying one or more genes that are not part of the central metabolism of the cell, and is usually in the form of a circular double-stranded DNA molecule. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear, circular, or supercoiled, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. Preferably, the plasmids or vectors of the present invention are stable and self-replicating.

[0133] The term "integrated" as used herein refers to genetic elements that are placed, through molecular biology techniques, into a chromosome of a host cell. For example, genetic elements can be placed into the chromosomes of the host cell as opposed to in a vector such as a plasmid carried by the host cell. Methods for integrating genetic

elements into the genome of a host cell are well known in the art and include homologous recombination.

[0134] The term "domain' as used herein refers to a part of a molecule or structure that shares common physical or chemical features, for example hydrophobic, polar, globular, helical domains or properties, *e.g.*, a DNA binding domain or an ATP binding domain. Domains can be identified by their homology to conserved structural or functional motifs. Examples of cellobiohydrolase (CBH) domains include the catalytic domain (CD) and the cellulose binding domain (CBD).

[0135] A nucleic acid molecule is "hybridizable to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified, *e.g.*, in Sambrook, J., Fritsch, E. F. and Maniatis, T. MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein (hereinafter "Maniatis"). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. For more stringent conditions, washes are performed at higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS are increased to 60°C. Another set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C. An additional set of highly stringent conditions are defined by hybridization at 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS.

[0136] Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches

between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of Tm for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher Tm) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating Tm have been derived (*see*, *e.g.*, Maniatis at 9.50-9.51). For hybridizations with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see*, *e.g.*, Maniatis, at 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferably a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

[0137] The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences.

[0138] As known in the art, "similarity" between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide.

[0139] "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, NY (1991). Preferred methods to determine identity are designed to give the best match

between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Multiple alignments of the sequences disclosed herein were performed using the Clustal method of alignment (Higgins and Sharp (1989) CABIOS. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

[0140] Suitable nucleic acid sequences or fragments thereof (isolated polynucleotides of the present invention) encode polypeptides that are at least about 70% to about 75% identical to the amino acid sequences reported herein, at least about 80%, about 85%, or about 90% identical to the amino acid sequences reported herein, or at least about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to the amino acid sequences reported herein. Suitable nucleic acid fragments are at least about 70%, about 75%, or about 80% identical to the nucleic acid sequences reported herein, at least about 80%, about 85%, or about 90% identical to the nucleic acid sequences reported herein, or at least about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to the nucleic acid sequences reported herein. Suitable nucleic acid fragments not only have the above identities/similarities but typically encode a polypeptide having at least 50 amino acids, at least 100 amino acids, at least 150 amino acids, at least 200 amino acids, or at least 250 amino acids.

[0141] A DNA or RNA "coding region" is a DNA or RNA molecule which is transcribed and/or translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. "Suitable regulatory regions" refer to nucleic acid regions located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding region, and which influence the transcription, RNA processing or stability, or translation of the associated coding region. Regulatory regions may include promoters, translation leader sequences, RNA processing site, effector binding site and stem-loop structure. The boundaries of the coding region are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding region can include, but is not limited to, prokaryotic

regions, cDNA from mRNA, genomic DNA molecules, synthetic DNA molecules, or RNA molecules. If the coding region is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding region.

[0142] An "isoform" is a protein that has the same function as another protein but which is encoded by a different gene and may have small differences in its sequence.

[0143] A "paralogue" is a protein encoded by a gene related by duplication within a genome.

[0144] An "orthologue" is gene from a different species that has evolved from a common ancestral gene by speciation. Normally, orthologues retain the same function in the course of evolution as the ancestral gene.

[0145] "Open reading frame" is abbreviated ORF and means a length of nucleic acid, either DNA, cDNA or RNA, that comprises a translation start signal or initiation codon, such as an ATG or AUG, and a termination codon and can be potentially translated into a polypeptide sequence.

[0146] "Promoter" refers to a DNA fragment capable of controlling the expression of a coding sequence or functional RNA. In general, a coding region is located 3' to a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of cellular development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity. A promoter is generally bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[0147] A coding region is "under the control" of transcriptional and translational control elements in a cell when RNA polymerase transcribes the coding region into mRNA, which is then trans-RNA spliced (if the coding region contains introns) and translated into the protein encoded by the coding region.

[0148] "Transcriptional and translational control regions" are DNA regulatory regions, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding region in a host cell. In eukaryotic cells, polyadenylation signals are control regions.

[0149] The term "operably associated" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably associated with a coding region when it is capable of affecting the expression of that coding region (*i.e.*, that the coding region is under the transcriptional control of the promoter). Coding regions can be operably associated to regulatory regions in sense or antisense orientation.

[0150] As used herein, the term "anaerobic" refers to an organism, biochemical reaction or process that is active or occurs under conditions of an absence of gaseous O_2.

[0151] "Anaerobic conditions" are defined as conditions under which the oxygen concentration in the fermentation medium is too low for the microorganism to use as a terminal electron acceptor. Anaerobic conditions may be achieved by sparging a fermentation medium with an inert gas such as nitrogen until oxygen is no longer available to the microorganism as a terminal electron acceptor. Alternatively, anaerobic conditions may be achieved by the microorganism consuming the available oxygen of fermentation until oxygen is unavailable to the microorganism as a terminal electron acceptor.

[0152] "Aerobic metabolism" refers to a biochemical process in which oxygen is used as a terminal electron acceptor to convert energy, typically in the form of ATP, from carbohydrates. Aerobic metabolism typically occurs, for example, via the electron transport chain in mitochondria in eukaryotes, wherein a single glucose molecule is metabolized completely into carbon dioxide in the presence of oxygen.

[0153] In contrast, "anaerobic metabolism" refers to a biochemical process in which oxygen is not the final acceptor of electrons generated. Anaerobic metabolism can be divided into anaerobic respiration, in which compounds other than oxygen serve as the

terminal electron acceptor, and substrate level phosphorylation, in which no exogenous electron acceptor is used and products of an intermediate oxidation state are generated via a "fermentative pathway."

[0154] In "fermentative pathways", the amount of NAD(P)H generated by glycolysis is balanced by the consumption of the same amount of NAD(P)H in subsequent steps. For example, in one of the fermentative pathways of certain yeast strains, NAD(P)H generated through glycolysis donates its electrons to acetaldehyde, yielding ethanol. Fermentative pathways are usually active under anaerobic conditions but may also occur under aerobic conditions, under conditions where NADH is not fully oxidized via the respiratory chain.

[0155] As used herein, the term "end-product" refers to a chemical compound that is not or cannot be used by a cell, and so is excreted or allowed to diffuse into the extracellular environment. Common examples of end-products from anaerobic fermentation include, but are not limited to, ethanol, acetic acid, formic acid, lactic acid, hydrogen and carbon dioxide.

[0156] As used herein, "cofactors" are compounds involved in biochemical reactions that are recycled within the cells and remain at approximately steady state levels. Common examples of cofactors involved in anaerobic fermentation include, but are not limited to, NAD^+ and $NADP^+$. In metabolism, a cofactor can act in oxidation-reduction reactions to accept or donate electrons. When organic compounds are broken down by oxidation in metabolism, their energy can be transferred to NAD^+ by its reduction to NADH, to $NADP^+$ by its reduction to NADPH, or to another cofactor, FAD^+, by its reduction to $FADH_2$. The reduced cofactors can then be used as a substrate for a reductase.

[0157] As used herein, a "pathway" is a group of biochemical reactions that together can convert one compound into another compound in a step-wise process. A product of the first step in a pathway may be a substrate for the second step, and a product of the second step may be a substrate for the third, and so on. P athways of the present invention include, but are not limited to, the pyruvate metabolism pathway the lactate production pathway, the ethanol production pathway, the glycerol-production pathway, the nitrogen assimilation pathway, and the ammonium assimilation pathway.

[0158] The term "recombination" or "recombinant" refers to the physical exchange of DNA between two identical (homologous), or nearly identical, DNA molecules.

Recombination can be used for targeted gene deletion or to modify the sequence of a gene. The term "recombinant microorganism" and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or over-express endogenous polynucleotides, or to express heterologous polynucleotides, such as those included in a vector, or which have a modification in expression of an endogenous gene.

[0159] By "expression modification" it is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more polypeptides or polypeptide subunits, or activity of one or more polypeptides or polypeptide subunits is up regulated or down-regulated, such that expression, level, or activity, is greater than or less than that observed in the absence of the modification.

[0160] In one aspect of the invention, genes or particular polynucleotide sequences are partially, substantially, or completely deleted, silenced, inactivated, or down-regulated in order to inactivate the enzymatic activity they encode. Complete deletions provide maximum stability because there is no opportunity for a reverse mutation to restore function. Alternatively, genes can be partially, substantially, or completely deleted, silenced, inactivated, or down-regulated by insertion, deletion, removal or substitution of nucleic acid sequences that disrupt the function and/or expression of the gene.

[0161] As used herein, the term "down-regulate" includes the deletion or mutation of a genetic sequence, or insertion of a disrupting genetic element, coding or non-coding, such that the production of a gene product is lessened by the deletion, mutation, or insertion. It includes a decrease in the expression level (*i.e.*, molecular quantity) of an mRNA or protein. "Delete" or "deletion" as used herein refers to a removal of a genetic element such that a corresponding gene is completely prevented from being expressed. In some embodiments, deletion refers to a complete gene deletion. Down-regulation can also occur by engineering the repression of genetic elements by chemical or other environmental means, for example by engineering a chemically-responsive promoter element (or other type of conditional promoter) to control the expression of a desired gene product. Down-regulation can also occur through use of a weak promoter.

[0162] As used herein, the term "up-regulate" includes the insertion, reintroduction, mutation, or increased expression of a genetic sequence, such that the production of a gene product is increased by the insertion, reintroduction, or mutation. It includes an

increase in the expression level (*i.e.*, molecular quantity) of an mRNA or protein. "Insert" or "insertion" as used herein refers to an introduction of a genetic element such that a corresponding gene is expressed. Up-regulation can also occur by causing the increased expression of genetic elements through an alteration of the associated regulatory sequence. Up-regulation can occur by engineering the expression of genetic elements by chemical or other environmental means, for example by engineering a chemically-responsive promoter element (or other type of conditional promoter) to control the expression of a desired gene product. Up-regulation can also occur through use of a strong promoter.

[0163] As used herein, the term "glycerol-production pathway" refers to the collection of biochemical pathways that produce glycerol from DHAP. Components of the pathway consist of all substrates, cofactors, byproducts, intermediates, end-products, and enzymes in the pathway.

[0164] As used herein, the term "ethanol production pathway" refers the collection of biochemical pathways that produce ethanol from a carbohydrate source. Components of the pathway consist of all substrates, cofactors, byproducts, intermediates, end-products, and enzymes in the pathway.

[0165] As used herein, the term "nitrogen assimilation pathway" refers to the collection of biochemical pathways that result in the formation of organic nitrogen containing compounds from inorganic nitrogen compounds. Components of the pathway consist of all substrates, cofactors, byproducts, intermediates, end-products, and enzymes in the pathway.

[0166] As used herein, the term "ammonium assimilation pathway" refers to the collection of biochemical pathways that assimilate ammonia or ammonium (NH_4^+) into glutamate and/or glutamine. The ammonium assimilation pathway is part of the larger nitrogen assimilation pathway. Components of the pathway consist of all substrates, cofactors, byproducts, intermediates, end-products, and enzymes in the pathway.

[0167] As used herein, the term "glycolysis" or "glycolytic pathway" refers to the canonical pathway of basic metabolism in which a sugar such as glucose is broken down into more oxidized products, converting energy and compounds required for cell growth. Components of the pathway consist of all substrates, cofactors, byproducts, intermediates end-products, and enzymes in the pathway.

[0168] As used herein, the term "alcohol dehydrogenase" or "ADH" is intended to include the enzymes that catalyze the conversion of ethanol into acetylaldehyde. Very commonly, the same enzyme catalyzes the reverse reaction from acetaldehyde to ethanol, which is the direction more relevant to fermentation. Alcohol dehydrogenase includes those enzymes that correspond to EC 1.1.1.1 and 1.1.1.2 and exemplified by the enzymes disclosed in GenBank Accession No. U49975.

[0169] As used herein, the term "aldehyde dehydrogenase", "ALD" or "ALDH" is intended to include the enzymes that catalyze the oxidation of aldehydes. Aldehyde dehydrogenase enzymes include "acetaldehyde dehydrogenase", which catalyzes the conversion of acetaldehyde into acetyl-CoA. Very commonly, the same enzyme catalyzes the reverse reaction from acetyl-CoA to acetaldehyde, which is the direction more relevant to fermentation. Aldehyde dehydrogenase includes those enzymes that correspond to EC 1.2.1.3, 1.2.1.4 and 1.2.1.10.

[0170] As used herein, the term "glycerol-3-phosphate dehydrogenase" or "GPD" is intended to include those enzymes capable of converting dihydroxyacetone phosphate to glycerol-3-phosphate. GPD includes those enzymes that correspond to EC 1.1.1.8. In some embodiments, the GPD is GPD1 and/or GPD2 from *S. cerevisiae* (GDP1: SEQ ID NO: 4 and 5, GDP2: SEQ ID NO: 6 and 7).

[0171] As used herein, the term "glycerol-3-phosphate phosphatase" or "GPP" is intended to include those enzymes capable of converting glycerol-1-phosphate to glycerol. Glycerol-3-phosphate is intended to include those enzymes that correspond to EC 3.1.3.21. (GPP1: SEQ ID NO: 158 and 159, GPP2: SEQ ID NO 160 and 161)

[0172] As used herein, the term "formate dehydrogenase" or "FDH" is intended to include those enzymes capable of converting formate to bicarbonate (carbon dioxide). Formate dehydrogenase includes those enzymes that correspond to EC 1.2.1.43 and EC 1.2.1.2. In some embodiments, the FDH is from *S. cerevisiae* (FDH1: SEQ ID NO: 1 and 2, FDH2: SEQ ID NO: 3).

[0173] As used herein, the term "bifunctional" is intended to include enzymes that catalyze more than one biochemical reaction step. A specific example of a bifunctional enzyme used herein is an enzyme (adhE) that catalyzes both the alcohol dehydrogenase and acetaldehyde dehydrogenase reactions, and includes those enzymes that correspond to EC 1.2.1.10 and 1.1.1.1. In some embodiments, the bifunctional acetaldehyde-alcohol

dehydrogenase is from *B. adolescentis* (adhE: SEQ ID NO: 12 and 13). In some embodiments, the bifunctional enzyme is a NADPH specific bifunctional acetaldehyde-alcohol dehydrogenase, and includes those enzymes that correspond to EC 1.2.1.10 and 1.1.1.2. In some embodiments, the NADPH specific bifunctional acetaldehyde-alcohol dehydrogenase is from *L. mesenteroides* (SEQ ID NO: 14 and 15) or *O. oeni* (SEQ ID NO: 16 and 17).

[0174] As used herein, the term "pyruvate formate lyase" or "PFL" is intended to include the enzymes capable of converting pyruvate to formate and acetyl-CoA. PFL includes those enzymes that correspond to EC 2.3.1.54 and exemplified by SEQ ID NO: 8 and 9.

[0175] As used herein, the term "PFL-activating enzymes" is intended to include those enzymes capable of aiding in the activation of PFL. PFL-activating enzymes include those enzymes that correspond to EC 1.97.1.4 and are exemplified by SEQ ID NO: 10 and 11.

[0176] As used herein, the term "glutamate dehydrogenase", "GDH", or "GLDH" is intended to include those enzymes that convert glutamate to α-ketoglutarate, as well as those enzymes that catalyze the reverse reaction. The glutamate dehydrogenase can be NADPH-dependent (*e.g.* GDH1 or GDH3 in *S. cerevisiae*). The glutamate dehydrogenase can be NADH-dependent (*e.g.* GDH2 in *S. cerevisiae*). Glutamate dehydrogenases include those enzymes that correspond to EC 1.4.1.2 and EC 1.4.1.4. Glutamate dehydrogenases include those enzymes that correspond to accession numbers: M10590, S66436, S 66039.1, U12980, NP_015020, NP_010066, S66039.1 and AAC04972. In some embodiments, the glutamate dehydrogenase is from *S. cerevisiae* (GDH1; SEQ ID NOs: 25 and 25; GDH2: SEQ ID NOs: 26 and 27; GDH3: SEQ ID NOs: 30 and 31.) or *N. crassa* (GDH2: SEQ ID NOs: 28 29).

[0177] As used herein, the term "glutamate synthase" or "GLT" is intended to include those enzymes that convert L-glutamine and 2-oxoglutarate to L-glutamate, as well as those enzymes that catalyze the reverse reaction. Glutamate synthases include those enzymes that correspond to EC 1.4.1.14 and EC 1.4.1.13. In some embodiments, the glutamate synthase is GLT1 from *S. cerevisiae* (SEQ ID NOs: 32 and 33; accession numbers: X89221 and NP_010110.1).

[0178] As used herein, the term "glutamine synthase", "glutamine synthetase", or "GLN" is intended to include those enzymes that convert glutamate to glutamine. Glutamine

synthases include those enzymes that correspond to EC 6.3.1.2. In some embodiments, the glutamine synthase is GLN1 from *S. cerevisiae* (SEQ ID NOs: 34 and 35; accession numbers: M65157 and NP_015360.2).

[0179] As used herein, the term "urea-amido lyase" is intended to include those enzymes that convert urea to urea-1-carboxylate. Urea-amido lyases include those enzymes that correspond to EC 6.3.4.6. In some embodiments, the urea-amido lyase is DUR1/2 (DUR1,2) from *S. cerevisiae* (SEQ ID NOs: 36 and 37; accession numbers: M64926 and NP_009767.1)

[0180] As used herein, the term "urea transporter" is a membrane protein that transports urea across a cellular membrane. In some embodiments, the urea transporter is Dur3 or Dur4 from *S. cerevisiae* (DUR3: SEQ ID NOs: 38 and 39; accession numbers: AY693170 and NP_011847.1).

[0181] As used herein, the term "protease" is any enzyme that hydrolyzes the peptide bonds between amino acids together in a protein. An exoprotease is a protease that breaks the peptide bonds of terminal amino acids in a protein. An endoprotease is a protease that breaks the peptide bonds of non-terminal amino acids in a protein. Proteases include those enzymes that correspond to EC 3.4.23.41. Proteases include those enzymes that correspond to accession numbers: NP_001151278, NP_001150196, NP_001148706, NCU00338, XP_001908191, XP_369812, EU970094.1, NM_001156724, NM_001155234.1, XP_957809.2, XM_001908156.1, and XM_003717209.1. In some embodiments, the protease is from *Z. mays* (SEQ ID NOs: 40-45), *N. crassa* (SEQ ID NOs: 46-47), *P. anserine* (SEQ ID NOs: 48-49), or *M. oryzae* (SEQ ID NOs: 50-51).

[0182] As used herein, the term "glucoamylase" or "γ-amylase" refers to an amylase that acts on α-1,6-glycosidic bonds. Glucoamylases include those enzymes that correspond to EC 3.2.1.3. In some embodiments, the glucoamylase is *S. fibuligera* glucoamylase (glu-0111-CO) (SEQ ID NO: 162 and 163).

[0183] As used herein, the term "permease" refers to a membrane transport protein that facilitates the diffusion of a molecule through the use of passive transport in or out of a cell. In some embodiments, the permease is the amino acid permease GAP1 from *S. cerevisiae*. (SEQ ID NO: 52 and 53).

[0184] As used herein, the term "ammonium transporter" refers to permeases, and is intended to include the enzymes that are involved in the transport of ammonium and ammonia, and are exemplified by the *S. cerevisiae* MEP1, MEP2 and MEP3 enzymes (MEP 1: SEQ ID NOs: 18 and 19; MEP2: SEQ ID NOs: 20 and 21; MEP3: SEQ ID NOs: 22 and 23). Ammonium transporters include those enzymes that correspond to accession numbers: X77608, X83608, AY692775, NP_011636.3, NP_014257.1, and NP_015464.1.

[0185] As used herein, the term "URE2" refers to transcription factor known in the art by that name that represses the nitrogen catabolism of glutamate by controlling the transcription factor. URE2 is a regulator of GLN3. In some embodiments, the URE2 is from *S. cerevisiae* (SEQ ID NOs: 54 and 55).

[0186] As used herein, "AUA1" refers to a transcription factor known in the art by that name which is required for the negative regulation of Gap1. In some embodiments, the AUA1 is from *S. cerevisiae* (SEQ ID NOs: 56 and 57).

[0187] As used herein, "GLN3" refers to a transcription factor known in the art by that name that activates genes that are regulated by nitrogen catabolite metabolism. In some embodiments, the GLN3 is from *S. cerevisiae* (SEQ ID NOs: 156 and 157). The term "feedstock" is defined as a raw material or mixture of raw materials supplied to a microorganism or fermentation process from which other products can be made. For example, a carbon source, such as biomass or the carbon compounds derived from biomass are a feedstock for a microorganism that produces a product in a fermentation process. A feedstock can contain nutrients other than a carbon source.

[0188] Biomass can include any type of biomass known in the art or described herein. The terms "lignocellulosic material," "lignocellulosic substrate" and "cellulosic biomass" mean any type of carbon containing feed stock including woody biomass, such as recycled wood pulp fiber, sawdust, hardwood, softwood, grasses, sugar-processing residues, agricultural wastes, such as, but not limited to, rice straw, rice hulls, barley straw, corn cobs, cereal straw, wheat straw, canola straw, oat straw, oat hulls, corn fiber, stover, succulents, agave, or any combination thereof.

[0189] The term "yield" is defined as the amount of product obtained per unit weight of raw material and may be expressed as gram product per gram substrate (g/g). Yield may be expressed as a percentage of the theoretical yield. "Theoretical yield" is defined as the maximum amount of product that can be generated per a given amount of substrate as

dictated by the stoichiometry of the metabolic pathway used to make the product. For example, the theoretical yield for one typical conversion of glucose to ethanol is 0.51g EtOH per 1g glucose. As such, a yield of 4.8g ethanol from 10g of glucose would be expressed as 94% of theoretical or 94% theoretical yield.

[0190] The term "titer" is defined as the strength of a solution or the concentration of a substance in solution. For example, the titer of a product in a fermentation broth is described as gram of product in solution per liter of fermentation broth (g/L) or as g/kg broth.

[0191] As used herein, the term "flux" is the rate of flow of molecules through a metabolic pathway, akin to the flow of material in a process.

[0192] "Bacteria", or "eubacteria", refers to a domain of prokaryotic organisms. Bacteria include gram-positive (gram+) bacteria and gram-negative (gram-) bacteria.

[0193] "Yeast" refers to a domain of eukaryotic organisms that are unicellular fungi.

[0194] The terms "derivative" and "analog" refer to a polypeptide differing from the enzymes of the invention, but retaining essential properties thereof. Generally, derivatives and analogs are overall closely similar, and, in many regions, identical to the enzymes of the invention. The terms "derived from", "derivative" and "analog" when referring to enzymes of the invention include any polypeptides which retain at least some of the activity of the corresponding native polypeptide or the activity of its catalytic domain.

[0195] Derivatives of enzymes disclosed herein are polypeptides which may have been altered so as to exhibit features not found on the native polypeptide. Derivatives can be covalently modified by substitution (*e.g.* amino acid substitution), chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (*e.g.*, a detectable moiety such as an enzyme or radioisotope). Examples of derivatives include fusion proteins, or proteins which are based on a naturally occurring protein sequence, but which have been altered. For example, proteins can be designed by knowledge of a particular amino acid sequence, and/or a particular secondary, tertiary, and quaternary structure. Derivatives include proteins that are modified based on the knowledge of a previous sequence, natural or synthetic, which is then optionally modified, often, but not necessarily to confer some improved function. These sequences, or proteins, are then said to be derived from a particular protein or amino acid sequence.

In some embodiments of the invention, a derivative must retain at least about 50% identity, at least about 60% identity, at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 97% identity, or at least about 99% identity to the sequence the derivative is "derived from." In some embodiments of the invention, an enzyme is said to be derived from an enzyme naturally found in a particular species if, using molecular genetic techniques, the DNA sequence for part or all of the enzyme is amplified and placed into a new host cell.

[0196] "Isolated" from, as used herein, refers to a process whereby, using molecular biology techniques, genetic material is harvested from a particular organism often with the end goal of putting the general material into a non-native environment.

[0197] The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences.

[0198] As known in the art, "similarity" between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide.

[0199] "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, NY (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Multiple alignments of the sequences disclosed herein were performed using the Clustal method of alignment (Higgins and Sharp (1989) CABIOS. 5:151-153) with the default parameters (GAP

PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

[0200] Suitable nucleic acid sequences or fragments thereof (isolated polynucleotides of the present invention) encode polypeptides that are at least about 70% to 75% identical to the amino acid sequences disclosed herein, at least about 80%, at least about 85%, or at least about 90% identical to the amino acid sequences disclosed herein, or at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identical to the amino acid sequences disclosed herein. Suitable nucleic acid fragments are at least about 70%, at least about 75%, or at least about 80% identical to the nucleic acid sequences disclosed herein, at least about 80%, at least about 85%, or at least about 90% identical to the nucleic acid sequences disclosed herein, or at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% identical to the nucleic acid sequences disclosed herein. Suitable nucleic acid fragments not only have the above identities/similarities but typically encode a polypeptide having at least about 50 amino acids, at least about 100 amino acids, at least about 150 amino acids, at least about 200 amino acids, or at least about 250 amino acids.

Codon Optimization

[0201] In some embodiments of the present invention, exogenous genes may be codon-optimized in order to express the polypeptide they encode most efficiently in the host cell. Methods of codon optimization are well known in the art. (*See, e.g.* Welch *et al.* "Designing genes for successful protein expression." *Methods Enzymol* 2011. 498:43-66.)

[0202] In general, highly expressed genes in an organism are biased towards codons that are recognized by the most abundant tRNA species in that organism. One measure of this bias is the "codon adaptation index" or "CAI," which measures the extent to which the codons used to encode each amino acid in a particular gene are those which occur most frequently in a reference set of highly expressed genes from an organism. The Codon Adaptation Index is described in more detail in Sharp *et al.*, "The Codon Adaptation Index: a Measure of Directional Synonymous Codon Usage Bias, and Its Potential Applications." *Nucleic Acids Research* (1987) 15: 1281-1295.

[0203] A codon optimized sequence may be further modified for expression in a particular organism, depending on that organism's biological constraints. For example, large runs of "As" or "Ts" (*e.g.*, runs greater than 3, 4, 5, 6, 7, 8, 9, or 10 consecutive bases) can effect transcription negatively. Therefore, it can be useful to remove a run by, for example, replacing at least one nucleotide in the run with another nucleotide. Furthermore, specific restriction enzyme sites may be removed for molecular cloning purposes by replacing at least one nucleotide in the restriction site with another nucleotide. Examples of such restriction enzyme sites include PacI, AscI, BamHI, BglII, EcoRI and XhoI. Additionally, the DNA sequence can be checked for direct repeats, inverted repeats and mirror repeats with lengths of about 5, 6, 7, 8, 9 or 10 bases or longer. Runs of "As" or "Ts", restriction sites and/or repeats can be modified by replacing at least one codon within the sequence with the "second best" codons, *i.e.*, the codon that occurs at the second highest frequency for a particular amino acid within the particular organism for which the sequence is being optimized.

[0204] Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). The "genetic code" which shows which codons encode which amino acids is reproduced herein as Table 1. As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six triplets each, whereas tryptophan and methionine are coded for by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

TABLE 1: The Standard Genetic Code

	T	C	A	G
T	TTT Phe (F) TTC " TTA Leu (L) TTG "	TCT Ser (S) TCC " TCA " TCG "	TAT Tyr (Y) TAC " TAA **Ter** TAG **Ter**	TGT Cys (C) TGC TGA **Ter** TGG Trp (W)
C	CTT Leu (L) CTC " CTA " CTG "	CCT Pro (P) CCC " CCA " CCG "	CAT His (H) CAC " CAA Gln (Q) CAG "	CGT Arg (R) CGC " CGA " CGG "
A	ATT Ile (I) ATC " ATA " **ATG** Met (M)	ACT Thr (T) ACC " ACA " ACG "	AAT Asn (N) AAC " AAA Lys (K) AAG "	AGT Ser (S) AGC " AGA Arg (R) AGG "
G	GTT Val (V) GTC " GTA " GTG "	GCT Ala (A) GCC " GCA " GCG "	GAT Asp (D) GAC " GAA Glu (E) GAG "	GGT Gly (G) GGC " GGA " GGG "

[0205] Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference or codon bias, differences in codon usage between organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, inter alia, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

Host cells

[0206] In some embodiments of the invention, the host cell is a eukaryotic microorganism. In some embodiments, the host cell is a yeast. In some embodiments, the host cell is able to digest and ferment cellulose. In some embodiments, the host cell is

from the genus *Saccharomyces*. In some embodiments, the host cell is *Saccharomyces cerevisiae*.

[0207] In some embodiments, the host cells of the invention are cultured at a temperature above about 20°C, above about 25°C, above about 27°C, above about 30°C, above about 33°C, above about 35°C, above about 37°C, above about 40°C, above about 43°C, above about 45°C, or above about 47°C. In some embodiments, the host cells of the invention contain genetic constructs that lead to the down-regulation of one or more genes encoding a polypeptide at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to one or more of the polypeptides encoded SEQ ID NOs: 2, 5, 7, 25, 31, 55, 57, 159 and 161, and the polynucleotide sequence encoded by SEQ ID NO: 3. In some embodiments, the host cells of the invention contain genetic constructs that lead to the expression or up-regulation of a polypeptide encoding the activity associated with EC Nos.: 1.1.1.8, 3.1.3.21, 1.2.1.43, 1.2.1.2, 1.4.1.2, and 1.4.1.4.

[0208] In some embodiments, the host cells of the invention contain genetic constructs that lead to the expression or up-regulation of one or more genes encoding a polypeptide at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to one or more of the polypeptides encoded by SEQ ID NOs: 9, 11, 13, 15, 17, 19, 21, 23, 27, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 157, and 163. In some embodiments, the host cells of the invention contain genetic constructs that lead to the expression or up-regulation of a polypeptide encoding the activity associated with EC Nos.: 1.1.1.1, 1.1.1.2, 1.2.1.3, 1.2.1.4, 1.2.1.10, 2.3.1.54, 1.97.1.4, 1.4.1.2, 1.4.1.4, 1.1.1.14, 1.4.1.13, 6.3.1.2, 6.3.4.6, and 3.2.1.3.

[0209] In some embodiments, bifunctional acetaldehyde-alcohol dehydrogenase is up-regulated. In some embodiments, the up-regulated bifunctional acetaldehyde-alcohol dehydrogenase is from an enzyme that corresponds to an EC number selected from the group consisting of: EC 1.2.1.0 and 1.1.1.1. In some embodiments, the bifunctional acetaldehyde-alcohol dehydrogenase is a NADPH dependent bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.2. In some embodiments, the bifunctional acetaldehyde-alcohol dehydrogenase corresponds to a polypeptide selected from the

group consisting of SEQ ID NOs: 13, 15, and 17. In some embodiments, the bifunctional acetaldehyde-alcohol dehydrogenase is adhE.

[0210] In some embodiments, pyruvate formate lyase is up-regulated. In some embodiments, the up-regulated pyruvate formate lyase is from an enzyme that corresponds to EC 2.3.1.54. In some embodiments, the pyruvate formate lyase corresponds to a polypeptide encoded by SEQ ID NO: 2. In some embodiments, pyruvate formate lyase activating enzyme is up-regulated. In some embodiments, the up-regulated pyruvate formate lyase activating enzyme is from an enzyme that corresponds to EC 1.97.1.4. In some embodiments, the pyruvate formate lyase activating enzyme corresponds to a polynucleotide encoded by SEQ ID NO: 3.

[0211] In some embodiments, glutamate dehydrogenase is up-regulated. In some embodiments, the glutamate dehydrogenase that is up-regulated is NADH-dependent. In some embodiments, the up-regulated glutamate dehydrogenase corresponds to EC 1.4.1.2. In some embodiments, glutamate dehydrogenase from *S. cerevisiae* is up-regulated. In some embodiments, the glutamate dehydrogenase that is up-regulated is from *S. cerevisiae* is GDH2 and corresponds to a polypeptide corresponding to SEQ ID NO: 29. In some embodiments, glutamate synthase is up-regulated. In some embodiments, the up-regulated glutamate synthase corresponds to EC 1.4.1.14. In some embodiments, glutamate synthase from *S. cerevisiae* is up-regulated. In some embodiments, the glutamate dehydrogenase that is up-regulated is from *S. cerevisiae* is GLT1 and corresponds to a polypeptide corresponding to SEQ ID NO: 33. In some embodiments, glutamine synthase is up-regulated. In some embodiments, the up-regulated glutamine synthase corresponds to EC 6.3.1.2. In some embodiments, glutamine synthase from *S. cerevisiae* is up-regulated. In some embodiments, the glutamine dehydrogenase that is up-regulated is from *S. cerevisiae* is GLN1 and corresponds to a polypeptide corresponding to SEQ ID NO: 35.

[0212] In some embodiments, a urea-amido lyase is up-regulated. In some embodiments, the up-regulated urea-amido lyase corresponds to EC 6.3.4.6. In some embodiments, urea-amido lyase from *S. cerevisiae* is up-regulated. In some embodiments, the urea-amido lyase that is up-regulated is from *S. cerevisiae* is DUR1/2 and corresponds to a polypeptide corresponding to SEQ ID NO: 37.

[0213] In some embodiments, a protease is up-regulated. In some embodiments, the up-regulated protease corresponds to EC 3.4.23.41. In some embodiments, the protease is an endoprotease. In some embodiments, the protease is an exoprotease. In some embodiments, a protease from *Z. mays*, *N. crassa*, *P. anserine*, or *M. oryzae* is up-regulated. In some embodiments, the protease that is up-regulated corresponds to a polypeptide corresponding to SEQ ID NOs: 41, 43, 45, 47, 49 or 51. In some embodiments, a permease is up-regulated. In some embodiments, a permease from *S. cerevisiae* is up-regulated. In some embodiments, the permease that is up-regulated is GAP1 and corresponds to a polypeptide corresponding to SEQ ID NO: 53.

[0214] In some embodiments, a glucoamylase is up-regulated. In some embodiments, the up-regulated glucoamylase corresponds to EC 3.2.1.3.. In some embodiments, a glucoamylase from *S. fibuligera* is up-regulated. In some embodiments, the glucoamylase from *S. fibuligera* that is up-regulated corresponds to a polypeptide corresponding to SEQ ID NO: 163.

[0215] In some embodiments, an ammonium transporter is up-regulated. In some embodiments, an ammonium transporter from *S. cerevisiae* is up-regulated. In some embodiments, the ammonium transporter that is up-regulated is MEP1, MEP2, or MEP3 from *S. cerevisiae* and corresponds to a polypeptide corresponding to SEQ ID NOs: 19, 21, and 23. In some embodiments, a urea transporter is up-regulated. In some embodiments, a urea transporter from is from *S. cerevisiae*. In some embodiments, the urea transporter that is up-regulated is DUR3 or DUR4 from *S. cerevisiae* and corresponds to a polypeptide corresponding to SEQ ID NOs: 39.

[0216] In some embodiments, glycerol-3-phosphate dehydrogenase is down-regulated. In some embodiments, the down-regulated Gpd is from an enzyme that corresponds to EC 1.1.1.8. In some embodiments, the glycerol-3-phosphate dehydrogenase is selected from the group consisting of glycerol-3-phosphate dehydrogenase 1 ("Gpd1"), glycerol-3-phosphate dehydrogenase 2 ("Gpd2"), and combinations thereof. In some embodiments, the Gpd1 is from *S. cerevisiae* and corresponds to a polypeptide encoded by SEQ ID NO: 5. In some embodiments, the Gpd2 is from *S. cerevisiae* and corresponds to a polypeptide encoded by SEQ ID NO: 7. In some embodiments, formate dehydrogenase is down-regulated. In some embodiments, the down-regulated formate dehydrogenase corresponds to an EC number selected from the group consisting of: EC 1.2.1.43 and EC

1.2.1.2. In some embodiments, formate dehydrogenase from *S. cerevisiae* is down-regulated. In some embodiments, the formate dehydrogenase from *S. cerevisiae* corresponds to a polypeptide corresponding to SEQ ID NO: 2 or a polynucleotide corresponding to SEQ ID NO: 3. In some embodiments, glycerol-3-phosphate phosphatase is down-regulated. In some embodiments, the down-regulated glycerol-3-phosphate phosphatase corresponds to EC 3.1.3.21. In some embodiments, the down-regulated glycerol-3-phosphate phosphatase corresponds to a polynucleotide corresponding to SEQ ID NOs 158 or 160 or a polypeptide corresponding to SEQ ID NOs 159 or 161.

[0217] In some embodiments, glutamate dehydrogenase is down-regulated. In some embodiments, the glutamate dehydrogenase that is down-regulated is NADPH-dependent. In some embodiments, the down-regulated glutamate dehydrogenase corresponds to EC 1.4.1.4. In some embodiments, glutamate dehydrogenase that is down-regulated is from *S. cerevisiae*. In some embodiments, the glutamate dehydrogenase is from *S. cerevisiae* is GDH1 and corresponds to a polypeptide corresponding to SEQ ID NO: 25.

[0218] In some embodiments, a regulatory element is down-regulated. In some embodiments, the regulatory element that is down-regulated is from *S. cerevisiae*. In some embodiments, the regulatory element from *S. cerevisiae* is Ure2 and corresponds to a polypeptide corresponding to SEQ ID NO: 55. In some embodiments, the regulatory element from *S. cerevisiae* is Aua1 and corresponds to a polypeptide corresponding to SEQ ID NO: 57.

[0219] In some embodiments, bifunctional acetaldehyde-alcohol dehydrogenase (AdhE), *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd1 and Gpd2 are down-regulated. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd1, Gpd2, Fdh1 and Fdh2 are down-regulated. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, Gpd1, Gpd2, Fdh1 and Fdh2 are down-regulated, *GPD1* is expressed under the control of the *GPD2* promoter, and *GPD2* is expressed under the control of the *GPD1* promoter. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, Gpd1, Gpd2, Fdh1, Fdh2,

Gdh1 are down-regulated, *GPD1* is expressed under the control of the *GPD2* promoter, and *GPD2* is expressed under the control of the *GPD1* promoter. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme, and Glt1 are up-regulated, Gpd1, Gpd2, Fdh1, Fdh2, Gdh1 are down-regulated, *GPD1* is expressed under the control of the *GPD2* promoter, and *GPD2* is expressed under the control of the *GPD1* promoter. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme, and Gln1 are up-regulated, Gpd1, Gpd2, Fdh1, Fdh2, Gdh1 are down-regulated, *GPD1* is expressed under the control of the *GPD2* promoter, and *GPD2* is expressed under the control of the *GPD1* promoter. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme, Gln1 and Glt1 are up-regulated, Gpd1, Gpd2, Fdh1, Fdh2, Gdh1 are down-regulated, *GPD1* is expressed under the control of the *GPD2* promoter, and *GPD2* is expressed under the control of the *GPD1* promoter. In some embodiments, the regulatory element Ure2 is down-regulated. In some embodiments, the regulatory element Aua1 is down-regulated. In some embodiments, Gln3 is up-regulated.

[0220] In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd2, Fdh1, and Fdh2 are down-regulated. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd2, Fdh1, Fdh2, and Gdh1 are down-regulated. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd1, Fdh1, and Fdh2 are down-regulated. In some embodiments, AdhE, *B. adolescentis* pyruvate formate lyase, and *B adolescentis* pyruvate formate lyase activating enzyme are up-regulated, and Gpd1, Fdh1, Fdh2, and Gdh1 are down-regulated. In some embodiments, Dur1/2 is additionally expressed. In some embodiments, Dur1/2 is expressed from the *TEF2* promoter. In some embodiments, Dur1/2 is expressed from the *HXT7* promoter. In some embodiments, Dur1/2 is expressed from the *GPM1* promoter. In some embodiments, Dur1/2 is expressed from the *ADH1* promoter. In some embodiments, Dur1/2 is expressed from the *HXT7/TEF2* promoters. In some embodiments, Gln3 is up-regulated. In some

embodiments, GPD1 is expressed from the GPD2 promoter. In some embodiments, GPD2 is expressed from a GPD1 promoter. Ethanol Production

[0221] For a microorganism to produce ethanol most economically, it is desired to produce a high yield. In one embodiment, the only product produced is ethanol. Extra products lead to a reduction in product yield and an increase in capital and operating costs, particularly if the extra products have little or no value. Extra products also require additional capital and operating costs to separate these products from ethanol.

[0222] Ethanol production can be measured using any method known in the art. For example, the quantity of ethanol in fermentation samples can be assessed using HPLC analysis. Additionally, many ethanol assay kits are commercially available, for example, alcohol oxidase enzyme based assays. Methods of determining ethanol production are within the scope of those skilled in the art from the teachings herein.

[0223] In some embodiments of the invention where redirected carbon flux generates increased ethanol production, the ethanol output can be improved by growth-coupled selection. For example, continuous culture or serial dilution cultures can be performed to select for cells that grow faster and/or produce ethanol (or any desired product) more efficiently on a desired feedstock.

[0224] One embodiment of the present invention relates to a method of producing ethanol using a microorganism described herein wherein the microorganism is cultured in the presence of a carbon containing feedstock for sufficient time to produce ethanol and, optionally, extracting the ethanol. In some embodiments, nitrogen is added to the culture containing the recombinant microorganism and the feedstock.

[0225] Ethanol may be extracted by methods known in the art. (*See, e.g.*, U.S. Appl. Pub. No. 2011/0171709.)

[0226] Another embodiment of the present invention relates to a method of producing ethanol using a co-culture composed of at least two microorganisms in which at least one of the organisms is an organism described herein, and at least one of the organisms is a genetically distinct microorganism. In some embodiments, the genetically distinct microorganism is a yeast or bacterium. In some embodiments the genetically distinct microorganism is any organism from the genus *Issatchenkia*, *Pichia*, *Clavispora*, *Candida*, *Hansenula*, *Kluyveromyces*, *Saccharomyces*, *Trichoderma*, *Thermoascus*,

Escherichia, *Clostridium*, *Caldicellulosiruptor*, *Thermoanaerobacter* and *Thermoanaerobacterium*.

[0227] In some embodiments, the recombinant microorganism produces about 2% to about 3% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 2% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 5% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 7% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 10% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 15% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 20% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 30% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 50% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 75% higher ethanol titer than a wildtype, non-recombinant organism; at least about 1% to at least about 100% higher ethanol titer than a wildtype, non-recombinant organism. In some embodiments, the recombinant microorganism produces at least about 0.5g/L ethanol to at least about 2 g/L ethanol, at least about 0.5g/L ethanol to at least about 3 g/L ethanol, at least about 0.5g/L ethanol to at least about 5 g/L ethanol, at least about 0.5g/L ethanol to at least about 7 g/L ethanol, at least about 0.5g/L ethanol to at least about 10 g/L ethanol, at least about 0.5g/L ethanol to at least about 15 g/L ethanol, at least about 0.5g/L ethanol to at least about 20 g/L ethanol, at least about 0.5g/L ethanol to at least about 30 g/L ethanol, at least about 0.5g/L ethanol to at least about 40 g/L ethanol, at least about 0.5g/L ethanol to at least about 50 g/L ethanol, at least about 0.5g/L ethanol to at least about 75 g/L ethanol, at least about 0.5g/L ethanol to at least about 99 g/L ethanol, at least about 0.5 g/L ethanol to at least about 125 g/L ethanol, or at least about 0.5 g/L to at least about 150 g/L ethanol per at least about 24 hour, at least about 48 hour, or at least about 72 hour incubation on a carbon-containing feed stock, such as corn mash.

[0228] In some embodiments, the recombinant microorganism produces ethanol at least about 55% to at least about 75% of theoretical yield, at least about 50% to at least about 80% of theoretical yield, at least about 45% to at least about 85% of theoretical yield, at least about 40% to at least about 90% of theoretical yield, at least about 35% to at least

about 95% of theoretical yield, at least about 30% to at least about 99% of theoretical yield, or at least about 25% to at least about 99% of theoretical yield. In some embodiments, methods of producing ethanol can comprise contacting a biomass feedstock with a host cell or co-culture of the invention and additionally contacting the biomass feedstock with externally produced saccharolytic enzymes. In some embodiments, the host cells are genetically engineered (*e.g.*, transduced, transformed, or transfected) with the polynucleotides encoding saccharolytic enzymes.

[0229] An "amylolytic enzyme" can be any enzyme involved in amylase digestion, metabolism and/or hydrolysis. The term "amylase" refers to an enzyme that breaks starch down into sugar. Amylase is present in human saliva, where it begins the chemical process of digestion. Foods that contain much starch but little sugar, such as rice and potato, taste slightly sweet as they are chewed because amylase turns some of their starch into sugar in the mouth. The pancreas also makes amylase (α-amylase) to hydrolyse dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds. Some amylases, such as γ-amylase (glucoamylase), also act on α-1,6-glycosidic bonds. Amylase enzymes include α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and γ-amylase (EC 3.2.1.3). The α-amylases are calcium metalloenzymes, unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme and its optimum pH is about 6.7-7.0. Another form of amylase, β-amylase is also synthesized by bacteria, fungi, and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. Many microbes produce amylase to degrade extracellular starches. In addition to cleaving the last α(1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose, γ-amylase will cleave α(1-6) glycosidic linkages. Another amylolytic enzyme is alpha-glucosidase that acts on maltose and other short malto-oligosaccharides produced by alpha-, beta- , and gamma-amylases, converting them to glucose. Another amylolytic

enzyme is pullulanase. Pullulanase is a specific kind of glucanase, an amylolytic exoenzyme, that degrades pullulan. Pullulan is regarded as a chain of maltotriose units linked by alpha-1,6-glycosidic bonds. Pullulanase (EC 3.2.1.41) is also known as pullulan-6-glucanohydrolase (debranching enzyme). Another amylolytic enzyme, isopullulanase, hydrolyses pullulan to isopanose (6-alpha-maltosylglucose). Isopullulanase (EC 3.2.1.57) is also known as pullulan 4-glucanohydrolase. An "amylase" can be any enzyme involved in amylase digestion, metabolism and/or hydrolysis, including α-amylase, β -amylase, glucoamylase, pullulanase, isopullulanase, and alpha-glucosidase.

[0230] In some embodiments, the recombinant microorganisms of the invention further comprise one or more native and/or heterologous enzymes which encodes a saccharolytic enzyme, including amylases, cellulases, hemicellulases, cellulolytic and amylolytic accessory enzymes, inulinases, levanases, and pentose sugar utilizing enzymes. In one aspect, the saccharolytic enzyme is an amylase, where the amylase is selected from *H. grisea*, *T. aurantiacus*, *T. emersonii*, *T. reesei*, *C. lacteus*, *C. formosanus*, *N. takasagoensis*, *C. acinaciformis*, *M. darwinensis*, *N. walkeri*, *S. fibuligera*, *C. luckowense R. speratus*, *Thermobfida fusca*, *Clostridum thermocellum*, *Clostridium cellulolyticum*, *Clostridum josui*, *Bacillus pumilis*, *Cellulomonas fimi*, *Saccharophagus degradans*, *Piromyces equii*, *Neocallimastix patricarum* or *Arabidopsis thaliana*. In another aspect, the saccharolytic enzyme is a glucoamylase (glu-0111-CO) from *S. fibuligera*.

[0231] The term "xylanolytic activity" is intended to include the ability to hydrolyze glycosidic linkages in oligopentoses and polypentoses. The term "xylanase" is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. As such, it plays a major role in micro-organisms thriving on plant sources (mammals, conversely, do not produce xylanase). Additionally, xylanases are present in fungi for the degradation of plant matter into usable nutrients. Xylanases include those enzymes that correspond to E.C. Number 3.2.1.8. A "xylose metabolizing enzyme" can be any enzyme involved in xylose digestion, metabolism and/or hydrolysis, including a xylose isomerase, xylulokinase, xylose reductase, xylose dehydrogenase, xylitol dehydrogenase, xylonate dehydratase, xylose transketolase, and a xylose transaldolase protein.

[0232] The term "pectinase" is a general term for enzymes, such as pectolyase, pectozyme and polygalacturonase, commonly referred to in brewing as pectic enzymes. These enzymes break down pectin, a polysaccharide substrate that is found in the cell walls of plants. One of the most studied and widely used commercial pectinases is polygalacturonase. Pectinases are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juice from fruit, including apples and sapota. Pectinases have also been used in wine production since the 1960s.

[0233] A "saccharolytic enzyme" can be any enzyme involved in carbohydrate digestion, metabolism and/or hydrolysis, including amylases, cellulases, hemicellulases, cellulolytic and amylolytic accessory enzymes, inulinases, levanases, and pentose sugar utilizing enzymes.

[0234] A "pentose sugar utilizing enzyme" can be any enzyme involved in pentose sugar digestion, metabolism and/or hydrolysis, including xylanase, arabinase, arabinoxylanase, arabinosidase, arabinofuranosidase, arabinoxylanase, arabinosidase, and arabinofuranosidase, arabinose isomerase, ribulose-5-phosphate 4-epimerase, xylose isomerase, xylulokinase, xylose reductase, xylose dehydrogenase, xylitol dehydrogenase, xylonate dehydratase, xylose transketolase, and/or xylose transaldolase.

Glycerol production

[0235] In some embodiments of the invention where redirected carbon flux generates increased ethanol production, the glycerol output can be decreased by growth-coupled selection. For example, continuous culture or serial dilution cultures can be performed to select for cells that produce less glycerol on a desired feedstock. Glycerol can be measured, for example, by HPLC analysis of metabolite concentrations.

[0236] In some embodiments, the recombinant microorganism produces at least about 20% to at least about 30% less glycerol than a wildtype, non-recombinant organism; at least about 30% to at least about 50% less glycerol than a wildtype, non-recombinant organism; at least about 40% to at least about 60% less glycerol than a wildtype, non-recombinant organism; at least about 50% to at least about 70% less glycerol than a wildtype, non-recombinant organism; at least about 60% to at least about 80% less glycerol than a wildtype, non-recombinant organism; at least about 70% to at least about 90% less glycerol than a wildtype, non-recombinant organism; at least about 75% to at least about 95% less glycerol than a wildtype, non-recombinant organism; at least about

70% to at least about 99% less glycerol than a wildtype, non-recombinant organism; at least about 15% to at least about 30% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 40% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 50% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 60% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 70% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 80% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 90% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 99% less glycerol than a wildtype, non-recombinant organism; at least about 10% to at least about 100% less glycerol than a wildtype, non-recombinant organism; at least about 5% to at least about 100% less glycerol than a wildtype, non-recombinant organism; at least about 1% to at least about 100% less glycerol than a wildtype, non-recombinant organism. In some embodiments, the recombinant microorganism produces no glycerol. In some embodiments, the recombinant microorganism has a growth rate at least about 1/2 to at least about equal to the growth rate of a wildtype, non-recombinant organism, at least about 1/4 to at least about equal to the growth rate of a wildtype, non-recombinant organism, at least about 1/8 to at least about equal to the growth rate of a wildtype, non-recombinant organism, at least about 1/10 to at least about equal to the growth rate of a wildtype, non-recombinant organism, at least about 1/25 to at least about equal to the growth rate of a wildtype, non-recombinant organism, at least about 1/50 to at least about equal to the growth rate of a wildtype, non-recombinant organism or at least about 1/100th to at least about equal to the growth rate of a wildtype, non-recombinant organism.

[0237] A wildtype-non-recombinant organism produces glycerol at a rate of at least about 8-11 mM glycerol per gram dry cell weight (DCW) during anaerobic growth. In some embodiments, glycerol production is reduced to a rate of between 1-10 mM glycerol per gram dry cell weight during anaerobic growth.

EXAMPLES

[0238] Strains used in the following examples were created using Mascoma Assemblies ("MAs"). Schematic diagrams of the MAs can be seen in Figures 6-44. Plasmids used to

make the MAs can be seen in Figures 45-68 and Table 2. Primers used to create the MAs can be seen in Table 3 below and in SEQ ID NOs: 66-155. Strains used in the invention can be seen in Table 4 below. For a general description of molecular methods that could be used to create the strains, see U.S. Application No 61/728,450.

Table 2. Plasmids used to make the MAs.

Plasmid ID	Description
pMU2873	AGTEF pro-KAN-AGTEF ter/HXT2 pro-TDK-ACT1 ter
pMU2879	AGTEF pro-cloNAT-AGTEF ter/HXT2 pro-TDK-ACT1 ter
pMU2908	PGK1 pro-*S. cerevisiae* GDH2-ENO1 ter
pMU2909	ADH1 pro-*S. cerevisiae* GDH2-PDC1 ter
pMU2911	ADH1 pro-GLN1-PDC1 ter
pMU2913	PGK1 pro-GLT1-ENO1 ter
pMU3409	TEF2 pro-DUR1,2-ADH3 ter
pMU3410	HXT7 pro-DUR1,2-PMA1t
pMU3411	ADH1 pro-DUR1,2-PDC1 ter
pMU3459	ADH1 pro-DUR3-PDC1 ter
pMU3460	ADH1 pro-MEP1-PDC1 ter
pMU3461	ADH1 pro-MEP2-PDC1 ter
pMU3463	ADH1 pro-GAP1-PDC1 ter
pMU3464	TEF2 pro-DUR3-ADH3 ter
pMU3465	TEF2 pro-MEP1-ADH3 ter
pMU3466	TEF2 pro-MEP2-ADH3 ter
pMU3468	TEF2 pro-GAP1-ADH3 ter
pMU3471	TPI pro-DUR3-FBA1 ter
pMU3472	TPI pro-MEP1-FBA1 ter
pMU3473	TPI pro-MEP2-FBA1 ter
pMU3475	TPI pro-GAP1-FBA1 ter
pMU3597	ADH1 pro-*N. crassa* GDH2-PDC1 ter
pMU3605	ADH1 pro-MEP3-PDC1 ter
pMU3606	TEF2 pro-MEP3-ADH3ter
pMU3607	TPI1 pro-MEP3-FBA1 ter

Table 3. Primers used to create MAs.

SEQ ID	Primer	Sequence 5' to 3'	Description
66	X14961	gcagttacctttagcaccaac	5' GDH1 5' flank
67	X14966	ggtgtaggtaagcagaatgaggag	3' GDH1 3' flank
68	X15464	GTCCATGTAAAATGATTGCTCCAATGATTGAAAGAGGTTTAGACATTGGCTCTTCATTG	ENOt+PDC1t
69	X15465	ctaagctcaatgaagagccaatgtctaaacctctttcaatcattggagcaatcatttta	PDC1t+ENOt
70	X18846	gtccatgtaaaatgattgctccaatgattgaaaagcacgcagcacgctgtatttacgtat	FCY3'+PDC1t
71	X18847	AATTAAATACGTAAATACAGCGTGCTGCGTGCTTTTCAATCATTGGAGCAATCATTTTA	PDC1t+FCY3'
72	X18858	agccagcttaaagagttaaaaatttcatagctactacttatttcccttcgagattatatct	pTPI+FCY5'
73	X18859	GTTCCTAGATATAATCTCGAAGGGAATAAGTAGTAGCTATGAAATTTTTAACTCTTTAA	FCY5'+pTPI
74	X18860	acatcatctttaacttgaatttattctctagcagcacgcagcacgctgtatttacgtat	FCY3'+FBA1t
75	X18861	AATTAAATACGTAAATACAGCGTGCTGCGTGCTGCTAGAGAATAAATTCAAGTTAAAAG	FBA1t+FCY3'
76	X18869	AGATCCTGTGGTAGTGCTGTCTGAACAGAA	FCY3' for 2kb
77	X18955	ataaaattaaatacgtaaatacagcgtgctgcgtgctcgattttttctaaacgcgtgga	pADH1+FCY3' rev
78	X19513	acttggtgcggtccatgtaaaatgattgctccaatgattgaaaatgaggaaggaaatccaa	ADH3t+PDC1t
79	X19514	TGAAGGTCATTAGGATTTGGATTTCTTCCTCATTTTCAATCATTGGAGCAATCATTTTAC	PDC1t+ADH3t
80	X19551	agccagcttaaagagttaaaaatttcatagctagggcgccataaccaaggtatctatag	pTEF2+FCY5'
81	X19552	TGGCGGTCTATAGATACCTTGGTTATGGCGCCCTAGCTATGAAATTTTTAACTCTTTAAG	FCY5'+pTEF2
82	X19721	aaagaaatgtcagagccagaatttcaacaagctaagctttctaactgatctatccaaaa	pPGK+GDH15'
83	X19722	TTTTCAGTTTTGGATAGATCAGTTAGAAAGCTTAGCTTGTTGAAATTCTGGCTCTGACAT	GDH15'+pPGK
84	X19726	atccgaaatattccacggtttagaaaaaaatcggatgctatgtttgaccaaggtgatgta	GDH13'+pADH1
85	X19727	TTAAAATACATCACCTTGGTCAAACATAGCATCCGATTTTTTTCTAAACCGTGGAATATT	pADH1+GDH13'
86	X19948	aaagaaatgtcagagccagaatttcaacaagctgatgctatgtttgaccaaggtgatgta	GDH13'+GDH15' for deletion
87	X19949	TTAAAATACATCACCTTGGTCAAACATAGCATCAGCTTGTTGAAATTCTGGCTCTGACAT	GDH15'+GDH13' for deletion
88	X19950	atccgaaatattccacggtttagaaaaaaatcgagcacgcagcacgctgtatttacgtat	FCY3'+pADH1
89	X19967	tgaaggtcattaggatttggatttcttcctcataaattagtgtgtgtgcattatatatat	PMA1t+ADH3t
90	X19968	TTTTTAATATATATAATGCACACACACTAATTTATGAGGAAGAAATCCAAATCCTAATGA	ADH3t+PMA1t
91	X19969	AATTAAATACGTAAATACAGCGTGCTGCGTGCTCCAGAAAGGCAACGCAAAATTTTTTT	pHXT7+FCY3'

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SEQ ID	Primer	Sequence 5' to 3'	Description
92	X19970	ccctggaaaaaaaaattttgcgttgcctttctggagcacgcagcacgctgtatttacgtat	FCY3'+pHXT7
93	X20022	aggtagacgctacagtcacaggtgtcacaact	URE2 5' flank
94	X20023	GGACGAGGCAAGCTAAACAGATCTCTAGACCTATTGGTGTACAACTTAATTTGCAGCTTA	URE2 5' flank
95	X20024	ccgttcttttcttggactatcatgtagtctcaggctgctttaaaaaccaagaaagaaag	URE2 3' flank
96	X20025	GAGTGGGATGCGCATATAGTGCATGAACCTAT	URE2 3' flank
97	X20026	ttgtttaagctgcaaattaagttgtacaccaaaggctgctttaaaaacaagaaagaaag	URE2 3' + 5' for deletion
98	X20027	CTTCTTCTTTCTTTCTTGTTTTTAAAGCAGCCTTTGGTGTACAACTTAATTTGCAGCTTA	URE2 5' + 3' for deletion
99	X20028	ttgtttaagctgcaaattaagttgtacaccaaaggctagagatctgtttagcttgcc	pAGTEF+URE2 5'
100	X20029	CTTCTTCTTTCTTTCTTGTTTTTAAAGCAGCCTGAGACTACATGATAGTCCAAAGAAAAG	pHXT2rc+URE2 3'
101	X20043	ATAAAATTAAATACGTAAATACAGCGTGCTGCGTGCTATGAGGAAGAAATCCAAATCCT	ADH3 t tails for FCY1 3' flank
102	X20044	tgaaggtcattaggatttggatttcttcctcatagcacgcagcacgctgtatttacgta	FCY 3' flank tails for ADH3t
103	X20282	agccagcttaaagagttaaaaatttcatagctaccagaaaggcaacgcaaaatttttt	pHXT7+FCY5'
104	X20283	CCCTGGAAAAAAAATTTTGCGTTGCCTTTCTGGTAGCTATGAAATTTTTAACTCTTTAAG	FCY5'+pHXT7
105	X20284	ttttaatatatatnatgcacacacactaatttagcacgcagcacgctgtatttacgtat	FCY3'+PMA1t
106	X20285	AATTAAATACGTAAATACAGCGTGCTGCGTGCTAAATTAGTGTGTGTGCATTATATATAT	PMA1t+FCY3'
107	X20286	agccagcttaaagagttaaaaatttcatagctatgtggtagaattcaaaagactatgtga	pGPM1+FCY5'
108	X20287	ATGGCATCACATAGTCTTTTGAATTCTACCACATAGCTATGAAATTTTTAACTCTTTAAG	FCY5'+pGPM1
109	X20288	ttttaatattgctttcaattactgttattaaaagcacgcagcacgctgtatttacgtat	FCY3'+TPIt
110	X20289	AATTAAATACGTAAATACAGCGTGCTGCGTGCTTTTAATAACAGTAATTGAAAAGCAATA	TPIt+FCY3'
111	X20620	ggtgattggaatggttatggttccggaatcgc	AUA1 5' Flank
112	X20621	GGACGAGGCAAGCTAAACAGATCTCTAGACCTATATACTACATAGAAAGCAATTAAAAGA	AUA15'+pAGTEF
113	X20622	ccgttcttttcttggactatcatgtagtctcctccaccttaacaaaccccgcaccaacac	AUA13'+pHXT2rc
114	X20623	GTCATATGGCCTCTTAACGTGGTCCTTTGTGG	AUA1 3' Flank
115	X20630	ttttttatctttttaattgctttctatgtagtatatataggtctagagatctgtttagcttgcc	pAGTEF+AUA1 5'
116	X20631	TACTTGGTGTTGGTGCGGGTTTGTTAGGTGGAGGAGACTACATGATAGTCCAAAGAAAAG	pHXT2rc+AUA13'
117	X20632	ttttatctttttaattgctttctatgtagtatatctccacctttaacaaaccccgcaccaacac	AUA13'+AUA15'
118	X20633	TACTTGGTGTTGGTGCGGGTTTGTTAGGTGGAGTATACTACATAGAAAGCAATTAAAAGA	AUA15'+AUA13'
119	X21123	gcgacatgtgatgagattgcatgcacctccacagaa	GDH2 5' Flank

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SEQ ID	Primer	Sequence 5' to 3'	Description
120	X21124	GGACGAGGCAAGCTAAACAGATCTCTAGACCTATCTTTATTCTTTTTATTGTTGTGAATT	GDH2 5' Flank+pAGTEF
121	X21125	ccgtttcttttcttggactatcatgtagtctcgcttcaataaaattgttttgtataaat	GDH2 3' Flank+pHXT2rc
122	X21126	GGCAGCTATCTCTACTATCCCGTTTAGTACTATCC	GDH2 3' Flank
123	X21127	atattaaatttcacaacaataaaaagaataaagataggtctagagatctgtttagcttgcc	pAGTEF+GDH2 5'
124	X21128	GAACTAATTTATACAAAACAATTTTATTGAAGCGAGACTACATGATAGTCCAAAGAAAAG	pHXT2rc+GDH2 3'
125	X21133	atattaaatttcacaacaataaaaagaataaagagcttcaataaaattgtttgtataaa	GDH2 3'+GDH2 5' for deletion
126	X21135	gcattgattgtctatcagagcatatcaaggtggt	GDH3 5' Flank
127	X21136	GGACGAGGCAAGCTAAACAGATCTCTAGACCTACGGTGACTGTTGCTACTTCCCTATATA	GDH3 5' Flank+pAGTEF
128	X21137	ccgtttcttttcttggactatcatgtagtctcccgtaagcgctatttcttttgttcg	GDH3 3' Flank+pHXT2rc
129	X21138	GGCTAGGACCCCGTAAGGAGGAAAGAATAGGCAAG	GDH3 3' Flank
130	X21139	tatatatatatagggaagtagcaacagtcaccgtaggtctagagatctgtttagcttgcc	pAGTEF+GDH3 5'
131	X21140	TAGTTACGAACAAAAAGAAAATAGCGCTTACGGGAGACTACATGATAGTCCAAAGAAAAG	pHXT2rc+GDH3 3'
132	X21147	tatatatatatagggaagtagcaacagtcaccgccgtaagcgctatttcttttgttcg	GDH3 3'+GDH3 5'
133	X21148	TAGTTACGAACAAAAAGAAAATAGCGCTTACGGCGGTGACTGTTGCTACTTCCCTATATA	GDH3 5'+GDH3 3'
134	X21179	ttgtttaagctgcaaattaagttgtacaccaagggcgccatacccaggtatctatataga	pTEF2+URE2 5'
135	X21180	TGGCGGTCTATAGATACCTTGGTTATGGCGCCCTTGGTGTACAACTTAATTTGCAGCTTA	URE2 5'+pTEF2
136	X21181	CTTCTTCTTTCTTTCTTGTTTTTAAAGCAGCCTCGATTTTTTTCTAAACCGTGGAATATT	pADH1rc+URE2 3'
137	X21182	atccgaaatatttccacggtttagtaaaaaaatcgaggctgctttaaaaacttagaaagaaag	URE2 3'+pADH1rc
138	X21289	aaagaaatgtcagagccagaatttcaacaagctaggtctagagatctgtttagcttgcct	pAGTEF+GDH1 5'
139	X21290	TTAAAATACATCACCTTGGTCAAACATAGCATCGAGACTACATGATAGTCCAAAGAAAAG	pHXT2rc+GDH1 3'
140	X21291	GGGACGAGGCAAGCTAAACAGATCTCTAGACCTAGCTTGTTGAAATTCTGGCTCTGACAT	GDH1 5'+pAGTEF
141	X21292	ccgtttcttttcttggactatcatgtagtctcgatgctatgtttgaccaaggtgatgt	GDH1 3'+pHXT2rc
142	X21319	ttgtttaagctgcaaattaagttgtacaccaacgatttttttctaaaccgtggaatatt	pADH1+URE2 5'
143	X21320	ATCCGAAATATTCCACGGTTTAGAAAAAAATCGTTGGTGTACAACTTAATTTGCAGCTTA	URE2 5'+pADH1
144	X21321	ttttcagttttggatagatcagttagaaagcttaggctgctttaaaaacaagaaagaaag	URE2 3'+PGKprc
145	X21322	CTTCTTCTTTCTTTCTTGTTTTTAAAGCAGCCTAAGCTTTCTAACTGATCTATCCAAAAC	PGKprc+URE2 3'
146	X21507	GAACTAATTTATACAAAACAATTTTATTGAAGCTCTTTATTCTTTTTATTGTTGTGAATT	GDH25'+GDH23' for deletion
147	X21735	agccagcttaaagtgttaaaaattcatagctacgatttttttctaaacgtggaatatt	pADH1+FCY5'

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SEQ ID	Primer	Sequence 5' to 3'	Description
148	X21736	ATCCGAAATATTCCACGGTTTAGAAAAAAATCGTAGCTATGAAATTTTTAACTCTTTAAG	FCY5'+pADH1
149	X21754	gccaaagtggattctcctactcaagctttgc	FCY5' Flank
150	X23319	aaagaaatgtcagagccagaatttcaacaagctcgatttttttctaaaccgtggaatatt	pADH1+GDH15'
151	X23320	ATCCGAAATATTCCACGGTTTAGAAAAAAATCGAGCTTGTTGAAATTCTGGCTCTGACAT	GDH1 5'+pADH1
152	X23321	gtccatgttaaaatgattgctccaatgattgaaagatgctatgtttgaccaaggtgatgta	GDH1 3'+PDC1t
153	X23322	TTAAAATACATCACCTTGGTCAAACATAGCATCTTTCAATCATTGGAGCAATCATTTTAC	PDC1t+GDH13'
154	X23408	TTTTCAGTTTTGGATAGATCAGTTAGAAAGCTTTAGCTATGAAATTTTTAACTCTTTAAG	FCY5'+pPGK
155	X23409	agccagcttaaagagttaaaaatttcatagctaaagctttctaactgatctatccaaaac	pPGK+FCY5'

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Table 4. Strain genotypes. Strains shown in examples are highlighted grey.

Strain	Description	Genotype	Associated MA Cassette(s)
MZ390	Type Strain	WT	
M3465	Glycerol Reduction Strain	*Δfdh1::MA0608Δfdh2::MA0280Δgpd2::MA0289*	MA0280, MA0289, MA0608
M3467	Glycerol Reduction Strain	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290*	MA0280, MA0290, MA0608
M3469	Glycerol Reduction Strain	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293*	MA0280, MA0608, MA0293
M3624	Glycerol Reduction Strain	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286*	MA0286, MA0280, MA0290, MA0608
M4076	GDH1 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0631*	MA0286, MA0280, MA0290, MA0608, MA0631
M4117	S. cerevisiae GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0425*	MA0286, MA0280, MA0290, MA0608, MA0425
M4118	S. cerevisiae GLN1/GLT1 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426*	MA0286, MA0280, MA0290, MA0608, MA0426
M4312	URE2 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δure2::MA0622*	MA0286, MA0280, MA0290, MA0608, MA0622
M4373	GDH1 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd2::MA0289Δgdh1::MA0631*	MA0280, MA0289, MA0608, MA0631
M4375	GDH1 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgdh1::MA0631*	MA0280, MA0290, MA0608, MA0631
M4377	GDH1 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δgdh1::MA0631*	MA0280, MA0608, MA0293, MA0631
M4400	GDH1 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd2::MA0289Δgdh1::MA0888*	MA0280, MA0289, MA0608, MA0888
M4401	GDH1 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgdh1::MA0888*	MA0280, MA0290, MA0608, MA0888
M4402	GDH1 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δgdh1::MA0888*	MA0280, MA0608, MA0293, MA0888
M4406	URE2 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δure2::MA0622.1*	MA0286, MA0280, MA0290, MA0608, MA0622.1
M4427	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δfcy1::MA0464.1	MA0280, MA0290, MA0608, MA0464.1
M4428	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δfcy1::MA0465.1	MA0280, MA0290, MA0608, MA0465.1

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M4429	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δfcy1::MA0467.1	MA0280, MA0290, MA0608, MA0467.1
M4430	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δfcy1::MA0454.14	MA0280, MA0290, MA0608, MA0454.14
M4431	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δfcy1::MA0464.1	MA0280, MA0293, MA0608, MA0464.1
M4432	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δfcy1::MA0465.1	MA0280, MA0293, MA0608, MA0465.1
M4433	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δfcy1::MA0467.1	MA0280, MA0290, MA0608, MA0467.1
M4434	DUR1,2 over expression	Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δfcy1::MA0454.14	MA0280, MA0290, MA0608, MA0454.14
M4469	*S. cerevisiae* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd2::MA0289Δgdh1::MA0425*	MA0280, MA0289, MA0608, MA0425
M4471	*S. cerevisiae* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δgdh1::MA0425*	MA0280, MA0608, MA0293, MA0425
M4507	AUA1 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δaua1::MA0617*	MA0286, MA0280, MA0290, MA0608, MA0617
M4538	GDH1 marked deletion	*Δgdh1::MA0631*	MA0631
M4540	AUA1 marked deletion	*Δaua1::MA0617*	MA0617
M4542	URE2 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δure2::MA0622*	MA0280, MA0608, MA0293, MA0622
M4571	AUA1 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δaua1::MA0617.1*	MA0286, MA0280, MA0290, MA0608, MA0617.1
M4573	GDH1 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0888*	MA0286, MA0280, MA0290, MA0608, MA0888
M4590	GDH1 clean deletion	*Δgdh1::MA0888*	MA0888
M4591	AUA1 clean deletion	*Δaua1::MA0617.1*	MA0617.1
M4592	URE2 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δure2::MA0622.1*	MA0280, MA0608, MA0293, MA0622.1
M4614	URE2 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0425Δure2::MA0622*	MA0286, MA0280, MA0290, MA0608, MA0425, MA0622
M4615	URE2 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δure2::MA0622*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0622
M4616	URE2 marked deletion	*Δure2::MA0622*	MA0622

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M4622	*S. cerevisiae* GDH2 over expression	*Δgdh1::MA0425*	MA0425
M4623	*S. cerevisiae* GLN1/GLT1 over expression	*Δgdh1::MA0426*	MA0426
M4624	*S. cerevisiae* GLN1/GLT1 over expression	*Δgdh1::MA0426*	MA0426
M4625	*S. cerevisiae* GLN1/GLT1 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd2::MA0289Δgdh1::MA0426*	MA0280, MA0289, MA0608, MA0426
M4625	*S. cerevisiae* GLN1/GLT1 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δgdh1::MA0426*	MA0280, MA0608, MA0293, MA0426
M4626	GLN1/GLT1 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0293Δgdh1::MA0426*	MA0280, MA0608, MA0293, MA0426
M4654	GDH3 marked deletion	*Δgdh3::MA0615*	MA0615
M4655	GDH2 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δgdh2::MA0616*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0616
M4656	GDH3 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δgdh3::MA0615*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0615
M4657	GDH3 marked deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0425Δgdh3::MA0615*	MA0286, MA0280, MA0290, MA0608, MA0425, MA0615
M4674	URE2 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0425Δure2::MA0622.1*	MA0286, MA0280, MA0290, MA0608, MA0425, MA0622.1
M4675	URE2 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δure2::MA0622.1*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0622.1
M4676	URE2 clean deletion	*Δure2::MA0622.1*	MA0622.1
M4677	GDH2 marked deletion	*Δgdh2::MA0616*	MA0616
M4690	GDH3 clean deletion	*Δgdh3::MA0615.1*	MA0615.1
M4691	GDH2 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δgdh2::MA0616.1*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0616.1

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M4692	GDH3 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0426Δgdh3::MA0615.1*	MA0286, MA0280, MA0290, MA0608, MA0426, MA0615.1
M4693	GDH3 clean deletion	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0425Δgdh3::MA0615.1*	MA0286, MA0280, MA0290, MA0608, MA0425, MA0615.1
M4694	GDH2 clean deletion	*Δgdh2::MA0616.1*	MA0616.1
M4748	DUR3 over expression	*Δfcy1::MA0464*	MA0464
M4749	GAP1 over expression	*Δfcy1::MA0464.4*	MA0464.4
M4750	MEP1 over expression	*Δfcy1::MA0464.2*	MA0464.2
M4751	DUR1,2 over expression	*Δfcy1::MA0465.1*	MA0465.1
M4752	MEP1 over expression	*Δfcy1::MA0434.2*	MA0434.2
M4753	MEP2 over expression	*Δfcy1::MA0434.3*	MA0434.3
M4754	DUR3 over expression	*Δfcy1::MA0434*	MA0434
M4755	GAP1 over expression	*Δfcy1::MA0434.4*	MA0434.4
M4756	MEP2 over expression	*Δfcy1::MA0464.2*	MA0464.2
M4810	DUR3 over expression	*Δfcy1::MA0467*	MA0467
M4811	DUR3 over expression	*Δfcy1::MA0467*	MA0467
M4812	MEP1 over expression	*Δfcy1::MA0467.2*	MA0467.2
M4813	MEP1 over expression	*Δfcy1::MA0467.2*	MA0467.2
M4814	GAP1 over expression	*Δfcy1::MA0467.4*	MA0467.4
M4815	GAP1 over expression	*Δfcy1::MA0467.4*	MA0467.4
M5841	*N. crassa* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0837*	MA0280, MA0608, MA0286, MA0290, MA0837
M5842	*N. crassa* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0837*	MA0280, MA0608, MA0286, MA0290, MA0837
M5843	*N. crassa* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0837*	MA0280, MA0608, MA0286, MA0290, MA0837
M5844	*N. crassa* GDH2 over expression	*Δfdh1::MA0608Δfdh2::MA0280Δgpd1::MA0290Δgpd2::MA0286Δgdh1::MA0837*	MA0280, MA0608, MA0286, MA0290, MA0837

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Example 1:

Deletion of GDH1 and overexpression of GDH2 or GLT1/GLN1

[0239] M3624 (Δ*gpd1*::GPD2-*B.adolescentis*pflA/pFlB/adhEΔ*gpd2*::GPD1-*B. adolescentis*pflA/pflB/adhEΔ*fdh1*Δ*fdh2*::*B.adolescentis*pflA/pflB/adhE) has an approximately 85% reduction in glycerol formation when grown on > 30% solids corn mash. However, the strain is unable to complete the fermentation even after extended incubation periods. Two modifications of the ammonium assimilation pathway were constructed in M3624 and evaluated for fermentation performance. The modifications were a deletion of *GDH1* and over-expression of Gdh2, resulting in strain M4117 (M3634 Gdh2; *Δgdh1*). The second modification was a deletion of GDH1 and overexpression of GLT1 and GLN1, resulting in strain M4118 (M3634 Glt1; Gln1; *Δgdh1*). These strains were compared to M3624 and the conventional yeast control (M2390 (a wild type unmodified strain isolated from industrial sources)) following fermentation of 31% solids corn mash.

[0240] An industrial corn mash was prepared to a final solids concentration of 31% supplemented with penicillin (0.006mg/mL) and urea (0.5 g/l). Glucoamylase was added at a concentration of 0.6 AGU/gTS. Fermentation was stopped by addition of each strain to an final starting concentration of 0.1 g/l. Vials were capped with a rubber stopper and sealed. A 23-gauge needle was inserted through the stopper to vent and for the safety of the experiment. Vials were incubated at 35°C with shaking at 125rpm. At the termination of the experiment samples were prepared for HPLC analysis of ethanol and residual sugars.

[0241] The results in Figure 69 illustrate that both M4117 and M4118 reach a much higher final ethanol titer than M3624, which was unable to complete the fermentation. Relative to M2390 both M4117 and M4118 had ethanol titers that were 4.2% and 5.2% higher respectively.

Example 2:

Deletion of *GDH1*

[0242] As shown in Figure 5, M3465 (Δ*gpd2*::*B.adolescentis*pflA/pflB/adhEΔ*fdh1*Δ *fdh2*::*B.adolescentis*pflA/pflB/adhE), M3467 (Δfdh1Δfdh2::PFK-pro-adhE-HXT-ter ENO1-pro-pflB-ENO1-ter ADH1-pro-adhE-PDC10-ter TPI-pro-pflA-FBA-ter Δgpd1::GPD2::PFK-pro-adhE-HXT-ter ENO1-pro-pflB-ENO1-ter ADH1-pro-adhE-

PDC10-ter TPI-pro-pflA-FBA-ter) and M3469 (*Δgpd1::B.adolescentis* pflA/pflB/adhE *fdh1Δfdh2Δ::B.adolescentis*pflA/pflB/adhE) have degrees of glycerol reduction ranging from 20% to ~45% relative to the control strain M2390. A clean deletion of GDH1 was constructed in each of these backgrounds resulting in M4400 (M3465 *Δgdh1*), M4401(M3467 *Δgdh1*) and M4402 (M3469 *Δgdh1*) and compared to the conventional yeast control (M2390) following fermentation of 31% solids corn mash (The fermentation was performed as described in Example 1). As shown in Figure 70, all three glycerol reduction strains engineered with a deletion of GDH1 reached a higher ethanol titer than their respective parent strain.

Example 3:

Overexpression of DUR1/2

[0243] Four different DUR1/2 expression cassettes were constructed in both M3467 *Δfdh1Δfdh2::PFK-pro-adhE-HXT-ter ENO1-pro-pflB-ENO1-ter ADH1-pro-adhE-PDC10-ter TPI-pro-pflA-FBA-ter Δgpd1::GPD2::PFK-pro-adhE-HXT-ter ENO1-pro-pflB-ENO1-ter ADH1-pro-adhE-PDC10-ter TPI-pro-pflA-FBA-ter*) and M3469 (*Δgpd1::B. adolescentis* pflA/pflB/adhE *fdh1Δfdh2Δ::B.adolescentis*pflA/pflB/adhE) resulting in strains M4427-M3343 (Table 5). These strains were compared to their parent strain and the conventional yeast control (M2390) following fermentation of 31% solids corn mash (The fermentation was performed as described in Example 1). As shown in Figure 71, all strains containing an overexpression of DUR1/2 reached the same or higher ethanol titers than their respective parent strain but the TEF2 and ADH1 promoter appeared particularly affective. Promoters and terminators used and that could be used include: *S. cerevisiae* TEF 2 promoter: SEQ ID NO: 58, *S. cerevisiae* HXT7 promoter: SEQ ID NO: 59, *S. cerevisiae*, *S. cerevisiae* ADH1 promoter: SEQ ID NO: 60, *S. cerevisiae* TP1 promoter: SEQ ID NO: 61, *S. cerevisiae* FBA1 terminator: SEQ ID NO: 62, *S. cerevisiae* PDC1 terminator: SEQ ID NO: 63, *S. cerevisiae* PMA1 terminator: SEQ IS NO: 64, and *S. cerevisiae ADH3* terminator: SEQ ID NO: 65.

Table 5. Description of constructions and strain designations containing over-expression of DUR1/2

Parent strain	Strain designation	Genetic modification
M3467	M4427	MA0464.1 : expression of DUR1,2 from the TEF2 promoter
M3467	M4428	MA0465.1 : expression of DUR1,2 from the HXT7 promoter
M3467	M4429	MA467.1 : expression of DUR1,2 from the ADH1 promoter
M3467	M4430	MA454.14 : expression of DUR1,2 from the HXT7/TEF2 promoters
M3469	M4431	MA0464.1 : expression of DUR1,2 from the TEF2 promoter
M3469	M4432	MA0465.1 : expression of DUR1,2 from the HXT7 promoter
M3469	M4433	MA467.1 : expression of DUR1,2 from the ADH1 promoter
M3469	M4434	MA454.14 : expression of DUR1,2 from the HXT7/TEF2 promoters

Example 4

Deletion of URE2

[0244] To evaluate an alteration in the *S. cerevisiae* nitrogen catabolite repression system in glycerol reduction backgrounds, a deletion of *URE2* was constructed in M3624 (Example 1), creating strain M4406 (M3624 *Δure2*). This strain was compared to M3624 and the conventional yeast control (M2390) following fermentation of 31% solids corn mash (The fermentation was performed as described in Example 1). As shown in Figure 72, M4406 reached a higher titer than M3624 however a yield increase over the conventional strain was not observed and there was ~15 g/l residual glucose. This is an indication that additional modifications to the NCR system may give improved performance or that an adaptation of M4406 may be required to obtain the potential yield increase.

Example 5

Regulation of nitrogen utilization

[0245] Preferred nitrogen sources generally repress transcription of genes required to utilize non-preferred nitrogen sources. Urea is added as a supplemental nitrogen source in corn mash fermentation; however, there are significant quantities of amino acids and ammonia, both of which are preferred nitrogen sources over urea. Expression of the urea transporter (Dur3) and the urea:amido lyase responsible for intracellular degradation (Dur1/2) may be repressed in the presence amino acids and ammonia as part of a phenomenon referred to as Nitrogen Catabolite Repression (NCR). This repression could slow the rate of urea uptake or require larger quantities to be added. It would be an

economic benefit to a corn ethanol producer if constitutive expression of Dur3 and Dur1,2 allowed them to either reduce the amount of urea needed or accelerate fermentation rate.

[0246] The NCR is controlled by Ure2 and four transcription factors known as Gln3, Gat1, Dal80, and Gzf3. Ure2 participates in repressing gene expression in the presence of non-preferred nitrogen source. It has been observed that deletion of *URE2* activates the expression of genes involved in the uptake of non-preferred nitrogen sources. Inactivation of Ure2 results in dephosphorylation and nuclear localization of the transcription factor Gln3.

Example 5A

Deletion of URE2 deletion of URE2 results in nuclear localization of GLN3 and activation of NCR sensitive genes.

[0247] To evaluate an alteration in the *S. cerevisiae* nitrogen catabolite repression system in glycerol reduction backgrounds, a deletion of Ure2 is constructed as in Example 4. A deletion of *URE2* will be constructed in M3624 (Example 1). Strains in which *URE2* is deleted show a nuclear localization of Gln3, and an activation of NCR sensitive genes, including Dur3 and Dur1/2.

Example 5B

Overexpression GLN3 results in activation of NCR sensitive genes.

[0248] To evaluate an alteration in the *S. cerevisiae* nitrogen catabolite repression system in glycerol reduction backgrounds, Gln3 (SEQ ID NOs: 156 and 157) is overexpressed. Strains in which Gln3 is overexpressed show an activation of NCR sensitive genes, including Dur3 and Dur1/2.

Example 6

Deletion of GDH1 and expression of *S. cerevisiae* GDH2

[0249] The results show that strain M3624 (Example 1) was able to reach a slightly higher titer than strain M2390 (WT), producing 1.5 g/l more ethanol (Figure 73). To create strain M4117 (M3634 Gdh2; *Δgdh1*), the *GDH1* gene was deleted and replaced with 4 copies of the *S. cerevisiae* GDH2 gene expression cassette. The results in Figure 73 below demonstrate that when compared to M3624, M4117 had a clear improvement of 3.7 g/l more ethanol. The data shown in Figure 74 shows that M3624 makes 1.3 g/l glycerol which is 87% less than the wild type strain M2390, which made 10 g/l. The deletion of GDH1 and addition of the *S. cerevisiae* GDH2 expression cassette decreased

the glycerol titers to around 1 g/l. These results illustrate that the combination of glycerol reduction through formate production is synergistic with modifications to the ammonium assimilation pathway.

Example 7

Deletion of GDH1 and expression of *N. crassa* GDH2

[0250] To create strains M5841-M5844, the GDH1 gene was deleted and replaced with 4 copies of the *N. crassa* GDH2 gene expression cassette in Figure 10. Each strain resulted from independent colonies and have the same genotype (Table 6). The results shown in Figure 73 demonstrate that the addition of the *N. crassa* GDH2 to M3624 resulted in titers that were between 3.6g/l and 4.3 g/1 higher than M3624. The data shown in Figure 74 shows that M3624 makes 1.3 g/l glycerol which is 87% less than the wild type strain M2390, which made 10 g/l. The deletion of GDH1 and addition of the *N. crassa* GDH2 expression cassette decreased the glycerol titers to around 1 g/l. These results support the conclusion that a combination of glycerol reduction through formate production is synergistic with modifications to the ammonitun assimilation pathway, even when using a heterologous expression of GDH2.

Table 6. Glycerol deletion strains which further comprise a deletion of gdh1 and an expression of Gdh2.

Strain	Genotype
M2390	WT
M3624	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2p
M4117	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2pAScgdh1::4gdh2
M5841	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2pANcrassagdh1::4gdh2
M5842	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2pANcrassagdh1::4gdh2
M5843	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2pANcrassagdh1::4gdh2
M5844	Δfdh1Δfdh2::4a2pAgpd1::gpd24a2pAgpd2::gpd14a2pANcrassagdh1::4gdh2

[0251] The strains in Table 6 were inoculated in vials containing 4m1 industrial com mash (mini-vials). The fermentation was allowed to proceed for 68hrs and samples were run on an HPLC to obtain ethanol and glycerol values.

[0252] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We Claim:

1. A recombinant yeast comprising:
   a. at least one engineered genetic modification that leads to the up-regulation of one or more native and/or heterologous enzymes that function in one or more ethanol production pathways;
   b. at least one engineered genetic modification that leads to the down-regulation of an enzyme in a glycerol-production pathway; and,
   c. at least one engineered genetic modification that leads to the up-regulation or down-regulation of an enzyme in a nitrogen-assimilation pathway;

wherein the up-regulated enzyme that acts in the ethanol production pathway is pyruvate formate lyase (EC 2.3.1.54), pyruvate formate lyase activating enzyme (EC 1.91.1.4), and/or bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having both of the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.1;

wherein the down-regulated enzyme that acts in the glycerol production pathway is at least one enzyme selected from the group consisting of: a glycerol-3-phosphate dehydrogenase 1 polynucleotide (GPD1) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 1 polypeptide (Gpd1) (EC 1.1.1.8), a glycerol-3-phosphate dehydrogenase 2 polynucleotide (GPD2) (EC 1.1 1.8), a glycerol-3-phosphate dehydrogenase 2 polypeptide (Gpd2) (EC 1.1 1 8), a glycerol-3-phosphate phosphatase I polynucleotide (GPP1) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase polypeptide 1 (Gpp1) (EC 3.1.3.21), a glycerol-3-phosphate phosphatase 2 polynucleotide (GPP2) (EC 3.1.3.21), and a glycerol-3-phosphate phosphatase polypeptide 2 (Gpp2) (EC 3.1.3.21); and

wherein the down-regulated enzyme that acts in the nitrogen-assimilation pathway is glutamate dehydrogenase (Gdh) (EC 1.4.1.4) and wherein the up-regulated enzyme in the nitrogen-assimilation pathway is at least one enzyme selected from the group consisting of glutamate dehydrogenase (Gdh) (EC 1.4.1.2), glutamate synthase (Glt) (EC 1.4.1.14), glutamine synthase (Gln) (EC 6.3.1.2), an ammonium transporter, a urea amido lyase (EC 6.3.4.6), and a urea transporter.

2. The recombinant yeast of claim 1, wherein the down-regulated enzyme glutamate dehydrogenase (Gdh) in the nitrogen-assimilation pathway is a glutamate dehydrogenase 1 (Gdh1) or a glutamate dehydrogenase 3 (Gdh3).

3. The recombinant yeast of claim 2, wherein the Gdh1 or Gdh3 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence selected from the group consisting of: SEQ ID NOs: 25 and 31 and having the same biological activity as the polypeptide of SEQ ID NO: 25 and SEQ ID NO: 31 respectively.

4. The recombinant yeast of claim 3, wherein the Gdh1 or Gdh3 polypeptide sequence is at least 90% identical to a polypeptide sequence selected from the group consisting of: SEQ ID NOs: 25 and 31.

5. The recombinant yeast of claim 3 or claim 4, wherein the Gdh1 or Gdh3 polypeptide sequence is at least 95% identical to a polypeptide sequence selected from the group consisting of: SEQ ID NOs: 25 and 31.

6. The recombinant yeast of any one of claims 3 to 5, wherein the Gdh1 or Gdh3 polypeptide sequence is 100% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 25 and 31.

7. The recombinant yeast of claim 1, wherein the up-regulated enzyme glutamate dehydrogenase (Gdh) in the nitrogen-assimilation pathway is glutamate dehydrogenase 2 (Gdh2).

8. The recombinant yeast of claim 7, wherein the Gdh2 is isolated from an organism from a genus selected from the group consisting of *Saccharomyces* and *Neurospora.*

9. The recombinant yeast of claim 7, wherein the Gdh2 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence selected from the group consisting of: SEQ ID NOs: 27 and 29 and having the same biological activity as the polypeptide of SEQ ID NO: 27 and SEQ ID NO: 29 respectively.

10. The recombinant yeast of claim 9, wherein the Gdh2 polypeptide sequence is at least 90% identical to the polypeptide sequence selected from the group consisting of: SEQ ID NOs: 27 and 29.

11. The recombinant yeast of claim 9 or claim 10, wherein the Gdh2 polypeptide sequence is at least 95% identical to the polypeptide sequence selected from the group consisting of: SEQ ID NOs: 27 and 29.

12. The recombinant yeast of any one of claims 9 to 11, wherein the Gdh2 polypeptide sequence is 100% identical to the polypeptide sequence selected from the group consisting of: SEQ ID NOs: 27 and 29.

13 The recombinant yeast of claim 1, wherein the up-regulated enzyme glutamate synthase (Glt) in the nitrogen-assimilation pathway is a Glt1 isolated from an organism from the genus *Saccharomyces*.

14. The recombinant yeast of claim 13, wherein the Glt1 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 33 and having the same biological activity as the polypeptide of SEQ ID NO: 33.

15. The recombinant yeast of claim 13 or claim 14, wherein the Glt1 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 33.

16. The recombinant yeast of any one of claims 13 to 15, wherein the Glt1 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 33.

17. The recombinant yeast of any one of claims 13 to 16, wherein the Glt1 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 33.

18. The recombinant yeast of claim 1, wherein the up-regulated enzyme glutamine synthase (Gln) in the nitrogen-assimilation pathway is a glutamine synthase (Gln1) isolated from an organism from the genus *Saccharomyces*.

19. The recombinant yeast of claim 18, wherein the Gln1 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 35 and having the same biological activity as the polypeptide of SEQ ID NO: 35.

20. The recombinant yeast of claim 18 or claim 19, wherein the Gln1 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 35.

21. The recombinant yeast of any one of claims 18 to 20, wherein the Gln1 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 35.

22. The recombinant yeast of any one of claims 18 to 21, wherein the Gln1 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 35.

23. The recombinant yeast of claim 1, wherein the up-regulated enzyme ammonium transporter in the nitrogen-assimilation pathway is a MEP protein.

24. The recombinant yeast of claim 23, wherein the MEP protein is isolated from an organism from the genus *Saccharomyces*.

25. The recombinant yeast of claim 23 or claim 24, wherein the MEP protein is selected from the group consisting of: Mep1, Mep2, and Mep3.

26. The recombinant yeast of claim 25, wherein the Mep1 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 19 and having the same biological activity as the polypeptide of SEQ ID NO: 19.

27. The recombinant yeast of claim 25 or claim 26, wherein the Mep1 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 19 and having the same biological activity as the polypeptide of SEQ ID NO: 19.

28. The recombinant yeast of any one of claims 25 to 27, wherein the Mep1 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 19 and having the same biological activity as the polypeptide of SEQ ID NO: 19.

29. The recombinant yeast of any one of claims 25 to 28, wherein the Mep1 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 19.

30. The recombinant yeast of claim 25, wherein the Mep2 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 21 and having the same biological activity as the polypeptide of SEQ ID NO: 21.

31. The recombinant yeast of claim 30, wherein the Mep2 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 21.

32. The recombinant yeast of claim 30 or claim 31, wherein the Mep2 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 21.

33. The recombinant yeast of any one of claims 30 to 32, wherein the Mep2 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 21.

34. The recombinant yeast of claim 25, wherein the Mep3 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 23 and having the same biological activity as the polypeptide of SEQ ID NO: 23.

35. The recombinant yeast of claim 34, wherein the Mep3 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 23.

36. The recombinant yeast of claim 34 or claim 35, wherein the Mep3 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 23.

37. The recombinant yeast of any one of claims 34 to 36, wherein the Mep3 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 23.

38. The recombinant yeast of claim 1, wherein the up-regulated enzyme urea-amido lyase in the nitrogen assimilation pathway is Dur1/2.

39. The recombinant yeast of claim 38, wherein the Dur1/2 is isolated from an organism from the genus *Saccharomyces*.

40. The recombinant yeast of claim 38 or claim 39, wherein the Dur1/2 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 37 and having the same biological activity as the polypeptide of SEQ ID NO: 37.

41. The recombinant yeast of any one of claims 38 to 40, wherein the Dur1/2 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 37.

42. The recombinant yeast of any one of claims 38 to 41, wherein the Dur1/2 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 37.

43. The recombinant yeast of any one of claims 38 to 42, wherein the Dur1/2 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 37.

44. The recombinant yeast of claim 1, wherein the up-regulated enzyme urea transporter in the nitrogen assimilation pathway is a DUR3 or DUR4.

45. The recombinant yeast of claim 44, wherein the DUR3 or DUR4 is isolated from an organism from the genus *Saccharomyces*.

46. The recombinant yeast of claim 44, wherein the Dur3 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence: SEQ ID NO: 39 and having the same biological activity as the polypeptide of SEQ ID NO: 39.

47. The recombinant yeast of claim 46, wherein the Dur3 polypeptide sequence is at least 90% identical to a polypeptide sequence: SEQ ID NO: 39.

48. The recombinant yeast of claim 46 or claim 47, wherein the Dur3 polypeptide sequence is at least 95% identical to a polypeptide sequence: SEQ ID NO: 39.

49. The recombinant yeast of any one of claims 46 to 48, wherein the Dur3 polypeptide sequence is 100% identical to a polypeptide sequence: SEQ ID NO: 39.

50. The recombinant yeast of claim 1, wherein the up-regulated enzyme glutamine synthase (Gln) in the nitrogen assimilation pathway is a glutamine synthase 3 (Gln3).

51. The recombinant yeast of claim 50, wherein the Gln3 is a native Gln3.

52. The recombinant yeast of claim 50 or claim 51, wherein the Gln3 is isolated from the genus *Saccharomyces*.

53. The recombinant yeast of claim 50 or claim 51, wherein the Gln3 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 156 and having the same biological activity as the polypeptide of SEQ ID NO: 156.

54. The recombinant yeast of claim 53, wherein the Gln3 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 156.

55. The recombinant yeast of claim 53 or claim 54, wherein the Gln3 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 156.

56. The recombinant yeast of any one of claims 53 to 55, wherein the Gln3 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 156.

57. The recombinant yeast of claim 1, wherein the Gpd1 comprises a polypeptide sequence at least 80% identical to the polypeptide sequence SEQ ID NO: 5 and having the same biological activity as the polypeptide of SEQ ID NO: 5.

58. The recombinant yeast of claim 57, wherein the Gpd1 polypeptide sequence is at least 90% identical to the polypeptide sequence: SEQ ID NO: 5.

59. The recombinant yeast of claim 57 or claim 58, wherein the Gpd1 polypeptide sequence is at least 95% identical to the polypeptide sequence SEQ ID NO: 5.

60. The recombinant yeast of any one of claims 57 to 59, wherein the Gpd1 polypeptide sequence is 100% identical to the polypeptide sequence: SEQ ID NO: 5.

61. The recombinant yeast of claim 1, wherein the Gpd2 in the glycerol production pathway comprises a polypeptide sequence at least 80% identical to the polypeptide sequence: SEQ ID NO: 7 and having the same biological activity as the polypeptide of SEQ ID NO: 7.

62. The recombinant yeast of claim 61, wherein the Gpd2 polypeptide sequence is at least 90% identical to the polypeptide sequence SEQ ID NO: 7.

63. The recombinant yeast of claim 61 or claim 62, wherein the Gpd2 polypeptide sequence is at least 95% identical to the polypeptide sequence SEQ ID NO: 7.

64. The recombinant yeast of any one of claims 61 to 63, wherein the Gpd2 polypeptide sequence is 100% identical to the polypeptide sequence SEQ ID NO: 7.

65. The recombinant yeast of claim 1, wherein the Gpp1 comprises a polypeptide sequence at least 80% identical to the polypeptide sequence SEQ ID NO: 159 and having the same biological activity as the polypeptide of SEQ ID NO: 159.

66. The recombinant yeast of claim 65, wherein the Gpp1 polypeptide sequence is at least 90% identical to the polypeptide sequence SEQ ID NO: 159.

67. The recombinant yeast of claim 65 or claim 66, wherein the Gpp1 polypeptide sequence is at least 95% identical to the polypeptide sequence SEQ ID NO: 159.

68. The recombinant yeast of any one of claims 65 to 67, wherein the Gpp1 polypeptide sequence is 100% identical to the polypeptide sequence SEQ ID NO: 159.

69. The recombinant yeast of claim 1, wherein the Gpp2 comprises a polypeptide sequence at least 80% identical to the polypeptide sequence SEQ ID NO: 161 and having the same biological activity as the polypeptide of SEQ ID NO: 161.

70. The recombinant yeast of claim 69, wherein the Gpp2 polypeptide sequence is at least 90% identical to the polypeptide sequence SEQ ID NO: 161.

71. The recombinant yeast of claim 69 or claim 70, wherein the Gpp2 polypeptide sequence is at least 95% identical to the polypeptide sequence SEQ ID NO: 161.

72. The recombinant yeast of any one of claims 69 to 71, wherein the Gpp2 polypeptide sequence is 100% identical to the polypeptide sequence SEQ ID NO: 161.

73. The recombinant yeast of claim 1, wherein the pyruvate formate lyase comprises a polypeptide sequence at least 80% identical to the polypeptide sequence SEQ ID NO: 9 and having the same biological activity as the polypeptide of SEQ ID NO: 9.

74. The recombinant yeast of claim 73, wherein the pyruvate formate lyase polypeptide sequence is at least 90% identical to the polypeptide sequence SEQ ID NO: 9.

75. The recombinant yeast of claim 73 or claim 74, wherein the pyruvate formate lyase polypeptide sequence is at least 95% identical to the polypeptide sequence SEQ ID NO: 9.

76. The recombinant yeast of any one of claims 73 to 75, wherein the pyruvate formate lyase polypeptide sequence is 100% identical to the polypeptide sequence encoded by SEQ ID NO. 9.

77. The recombinant yeast of claim 1, wherein the pyruvate formate lyase activating enzyme comprises a polypeptide sequence at least 80% identical to the polypeptide sequence

SEQ ID NO: 11 and having the same biological activity as the polypeptide of SEQ ID NO: 11.

78. The recombinant yeast of claim 77, wherein the pyruvate formate lyase activating enzyme comprises a polypeptide sequence at least 90% identical to the polypeptide sequence SEQ ID NO: 11.

79. The recombinant yeast of claim 77 or claim 78, wherein the pyruvate formate lyase activating enzyme comprises a polypeptide sequence at least 95% identical to the polypeptide sequence SEQ ID NO: 11.

80. The recombinant yeast of any one of claims 77 to 79, wherein the pyruvate formate lyase activating enzyme is a polypeptide sequence 100% identical to the polypeptide sequence SEQ ID NO: 11.

81. The recombinant yeast of claim 1, wherein the bifunctional acetaldehyde-alcohol dehydrogenase comprises a polypeptide sequence at least 80% identical to the polypeptide sequence SEQ ID NO: 13 and having the same biological activity as the polypeptide of SEQ ID NO: 13.

82. The recombinant yeast of claim 81, wherein the bifunctional acetaldehyde-alcohol dehydrogenase comprises a polypeptide sequence at least 90% identical to the polypeptide sequence SEQ ID NO: 13.

83. The recombinant yeast of claim 81 or claim 82, wherein the bifunctional acetaldehyde-alcohol dehydrogenase comprises a polypeptide sequence at least 95% identical to the polypeptide sequence SEQ ID NO: 13.

84. The recombinant yeast of any one of claims 81 to 83, wherein the bifunctional acetaldehyde-alcohol dehydrogenase is a polypeptide sequence 100% identical to the polypeptide sequence SEQ ID NO: 13.

85. The recombinant yeast of any one of claims 1 to 84, wherein the up-regulated enzyme that acts in the ethanol production pathway is an NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase selected from a group of enzymes having both of the following Enzyme Commission Numbers: EC 1.2.1.10 and 1.1.1.2.

86. The recombinant yeast of claim 85, wherein the NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase is isolated from a genus selected from the group consisting of: *Leuconostoc* and *Oenococcus*.

87. The recombinant yeast of claim 86, wherein the NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase comprises a polypeptide sequence at least 80% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 15 and 17 and having the same biological activity as the polypeptide of SEQ ID NO: 15 and SEQ ID NO: 17 respectively.

88. The recombinant yeast of claim 86 or claim 87, wherein the NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase is a polypeptide sequence at least 90% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 15 and 17.

89. The recombinant yeast of any one of claims 85 to 88, wherein the NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase is a polypeptide sequence at least 95% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 15 and 17.

90. The recombinant yeast of any one of claims 85 to 89, wherein the NADPH-dependent bifunctional acetaldehyde-alcohol dehydrogenase is a polypeptide sequence 100% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 15 and 17.

91. The recombinant yeast of any one of claims 1 to 90, wherein the yeast further comprises a down-regulation in one or more native enzymes encoded by a formate dehydrogenase enzyme Fdh1 or Fdh2 selected from the group consisting of: EC 1.2.1.43 and EC 1.2.1.2.

92. The recombinant yeast of claim 91, wherein the Fdh1 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 2 and having the same biological activity as the polypeptide of SEQ ID NO: 2.

93. The recombinant yeast of claim 92, wherein the Fdh1 polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 2.

94. The recombinant yeast of claim 92, wherein the Fdh1 polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 2.

95. The recombinant yeast of claim 92, wherein the Fdh1 polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 2.

96. The recombinant yeast of any one of claims 91 to 95, wherein the Fdh2 comprises a polypeptide sequence at least 80% identical to a polypeptide sequence encoded by the polynucleotide sequence of SEQ ID NO: 3 and having the same biological activity as the polypeptide encoded by the polynucleotide sequence of SEQ ID NO: 3.

97. The recombinant yeast of claim 96, wherein the Fdh2 polypeptide sequence is at least 90% identical to a polypeptide sequence encoded by the polynucleotide sequence of SEQ ID NO: 3.

98. The recombinant yeast of claim 96, wherein the Fdh2 polypeptide sequence is at least 95% identical to a polypeptide sequence encoded by the polynucleotide sequence of SEQ ID NO: 3.

99. The recombinant yeast of claim 96, wherein the Fdh2 polypeptide sequence is 100% identical to a polypeptide sequence encoded by the polynucleotide sequence of SEQ ID NO: 3.

100. The recombinant yeast of any one of claims 1 to 99, wherein the recombinant yeast further comprises heterologous GPD1 polynucleotide operably linked to a native GPD2 promoter.

101. The recombinant yeast of any one of claims 1 to 100, wherein the recombinant yeast further comprises a heterologous GPD2 polynucleotide operably linked to a native GPD1 promoter.

102. The recombinant yeast of any one of claims 1 to 101, wherein the yeast further comprises an up-regulation or down-regulation of a regulatory element, wherein the regulatory element is selected from the group consisting of: Ure2 and Aual.

103. The recombinant yeast of claim 102, wherein the regulatory element is a polypeptide sequence at least 80% identical to a polypeptide sequence of SEQ ID NO: 55 and having the same biological activity as the polypeptide of SEQ ID NO: 55.

104. The recombinant yeast of claim 103, wherein the regulatory element is a polypeptide sequence at least 90% identical to a polypeptide sequence of SEQ ID NO: 55.

105. The recombinant yeast of claim 103, wherein the regulatory element is a polypeptide sequence at least 95% identical to a polypeptide sequence of SEQ ID NO: 55.

106. The recombinant yeast of claim 102, wherein the regulatory element is a polypeptide sequence identical to a polypeptide sequence of SEQ ID NO: 55.

107. The recombinant yeast of claim 102, wherein the regulatory element is a polypeptide sequence at least 80% identical to a polypeptide sequence of SEQ ID NO: 57 and having the same biological activity as the polypeptide of SEQ ID NO: 57.

108. The recombinant yeast of claim 107, wherein the regulatory element is a polypeptide sequence at least 90% identical to a polypeptide sequence of SEQ ID NO: 57.

109. The recombinant yeast of claim 107 or claim 108, wherein the regulatory element is a polypeptide sequence at least 95% identical to a polypeptide sequence of SEQ ID NO: 57.

110. The recombinant yeast of any one of claims 107 to 109, wherein the regulatory element is a polypeptide sequence identical to the polypeptide sequence of SEQ ID NO: 57.

111. The recombinant yeast of any one of claims 1 to 110, wherein the yeast further comprises at lest one additional up-regulated enzyme and wherein said additional up-regulated enzyme is a glucoamylase enzyme with an EC number 3.2.1.3.

112. The recombinant yeast of claim 111, wherein the up-regulated enzyme is isolated from the genus *Saccharomycopsis*.

113. The recombinant yeast of claim 111, wherein the glucoamylase comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 163 and having the same biological activity as the polypeptide of SEQ ID NO: 163.

114. The recombinant yeast of claim 113, wherein the glucoamylase polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 163.

115. The recombinant yeast of claim 113 or claim 114, wherein the glucoamylase polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 163.

116. The recombinant yeast of any one of claims 113 to 115, wherein the glucoamylase polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 163.

117. The recombinant yeast of any one of claims 1 to 110, wherein the yeast further comprises at least one additional up-regulated enzyme and wherein the up-regulated enzyme is a permease.

118. The recombinant yeast of claim 117, wherein the permease is isolated from the genus *Saccharomyces*.

119. The recombinant yeast of claim 117, wherein the permease comprises a polypeptide sequence at least 80% identical to a polypeptide sequence SEQ ID NO: 53 and having the same biological activity as the polypeptide of SEQ ID NO: 53.

120. The recombinant yeast of claim 119, wherein the permease polypeptide sequence is at least 90% identical to a polypeptide sequence SEQ ID NO: 53.

121. The recombinant yeast of claim 119 or claim 120, wherein the permease polypeptide sequence is at least 95% identical to a polypeptide sequence SEQ ID NO: 53.

122. The recombinant yeast of any one of claims 119 to 121, wherein the permease polypeptide sequence is 100% identical to a polypeptide sequence SEQ ID NO: 53.

123. The recombinant yeast of any one of claims 1 to 110, wherein the yeast further comprises at lest one additional up-regulated enzyme and wherein said additional up-regulated enzyme is a protease with EC number: 3.4.23.41.

124. The recombinant yeast of claim 123, wherein the protease is isolated from a genus selected from the group consisting of *Zea*, *Neurospora*, *Podospora*, and *Magnaporthe*.

125. The recombinant yeast of claim 123, wherein the protease comprises a polypeptide sequence at least 80% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 41, 43, 45, 47, 49, and 51, and having the same biological activity as the polypeptide of SEQ ID NOs: 41, 43, 45, 47, 49 and 51 respectively.

126. The recombinant yeast of claim 125, wherein the protease polypeptide sequence is at least 90% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 41, 43, 45, 47, 49, and 51.

127. The recombinant yeast of claim 125 or claim 126, wherein the protease polypeptide sequence is at least 95% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 41, 43, 45, 47, 49, and 51.

128. The recombinant yeast of any one of claims 124 to 127, wherein the protease polypeptide sequence is 100% identical to a polypeptide sequence selected from the group consisting of SEQ ID NOs: 41, 43, 45, 47, 49, and 51.

129. The recombinant yeast of any one of claims 1 to 128, wherein the up-regulated or down-regulated enzymes are under the control of a heterologous promoter.

130. The recombinant yeast of claim 129, wherein the heterologous promoter is selected from the group consisting of: TEF2 (SEQ ID NO: 58), HXT7 (SEQ ID NO: 59), ADH1 (SEQ ID NO: 60), and TP1 (SEQ ID NO: 61).

131. The recombinant yeast of any one of claims 1 to 130, wherein the yeast is from the genus *Saccharomyces*.

132. The recombinant yeast of claim 131, wherein the yeast is *Saccharomyces cerevisiae*.

133. The recombinant yeast of any one of claims 1 to 132, wherein the yeast produces ethanol at a higher yield than an otherwise identical yeast lacking the genetic modifications.

134. The recombinant yeast of any one of claims 1 to 133, wherein the yeast produces an ethanol titer from 1% to 10% more than an otherwise identical yeast lacking the genetic modifications.

135. The recombinant yeast of any one of claims 1 to 134, wherein the yeast produces an ethanol titer of at least 125 g/L.

136. The recombinant yeast of any one of claims 1 to 135, wherein the yeast produces glycerol at a lower yield than an otherwise identical yeast lacking the genetic modifications.

137. The recombinant yeast of any one of claims 1 to 136, wherein the yeast produces a glycerol titer of from 10 to 100% less than an otherwise identical yeast lacking the genetic modifications.

138. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

139. The recombinant yeast of claim 138, wherein Gdh1 is down-regulated.

140. The recombinant yeast of claim 138 or claim 139, wherein Glt1 is up-regulated.

141. The recombinant yeast of any one of claims 138 to 140, wherein Gln1 is up-regulated.

142. The recombinant yeast of any one of claims 138 to 141, wherein Aual is down-regulated.

143. The recombinant yeast of any one of claims 138 to 142, wherein Urel is down-regulated.

144. The recombinant yeast of any one of claims 138 to 143, further comprising Dur1/2.

145. The recombinant yeast of any one of claims 138 to 144, further comprising an up-regulated enzyme selected from the group consisting of: Dur3 and Dur4.

146. The recombinant yeast of any one of claims 138 to 145, further comprising an up-regulated enzyme selected from the group consisting of Mep1, Mep2, and Mep3.

147. The recombinant yeast of any one of claims 138 to 146, further comprising an up-regulated Gln3.

148. The recombinant yeast of any one of claims 138 to 147, further comprising GPD1 expressed from a GPD2 promoter.

149. The recombinant yeast of any one of claims 138 to 148, further comprising a GPD2 expressed from a GPD1 promoter.

150. A recombinant yeast comprising down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

151. The recombinant yeast of claim 150, wherein Gdh1 is down-regulated.

152. The recombinant yeast of claim 150 or claim 151, wherein Glt1 is up-regulated.

153. The recombinant yeast of any one of claims 150 to 152, wherein Gln1 is up-regulated.

154. The recombinant yeast of any one of claims 150 to 153, wherein Aual is down-regulated.

155. The recombinant yeast of any one of claims 150 to 154, wherein Urel is down-regulated.

156. The recombinant yeast of any one of claims 150 to 155, further comprising Dur1/2.

157. The recombinant yeast of any one of claims 150 to 156, further comprising an up-regulated enzyme selected from the group consisting of: Dur3 and Dur4.

158. The recombinant yeast of any one of claims 150 to 157, further comprising an up-regulated enzyme selected from the group consisting of: Mep1, Mep2, and Mep3.

159. The recombinant yeast of any one of claims 150 to 158, further comprising an up-regulated Gln3.

160. The recombinant yeast of any one of claims 150 to 159, further comprising GPD1 expressed from a GPD2 promoter.

161. The recombinant yeast of any one of claims 150 to 160, further comprising a GPD2 expressed from a GPD1 promoter.

162. A recombinant yeast comprising: down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

163. The recombinant yeast of claim 162, wherein Gdh1 is down-regulated.

164. The recombinant yeast of claim 162 or claim 163, wherein Glt1 is up-regulated.

165. The recombinant yeast of any one of claims 162 to 164, wherein Gln1 is up-regulated.

166. The recombinant yeast of any one of claims 162 to 165, wherein Aual is down-regulated.

167. The recombinant yeast of any one of claims 162 to 166, wherein Urel is down-regulated.

168. The recombinant yeast of any one of claims 162 to 167, further comprising Dur1/2.

169. The recombinant yeast of any one of claims 162 to 168, further comprising an up-regulated enzyme selected from the group consisting of: Dur3 and Dur4.

170. The recombinant yeast of any one of claims 162 to 169, further comprising an up-regulated enzyme selected from the group consisting of: Mep1, Mep2, and Mep3.

171. The recombinant yeast of any one of claims 162 to 170, further comprising an up-regulated Gln3.

172. The recombinant yeast of any one of claims 162 to 171, further comprising GPD1 expressed from a GPD2 promoter.

173. The recombinant yeast of any one of claims 162 to 172, further comprising a GPD2 expressed from a GPD1 promoter.

174. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme,

GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, and up-regulated Gdh2.

175. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, up-regulated Glt1 and up-regulated Gln1.

176. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, GPD2 under the control of the GPD1 promoter, up-regulated Glt1 and up-regulated Gln1.

177. A recombinant yeast comprising: down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

178. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and GPD2 under the control of the GPD1 promoter.

179. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, down-regulated Gdh1, up-regulated AdhE, up-regulated pyruvate formate lyase, and an up-regulated pyruvate formate lyase-activating enzyme.

180. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, upregulated-DUR/12, and GPD2 under the control of the GPD1 promoter.

181. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, and up-regulated-DUR/12.

182. The recombinant yeast of claim 181, wherein the DUR1/2 is driven by a TEF2 promoter.

183. The recombinant yeast of claim 181, wherein the DUR1/2 is driven by an HXT7 promoter.

184. The recombinant yeast of claim 181, wherein the DUR1/2 is driven by a ADH1 promoter.

185. The recombinant yeast of claim 181, wherein the DUR1/2 is driven by an HXT7/TEF2 promoter.

186. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, down-regulated Ure2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, GPD1 under the control of the GPD2 promoter, and GPD2 under the control of the GPD1 promoter.

187. The recombinant yeast of claim 186, further comprising an up-regulated DUR3.

188. The recombinant yeast of claim 186 or claim 187, further comprising an upregulated DUR1/2.

189. The recombinant yeast of any one of claims 174 to 188, further comprising an up-regulated Gln3.

190. A recombinant yeast comprising: down-regulated Gpd1, down-regulated Gpd2, down-regulated Fdh1, down-regulated Fdh2, up-regulated AdhE, up-regulated pyruvate formate lyase, an up-regulated pyruvate formate lyase-activating enzyme, up-regulated GDH2, GPD1 under the control of the GPD2 promoter, and GPD2 under the control of the GPD1 promoter.

191. A composition comprising a recombinant yeast from any one of claims 1 to 190 and a carbon-containing feedstock.

192. The composition of claim 191, wherein the feedstock is selected from the group consisting of woody biomass, grasses, sugar-processing residues, municipal waste, agricultural wastes and any combination thereof.

193. The composition of claim 192, wherein the feedstock comprises recycled wood pulp fiber, sawdust, hardwood, softwood, rice straw, rice hulls, barley straw, corn cobs, cereal straw, wheat straw, canola straw, oat straw, oat hulls, corn fiber, stover, succulents, agave, cane bagasse, switchgrass, miscanthus, paper sludge, municipal waste or any combination thereof.

194. A method of producing a fermentation product comprising:

a. providing the composition of any one of claims 191 to 193;
b. contacting the composition with a carbon containing feedstock, wherein the recombinant yeast is capable of fermenting the carbon containing feedstock to yield the fermentation product; and,
c. optionally recovering the fermentation production.

195. A method of producing ethanol comprising:

a. providing the recombinant yeast of any one of claims 1 to 190;
b. culturing the recombinant yeast in the presence of a carbon containing feedstock for sufficient time to produce ethanol; and, optionally,
c. extracting the ethanol.

196. A method of reducing glycerol production comprising providing a recombinant yeast of any one of claims 1 to 189, wherein the glycerol titer is from 10 to 100% less than the rate compared to an otherwise identical yeast lacking the genetic modifications.

197. The method of claim 194 or 195, wherein the glycerol production is reduced as compared to an otherwise identical yeast lacking the genetic modifications, and wherein the ethanol titer increased by at least 1 to 10% when the recombinant yeast is cultured in the presence of a carbon containing feedstock for a sufficient time to produce ethanol.

198. The method of any one of claims 194 to 197, wherein nitrogen is added to the culture containing the recombinant yeast and the feedstock.

199. A co-culture comprising at least two host cells wherein

a. one of the host cells comprises a recombinant yeast from any one of claims 1 to 190; and,

b. another host cell that is genetically distinct from (a).

200. The co-culture of claim 199, wherein the genetically distinct host cell is a yeast or bacterium.

201. The co-culture of claim 199 or claim 200, wherein the genetically distinct host cell is any organism from the genus *Saccharomyces, Issatchenkia, Pichia, Clavispora, Candida, Hansenula, Kluyveromyces, Trichoderma, Thermoascus, Escherichia, Clostridium, Caldicellulosiruptor, Zymomonas, Thermoanaerobacter* or *Thermoanaerobacterium*.

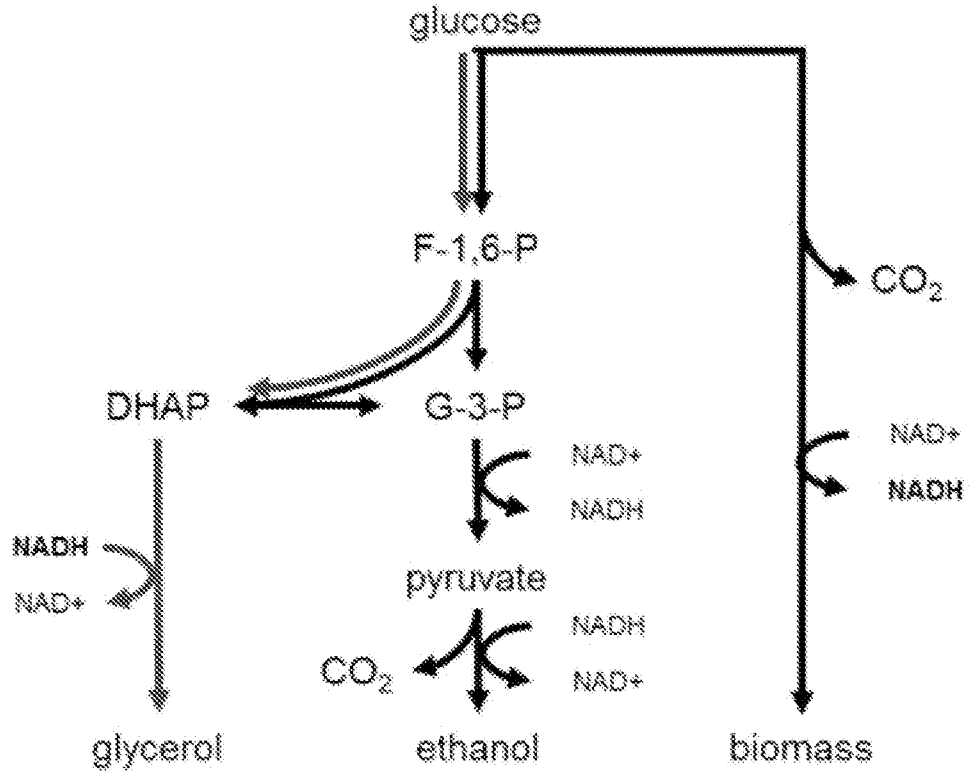

glucose
F-1,6-P
CO_2
DHAP
G-3-P
NAD+
NADH
NAD+
NADH
NADH
NAD+
pyruvate
NADH
CO_2
NAD+
glycerol
ethanol
biomass

FIGURE 1

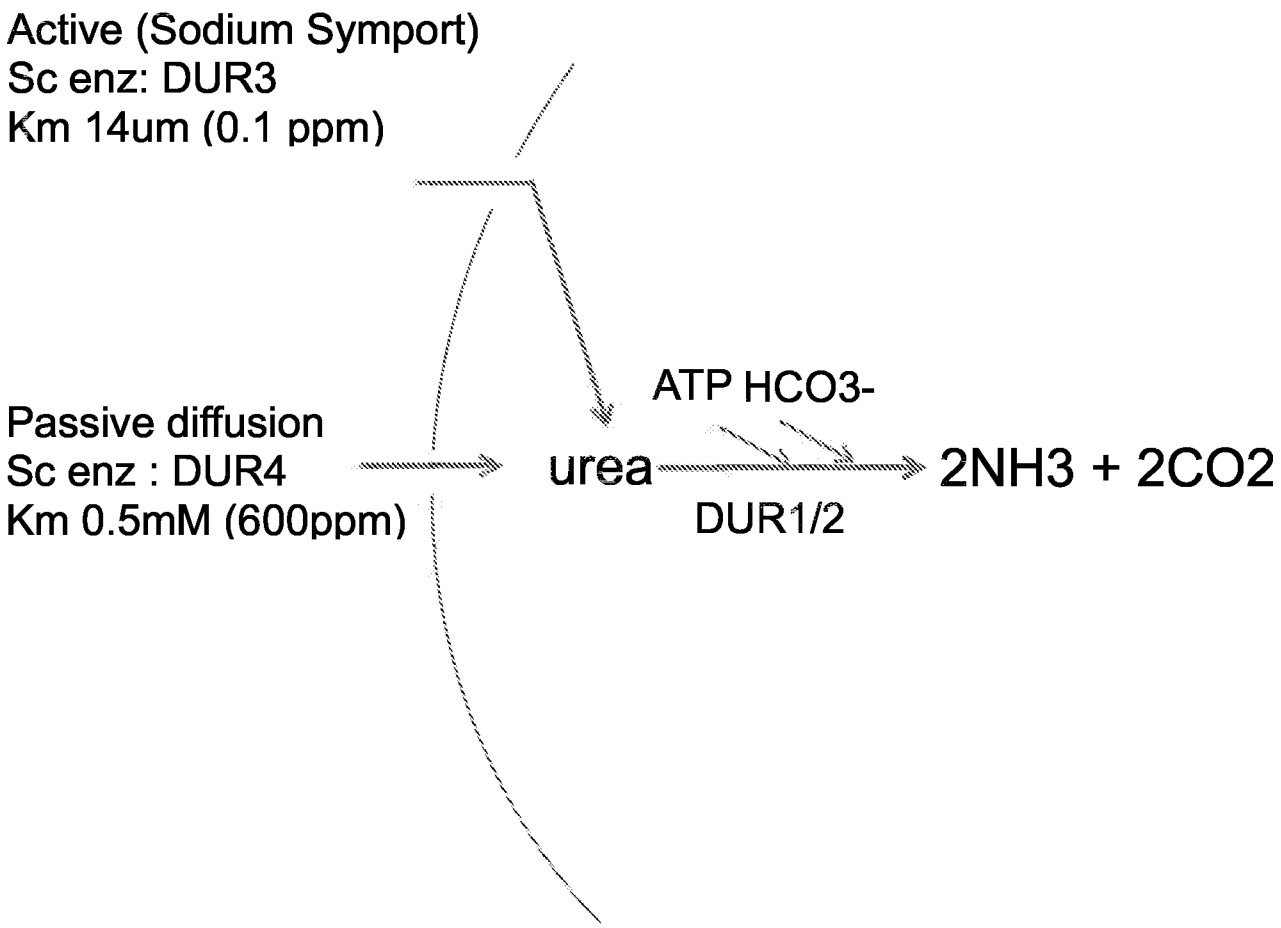

Active (Sodium Symport)
Sc enz: DUR3
Km 14um (0.1 ppm)
Passive diffusion
Sc enz : DUR4
Km 0.5mM (600ppm)
ATP HCO3-
urea
DUR1/2
2NH3 + 2CO2

FIGURE 2

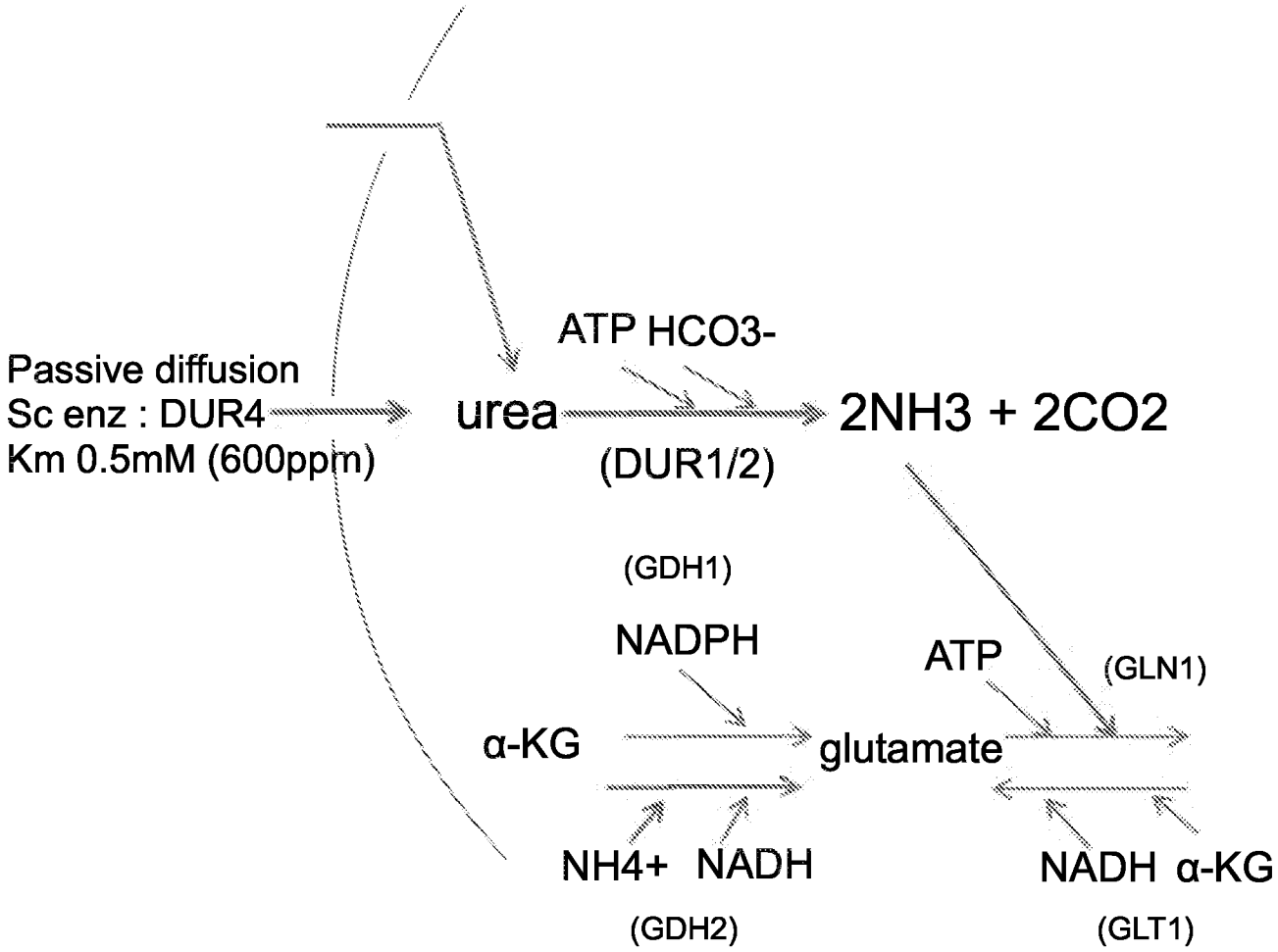

Passive diffusion
Sc enz : DUR4
Km 0.5mM (600ppm)
urea
ATP HCO3-
(DUR1/2)
2NH3 + 2CO2
(GDH1)
NADPH
α-KG
glutamate
ATP
(GLN1)
NH4+ NADH
(GDH2)
NADH α-KG
(GLT1)

FIGURE 3

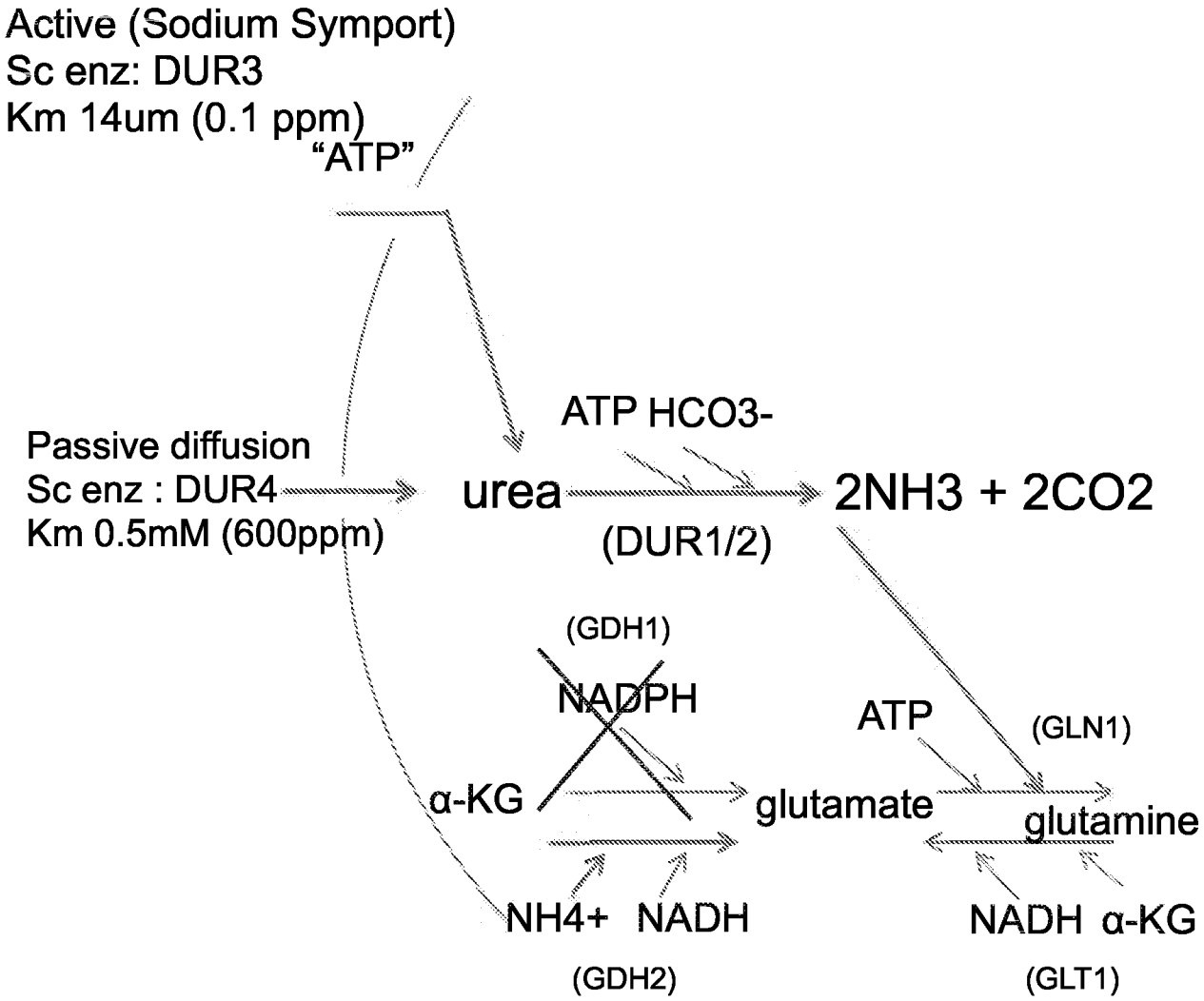

Active (Sodium Symport)
Sc enz: DUR3
Km 14um (0.1 ppm)
"ATP"
ATP HCO3-
Passive diffusion
Sc enz : DUR4
Km 0.5mM (600ppm)
urea
(DUR1/2)
2NH3 + 2CO2
(GDH1)
NADPH
ATP
(GLN1)
α-KG
glutamate
glutamine
NH4+ NADH
(GDH2)
NADH α-KG
(GLT1)

FIGURE 4

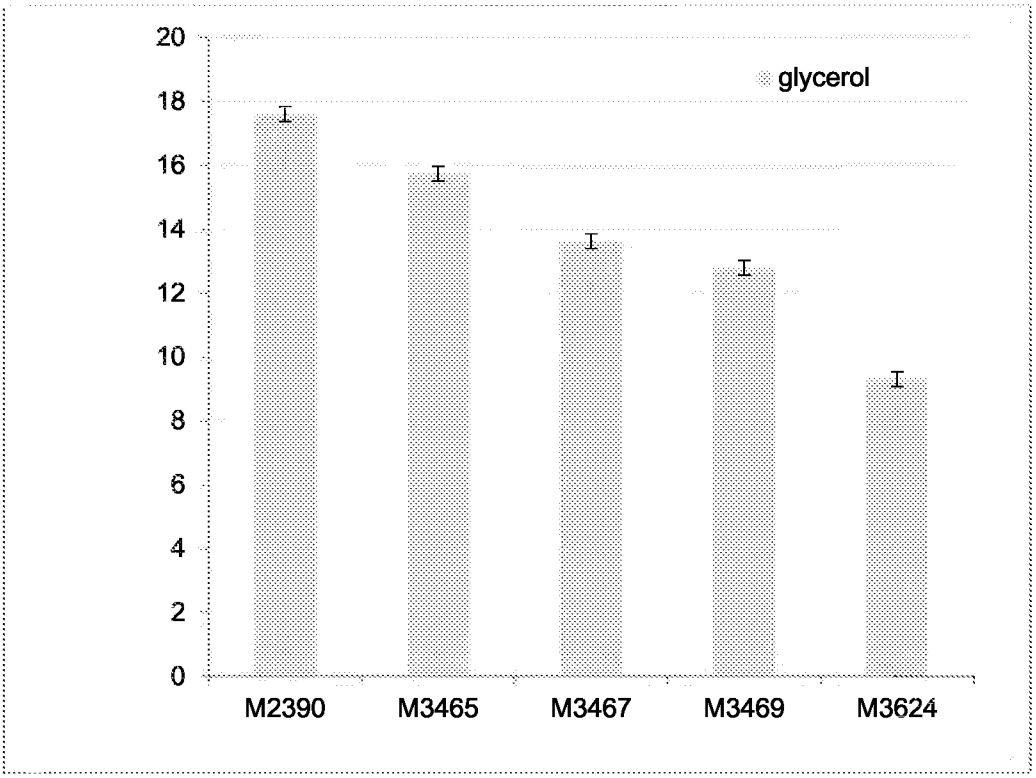

20
18
16
14
12
10
8
6
4
2
0
glycerol
M2390
M3465
M3467
M3469
M3624

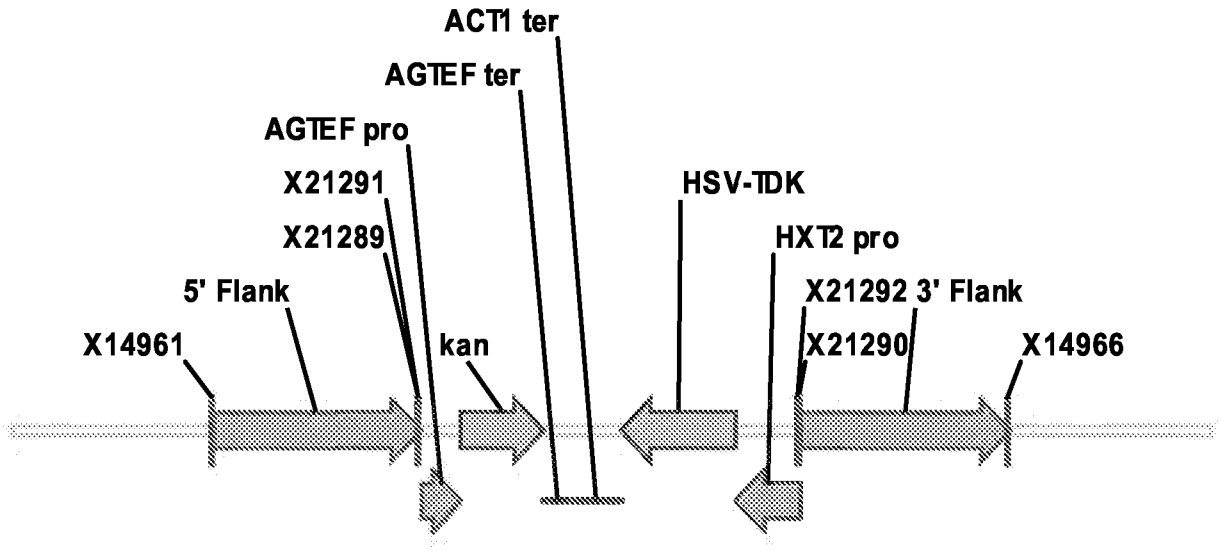
FIGURE 5

MA0631

Marked Deletion of GDH1 (just kan showed here – nat construct the same)

MA0631

Fragment	Primers	Template	Expected Size
GDH1 5' Flank	X14961/X21291	gDNA	2065bp
AGTEF pro-kan/nat/tdk-HXT2 pro	X21289/X21290	pMU2873/pMU2879	3820bp and 3583bp
GDH1 3' Flank	X21292/X14966	gDNA	2056bp



MA0631 GDH1::knt

11808 bp

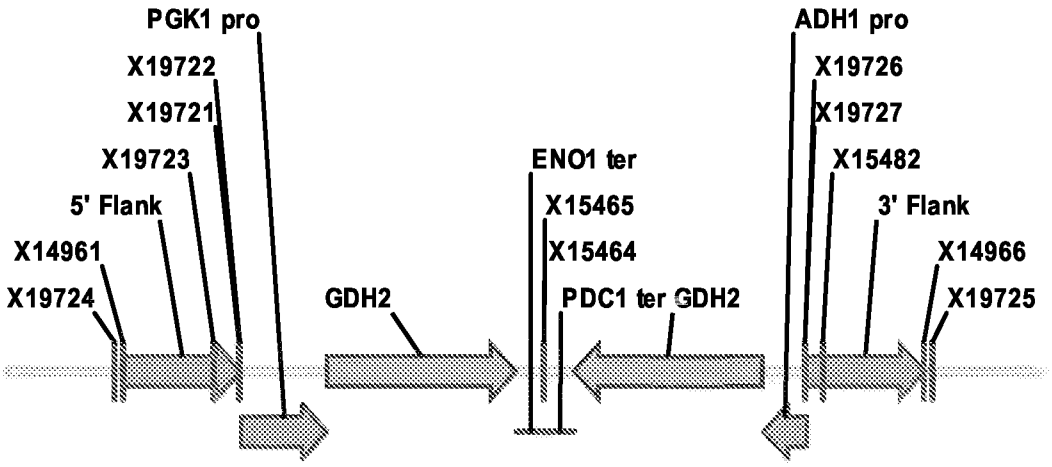
FIGURE 6

MA0425

4 copy *S. cerevisiae* GDH2 integrated at GDH1 locus

MA0425

Fragment	Primers	Template	Expected Size
GDH1 5' Flank	X14961/X19722	gDNA	2065bp
PGKpro-GDH2-ENO1ter	X19721/X15464	pMU2908	5283bp
ADH1pro-GDH2-PDC1ter	X15465/X19727	pMU2909	4546bp
GDH1 3' Flank	X19726/X14966	gDNA	2056bp



MA0425
17884bp

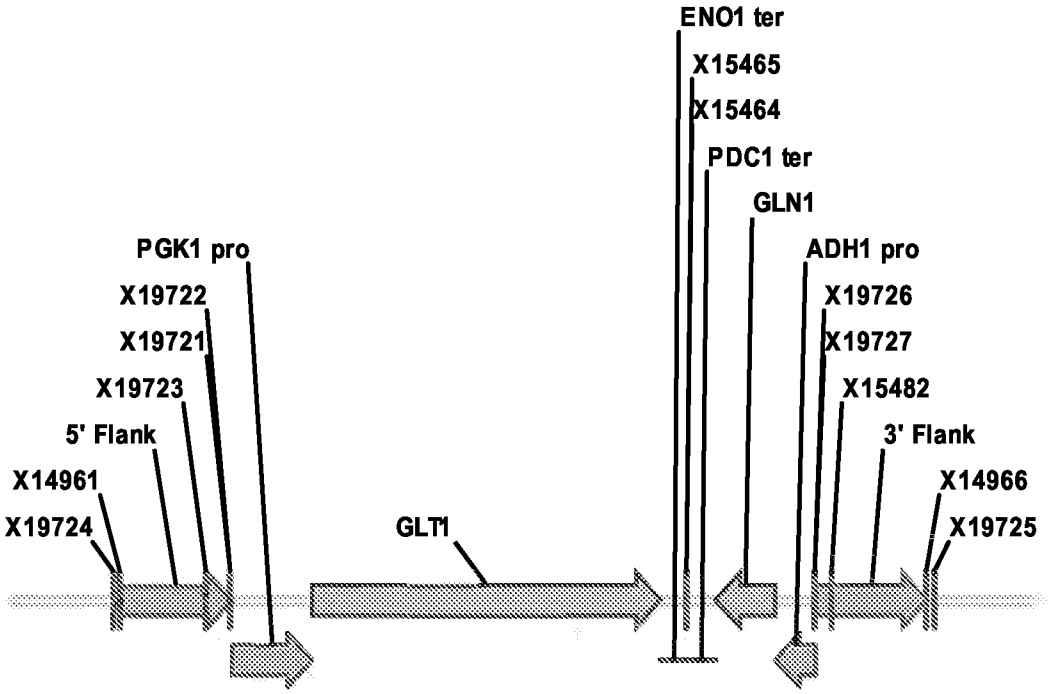
FIGURE 7

MA0426

2 copy *S. cerevisiae* GLT1/GLN1 integrated at GDH1 locus

MA0426

Fragment	Primers	Template	Expected Size
GDH1 5' Flank	X14961/X19722	M2390 gDNA	2065bp
PGKpro-GLT1-ENOter	X19721/X15464	pMU2913	8442bp
ADH1pro-GLN1-PDC1ter	X15465/X19727	pMU2911	2363bp
GDH1 3' Flank	X19726/X14966	M2390 gDNA	2056bp



MA0426

18860 bp

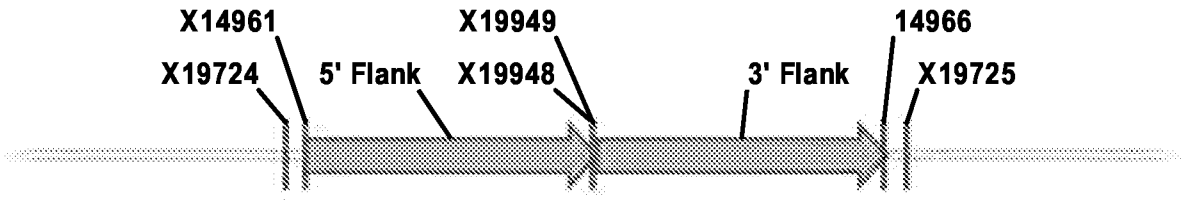
FIGURE 8

MA0888

Clean deletion of GDH1

MA0888

Fragment	Primers	Template	Expected Size
5' Flank	X14961/X19949	M2390 gDNA	2065bp
3' Flank	X19948/X14966	M2390 gDNA	2056bp



MA0888 GDH1::deletion

8055bp

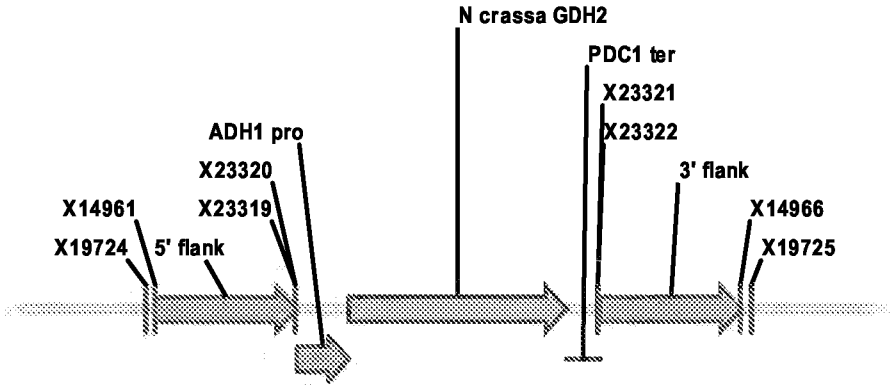
FIGURE 9

MA0837

2 copy *N. crassa* GDH2 integrated at GDH1 locus

MA0837

Fragment	Primers	Template	Expected Size
GDH1 5' Flank	X14961/X23320	gDNA	2065bp
ADH1pro-GDH2-PDC1 ter	X23319/X23322	pMU3597	4403bp
GDH1 3' Flank	X23321/X14966	gDNA	2056bp



MA0837
12458 bp

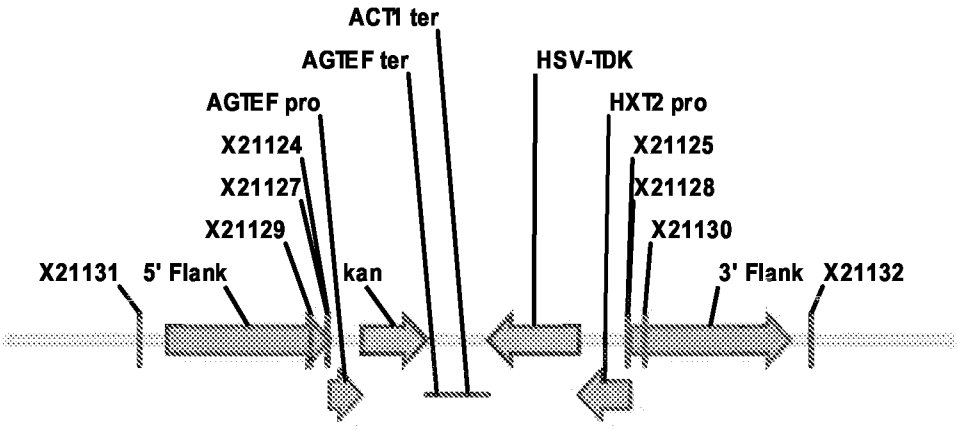
FIGURE 10

MA0616

GDH2 marked deletion

MA0616

Fragment	Primers	Template	Expected Size
GDH2 5' Flank	X21123/X21124	M2390 gDNA	2024bp
AGTEFpro-kan/nat/tdk-HXT2pro	X21127/X21128	pMU2873/pMU2879	3820bp and 3583bp
GDH2 3' Flank	X21125/X21126	M2390 gDNA	1990bp



MA0616
11754 bp

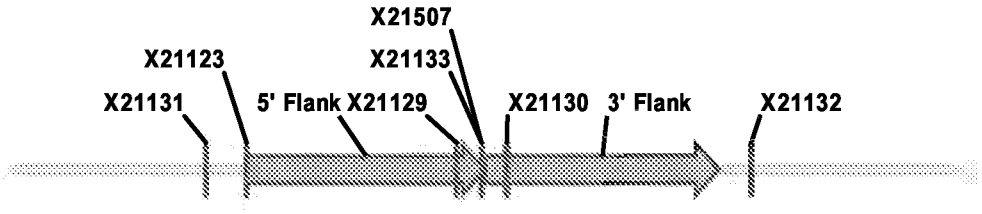
FIGURE 11

MA0616.1

GDH2 clean deletion

MA0616.1

Fragment	Primers	Template	Expected Size
GDH2 5' Flank	X21123/X21507	M2390 gDNA	2024bp
GDH2 3' Flank	X21133/X21126	M2390 gDNA	1990bp



MA0616.1
8000 bp

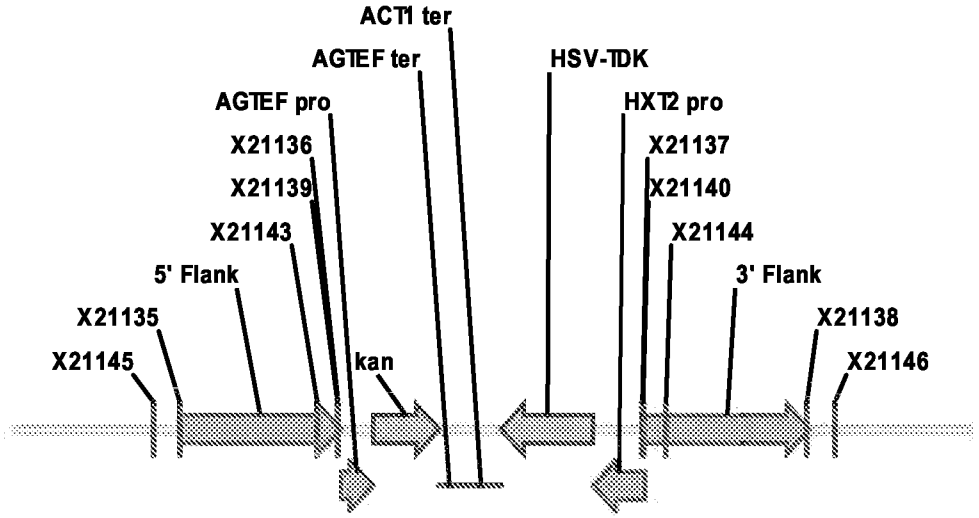
FIGURE 12

MA0615

GDH3 marked deletion

MA0615

Fragment	Primers	Template	Expected Size
GDH3 5' Flank	X21135/X21136	M2390 gDNA	1976bp
AGTEFpro-kan/nat/tdk-HXT2pro	X21139/X21140	pMU2873/pMU2879	3820bp and 3583bp
GDH3 3' Flank	X21137/X21138	M2390 gDNA	2015bp



MA0615
11730 bp

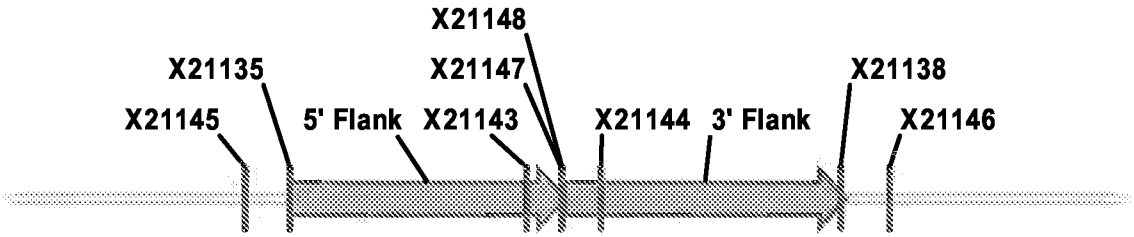
FIGURE 13

MA0615.1

GDH3 clean deletion

MA0615.1

Fragment	Primers	Template	Expected Size
GDH3 5' Flank	X21135/X21148	M2390 gDNA	1976bp
GDH3 3' Flank	X21147/X21138	M2390 gDNA	2015bp



MA0615.1

7976bp

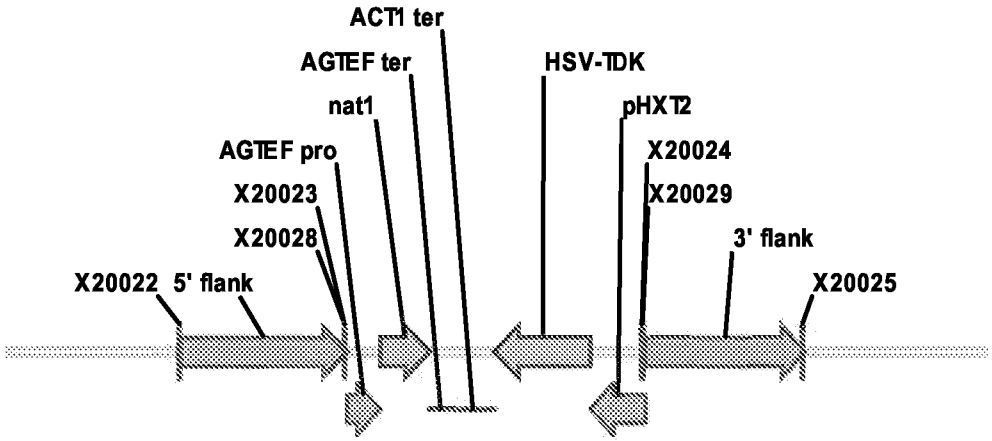
FIGURE 14

MA0622

URE2 marked deletion

MA0622

Fragment	Primers	Template	Expected Size
URE2 5' Flank	X20022/X20023	M2390 gDNA	1968bp
AGTEF pro-kan/nat/tdk-HXT2 pro	X20028/X20029	pMU2873, pMU2879	3820bp and 3583bp
URE2 3' Flank	X20024/X20025	M2390 gDNA	1885bp



MA0622
11517 bp

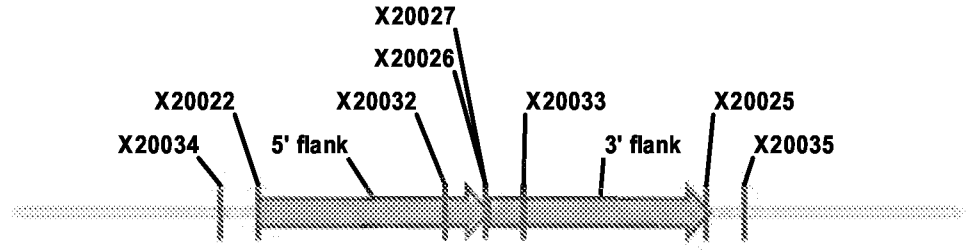
FIGURE 15

MA0622.1

URE2 clean deletion

MA0622.1

Fragment	Primers	Template	Expected Size
URE2 5' Flank	X20022/X20027	M2390 gDNA	1968bp
URE2 3' Flank	X20026/X20025	M2390 gDNA	1885bp



MA0622.1
8000 bp

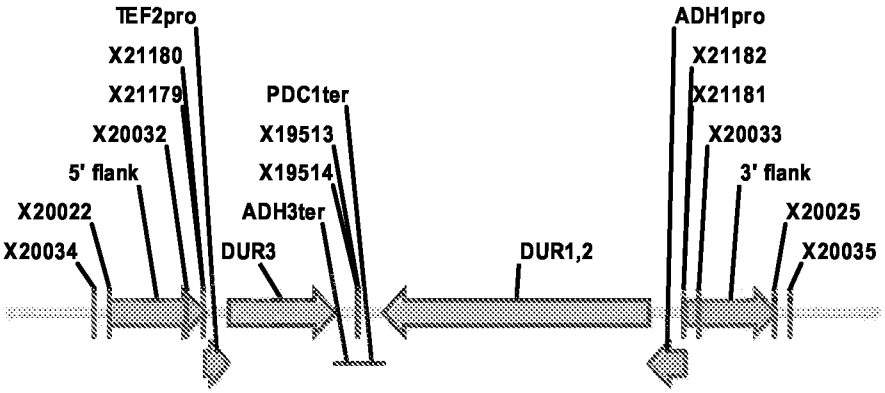
FIGURE 16

MA0580

DUR1,2,3 over expression at URE2 locus

MA0580

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21180	M2390 gDNA	1968bp
TEF2pro-DUR3-ADH3ter	X21179/X19513	pMU3464	3208bp
ADH1pro-DUR1,2-PDC1ter	X19514/X21181	pMU3411	6758bp
URE2 3' Flank	X21182/X20025	M2390 gDNA	1885bp



MA0580
17966 bp

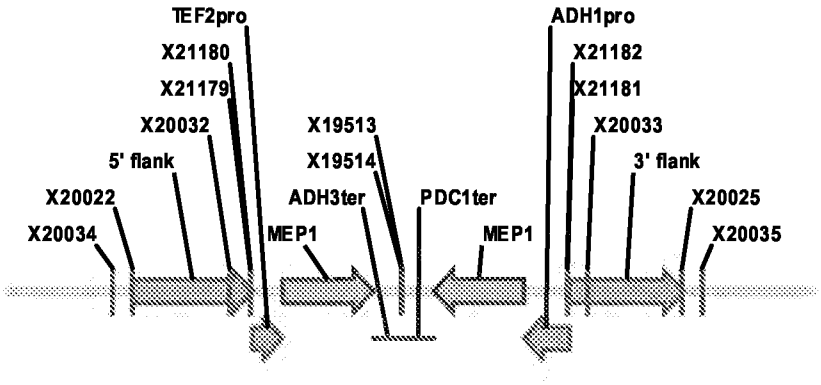
FIGURE 17

MA0581

MEP1 over expression at URE2 locus

MA0581

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21180	M2390 gDNA	1968bp
TEF2pro-MEP1-ADH3ter	X21179/X19513	pMU3465	2479bp
ADH1pro-MEP1-PDC1ter	X19514/X21181	pMU3460	2729bp
URE2 3' Flank	X21182/X20025	M2390 gDNA	1885bp



MA0581
13208 bp

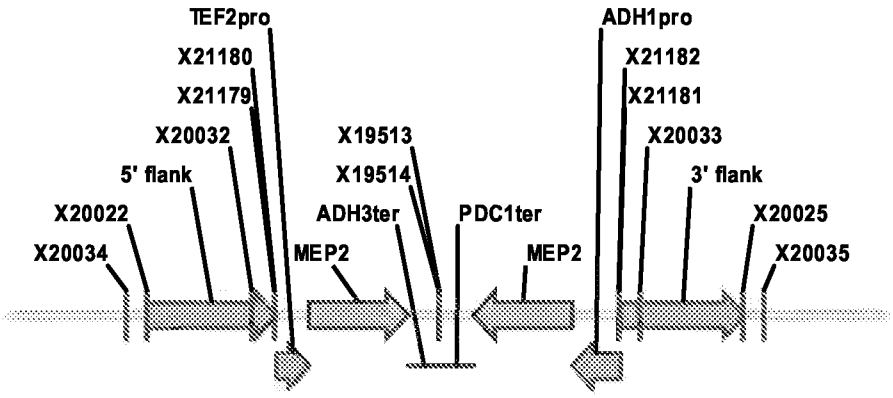
FIGURE 18

MA0582

MEP2 over expression at URE2 locus

MA0582

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21180	M2390 gDNA	1968bp
TEF2pro-MEP2-ADH3ter	X21179/X19513	pMU3465	2479bp
ADH1pro-MEP2-PDC1ter	X19514/X21181	pMU3461	2750bp
URE2 3' Flank	X21182/X20025	M2390 gDNA	1885bp



MA0582
13250 bp

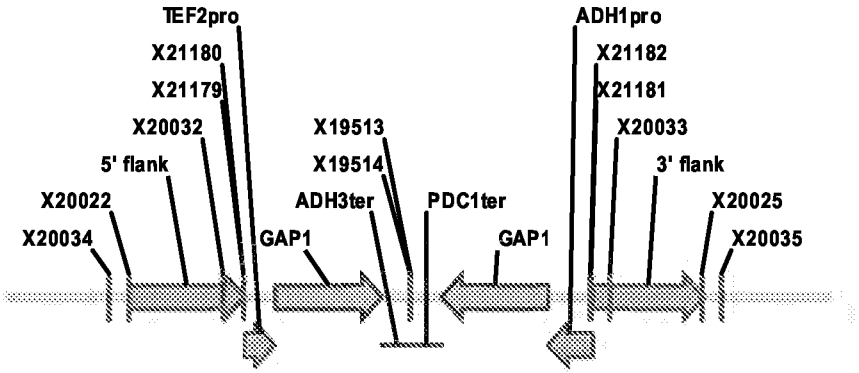
FIGURE 19

MA0583

GAP1 over expression at URE2 locus

MA0583

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21180	M2390 gDNA	1968bp
TEF2pro-GAP1-ADH3ter	X21179/X19513	pMU3468	2809bp
ADH1pro-GAP1-PDC1ter	X19514/X21181	pMU3463	3059bp
URE2 3' Flank	X21182/X20025	M2390 gDNA	1885bp



MA0583
13868 bp

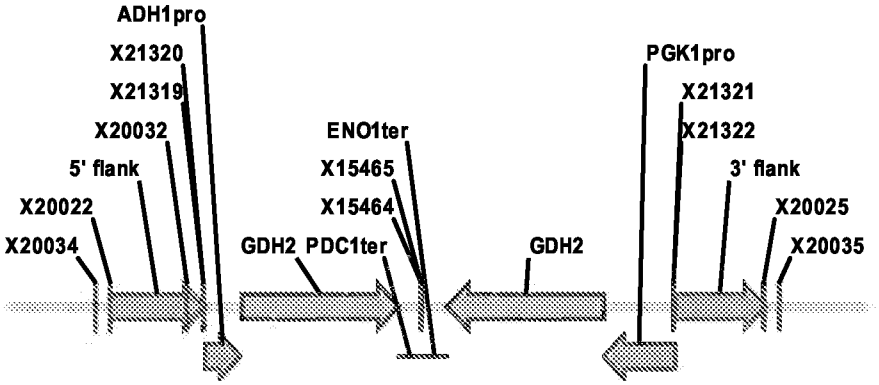
FIGURE 20

MA0584

S. cerevisiae GDH2 over expression at URE2 locus

MA0584

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21320	M2390 gDNA	1968bp
ADH1pro-GDH2-PDC1ter	X21319/X15465	pMU2909	4546bp
PGKpro-GDH2-ENO1ter	X15464/X21322	pMU2908	5283bp
URE2 3' Flank	X21321/X20025	M2390 gDNA	1885bp



MA0584
17829bp

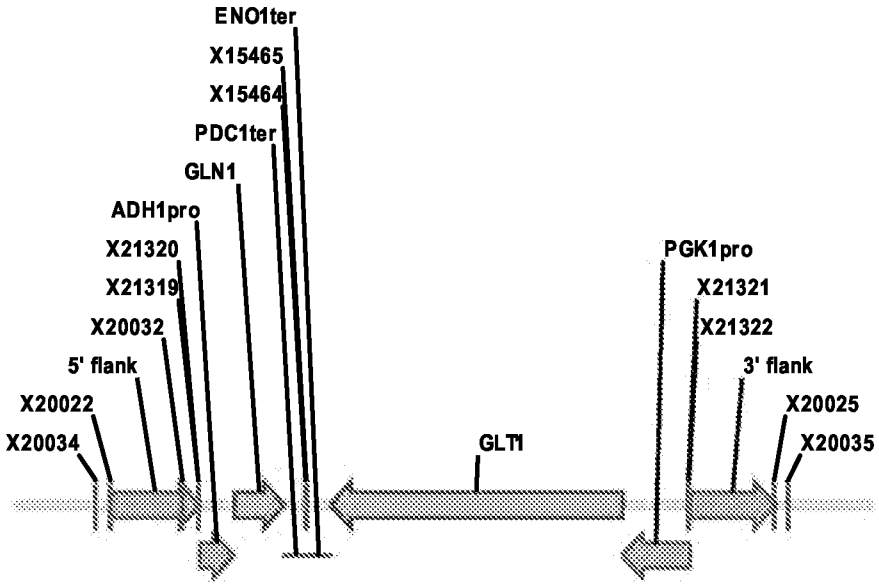
FIGURE 21

MA0585

GLT1/GLN1 over expression at the URE2 locus

MA0585

Fragment	Primers	Template	Exp size
URE2 5' Flank	X20022/X21320	M2390 gDNA	1968bp
ADH1pro-GLTI-PDC1ter	X21319/X15465	pMU2911	2363bp
PGKpro-GLN1-ENO1ter	X15464/X21322	pMU2913	8442bp
URE2 3' Flank	X21321/X20025	M2390 gDNA	1885bp



MA0585
18794 bp

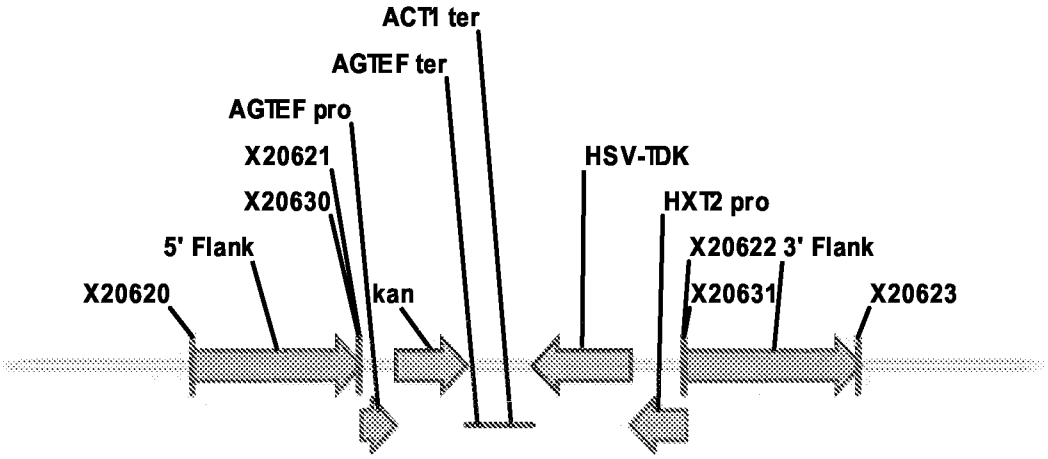
FIGURE 22

MA0617

AUA1 marked deletion

MA0617

Fragment	Primers	Template	Expected Size
AUA1 5' Flank	X20620/X20621	M2390 gDNA	1947bp
AGTEFpro-kan/nat/tdk-HXT2pro	X20630/X20631	pMU2873,pMU2879	3820bp and 3583bp
AUA1 3' Flank	X20622/X20623	M2390 gDNA	2015bp



MA0617
11754 bp

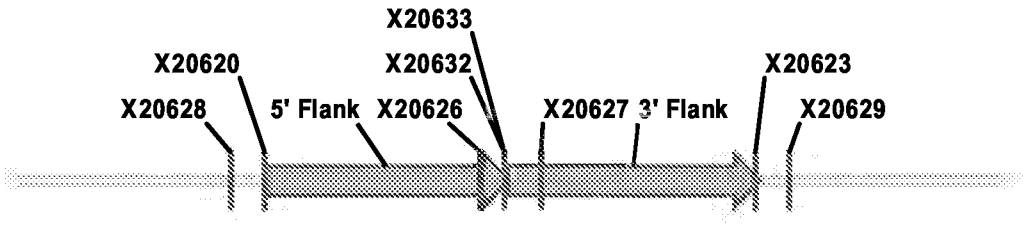
FIGURE 23

MA0617.1

AUA1 clean deletion

MA0617.1

Fragment	Primers	Template	Expected Size
AUA1 5' Flank	X20620/X20633	M2390 gDNA	1947bp
AUA1 3' Flank	X20632/X20623	M2390 gDNA	2015bp



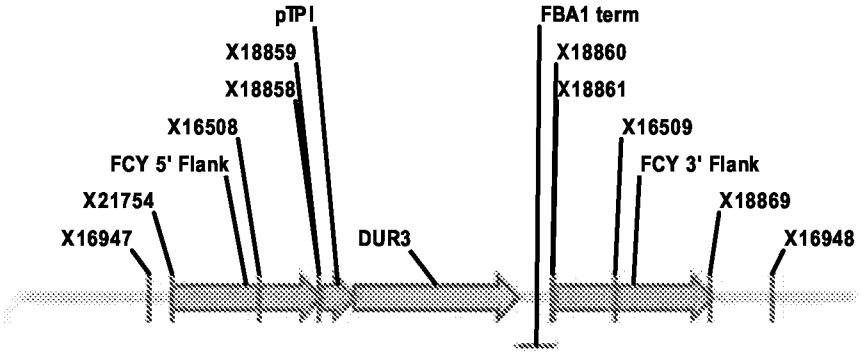
MA0617.1
8000 bp

FIGURE 24

MA0434

DUR3 over expression at FCY1 locus

MA0434	DUR3		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X18859	gDNA	2049bp
TP1pro-DUR3-FBA1ter	X18858/X18861	pMU3471	3157bp
FCY 3' Flank	X18860/X18869	gDNA	2166bp



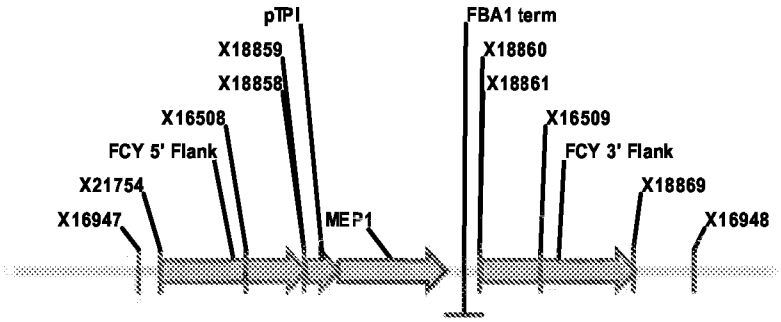
MA0434 with DUR3
11157bp

FIGURE 25

MA0434.2

MEP1 over expression at FCY1 locus

MA0434.2	MEP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X18859	gDNA	2049bp
TP1pro-MEP1-FBA1ter	X18858/X18861	pMU3472	2428bp
FCY 3' Flank	X18860/X18869	gDNA	2166bp



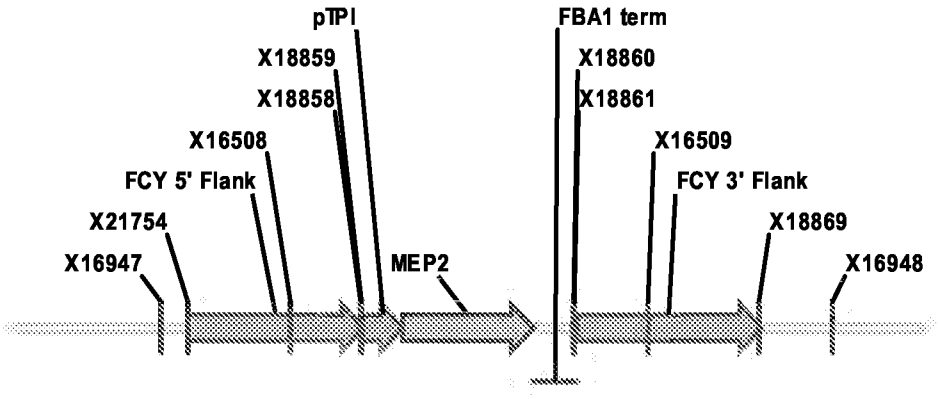
MA0434.2 with MEP1
10428 bp

FIGURE 26

MA0434.3

MEP2 over expression at FCY1 locus

MA0434.3	MEP2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X18859	gDNA	2049bp
TP1pro-MEP2-FBA1ter	X18858/X18861	pMU3473	2449bp
FCY 3' Flank	X18860/X18869	gDNA	2166bp



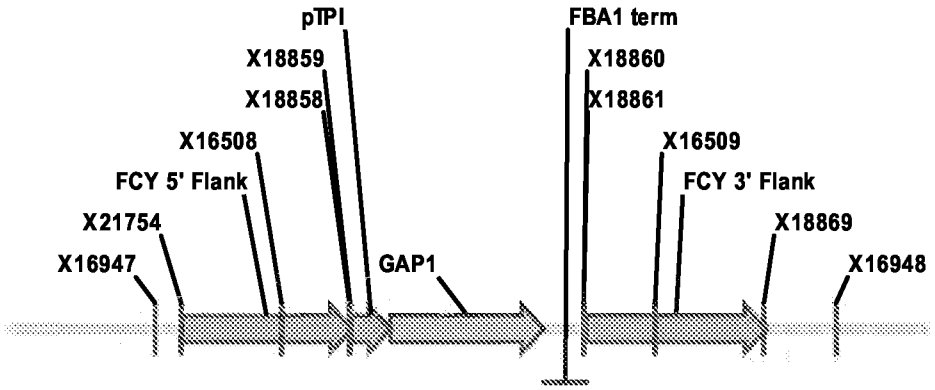
MA0434.3 with MEP2
10449 bp

FIGURE 27

MA0434.4

GAP1 over expression at FCY1 locus

MA0434.4	GAP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X18859	gDNA	2049bp
TP1pro-GAP1-FBA1ter	X18858/X18861	pMU3475	2758bp
FCY 3' Flank	X18860/X18869	gDNA	2166bp



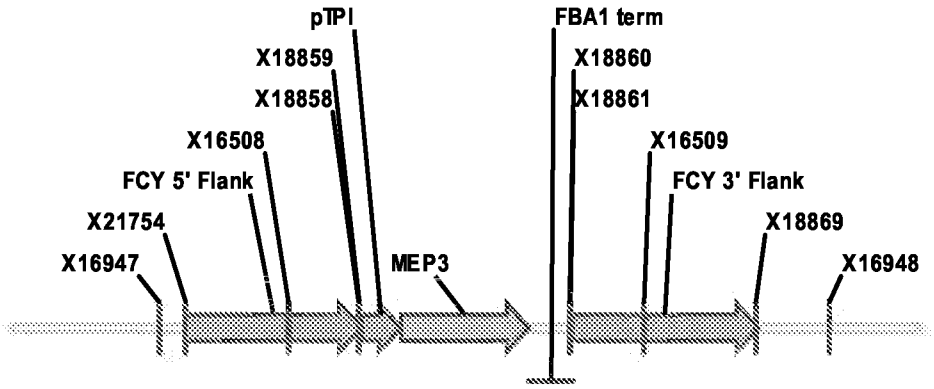
MA0434.4 with GAP1
10758 bp

FIGURE 28

MA0434.5

MEP3 over expression at FCY1 locus

MA0434.5	MEP3		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X18859	gDNA	2049bp
TP1pro-MEP3-FBA1ter	X18858/X18861	pMU3607	2420bp
FCY 3' Flank	X18860/X18869	gDNA	2166bp



MA0434.5 with MEP3
10419 bp

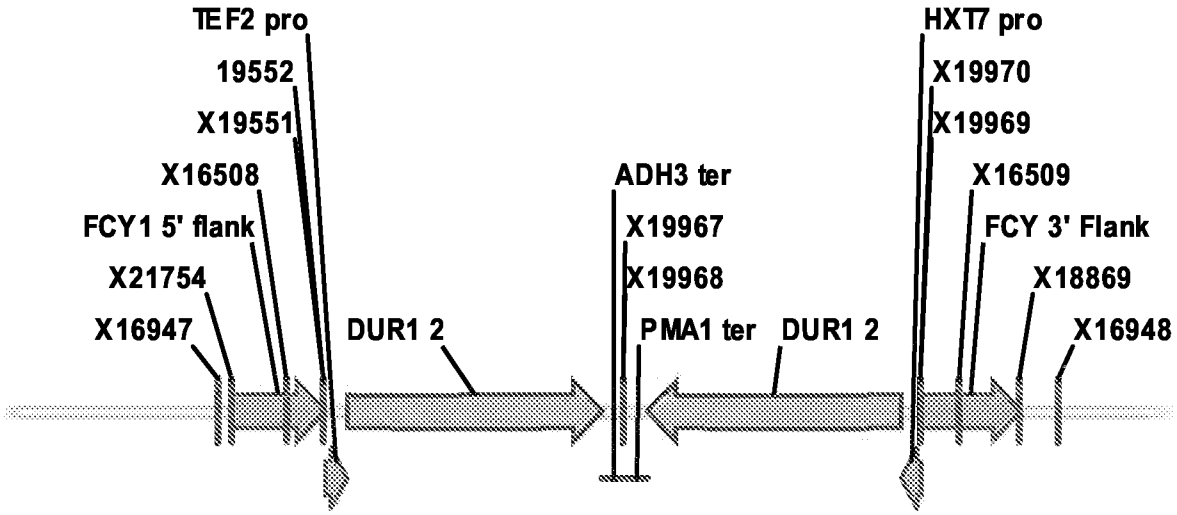
FIGURE 29

MA0454.14

DUR1,2 over expression at FCY1 locus

MA0454.14

Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	gDNA	2018bp
pTEF2/ADH3t	X19551/X19968	pMU3409	6508bp
pHXT7/PMA1trc	X19967/X19969	pMU3410	6458bp
FCY3' Flank	X19970/X18869	gDNA	2159bp



MA0454.14 with DUR1,2

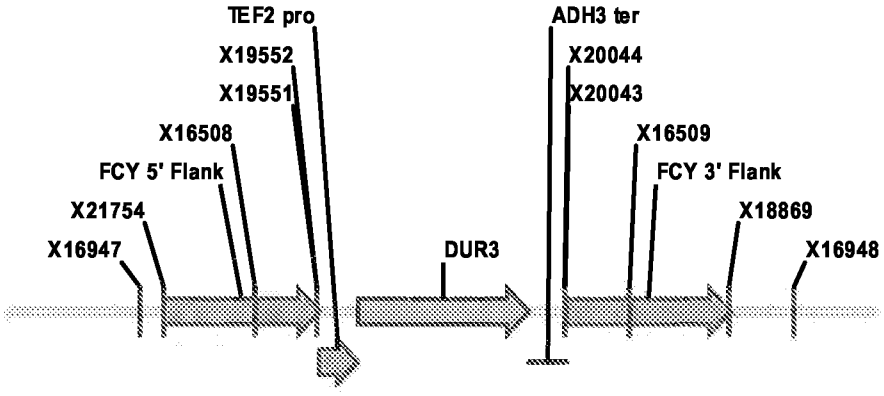
24889bp

FIGURE 30

MA0464

DUR3 over expression at FCY1 locus

MA0464	DUR3		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2018bp
TEF2pro-DUR3-ADH3ter	X19551/X20043	pMU3464	3208bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2133bp



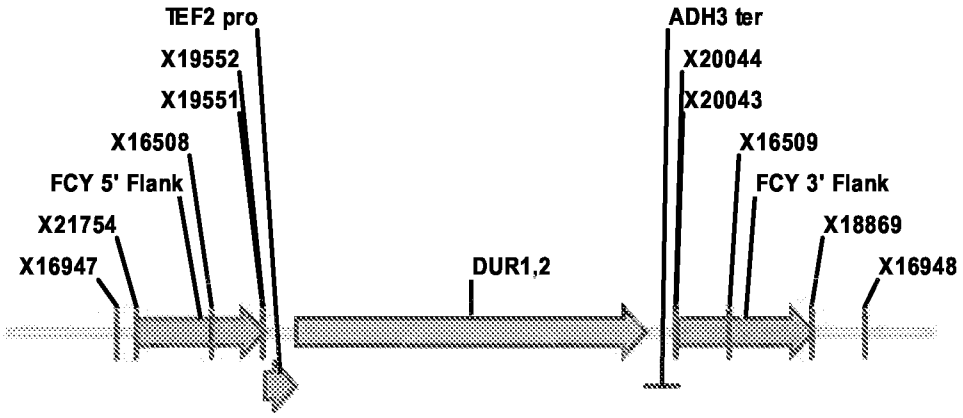
MA0464 with DUR3
11208 bp

FIGURE 31

MA0464.1

DUR1,2 over expression at FCY1 locus

MA0464.1	DUR1,2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2049bp
TEF2pro-DUR1,2-ADH3ter	X19551/X20043	pMU3409	6508bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2166bp



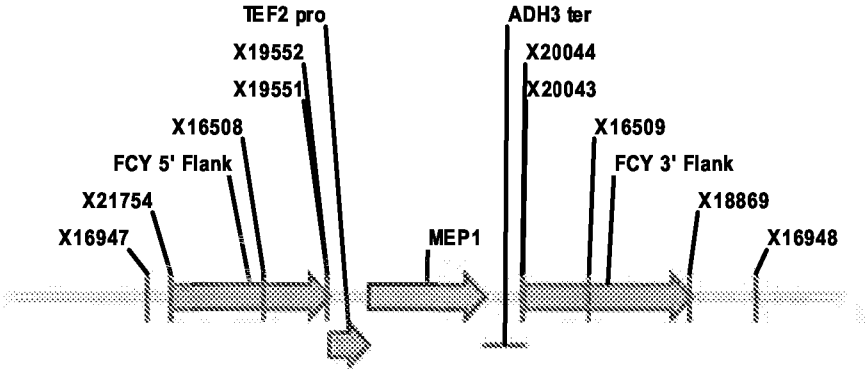
MA0464.1 with DUR1,2
14508 bp

FIGURE 32

MA0464.2

MEP1 over expression at FCY1 locus

MA0464.2	MEP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2049bp
TEF2pro-MEP1-ADH3ter	X19551/X20043	pMU3465	2479bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2166bp



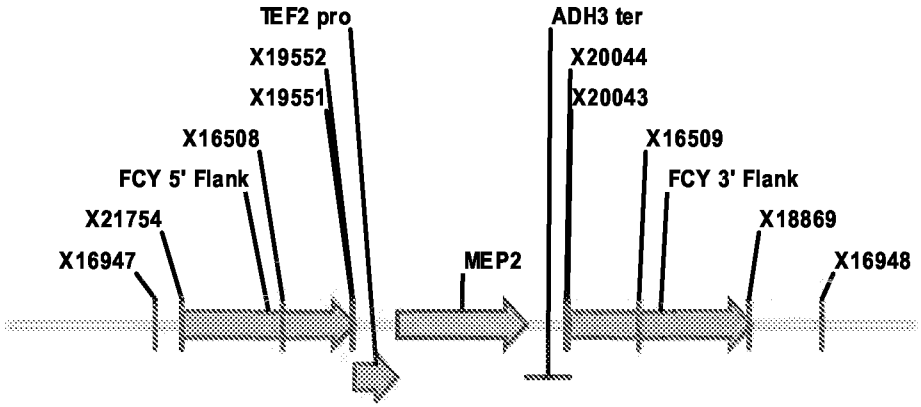
MA0464.2 with MEP1
10479 bp

FIGURE 33

MA0464.3

MEP2 over expression at FCY1 locus

MA0464.3	MEP2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2049bp
TEF2pro-MEP2-ADH3ter	X19551/X20043	pMU3466	2500bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2166bp



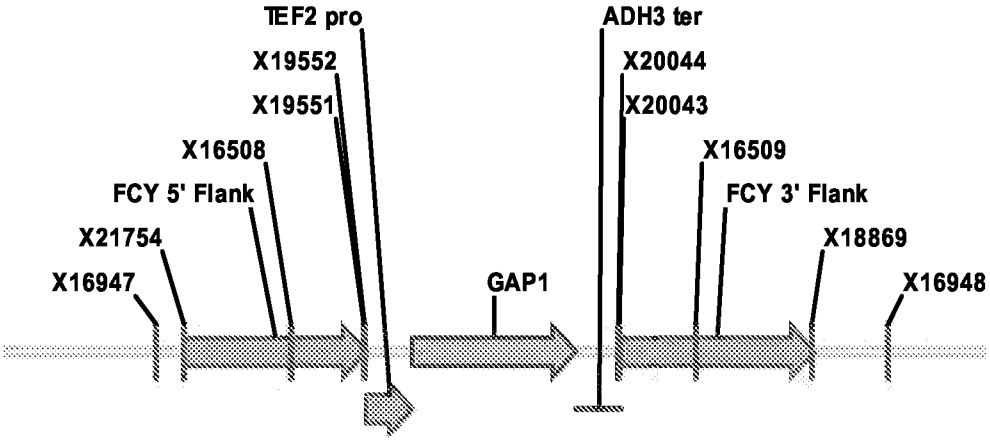
MA0464.3 with MEP2
10500 bp

FIGURE 34

MA0464.4

GAP1 over expression at FCY1 locus

MA0464.4	GAP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2049bp
TEF2pro-GAP1-ADH3ter	X19551/X20043	pMU3468	2809bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2166bp



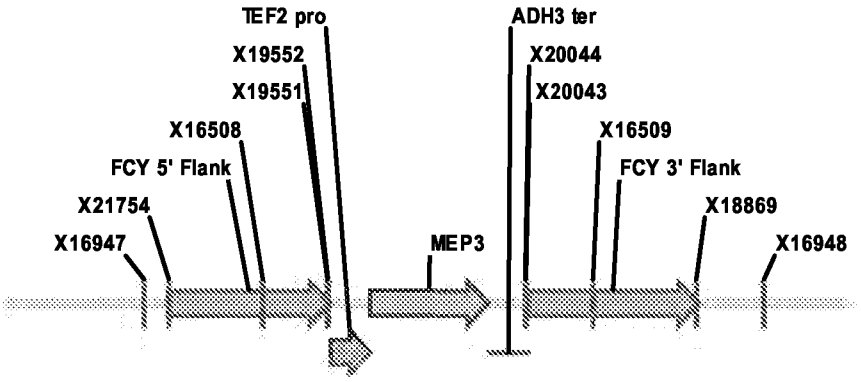
MA0464.4 with GAP1
10809bp

FIGURE 35

MA0464.5

MEP3 over expression at FCY1 locus

MA0464.5	MEP3		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X19552	M2390 gDNA	2049bp
TEF2pro-DUR1,2-ADH3ter	X19551/X20043	pMU3606	6508bp
FCY 3' Flank	X20044/X18869	M2390 gDNA	2166bp



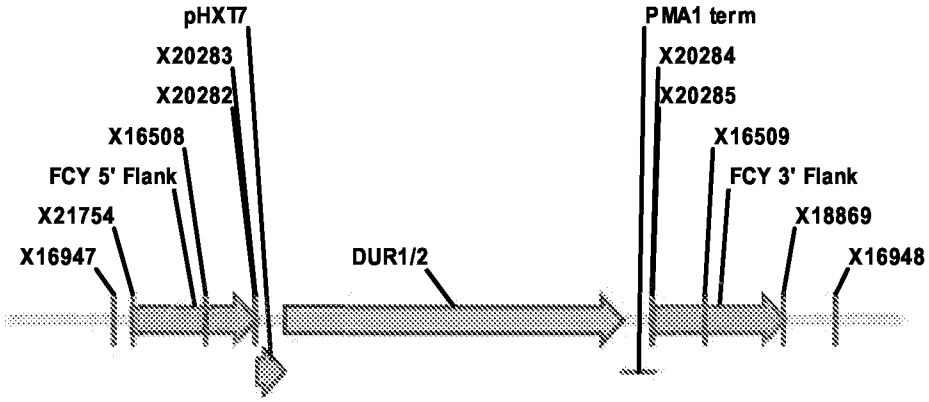
MA0464.5 with MEP3
10470 bp

FIGURE 36

MA0465.1

DUR1,2 over expression at FCY1

MA0465.1	DUR1,2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X20283	M2390 gDNA	2049bp
HXT7pro-DUR1,2-PMA1ter	X20282/X20285	pMU3410	6458bp
FCY 3' Flank	X20284/X18869	M2390 gDNA	2166bp



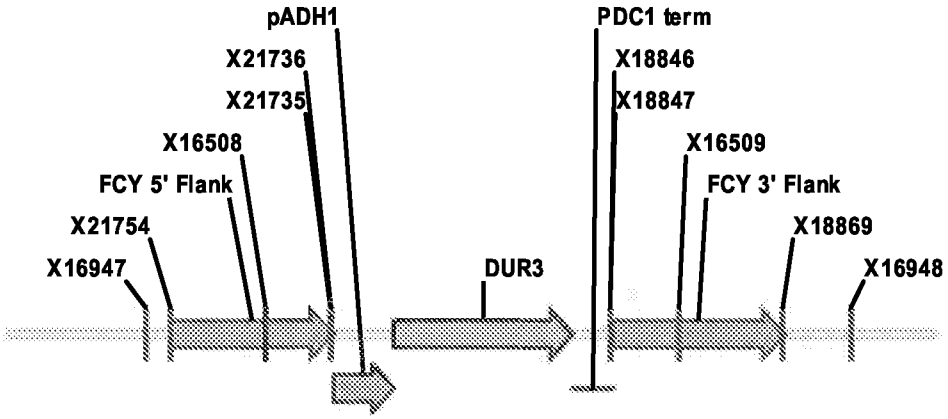
MA0465 with DUR1,2
14458 bp

FIGURE 37

MA0467

DUR3 over expression at FCY1 locus

MA0467	DUR3		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X21736	M2390 gDNA	2049bp
ADH1pro-DUR3-PDC1ter	X21735/X18847	pMU3459	3458bp
FCY 3' Flank	X18846/X18869	M2390 gDNA	2166bp



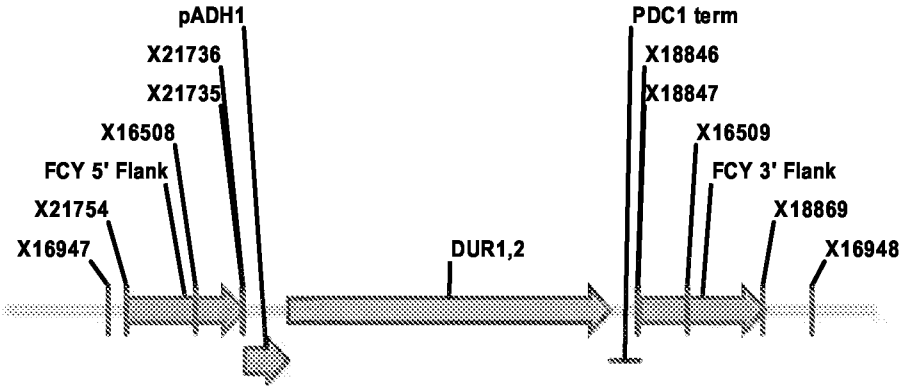
MA0467 with DUR3
11458 bp

FIGURE 38

MA0467.1

DUR1,2 over expression at FCY1 locus

MA0467.1	DUR1,2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X21736	M2390 gDNA	2049bp
ADH1pro-DUR1,2-PDC1ter	X21735/X18847	pMU3411	6758bp
FCY 3' Flank	X18846/X18869	M2390 gDNA	2166bp



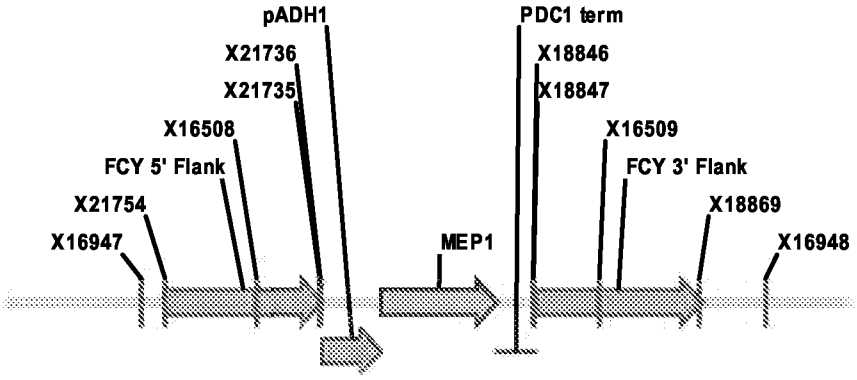
MA0467.1 with DUR1,2
14758 bp

FIGURE 39

MA0467.2

MEP1 over expression at FCY1 locus

MA0467.2	MEP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X21736	M2390 gDNA	2049bp
ADH1pro-MEP1-PDC1ter	X21735/X18847	pMU3460	2729bp
FCY 3' Flank	X18846/X18869	M2390 gDNA	2166bp



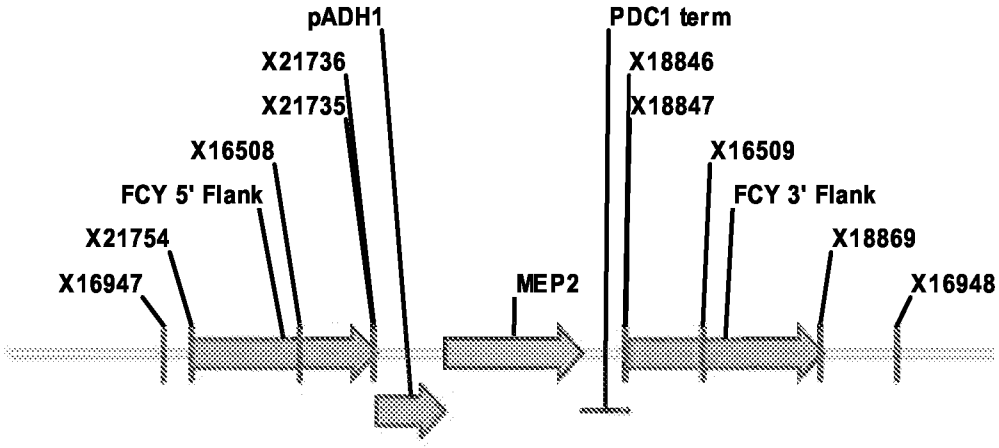
MA0467.2 with MEP1
10729 bp

FIGURE 40

MA0467.3

MEP2 over expression at FCY1 locus

MA0467.3	MEP2		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X21736	M2390 gDNA	2049bp
ADH1pro-MEP2-PDC1ter	X21735/X18847	pMU3461	2750bp
FCY 3' Flank	X18846/X18869	M2390 gDNA	2166bp



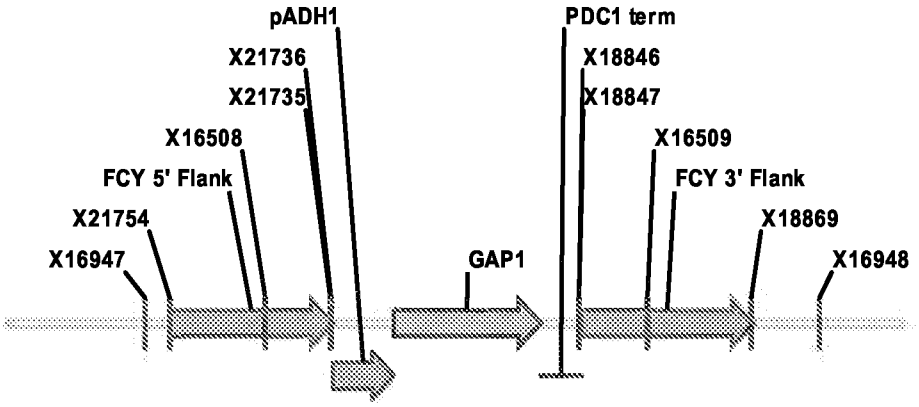
MA0467.3 with MEP2
10750 bp

FIGURE 41

MA0467.4

GAP1 over expression at FCY1 locus

MA0467.4	GAP1		
Fragment	Primers	Template	Exp Size
FCY 5' Flank	X21754/X21736	M2390 gDNA	2049bp
ADH1pro-GAP1-PDC1ter	X21735/X18847	pMU3463	3059bp
FCY 3' Flank	X18846/X18869	M2390 gDNA	2166bp



MA0467.4 with GAP1
11059 bp

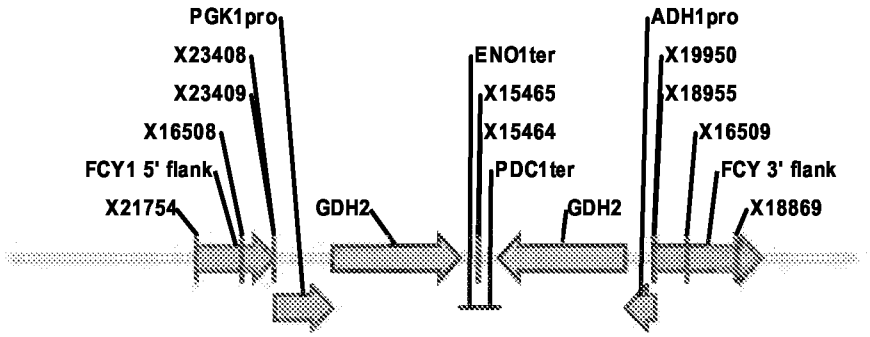
FIGURE 42

MA0881

S. cerevisiae GDH2 over expression at FCY1 locus

MA0881

Fragment	Primers	Template	Expected Size
5' Flank	X21754/X23408	M2390 gDNA	2049bp
pPGK-GDH2-ENO1t	X23409/X15464	pMU2908	5283bp
pADH-GDH2-PDC1trc	X15465/X18955	pMU2909	4546bp
3' Flank	X19950/X18869	M2390 gDNA	2166bp



MA0881
21752 bp

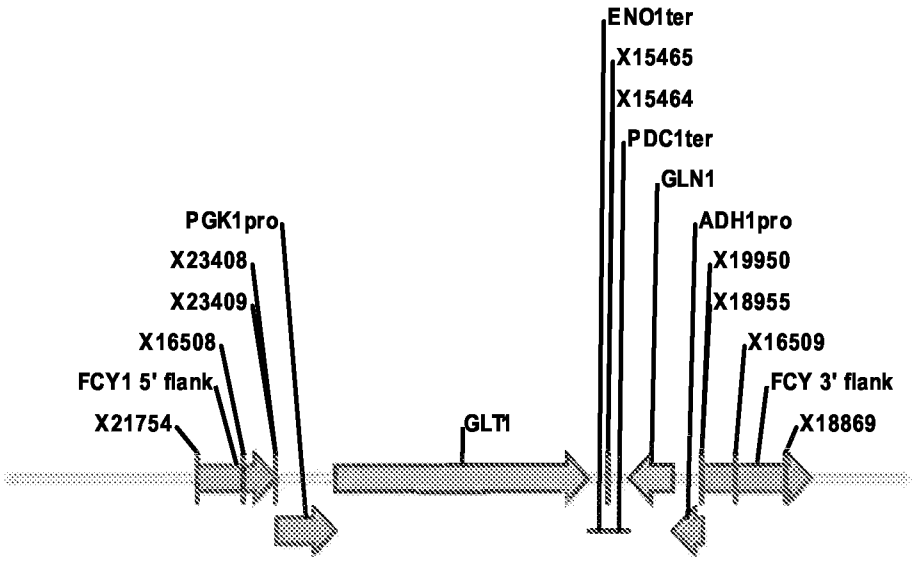
FIGURE 43

MA0881.1

GLT1/GLN1 over expression at FCY1 locus

MA0881.1

Fragment	Primers	Template	Expected Size
5' Flank	X21754/X23408	M2390 gDNA	2049bp
pPGK-GLT1-ENO1t	X23409/X15464	pMU2913	8442bp
pADH-GLN1-PDC1trc	X15465/X18955	pMU2911	2363bp
3' Flank	X19950/X18869	M2390 gDNA	2166bp



MA0881.1

22745 bp

FIGURE 44

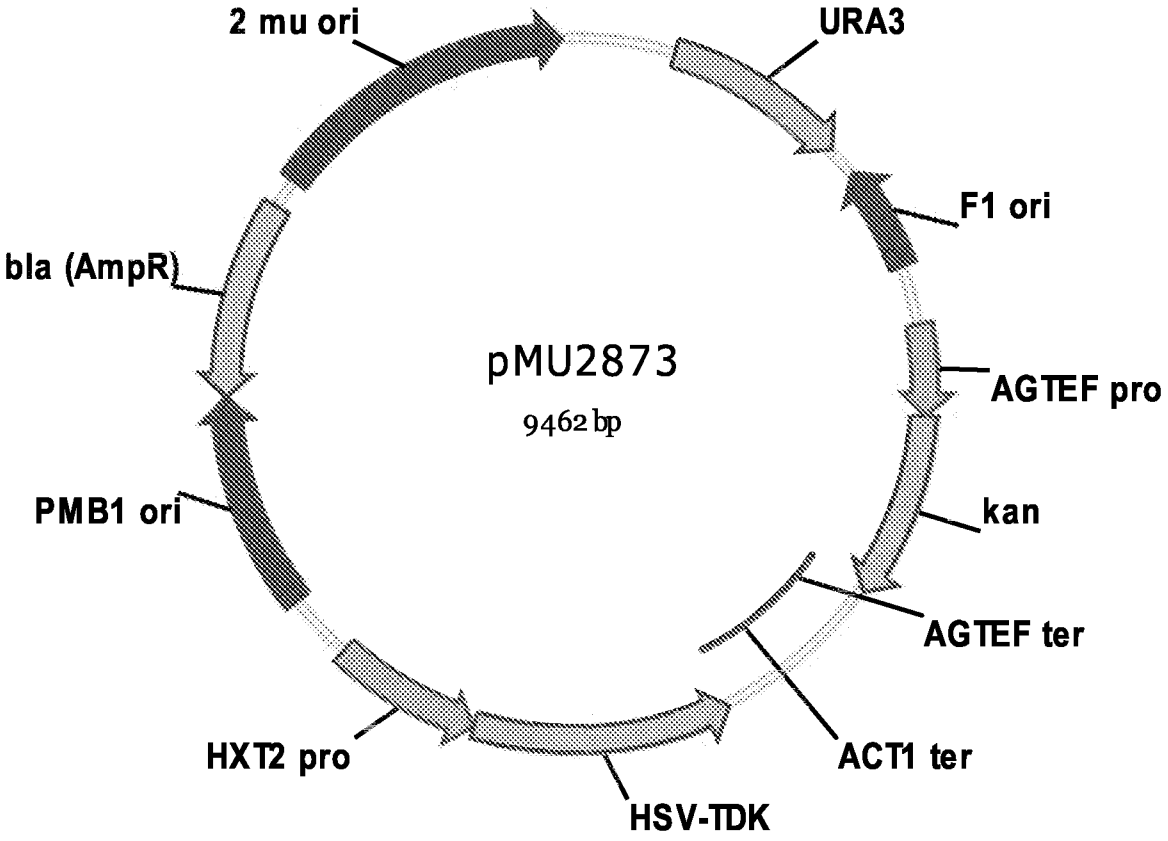

2 mu ori
URA3
F1 ori
bla (AmpR)
pMU2873
9462 bp
AGTEF pro
PMB1 ori
kan
AGTEF ter
HXT2 pro
ACT1 ter
HSV-TDK

FIGURE 45

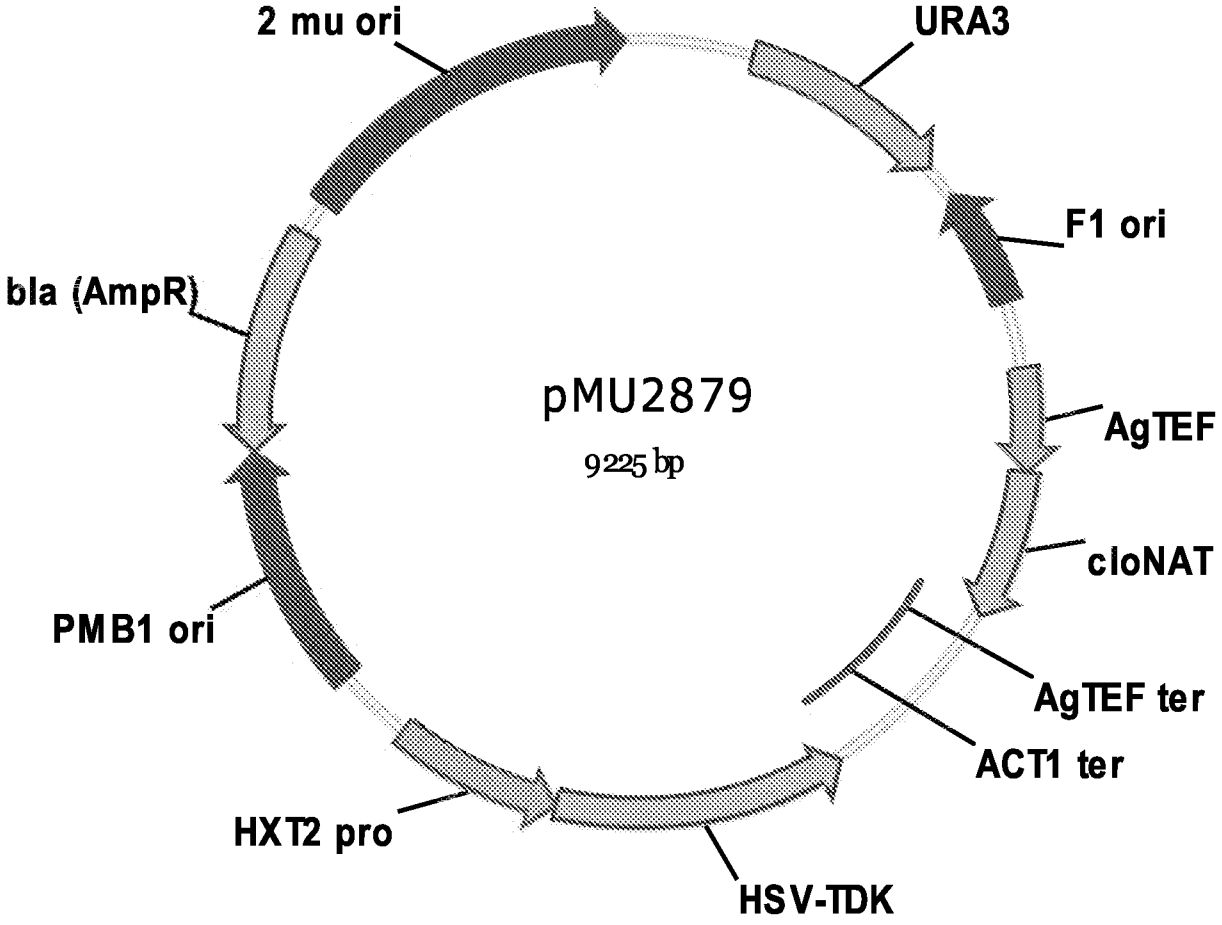

2 mu ori
URA3
F1 ori
bla (AmpR)
pMU2879
9225 bp
AgTEF
cloNAT
PMB1 ori
AgTEF ter
ACT1 ter
HXT2 pro
HSV-TDK

FIGURE 46

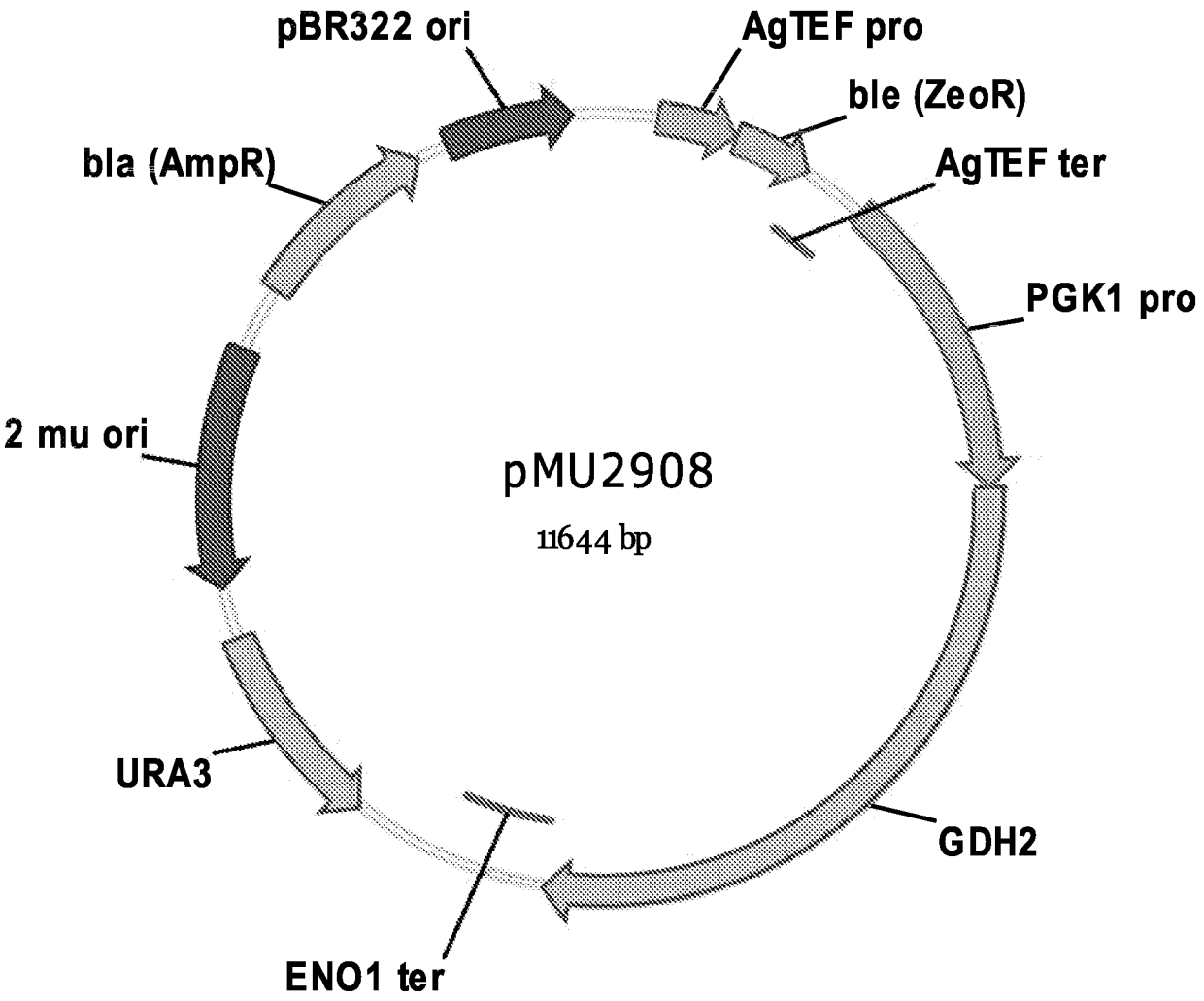

pBR322 ori
AgTEF pro
ble (ZeoR)
bla (AmpR)
AgTEF ter
PGK1 pro
2 mu ori
pMU2908
11644 bp
URA3
GDH2
ENO1 ter

FIGURE 47

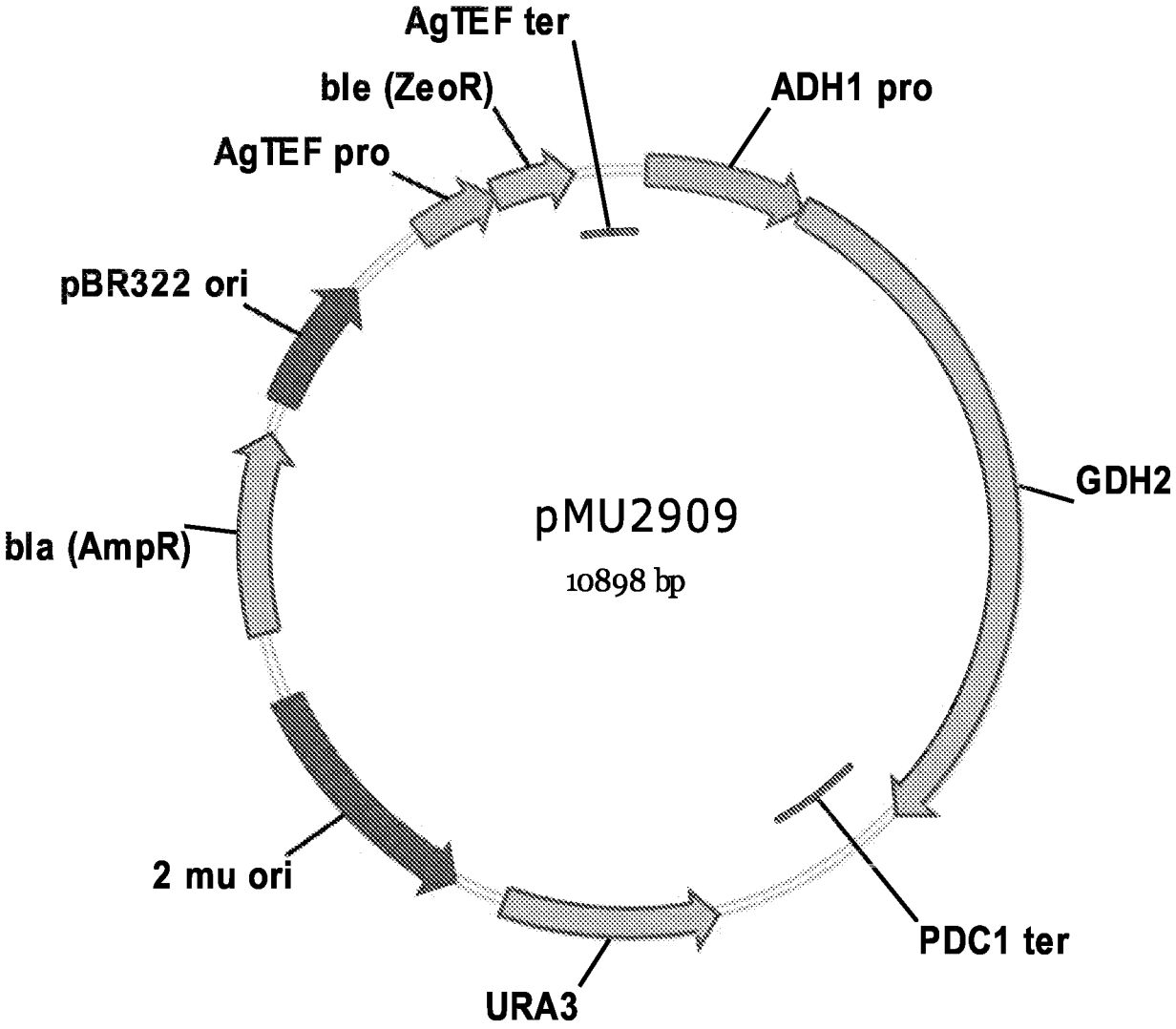

AgTEF ter
ble (ZeoR)
ADH1 pro
AgTEF pro
pBR322 ori
GDH2
pMU2909
10898 bp
bla (AmpR)
2 mu ori
PDC1 ter
URA3

FIGURE 48

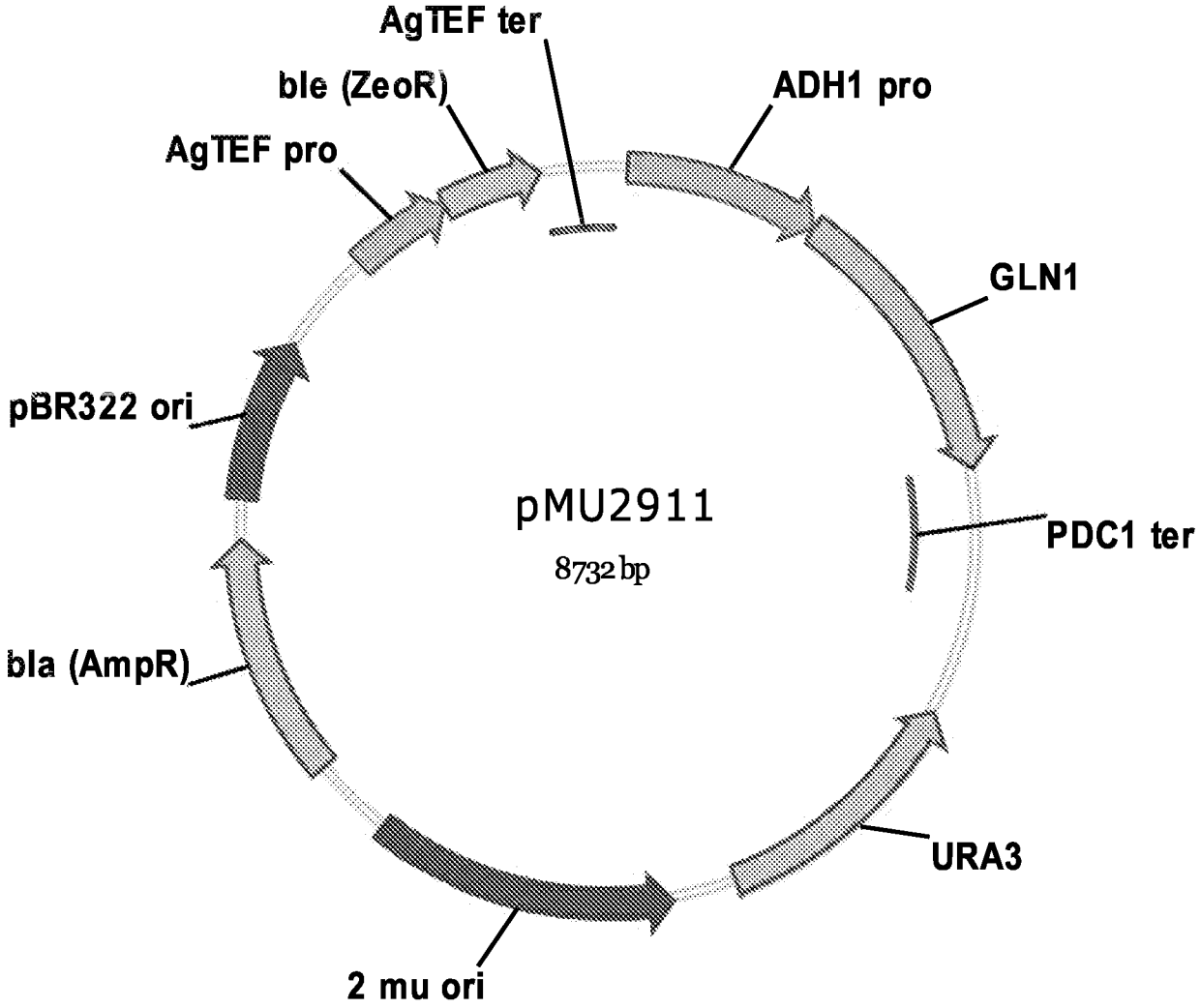

AgTEF ter
ble (ZeoR)
ADH1 pro
AgTEF pro
GLN1
pBR322 ori
pMU2911
8732 bp
PDC1 ter
bla (AmpR)
URA3
2 mu ori

FIGURE 49

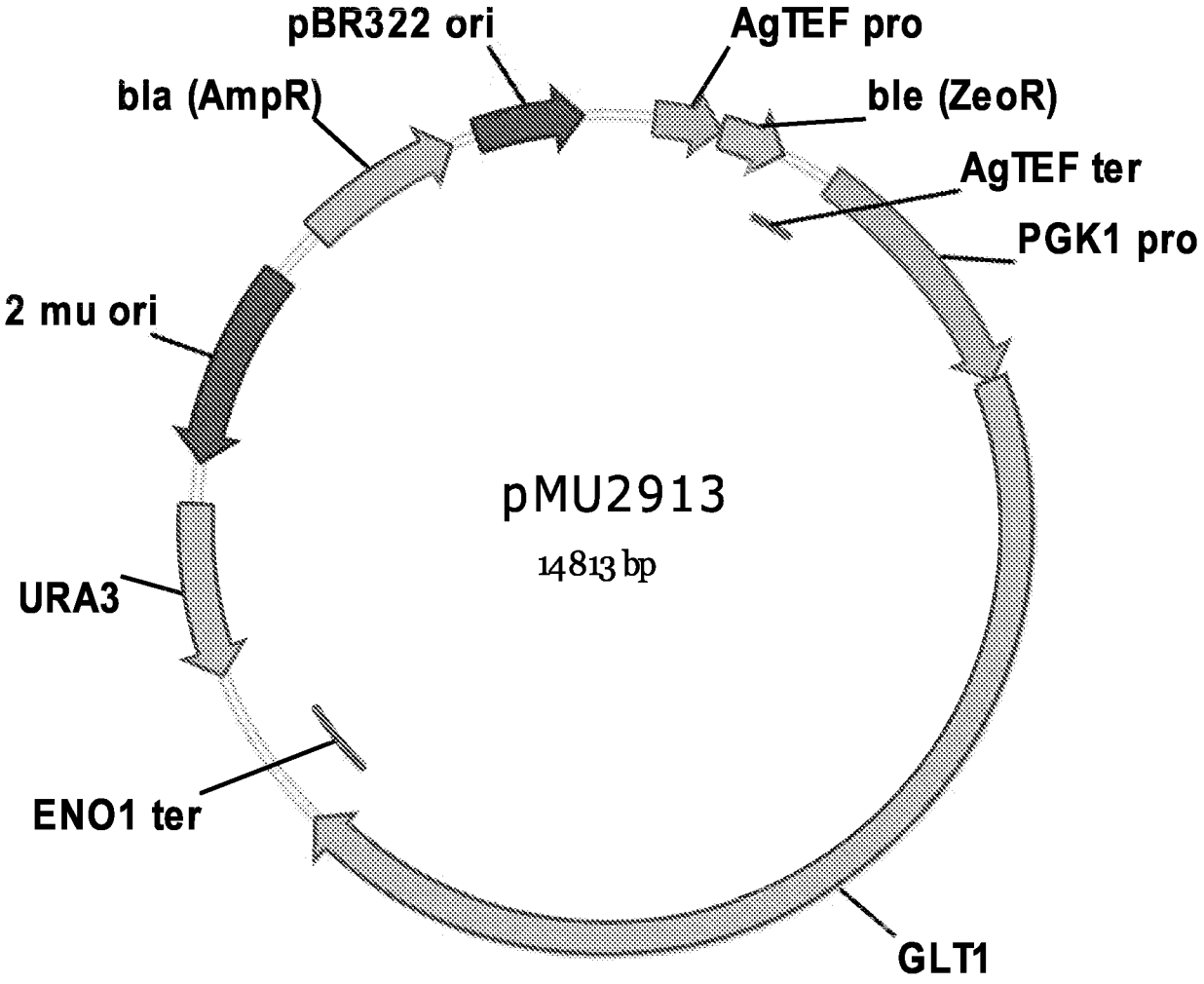

pBR322 ori
AgTEF pro
bla (AmpR)
ble (ZeoR)
AgTEF ter
PGK1 pro
2 mu ori
pMU2913
14813 bp
URA3
ENO1 ter
GLT1

FIGURE 50

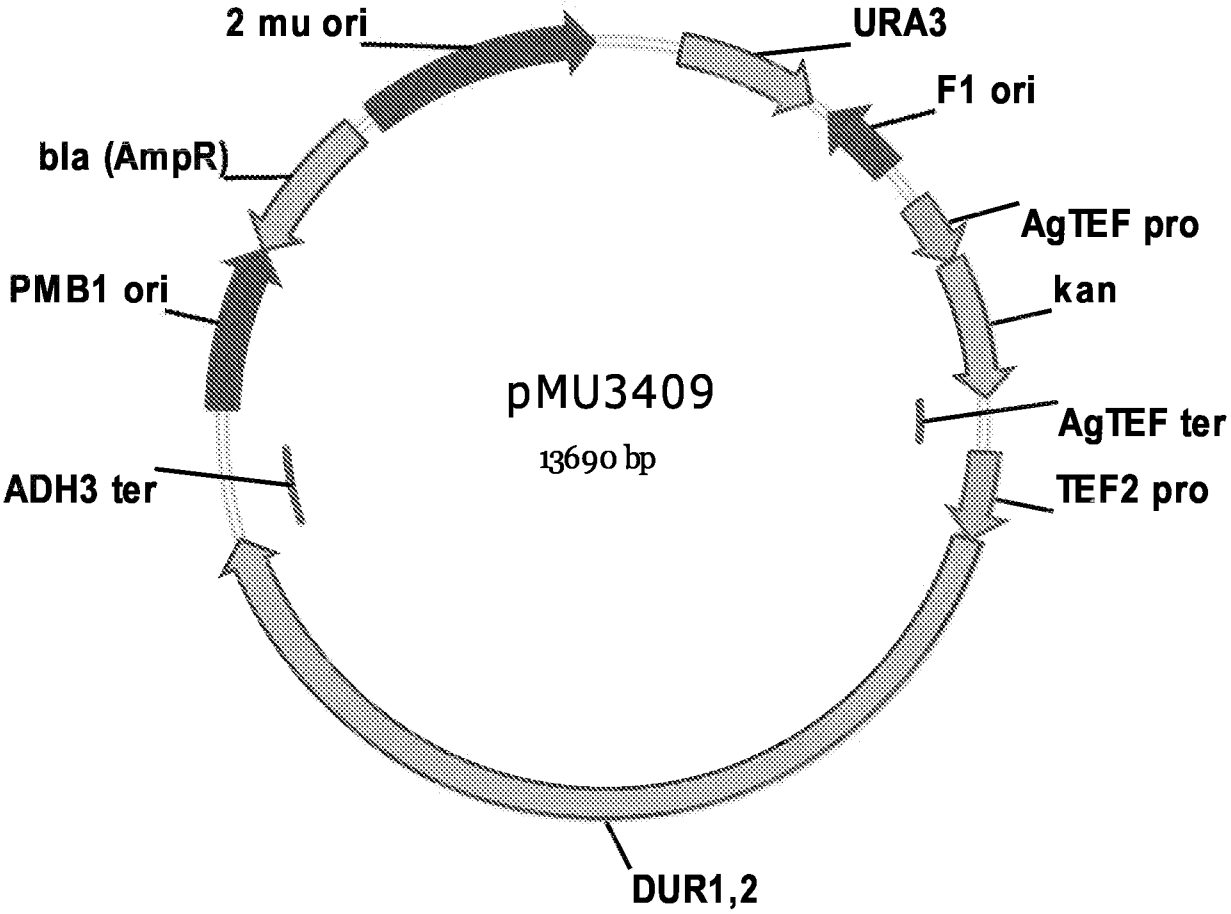

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
PMB1 ori
kan
pMU3409
13690 bp
AgTEF ter
ADH3 ter
TEF2 pro
DUR1,2

FIGURE 51

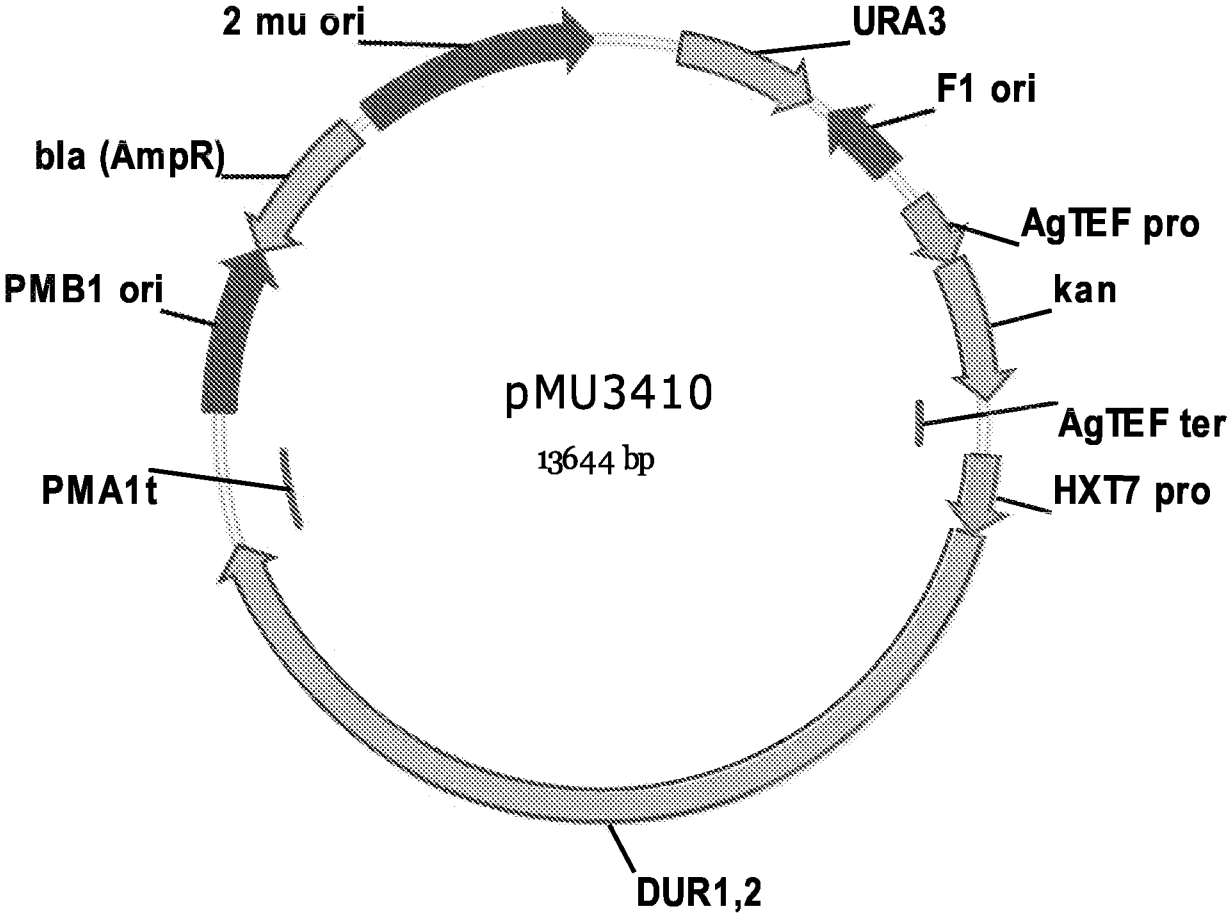

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
PMB1 ori
kan
pMU3410
13644 bp
AgTEF ter
PMA1t
HXT7 pro
DUR1,2

FIGURE 52

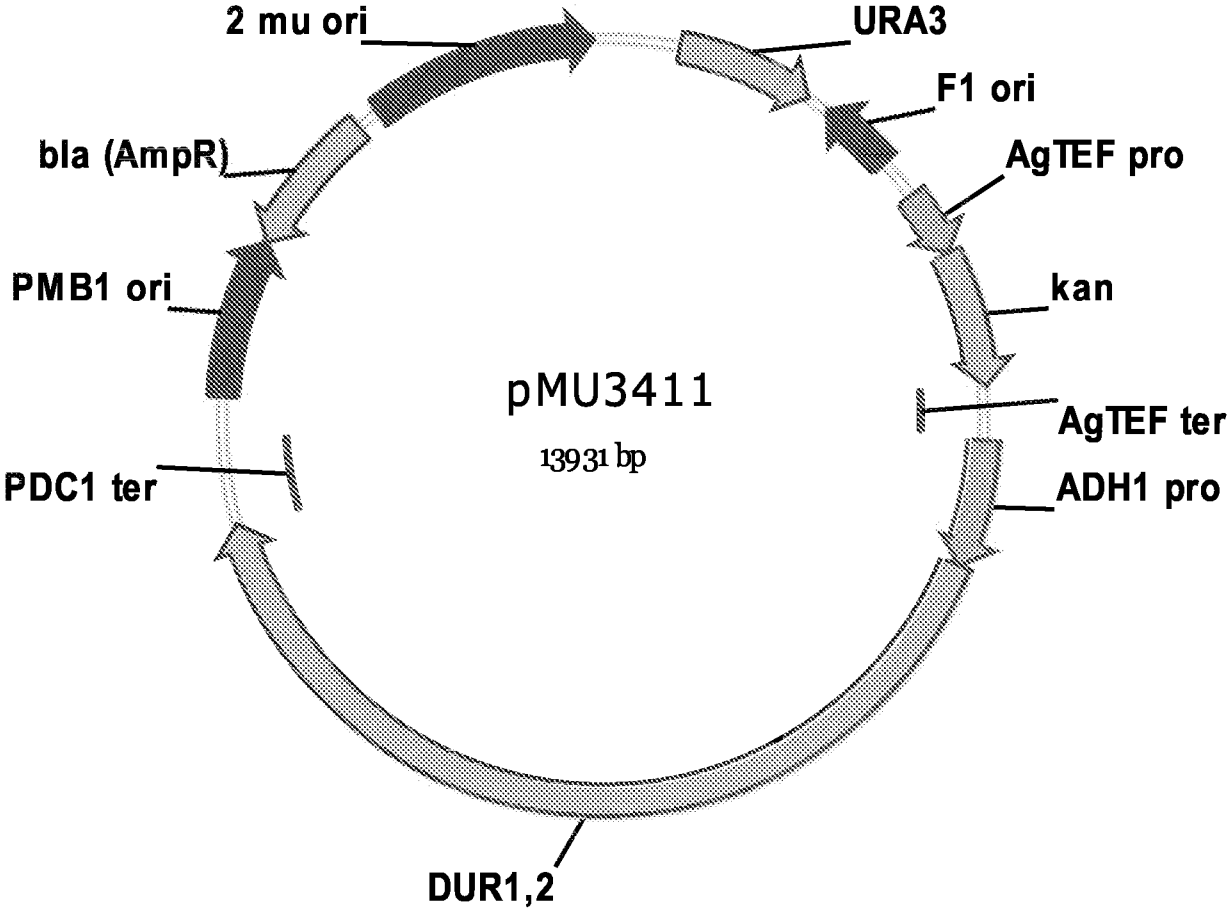

2 mu ori
URA3
F1 ori
AgTEF pro
bla (AmpR)
kan
PMB1 ori
pMU3411
13931 bp
AgTEF ter
PDC1 ter
ADH1 pro
DUR1,2

FIGURE 53

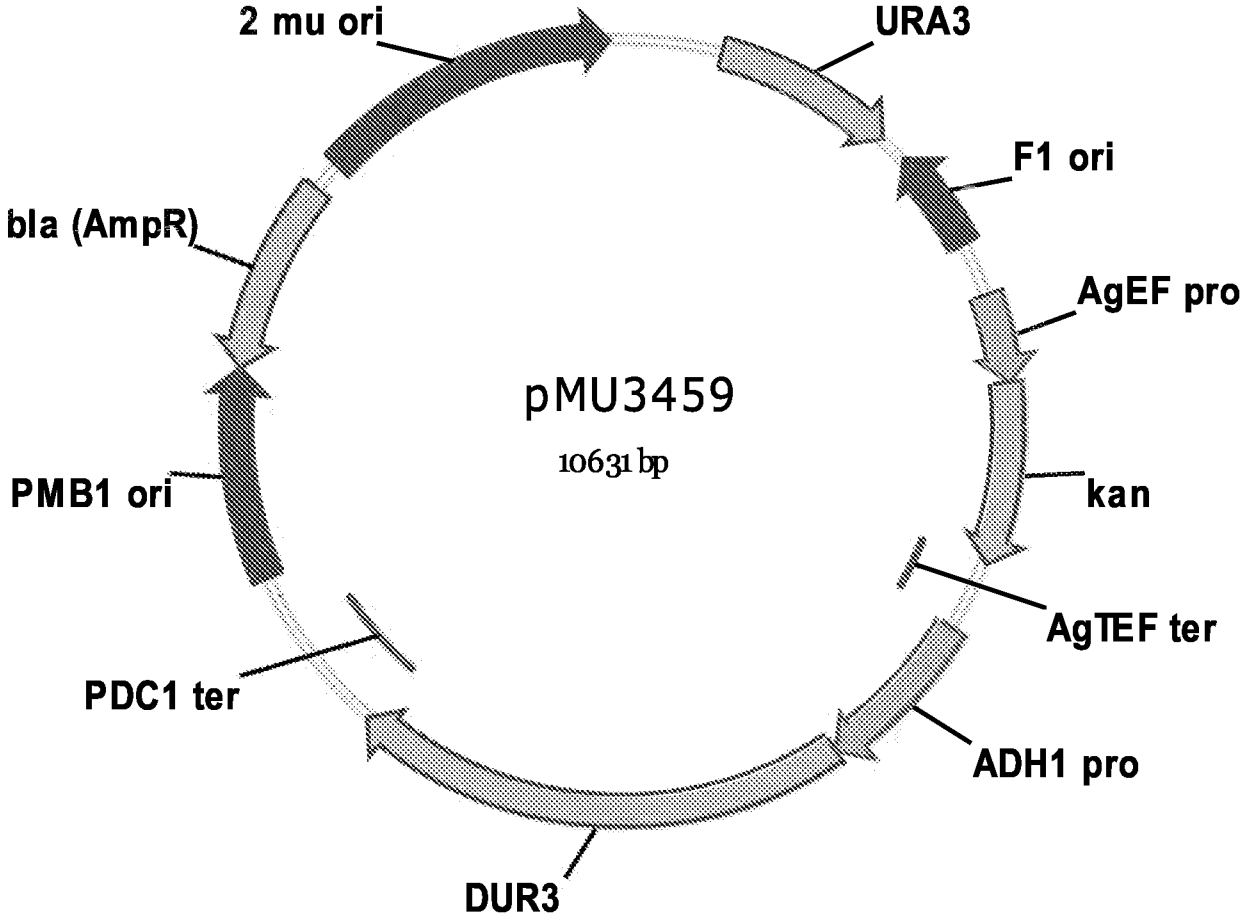

2 mu ori
URA3
F1 ori
bla (AmpR)
AgEF pro
pMU3459
10631 bp
PMB1 ori
kan
AgTEF ter
PDC1 ter
ADH1 pro
DUR3

FIGURE 54

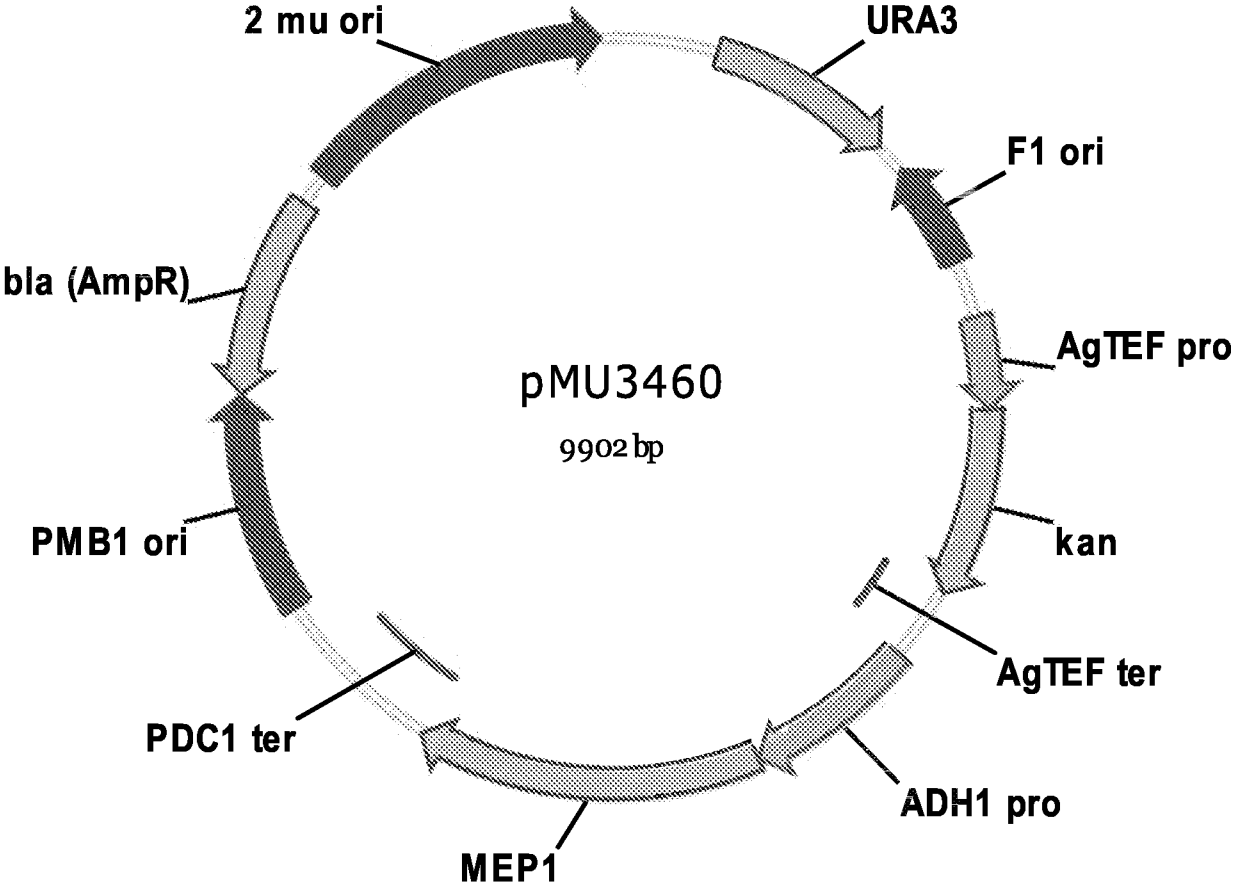

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3460
9902 bp
PMB1 ori
kan
AgTEF ter
PDC1 ter
ADH1 pro
MEP1

FIGURE 55

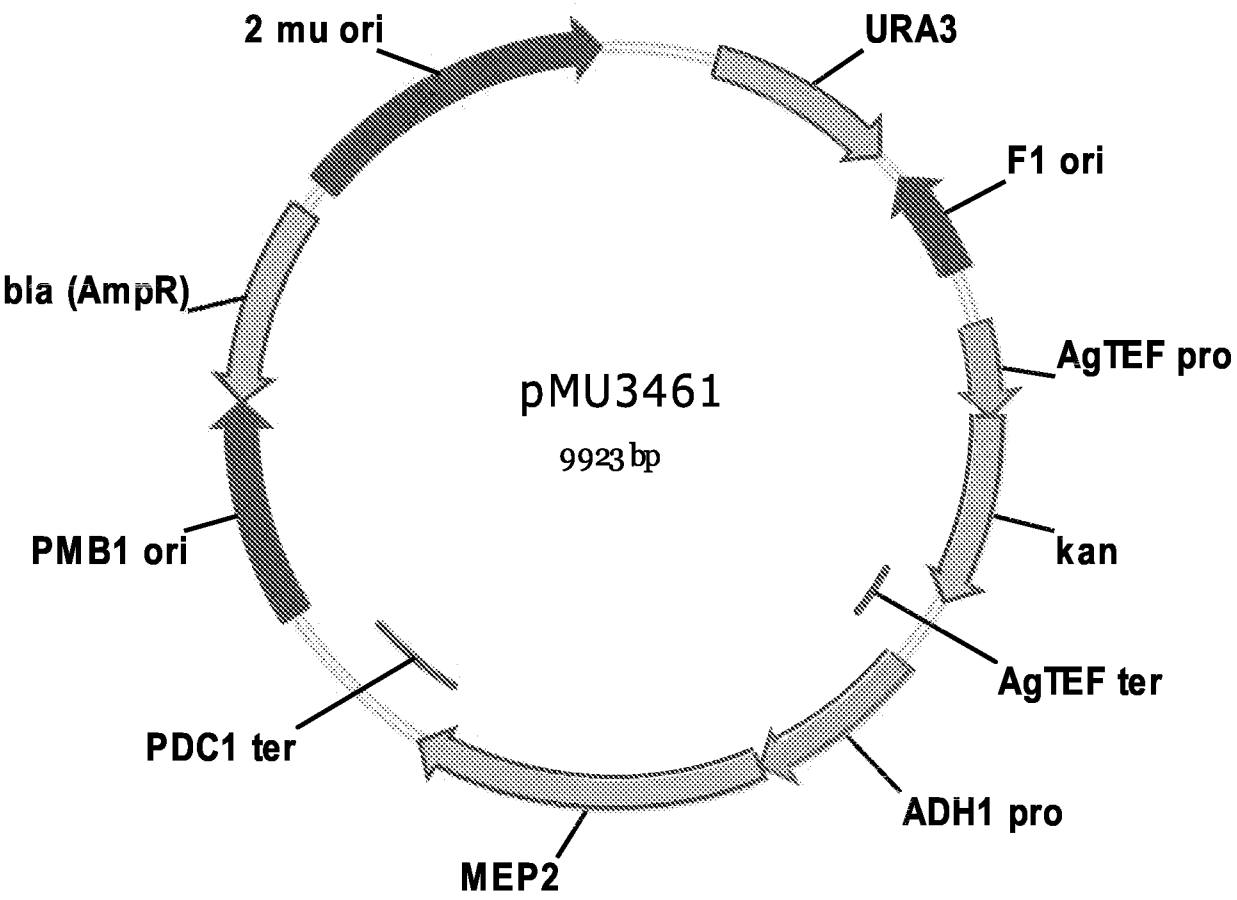

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3461
9923 bp
PMB1 ori
kan
AgTEF ter
PDC1 ter
ADH1 pro
MEP2

FIGURE 56

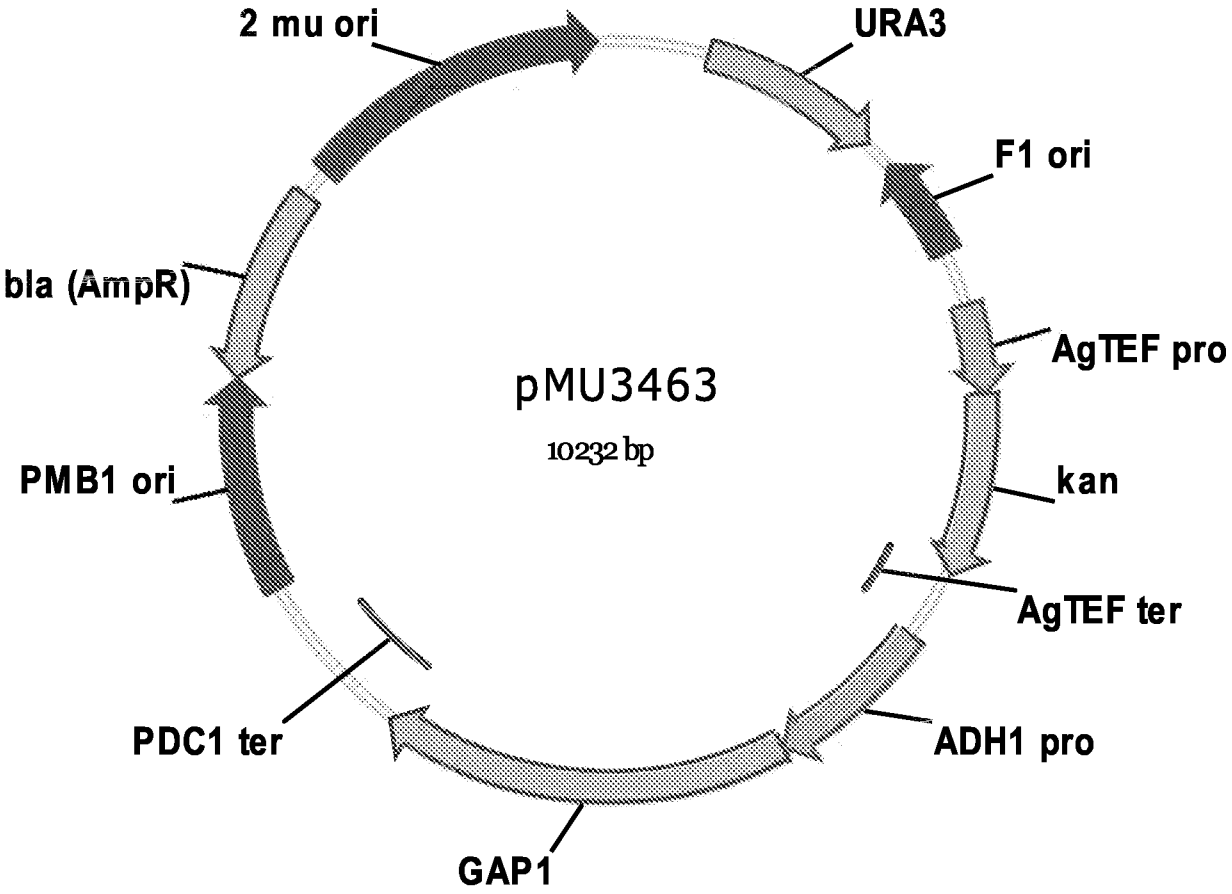

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3463
10232 bp
PMB1 ori
kan
AgTEF ter
PDC1 ter
ADH1 pro
GAP1

FIGURE 57

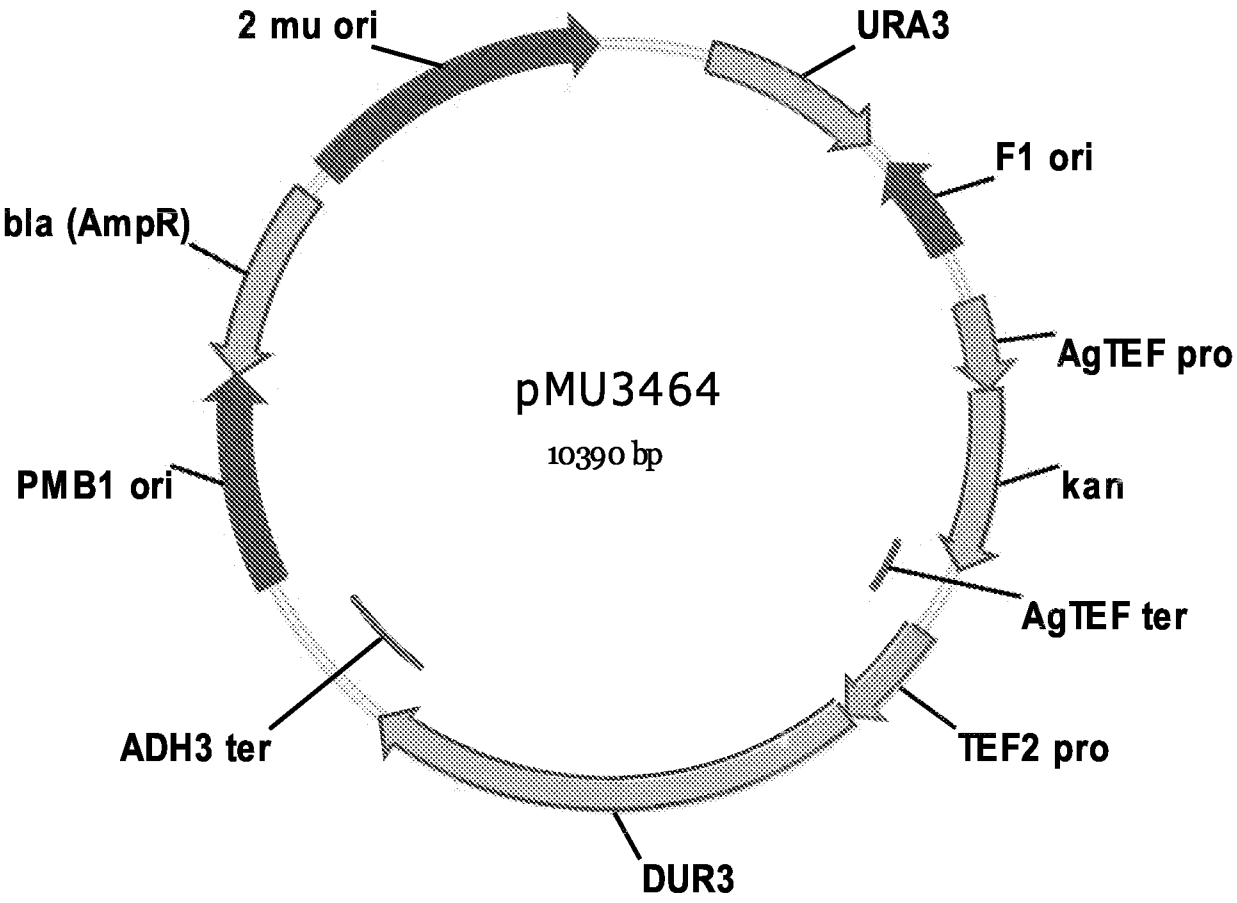

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3464
10390 bp
PMB1 ori
kan
AgTEF ter
ADH3 ter
TEF2 pro
DUR3

FIGURE 58

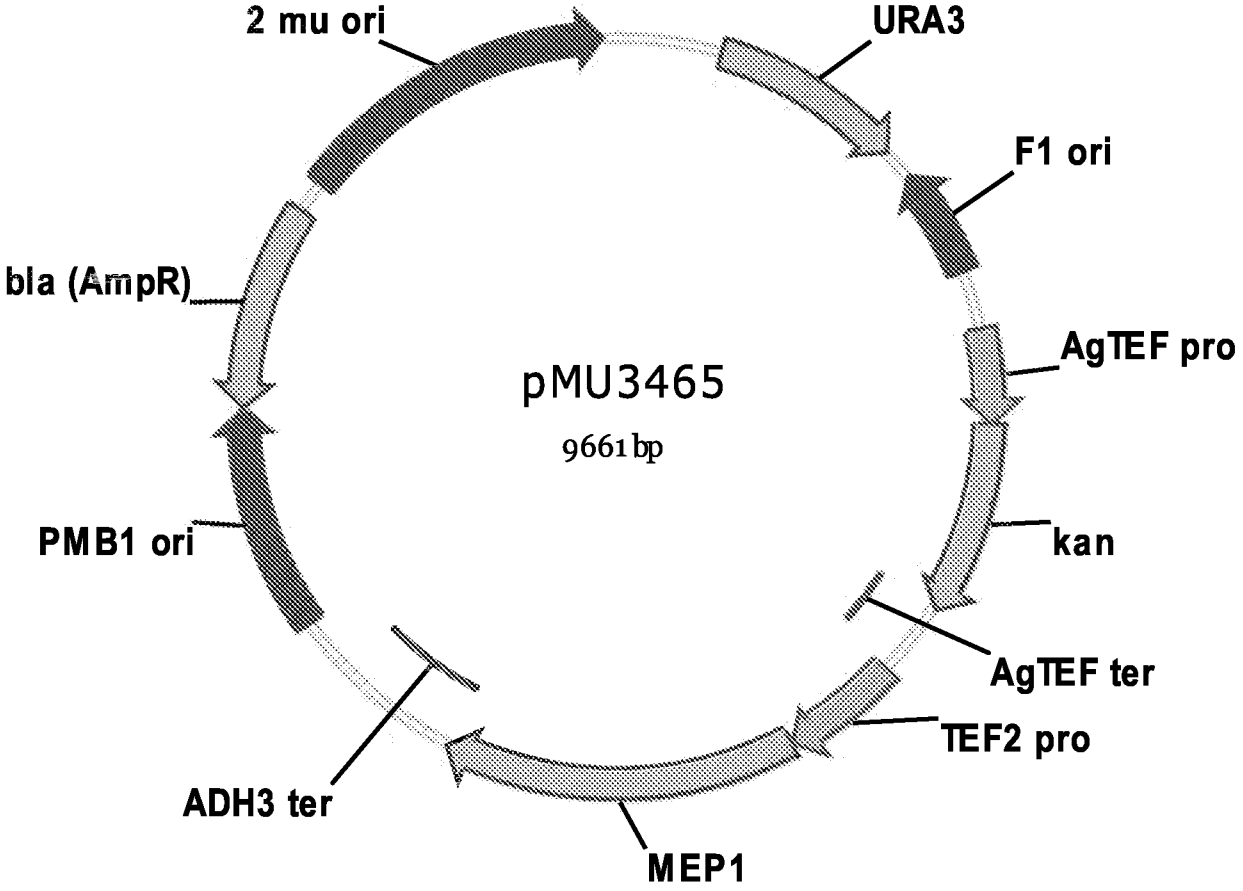

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3465
9661 bp
PMB1 ori
kan
AgTEF ter
TEF2 pro
ADH3 ter
MEP1

FIGURE 59

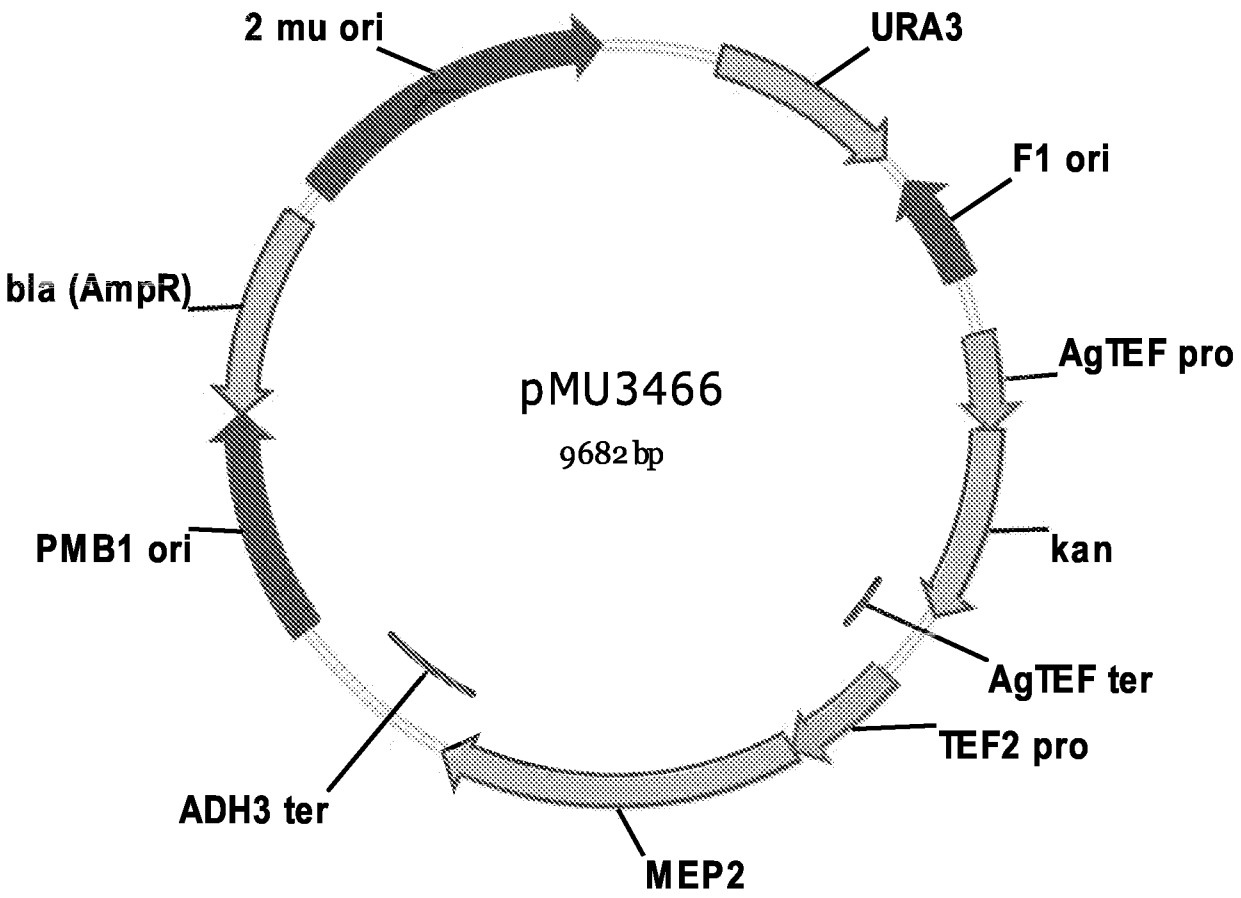

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3466
9682 bp
PMB1 ori
kan
AgTEF ter
TEF2 pro
ADH3 ter
MEP2

FIGURE 60

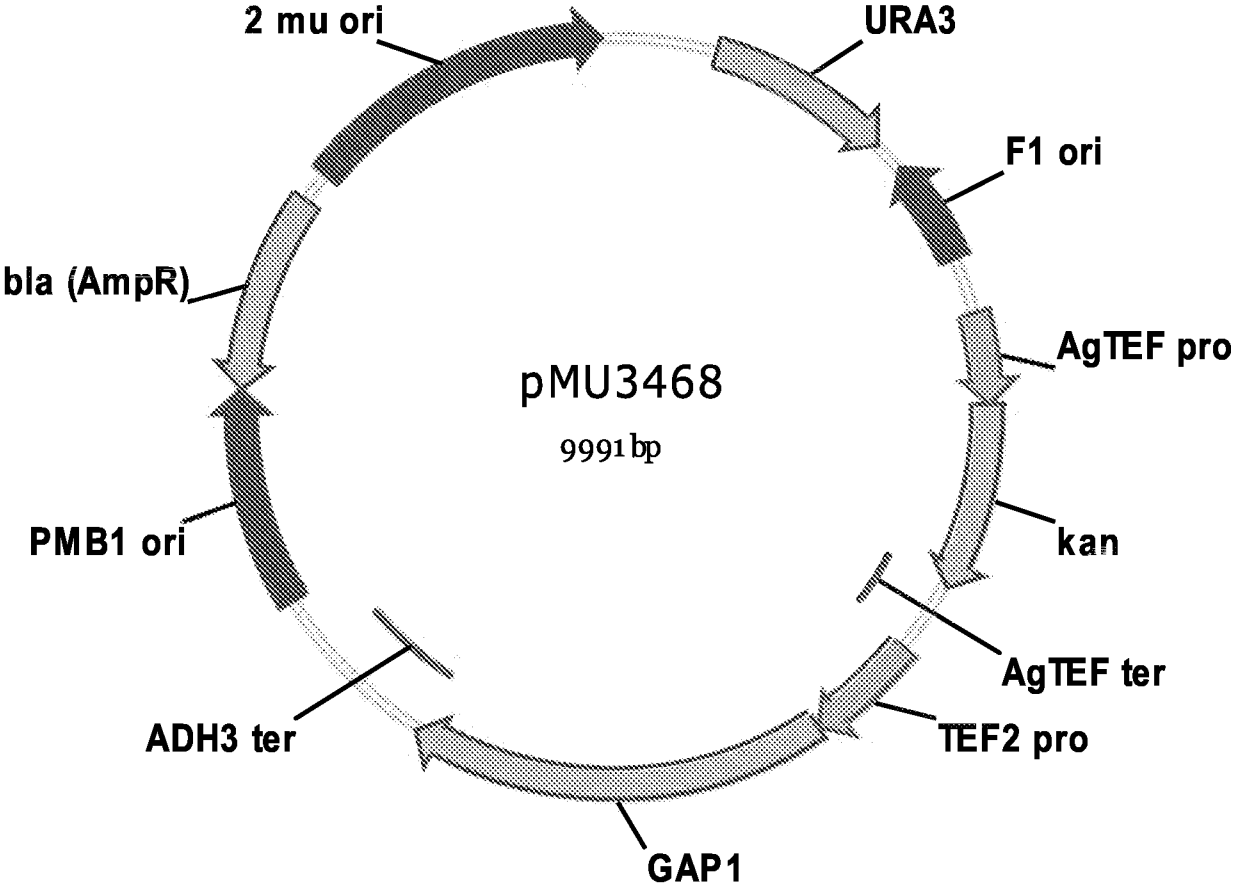

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3468
9991 bp
PMB1 ori
kan
AgTEF ter
ADH3 ter
TEF2 pro
GAP1

FIGURE 61

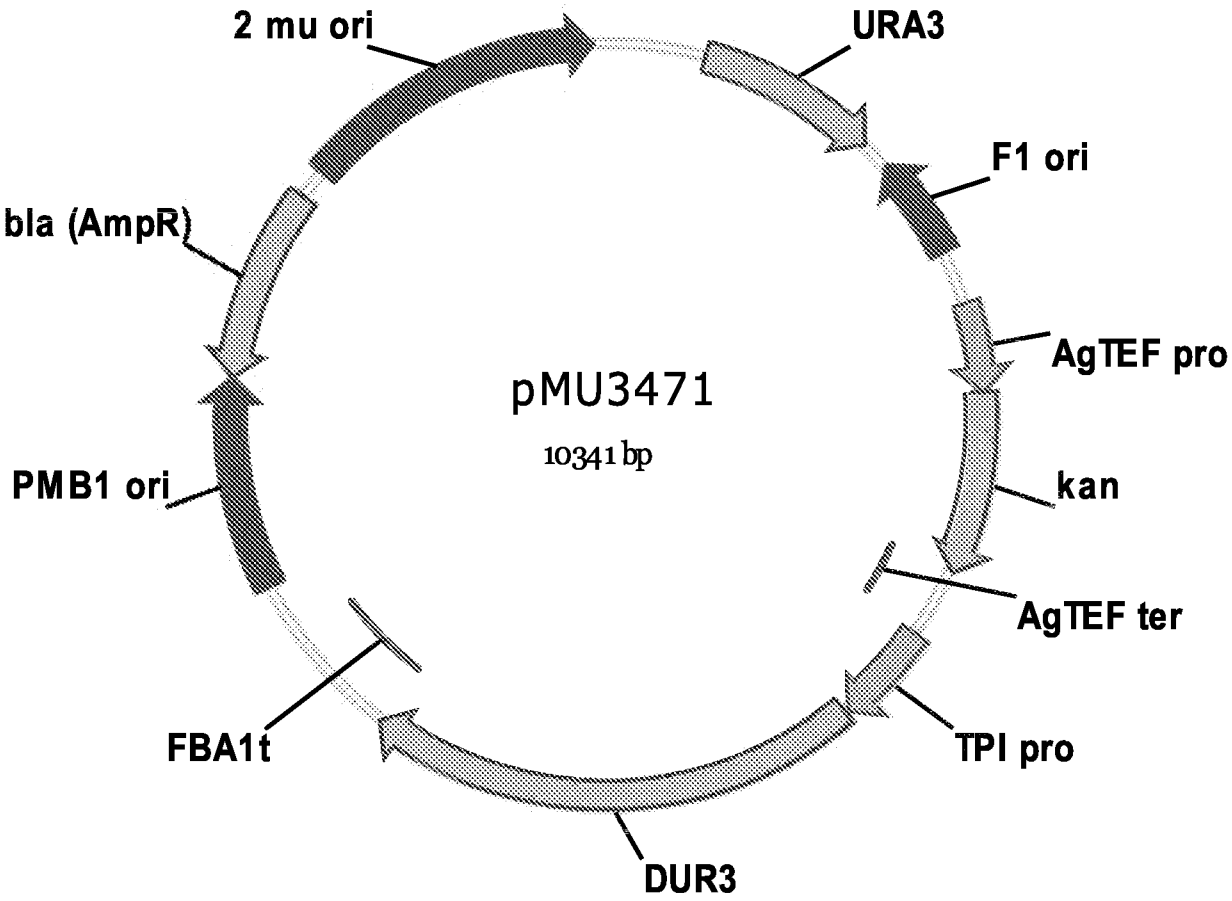

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3471
10341 bp
PMB1 ori
kan
AgTEF ter
FBA1t
TPI pro
DUR3

FIGURE 62

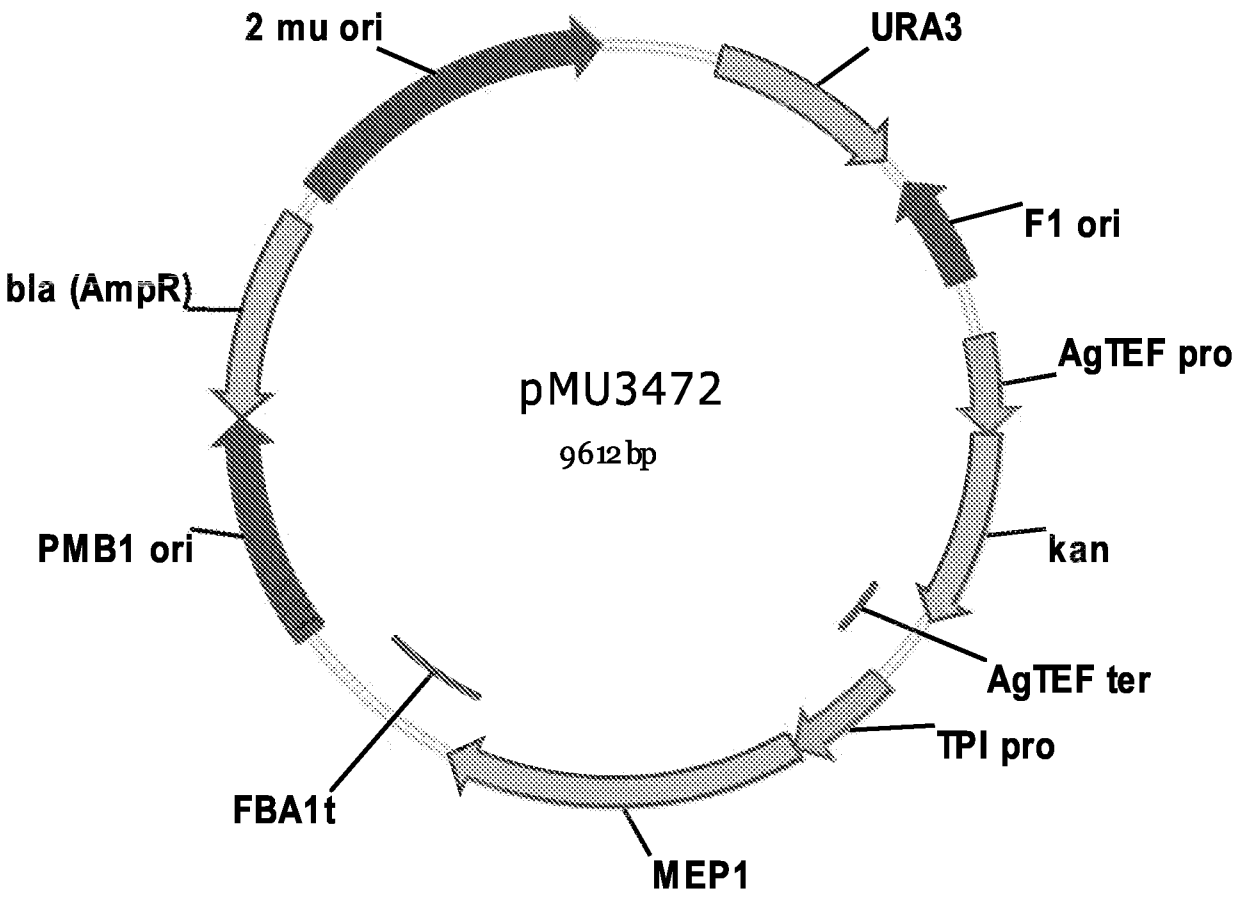

2 mu ori
URA3
F1 ori
AgTEF pro
kan
AgTEF ter
TPI pro
MEP1
FBA1t
PMB1 ori
bla (AmpR)
pMU3472
9612 bp

FIGURE 63

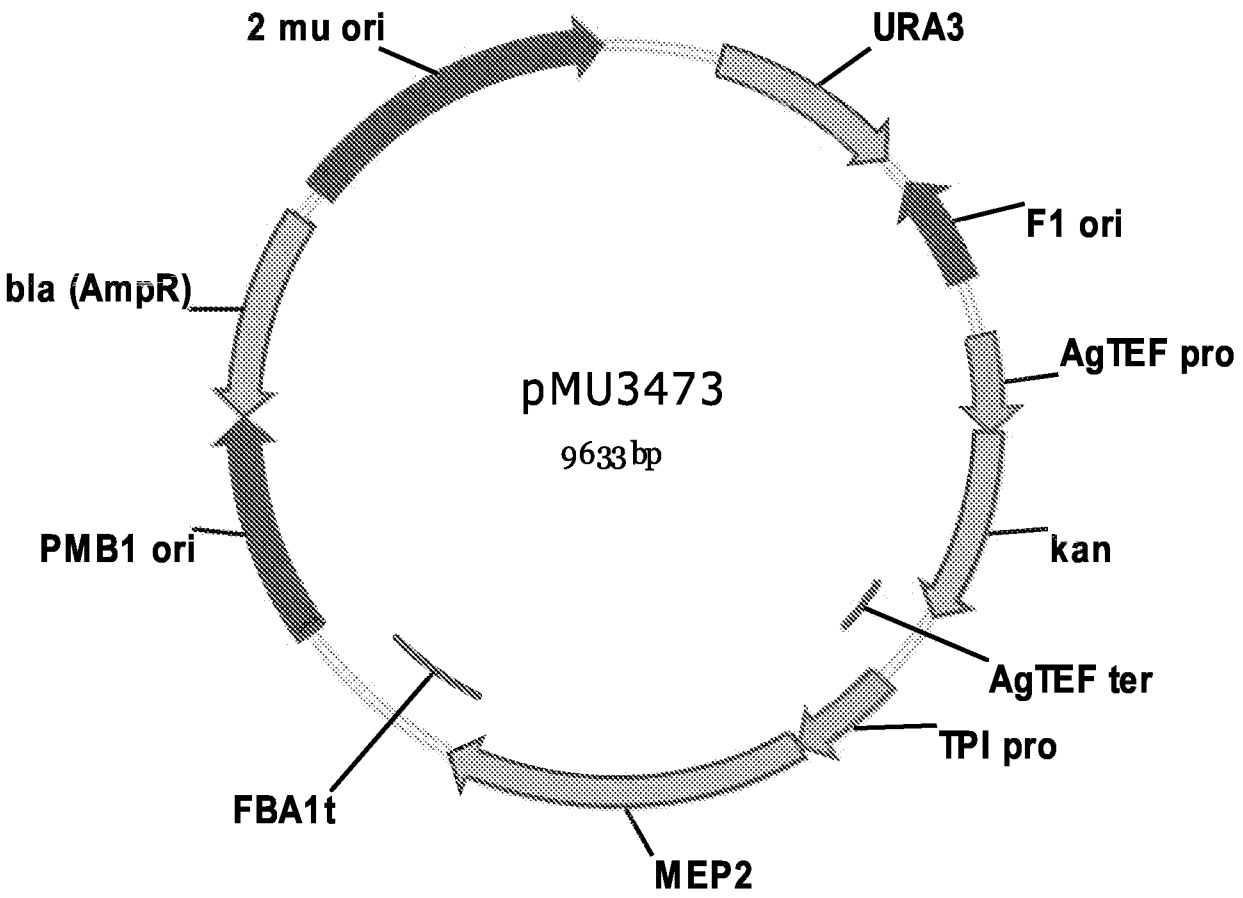

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3473
9633 bp
PMB1 ori
kan
AgTEF ter
TPI pro
FBA1t
MEP2

FIGURE 64

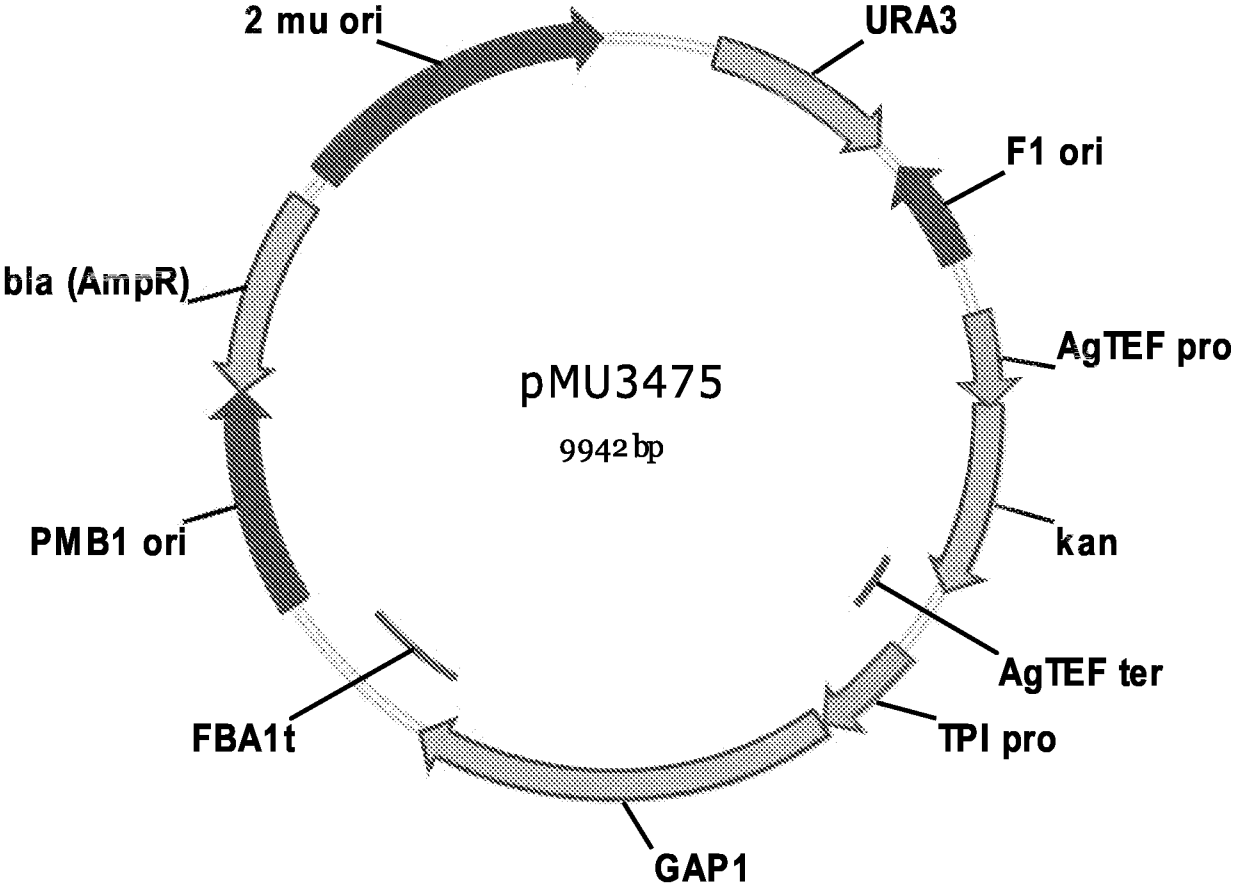

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3475
9942 bp
PMB1 ori
kan
AgTEF ter
FBA1t
TPI pro
GAP1

FIGURE 65

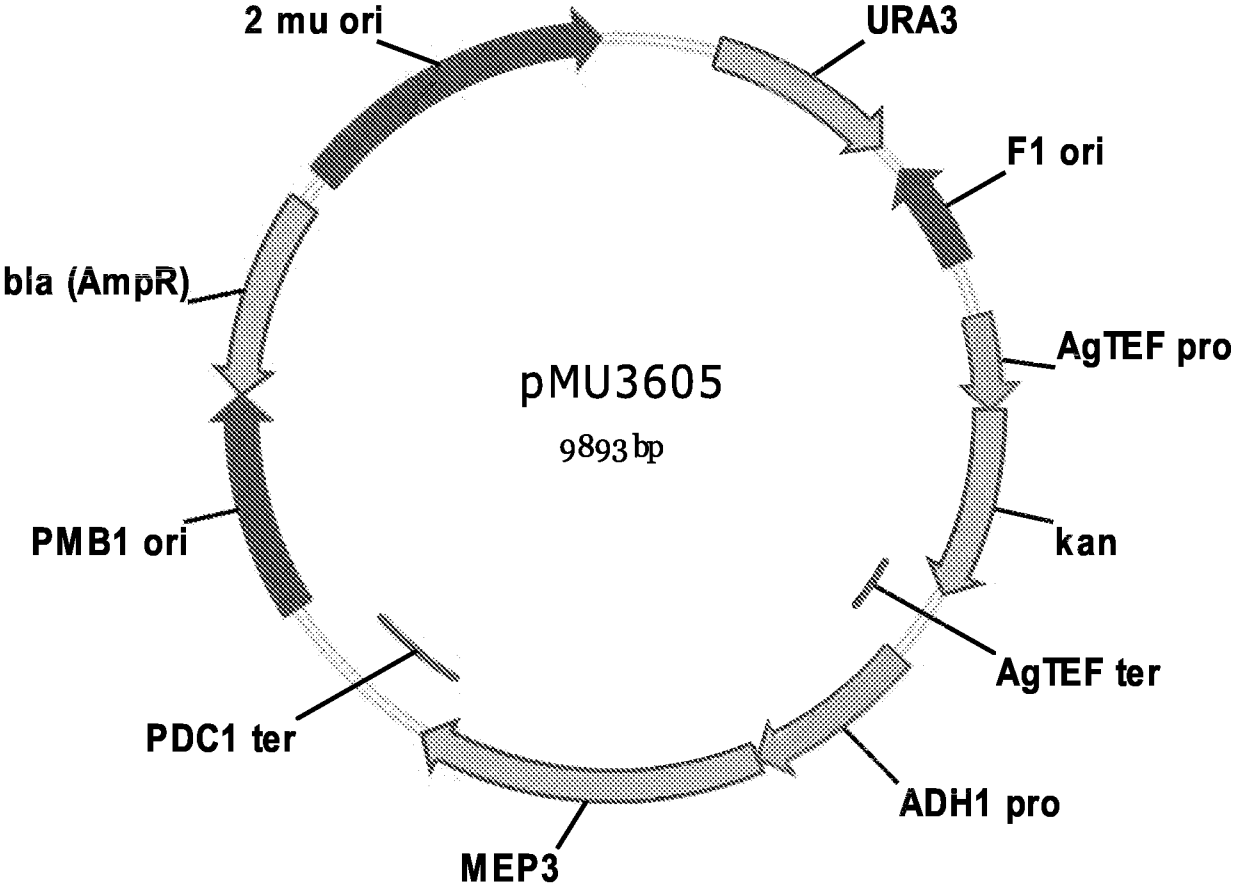

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3605
9893 bp
PMB1 ori
kan
AgTEF ter
PDC1 ter
ADH1 pro
MEP3

FIGURE 66

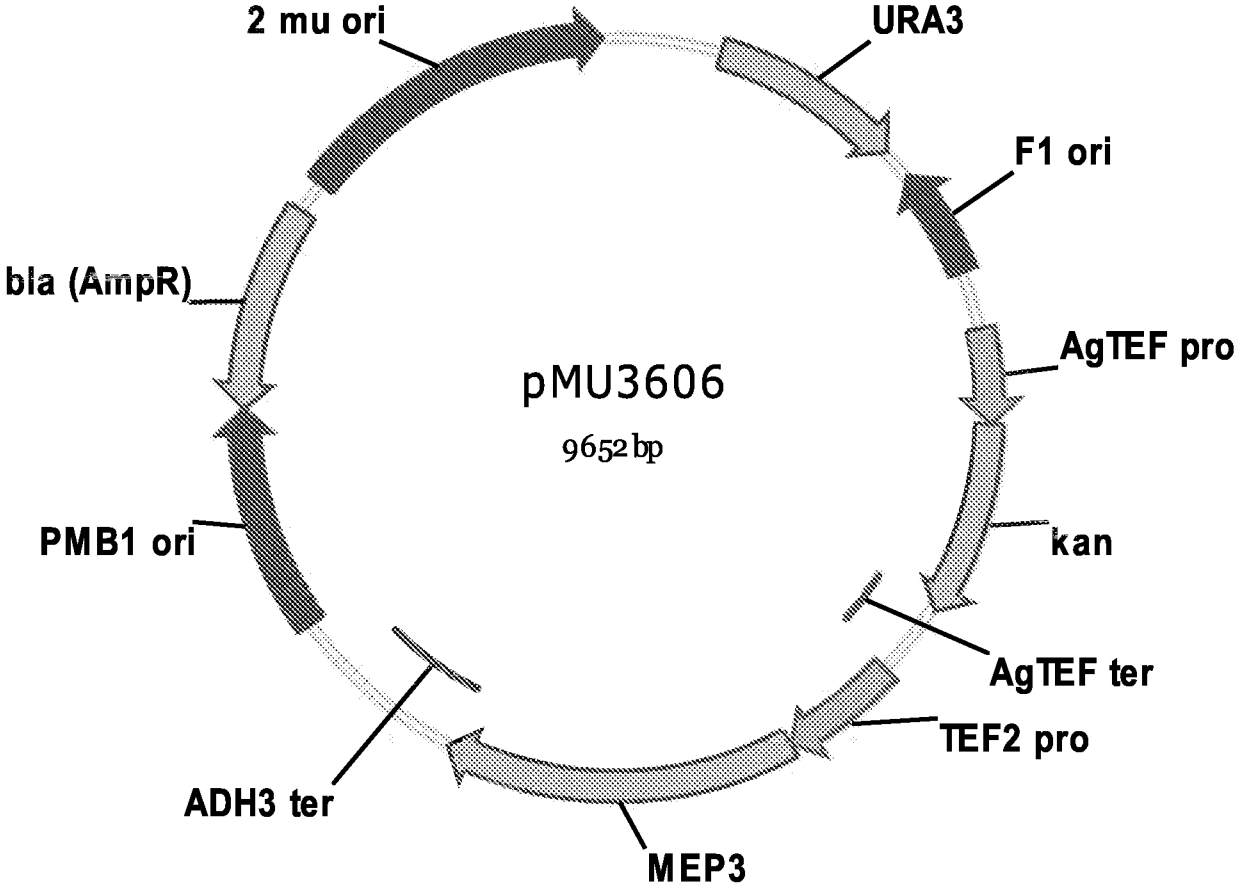

2 mu ori
URA3
F1 ori
bla (AmpR)
AgTEF pro
pMU3606
9652 bp
PMB1 ori
kan
AgTEF ter
TEF2 pro
ADH3 ter
MEP3

FIGURE 67

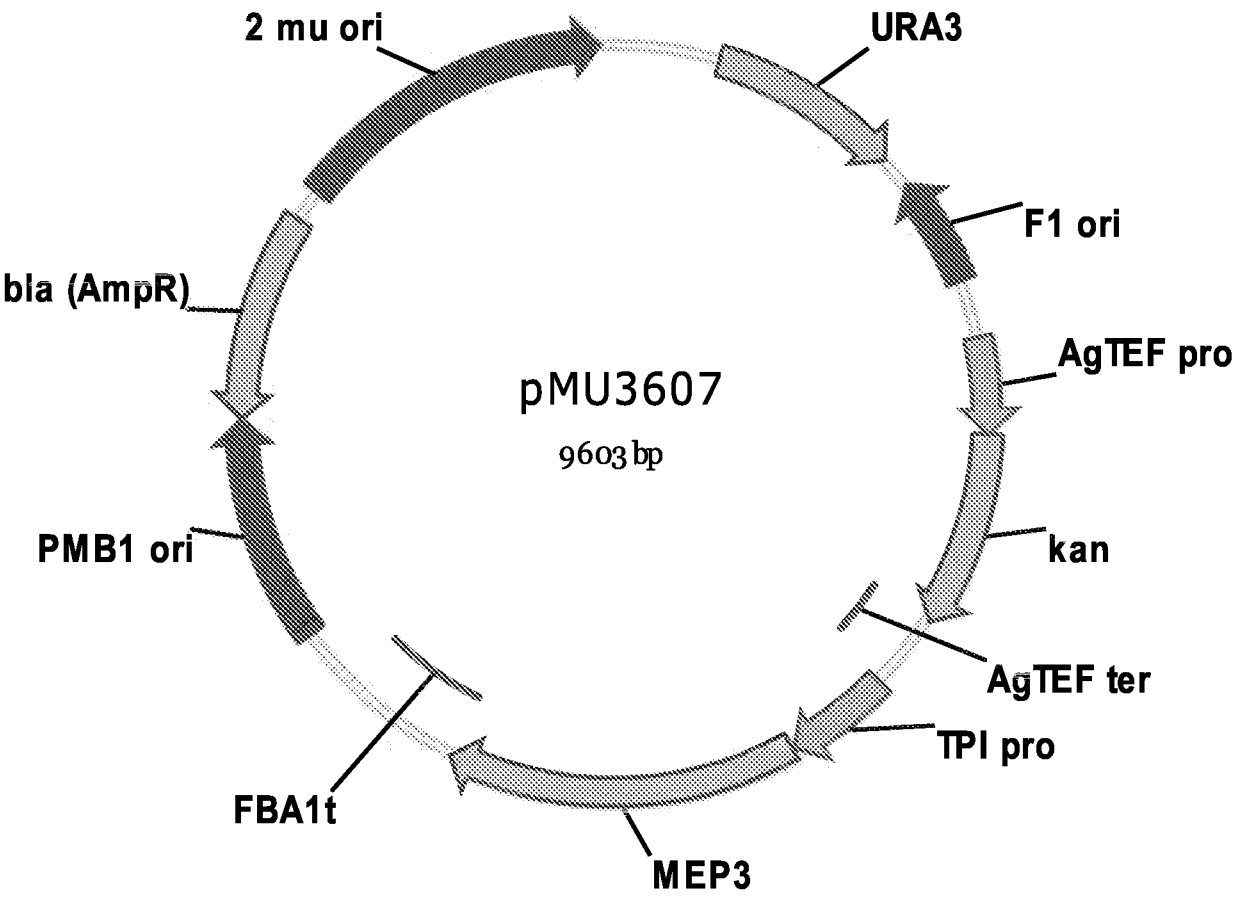

2 mu ori
URA3
F1 ori
AgTEF pro
kan
AgTEF ter
TPI pro
MEP3
FBA1t
PMB1 ori
bla (AmpR)
pMU3607
9603 bp

FIGURE 68

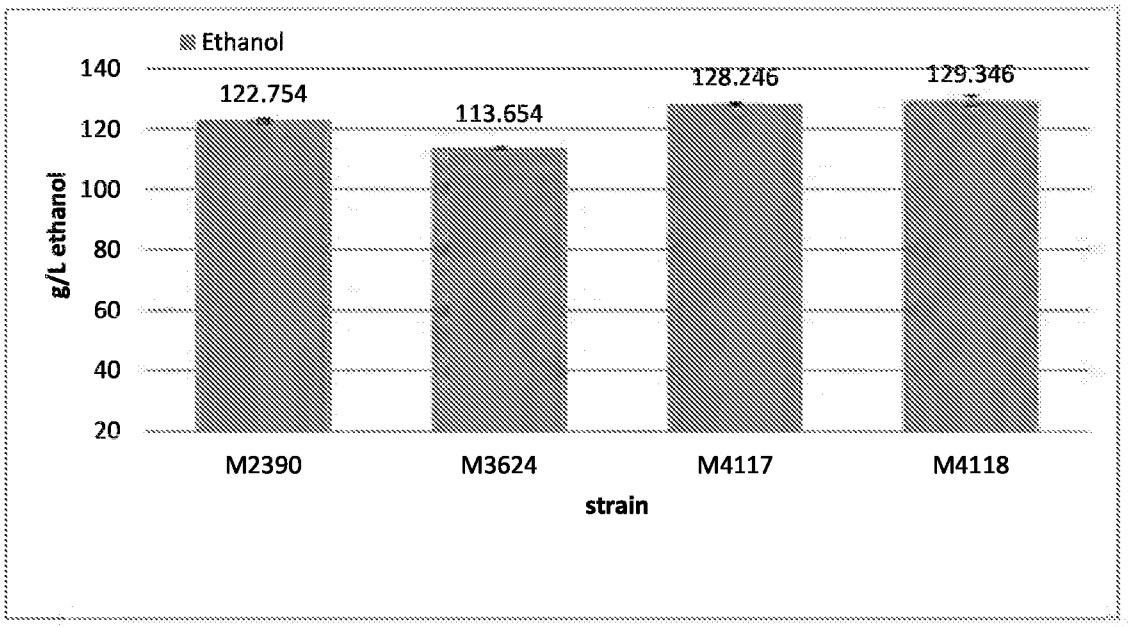

Ethanol
140
120
100
80
60
40
20
g/L ethanol
122.754
113.654
128.246
129.346
M2390
M3624
M4117
M4118
strain

FIGURE 69

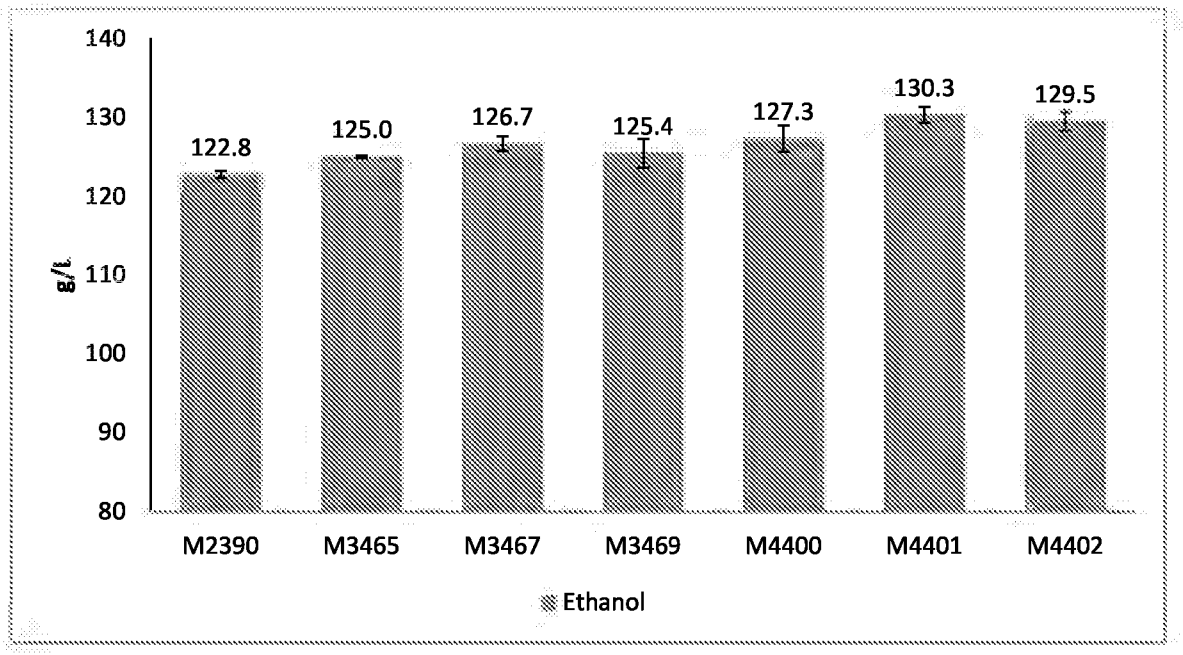

140
130
120
110
100
90
80
g/L
122.8
125.0
126.7
125.4
127.3
130.3
129.5
M2390
M3465
M3467
M3469
M4400
M4401
M4402
Ethanol

FIGURE 70

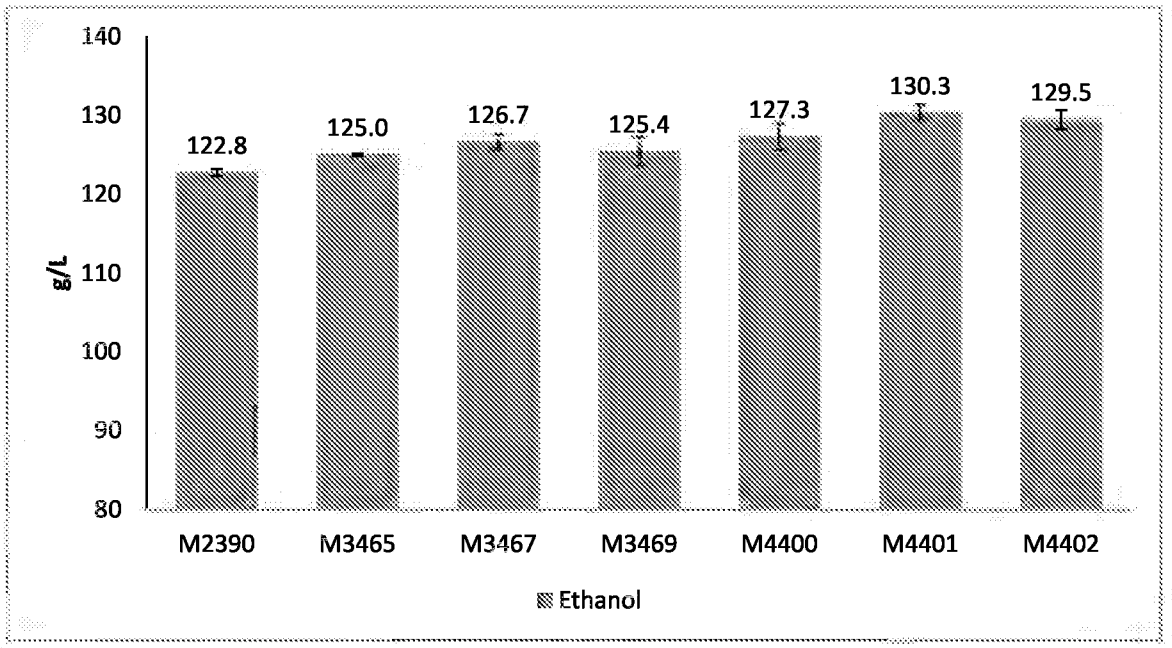

140
130
120
110
100
90
80
g/L
122.8
125.0
126.7
125.4
127.3
130.3
129.5
M2390
M3465
M3467
M3469
M4400
M4401
M4402
Ethanol

FIGURE 71

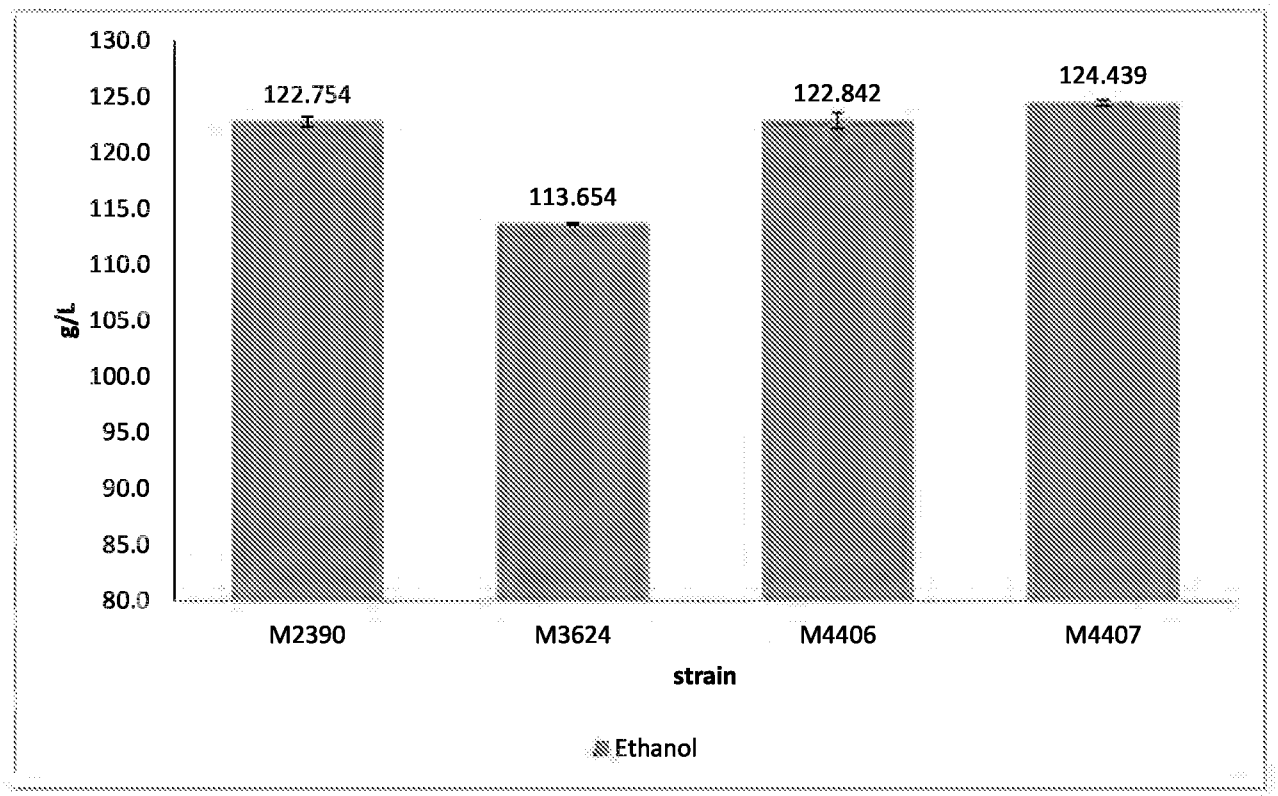

130.0
125.0
120.0
115.0
110.0
105.0
100.0
95.0
90.0
85.0
80.0
g/L
122.754
113.654
122.842
124.439
M2390
M3624
M4406
M4407
strain
Ethanol

FIGURE 72

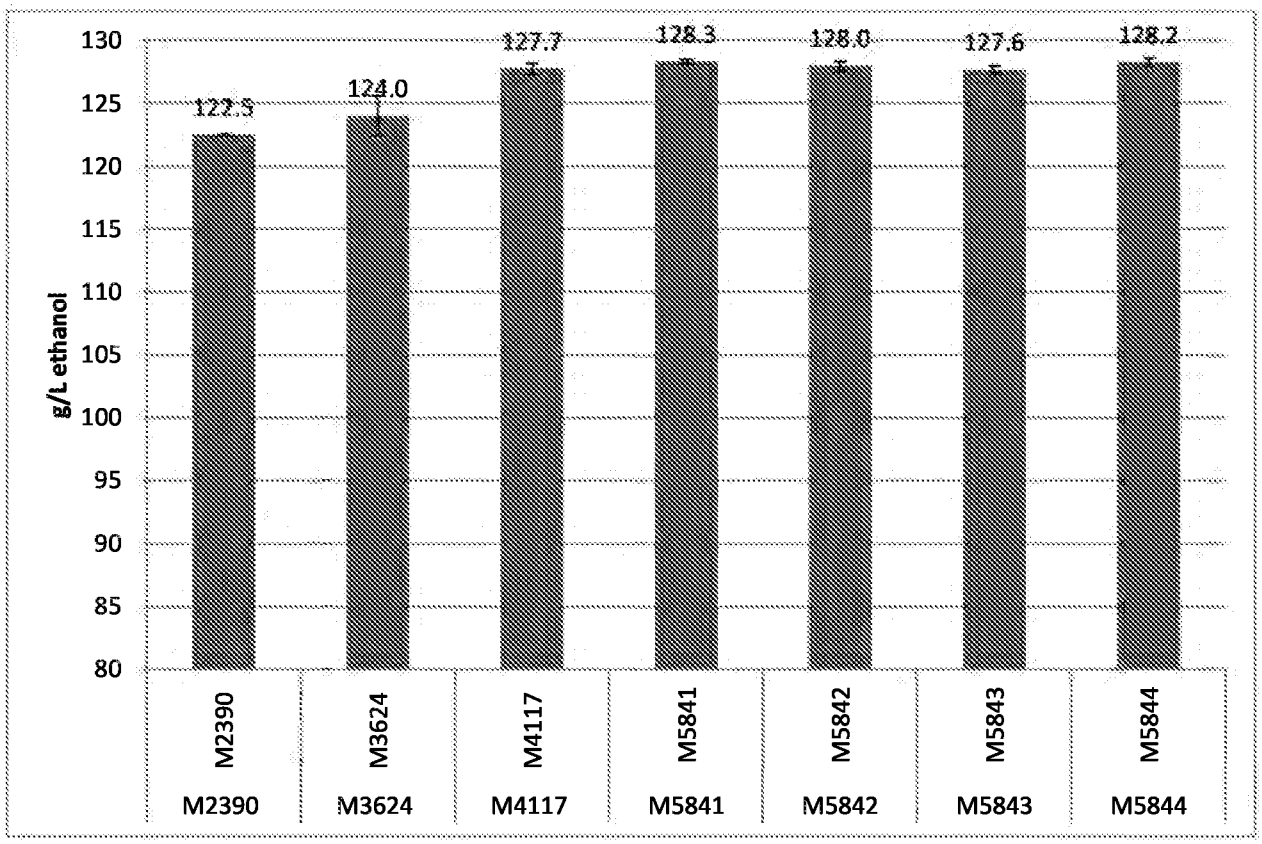

130
125
120
115
110
105
100
95
90
85
80
g/L ethanol
122.5
124.0
127.7
128.3
128.0
127.6
128.2
M2390
M3624
M4117
M5841
M5842
M5843
M5844
M2390
M3624
M4117
M5841
M5842
M5843
M5844

FIGURE 73

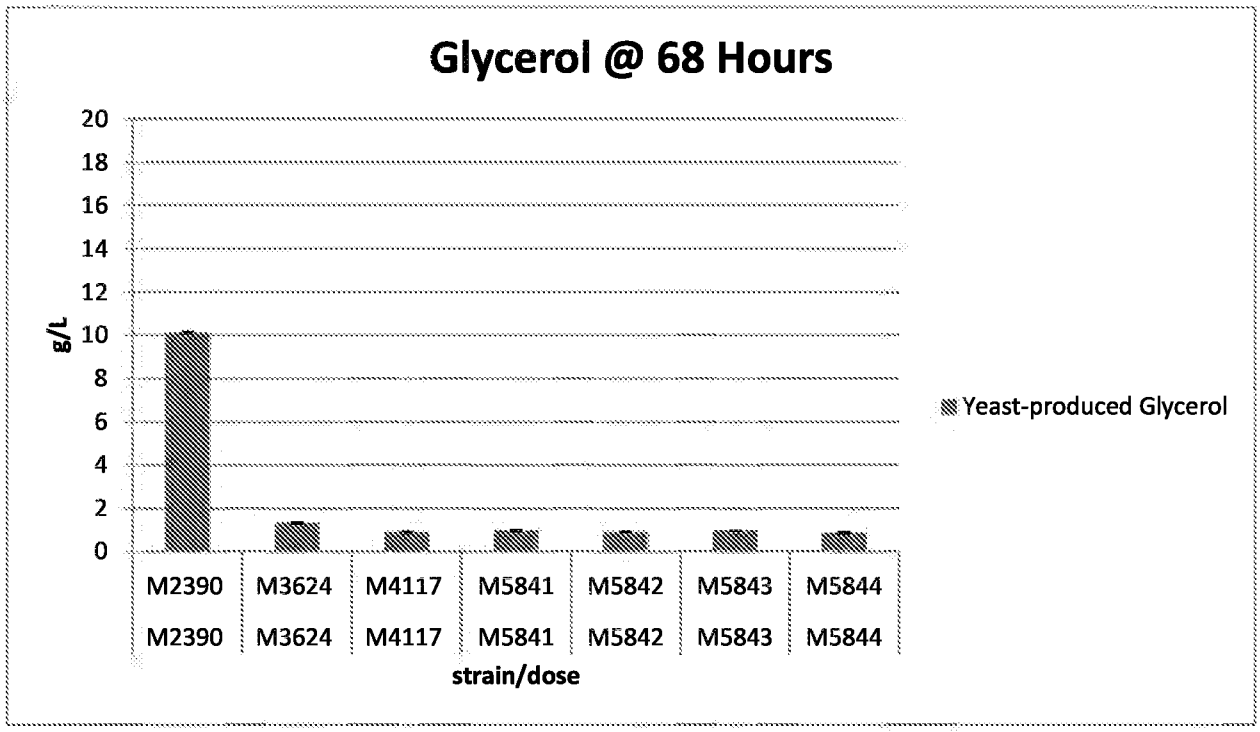
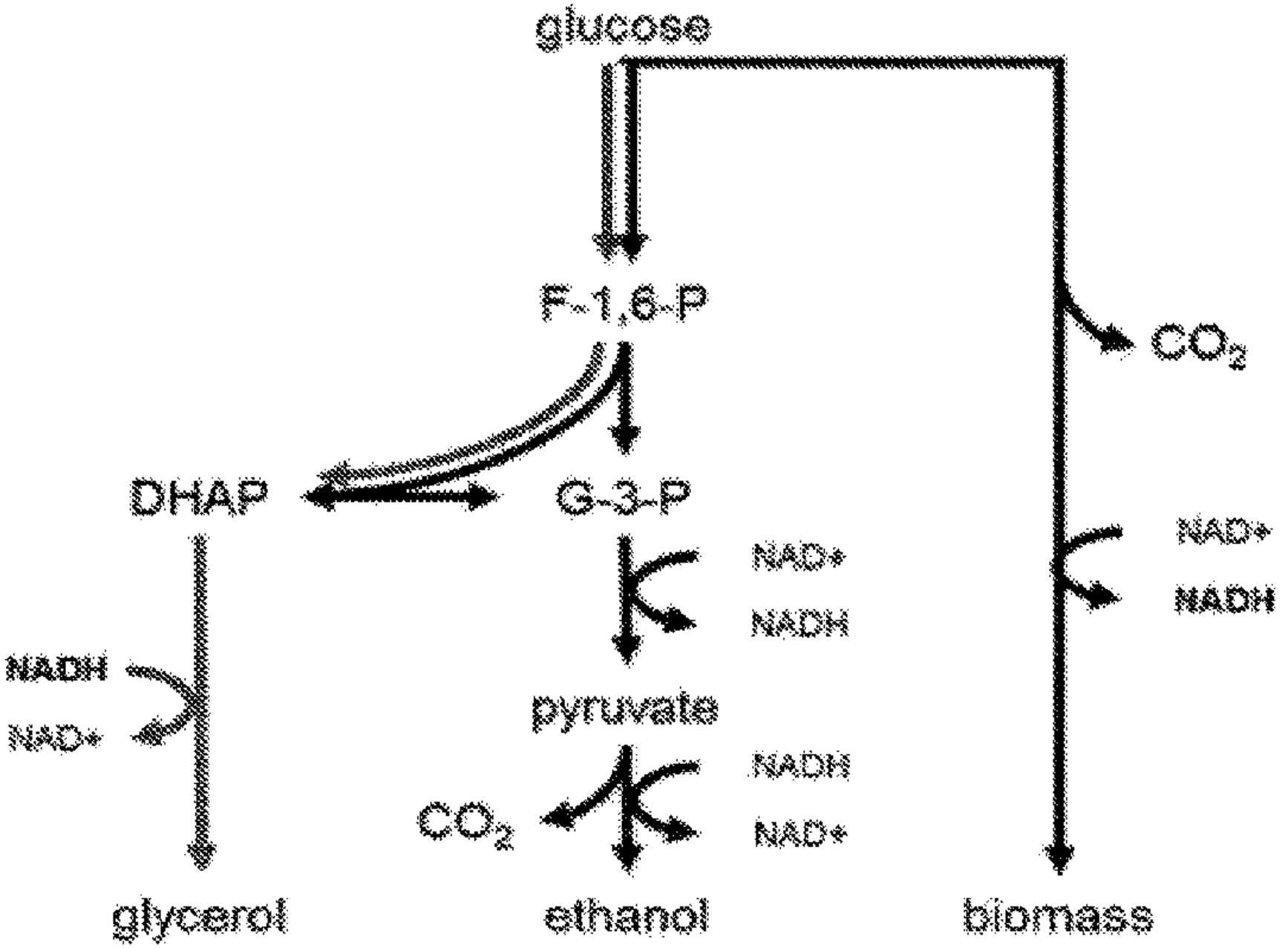

Glycerol @ 68 Hours
20
18
16
14
12
10
8
6
4
2
0
g/L
M2390
M3624
M4117
M5841
M5842
M5843
M5844
M2390
M3624
M4117
M5841
M5842
M5843
M5844
strain/dose
Yeast-produced Glycerol

FIGURE 74


glucose
F-1,6-P
CO_2
DHAP
G-3-P
NAD+
NADH
NAD+
NADH
NADH
NAD+
pyruvate
NADH
CO_2
NAD+
glycerol
ethanol
biomass