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(54) Title: COMPOSITIONS AND METHODS FOR 3-HYDROXYPROPIONIC ACID PRODUCTION

(57) Abstract: The present application discloses genetically modified yeast cells comprising an active 3-HP fermentation pathway, and the use of these cells to produce 3-HP.



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COMPOSITIONS AND METHODS FOR 3-HYDROXYPROPIONIC ACID PRODUCTIONREFERENCE TO SEQUENCE LISTING

[0001] This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

BACKGROUND

[0002] 3-hydroxypropionic acid (3-HP) is a three carbon carboxylic acid identified by the U.S. Department of Energy as one of the top 12 high-potential building block chemicals that can be made by fermentation. Alternative names for 3-HP, which is an isomer of lactic (2-hydroxypropionic) acid, include ethylene lactic acid and 3-hydroxypropionate. 3-HP is an attractive renewable platform chemical, with 100% theoretical yield from glucose, multiple functional groups that allow it to participate in a variety of chemical reactions, and low toxicity. 3-HP can be used as a substrate to form several commodity chemicals, such as 1,3-propanediol, malonic acid, acrylamide, and acrylic acid. Acrylic acid is a large-volume chemical (>7 billion lbs/year) used to make acrylate esters and superabsorbent polymers, and is currently derived from catalytic oxidation of propylene. Fermentative production of 3-HP would provide a sustainable alternative to petrochemicals as the feedstock for these commercially-significant chemicals, thus reducing energy consumption, US dependence on foreign oil, and the production of greenhouse gases.

[0003] Bacteria can be used to ferment sugars to organic acids. However, bacteria present certain drawbacks for large-scale organic acid production. As organic acids are produced, the fermentation medium becomes increasingly acidic. Lower pH conditions are actually preferable, because the resultant product is partially or wholly in the acid form. However, most bacteria that produce organic acids do not perform well in strongly acidic environments, and therefore either die or begin producing so slowly that they become economically unviable as the medium becomes more acidic. To prevent this, it becomes necessary to buffer the medium to maintain a higher pH. However, this makes recovery of the organic acid product more difficult and expensive.

[0004] There has been increasing interest in recent years around the use of yeast to ferment sugars to organic acids. Yeasts are used as biocatalysts in a number of industrial fermentations, and present several advantages over bacteria. While many bacteria are unable to synthesize certain amino acids or proteins that they need to grow and metabolize sugars efficiently, most yeast species can synthesize their necessary amino acids or proteins from inorganic nitrogen compounds. Yeasts are also not susceptible to

bacteriophage infection, which can lead to loss of productivity or of whole fermentation runs in bacteria.

[0005] Although yeasts are attractive candidates for organic acid production, they present several difficulties. First, pathway engineering in yeast is typically more difficult than in bacteria. Enzymes in yeast are compartmentalized in the cytoplasm, mitochondria, or peroxisomes, whereas in bacteria they are pooled in the cytoplasm. This means that targeting signals may need to be removed to ensure that all the enzymes of the biosynthetic pathway co-exist in the same compartment within a single cell. Control of transport of pathway intermediates between the compartments may also be necessary to maximize carbon flow to the desired product. Second, not all yeast species meet the necessary criteria for economic fermentation on a large scale. In fact, only a small percentage of yeasts possess the combination of sufficiently high volumetric and specific sugar utilization with the ability to grow robustly under low pH conditions. The U.S. Department of Energy has estimated that production rates of approximately 2.5 g/L/hour are necessary for economic fermentations of several organic acids, including 3-HP (<http://www1.eere.energy.gov/biomass/pdfs/35523.pdf>).

[0006] Although many yeast species naturally ferment hexose sugars to ethanol, few if any naturally produce significant yields of organic acids. This has led to efforts to genetically modify various yeast species to produce organic acids. Genetically modified yeast strains that produce lactic acid have been previously developed by disrupting or deleting the native pyruvate decarboxylase (PDC) gene and inserting a lactate dehydrogenase (LDH) gene to eliminate ethanol production (see, e.g., WO99/14335, WO00/71738, WO02/42471, WO03/049525, WO03/102152 and WO03/102201). This alteration diverts sugar metabolism from ethanol production to lactic acid production. The fermentation products and pathways for yeast differ from those of bacteria, and thus different engineering approaches are necessary to maximize yield. Other native products that may require elimination or reduction in order to enhance organic acid product yield or purity are glycerol, acetate, and diols. The reduction of glycerol in genetically altered yeast strains is described in, for example, WO07/106524.

[0007] Unlike lactic acid, 3-HP is not a major end product of any pathway known in nature, being found in only trace amounts in some bacteria and fungi. Thus, a greater deal of genetic engineering is necessary to generate yeast that produce 3-HP. A *Saccharomyces cerevisiae* strain was previously engineered to produce 3-HP at very low levels through a lactate intermediate (see WO02/042418). However, the tolerance level of wild-type *S. cerevisiae* is insufficient to make it an optimal host for 3-HP production. Therefore, there is a

need for improved yeast strains that generate 3-HP in a more cost-effective manner on an industrial scale.

SUMMARY

[0008] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP, pyruvate, and/or glycerol to 3-HP. In certain embodiments, the cells provided herein contain one or more 3-HP pathway genes encoding enzymes with PPC, PYC, AAT, ADC, BAAT, gabT, 3-HPDH, HIBADH, 4-hydroxybutyrate dehydrogenase, ACC, AAM, alanine dehydrogenase, aldehyde dehydrogenase, BCKA, KGD, 4-aminobutyrate aminotransferase, β -alanyl-CoA ammonia lyase, Co-A acylating malonate semialdehyde dehydrogenase, CoA synthetase, CoA transferase, glycerol dehydratase, IPDA, LDH, lactyl-CoA dehydratase, malate decarboxylase, malate dehydrogenase, malonyl-CoA reductase, OAA formatelyase, OAA dehydrogenase, pyruvate/alanine aminotransferase, PDH, 2-keto acid decarboxylase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, or 3-hydroxyisobutyryl-CoA hydrolase activity.

[0009] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP or pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with PPC, PYC, AAT, ADC, BAAT, gabT, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source. For example, the cells may contain a yeast PYC gene derived from *I. orientalis* or a bacterial PYC gene derived from *R. sphaeroides*, *R. etli*, *P. fluorescens*, *C. glutamicum*, or *S. meliloti*; a bacterial PPC gene derived from *E. coli*, *M. thermoautotrophicum*, or *C. perfringens*, a yeast AAT gene derived from *I. orientalis* or *S. cerevisiae* or a bacterial AAT gene derived from *E. coli*; a bacterial ADC gene derived from *S. avermitilis*, *C. acetobutylicum*, *H. pylori*, *B. licheniformis*, or *C. glutamicum*; a yeast BAAT gene derived from *I. orientalis* or *S. kluyveri* or a bacterial BAAT gene derived from *S. avermitilis*; a yeast gabT gene derived from *S. cerevisiae* or a bacterial gabT gene derived from *S. avermitilis*; a yeast 3-HPDH gene derived from *I. orientalis* or *S. cerevisiae* or a bacterial 3-HPDH gene derived from *E. coli* or *M. sedula*; a bacterial HIBADH gene derived from *A. faecalis*, *P. putida*, or *P. aeruginosa*; and/or a yeast 4-hydroxybutyrate dehydrogenase gene derived from *C. kluyveri* or a bacterial 4-hydroxybutyrate dehydrogenase gene derived from *R. eutropha*.

[0010] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP or pyruvate to 3-HP, wherein the

cells contain one or more genes encoding enzymes with PPC, malate dehydrogenase, and malate decarboxylase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0011] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP or pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with PPC, 2-keto acid decarboxylase, KGD, BCKA, indolepyruvate decarboxylase, 3-HPDH, HIBADH, or 4-hydroxybutyrate dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0012] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP or pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with PPC, OAA formatelyase, malonyl-CoA reductase, Co-A acylating malonate semialdehyde dehydrogenase, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0013] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with PDH, acetyl-CoA carboxylase, malonyl-CoA reductase, CoA acylating malonate semialdehyde dehydrogenase, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0014] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with alanine dehydrogenase, pyruvate/alanine aminotransferase, alanine 2,3 aminomutase, CoA transferase, CoA synthetase, β -alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, 3-hydroxyisobutyryl-CoA hydrolase, BAAT, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0015] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from pyruvate to 3-HP, wherein the cells

contain one or more genes encoding enzymes with LDH, CoA transferase, lactyl-CoA dehydratase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, and 3-hydroxyisobutyryl-CoA hydrolase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0016] Provided herein in certain embodiments are genetically modified yeast cells comprising an active 3-HP fermentation pathway from PEP or pyruvate to 3-HP, wherein the cells contain one or more genes encoding enzymes with glycerol dehydratase and aldehyde dehydrogenase activity. In certain embodiments, one or more of the 3-HP pathway genes are exogenous, and in these embodiments the genes may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source.

[0017] The genetically modified yeast cells provided herein may be any yeast species. In certain embodiments, the cells are Crabtree-negative, and in certain of these embodiments they belong to the genus *Issatchenkia*, *Candida*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, *Zygosaccharomyces*, or *Saccharomyces*. In certain of these embodiments, the cells may belong to the *I. orientalis*/*P. fermentans* clade or the *Saccharomyces* clade, and in these embodiments they may be *I. orientalis*, *C. lambica*, or *S. bulderi*. In certain embodiments, the yeast cells may be 3-HP resistant yeast cells. 3-HP resistance may be a native trait of the cells or it result from the cells having undergone mutation and/or selection before, during, or after introduction of genetic modifications related to an active 3-HP fermentation pathway, or a combination thereof. In certain embodiments, the yeast cells may exhibit a degree of tolerance to organic acids other than 3-HP, other fermentation products or byproducts, and/or various media components that is greater than that exhibited by wild-type yeast cells of the same species. In certain embodiments, the yeast cells have undergone mutation and/or selection, such that the mutated and/or selected cells possess a higher degree of resistance to 3-HP than a wild-type cell of the same species. In some of these embodiments, the cell has undergone mutation and/or selection before being genetically modified with the one or more exogenous 3-HP pathway genes. In some embodiments, the cell has undergone selection in the presence of lactic acid or 3-HP. In some embodiments, the selection is chemostat selection.

[0018] In addition to modifications related to an active 3-HP fermentation pathway, the cells provided herein may contain deletions or disruptions of one or more native genes. For example, the cells may contain deletions or disruptions of one or more PDC, ADH, GAL6, CYB2A, CYB2B, GPD, GPP, ALD, or PCK genes. In certain embodiments, these deletions or disruptions may be coupled to the introduction of one or more genes related to an active 3-HP fermentation pathway.

[0019] Provided herein in certain embodiments are methods of producing 3-HP using the genetically modified yeast cells provided herein by culturing the cells in the presence of at least one carbon source and isolating 3-HP from the culture medium. In certain of these embodiments, the carbon source may be selected from one or more of glucose, xylose, arabinose, sucrose, fructose, cellulose, glucose oligomers, and glycerol.

BRIEF DESCRIPTION OF DRAWING

- [0020]** Figure 1: Summary of select 3-HP fermentation pathways
- [0021]** Figure 2: Plasmid pMIBa107
- [0022]** Figure 3: Schematic representation of a targeted integration technique
- [0023]** Figure 4: Plasmid pGMEr125(a)
- [0024]** Figure 5: Plasmid pGMRr125(b)
- [0025]** Figure 6: Plasmid pGMEr121
- [0026]** Figure 7: Plasmid pMhCt074
- [0027]** Figure 8: Plasmid pMhCt083
- [0028]** Figure 9: Plasmid pMhCt087
- [0029]** Figure 10: Plasmid pMhCt075
- [0030]** Figure 11: Plasmid pMhCt077
- [0031]** Figure 12: Plasmid pMhCt095
- [0032]** Figure 13: Plasmid pMhCt096
- [0033]** Figure 14: Plasmid pMeJi310-2
- [0034]** Figure 15: Plasmid pMeJi312-2
- [0035]** Figure 16: Plasmid pGMEr126
- [0036]** Figure 17: Plasmid pGMEr130
- [0037]** Figure 18: Plasmid pGMEr137
- [0038]** Figure 19: Plasmid pACN5
- [0039]** Figure 20: Plasmid pACN23
- [0040]** Figure 21: Plasmid pHJJ27
- [0041]** Figure 22: Plasmid pACN43
- [0042]** Figure 23: Plasmid pHJJ75
- [0043]** Figure 24: Plasmid pHJJ76
- [0044]** Figure 25: Plasmid pJLJ49
- [0045]** Figure 26: Plasmid pJLJ62
- [0046]** Figure 27: Plasmid pMI458
- [0047]** Figure 28: Plasmid pCM208

[0048] Figure 29: Plasmid pJY39

[0049] Figure 30: Plasmid pMcTs64

[0050] Figure 31: Plasmid pMcTs65

[0051] Figure 32: Plasmid pJLJ8.

DETAILED DESCRIPTION

[0052] The following description of the invention is merely intended to illustrate various embodiments of the invention. As such, the specific modifications discussed are not to be construed as limitations on the scope of the invention. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the invention, and it is understood that such equivalent embodiments are to be included herein.

[0053] All references cited herein are incorporated by reference in their entirety.

Abbreviations

[0054] 3-HP, 3-hydroxypropionic acid; 3-HPA, 3-hydroxypropionaldehyde; 3-HPDH, 3-hydroxypropionic acid dehydrogenase; AAM, alanine 2,3 aminomutase; AAT, aspartate aminotransferase; ACC, acetyl-CoA carboxylase; ADC, aspartate 1-decarboxylase; AKG, alpha-ketoglutarate; ALD, aldehyde dehydrogenase; BAAT, β -alanine aminotransferase; BCKA, branched-chain alpha-keto acid decarboxylase; bp, base pairs; CYB2, L-(+)-lactate-cytochrome c oxidoreductase; CYC, iso-2-cytochrome c; EMS, ethane methyl sulfonase; ENO, enolase; gabT, 4-aminobutyrate aminotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase 3; GPD, glycerol 3-phosphate dehydrogenase; GPP, glycerol 3-phosphate phosphatase; HIBADH, 3-hydroxyisobutyrate dehydrogenase; IPDA, indolepyruvate decarboxylase; KGD, alpha-ketoglutarate decarboxylase; LDH, lactate dehydrogenase; MAE, malic enzyme; OAA, oxaloacetate; PCK, phosphoenolpyruvate carboxykinase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PGK, phosphoglycerate kinase; PPC, phosphoenolpyruvate carboxylase; PYC, pyruvate carboxylase; RKI, ribose 5-phosphate ketol-isomerase; TAL, transaldolase; TEF1, translation elongation factor-1; TEF2, translation elongation factor-2; TKL, transketolase, XDH, xylitol dehydrogenase; XR, xylose reductase, YP, yeast extract/peptone.

Description

[0055] Provided herein are genetically modified yeast cells for the production of 3-HP, methods of making these yeast cells, and methods of using these cells to produce 3-HP. "3-HP" as used herein includes salt and acid forms of 3-hydroxypropionic acid.

[0056] A number of 3-HP fermentation pathways are known in the art (see, e.g., US Patent No. 6,852,517; US Patent No. 7,309,597; US Pub. No. 2001/0021978; US Pub. No. 2008/0199926; WO02/42418; and WO10/031083, all incorporated by reference herein). 3-HP fermentation pathways operate via a series of intermediates that may include phosphoenolpyruvate (PEP), pyruvate, oxaloacetate (OAA), aspartate, β -alanine, malonate semialdehyde, malate, malonyl-CoA, acetyl-CoA, alanine, lactate, lactyl-CoA, acrylyl-CoA, glycerol, 3-hydroxypropionaldehyde (3-HPA), β -alanyl-CoA, 3-HP-CoA, and glycerate. An overview of several of the known 3-HP fermentation pathways is set forth in Figure 1.

[0057] As disclosed herein, a set of yeast cells from various species were tested for 3-HP resistance. Cells exhibiting 3-HP resistance were further evaluated based on their growth rates and glucose consumption rates in media containing varying concentrations of 3-HP. Based on these experiments, a set of ideal host cells for 3-HP production were identified. These host cells were then genetically modified to contain an active 3-HP fermentation pathway, resulting in genetically modified yeast cells that produce 3-HP under low pH conditions.

[0058] Provided herein in certain embodiments are genetically modified yeast cells having at least one active 3-HP fermentation pathway from PEP, pyruvate, and/or glycerol to 3-HP. A yeast cell having an "active 3-HP fermentation pathway" as used herein produces active enzymes necessary to catalyze each reaction in a 3-HP fermentation pathway, and therefore is capable of producing 3-HP in measurable yields when cultured under fermentation conditions in the presence of at least one fermentable sugar. A yeast cell having an active 3-HP fermentation pathway comprises one or more 3-HP pathway genes. A "3-HP pathway gene" as used herein refers to the coding region of a nucleotide sequence that encodes an enzyme involved in a 3-HP fermentation pathway.

[0059] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through PEP or pyruvate, OAA, aspartate, β -alanine, and malonate semialdehyde intermediates (see, e.g., US Pub. No. 2010/0021978, Figure 1). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of pyruvate carboxylase (PYC), PEP carboxylase (PPC), aspartate aminotransferase (AAT), aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), aminobutyrate aminotransferase (gabT), 3-HP dehydrogenase (3-HPDH), 3-hydroxyisobutyrate dehydrogenase (HIBADH), and 4-hydroxybutyrate dehydrogenase

genes. The 3-HP fermentation pathway genes may also include a PEP carboxykinase (PCK) gene that has been modified to produce a polypeptide that preferably catalyzes the conversion of PEP to OAA (native PCK genes generally produce a polypeptide that preferably catalyzes the reverse reaction of OAA to PEP).

[0060] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through PEP or pyruvate, OAA, and malate intermediates (see, e.g., US Pub. No. 2010/0021978, Figure 4). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of PPC, PYC, malate dehydrogenase, and malate decarboxylase genes. The 3-HP fermentation pathway genes may also include a PCK gene that has been modified to produce a polypeptide that preferably catalyzes the conversion of PEP to OAA.

[0061] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through PEP or pyruvate, OAA, and malonate semialdehyde intermediates (see, e.g., US Pub. No. 2010/0021978, Figure 1). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of PPC, PYC, 2-keto acid decarboxylase, alpha-ketoglutarate (AKG) decarboxylase (KGD), branched-chain alpha-keto acid decarboxylase (BCKA), indolepyruvate decarboxylase (IPDA), 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase genes. The 3-HP fermentation pathway genes may also include a PCK gene that has been modified to produce a polypeptide that preferably catalyzes the conversion of PEP to OAA. Further, the 3-HP fermentation pathway genes may include a PDC gene and/or benzoylformate decarboxylase gene that has been modified to encode a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde.

[0062] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through PEP or pyruvate, OAA, malonyl-CoA, and malonate semialdehyde intermediates, wherein the malonate semialdehyde intermediate is optional (see, e.g., US Pub. No. 2010/0021978, Figure 2). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of PPC, PYC, OAA formate lyase, malonyl-CoA reductase, CoA acylating malonate semialdehyde dehydrogenase, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase genes. The 3-HP fermentation pathway genes may also include a PCK gene that has been modified to produce a polypeptide that preferably catalyzes the conversion of PEP to OAA. Further, the 3-HP fermentation pathway genes may include an OAA dehydrogenase gene derived by modifying a 2-keto-acid dehydrogenase gene to produce a polypeptide that catalyzes the conversion of OAA to malonyl-CoA.

[0063] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through pyruvate, acetyl-CoA, malonyl-CoA, and malonate semialdehyde intermediates, wherein the malonate semialdehyde intermediate is optional (see, e.g., WO02/042418, Figure 44). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of pyruvate dehydrogenase (PDH), acetyl-CoA carboxylase (ACC), malonyl-CoA reductase, CoA acylating malonate semialdehyde dehydrogenase, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase genes.

[0064] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through pyruvate, alanine, β -alanine, β -alanyl-CoA, acrylyl-CoA, 3-HP-CoA, and malonate semialdehyde intermediates, wherein the β -alanyl-CoA, acrylyl-CoA, 3-HP-CoA, and malonate semialdehyde intermediates are optional (β -alanine can be converted to 3-HP via a malonate semialdehyde intermediate or via β -alanyl-CoA, acrylyl-CoA, and 3-HP-CoA intermediates (see, e.g., US Patent 7,309,597, Figure 1). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of alanine dehydrogenase, pyruvate/alanine aminotransferase, alanine 2,3 aminomutase, CoA transferase, CoA synthetase, β -alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, 3-hydroxyisobutyryl-CoA hydrolase, BAAT, 3-HPDH, HIBADH, and 4-hydroxybutyrate dehydrogenase genes.

[0065] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through pyruvate, lactate, lactyl-CoA, acrylyl-CoA, and 3-HP-CoA intermediates (see, e.g., WO02/042418, Figure 1). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of LDH, CoA transferase, CoA synthetase, lactyl-CoA dehydratase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, and 3-hydroxyisobutyryl-CoA hydrolase genes.

[0066] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through glycerol and 3-HPA intermediates (see, e.g., US Patent 6,852,517). In these embodiments, the yeast cells comprise a set of 3-HP fermentation pathway genes comprising one or more of glycerol dehydratase and aldehyde dehydrogenase genes.

[0067] In certain embodiments, the yeast cells provided herein have an active 3-HP fermentation pathway that proceeds through PEP or pyruvate, OAA, aspartate, β -alanine, β -alanyl-CoA, acrylyl-CoA, 3-HP-CoA, and alanine intermediates, wherein the OAA, aspartate, and alanine intermediates are optional (PEP or pyruvate can be converted to β -alanine via OAA and aspartate or via alanine) (see WO02/042418, Figure 54; US Patent 7,309,597, Figure 1). In these embodiments, the yeast cells comprise a set of 3-HP fermentation

pathway genes comprising one or more of PPC, PYC, AAT, ADC, CoA transferase, CoA synthetase, β -alanine-CoA ammonia lyase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, 3-hydroxyisobutyryl-CoA hydrolase, alanine dehydrogenase, pyruvate/alanine aminotransferase, and AAM genes. The 3-HP fermentation pathway genes may also include a PCK gene that has been modified to produce a polypeptide that preferably catalyzes the conversion of PEP to OAA.

[0068] The 3-HP fermentation pathway genes in the yeast cells provided herein may be endogenous or exogenous. "Endogenous" as used herein with regard to genetic components such as genes, promoters, and terminator sequences means that the genetic component is present at a particular location in the genome of a native form of a particular yeast cell. "Exogenous" as used herein with regard to genetic components means that the genetic component is not present at a particular location in the genome of a native form of a particular yeast cell. "Native" as used herein with regard to a yeast cell refers to a wild-type yeast cell of a particular yeast species. "Native" as used herein with regard to a metabolic pathway refers to a metabolic pathway that exists and is active in a native yeast cell.

[0069] An exogenous genetic component may have either a native or non-native sequence. An exogenous genetic component with a native sequence comprises a sequence identical to (apart from individual-to-individual mutations which do not affect function) a genetic component that is present in the genome of a native cell (i.e., the exogenous genetic component is identical to an endogenous genetic component). However, the exogenous component is present at a different location in the host cell genome than the endogenous component. For example, an exogenous PYC gene that is identical to an endogenous PYC gene may be inserted into a yeast cell, resulting in a modified cell with a non-native (increased) number of PYC gene copies. An exogenous genetic component with a non-native sequence comprises a sequence that is not found in the genome of a native cell. For example, an exogenous PYC gene from a particular species may be inserted into a yeast cell of another species. An exogenous gene is preferably integrated into the host cell genome in a functional manner, meaning that it is capable of producing an active protein in the host cell. However, in certain embodiments the exogenous gene may be introduced into the cell as part of a vector that is stably maintained in the host cytoplasm.

[0070] In certain embodiments, the yeast cells provided herein comprise one or more exogenous 3-HP fermentation pathway genes. In certain embodiments, the genetically modified yeast cells disclosed herein comprise a single exogenous gene. In other embodiments, the yeast cells comprise multiple exogenous genes. In these embodiments, the yeast cells may comprise multiple copies of a single exogenous gene and/or copies of two or more different exogenous genes. Yeast cells comprising multiple exogenous genes

may comprise any number of exogenous genes. For example, these yeast cells may comprise 1 to 20 exogenous genes, and in certain preferred embodiments they may comprise 1 to 7 exogenous genes. Multiple copies of an exogenous gene may be integrated at a single locus such that they are adjacent to one another. Alternatively, they may be integrated at several loci within the host cell's genome.

[0071] In certain embodiments, the yeast cells provided herein comprise one or more endogenous 3-HP fermentation pathway genes. In certain of these embodiments, the cells may be engineered to overexpress one or more of these endogenous genes, meaning that the modified cells express the endogenous gene at a higher level than a native cell under at least some conditions. In certain of these embodiments, the endogenous gene being overexpressed may be operatively linked to one or more exogenous regulatory elements. For example, one or more native or non-native exogenous strong promoters may be introduced into a cell such that they are operatively linked to one or more endogenous 3-HP pathway genes.

[0072] 3-HP fermentation pathway genes in the modified yeast cells provided herein may be operatively linked to one or more regulatory elements such as a promoter or terminator. As used herein, the term "promoter" refers to an untranslated sequence located upstream (i.e., 5') to the translation start codon of a gene (generally within about 1 to 1000 base pairs (bp), preferably within about 1 to 500 bp) which controls the start of transcription of the gene. The term "terminator" as used herein refers to an untranslated sequence located downstream (i.e., 3') to the translation finish codon of a gene (generally within about 1 to 1000 bp, preferably within about 1 to 500 bp, and especially within about 1 to 100 bp) which controls the end of transcription of the gene. A promoter or terminator is "operatively linked" to a gene if its position in the genome relative to that of the gene is such that the promoter or terminator, as the case may be, performs its transcriptional control function. Suitable promoters and terminators are described, for example, in WO99/14335, WO00/71738, WO02/42471, WO03/102201, WO03/102152 and WO03/049525 (all incorporated by reference herein in their entirety).

[0073] Regulatory elements linked to 3-HP fermentation pathway genes in the cells provided herein may be endogenous or exogenous. For example, an exogenous 3-HP fermentation pathway gene may be inserted into a yeast cell such that it is under the transcriptional control of an endogenous promoter and/or terminator. Alternatively, the exogenous 3-HP fermentation pathway gene may be linked to one or more exogenous regulatory elements. For example, an exogenous gene may be introduced into the cell as part of a gene expression construct that comprises one or more exogenous regulatory elements. In certain embodiments, exogenous regulatory elements, or at least the functional

portions of exogenous regulatory elements, may comprise native sequences. In other embodiments, exogenous regulatory elements may comprise non-native sequences. In these embodiments, the exogenous regulatory elements may comprise a sequence with a relatively high degree of sequence identity to a native regulatory element. For example, an exogenous gene may be linked to an exogenous promoter or terminator having at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% sequence identity to a native promoter or terminator. Sequence identity percentages for nucleotide or amino acid sequences can be calculated by methods known in the art, such as for example using BLAST (National Center for Biological Information (NCBI) Basic Local Alignment Search Tool) version 2.2.1 software with default parameters. For example, a sequences having an identity score of at least 90%, using the BLAST version 2.2.1 algorithm with default parameters is considered to have at least 90% sequence identity. The BLAST software is available from the NCBI, Bethesda, Maryland.

[0074] In certain aspects, a regulatory element (e.g., a promoter) linked to a 3-HP fermentation pathway gene in the cells provided herein may be foreign to the pathway gene. A regulatory element that is foreign to a pathway gene is a regulatory element that is not linked to the gene in its natural form. The skilled artisan can appreciate that a regulatory element foreign to a pathway gene can be endogenous or exogenous, depending on the pathway gene and its relation to the yeast cell. In some instances, an endogenous 3-HP fermentation pathway gene is operatively linked to a regulatory element (e.g., a promoter) that is foreign to the pathway gene. In other instances, an exogenous 3-HP fermentation pathway gene is operatively linked to an exogenous regulatory element (e.g., a promoter) that is foreign to the pathway gene.

[0075] In those embodiments wherein multiple exogenous genes are inserted into a host cell, each exogenous gene may be under the control of a different regulatory element, or two or more exogenous genes may be under the control of the same regulatory elements. For example, where a first exogenous gene is linked to a first regulatory element, a second exogenous gene may also be linked to the first regulatory element, or it may be linked to a second regulatory element. The first and second regulatory elements may be identical or share a high degree of sequence identity, or they be wholly unrelated.

[0076] Examples of promoters that may be linked to one or more 3-HP fermentation pathway genes in the yeast cells provided herein include, but are not limited to, promoters for PDC1, phosphoglycerate kinase (PGK), xylose reductase (XR), xylitol dehydrogenase (XDH), L-(+)-lactate-cytochrome c oxidoreductase (CYB2), translation elongation factor-1 (TEF1), translation elongation factor-2 (TEF2), enolase (ENO1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and orotidine 5'-phosphate decarboxylase (URA3)

genes. In these examples, the 3-HP fermentation pathway genes may be linked to endogenous or exogenous promoters for PDC1, PGK, XR, XDH, CYB2, TEF1, TEF2, ENO1, GAPDH, or URA3 genes. Where the promoters are exogenous, they may be identical to or share a high degree of sequence identity (i.e., at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%) with native promoters for PDC1, PGK, XR, XDH, CYB2, TEF1, TEF2, ENO1, GAPDH, or URA3 genes.

[0077] Examples of terminators that may be linked to one or more 3-HP fermentation pathway genes in the yeast cells provided herein include, but are not limited to, terminators for PDC1, XR, XDH, transaldolase (TAL), transketolase (TKL), ribose 5-phosphate ketol-isomerase (RKI), CYB2, or iso-2-cytochrome c (CYC) genes or the galactose family of genes (especially the GAL10 terminator). In these examples, the 3-HP fermentation pathway genes may be linked to endogenous or exogenous terminators for PDC1, XR, XDH, TAL, TKL, RKI, CYB2, or CYC genes or galactose family genes. Where the terminators are exogenous, they may be identical to or share a high degree of sequence identity (i.e., at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99%) with native terminators for PDC1, XR, XDH, TAL, TKL, RKI, CYB2, or CYC genes or galactose family genes. In certain embodiments, 3-HP fermentation pathway genes are linked to a terminator that comprises a functional portion of a native GAL10 gene native to the host cell or a sequence that shares at least 80%, at least 85%, at least 90%, or at least 95% sequence identity with a native GAL10 terminator.

[0078] Exogenous genes may be inserted into a yeast host cell via any method known in the art. In preferred embodiments, the genes are integrated into the host cell genome. Exogenous genes may be integrated into the genome in a targeted or a random manner. In those embodiments where the gene is integrated in a targeted manner, it may be integrated into the loci for a particular gene, such that integration of the exogenous gene is coupled to deletion or disruption of a native gene. For example, introduction of an exogenous 3-HP pathway gene may be coupled to deletion or disruption of one or more genes encoding enzymes involved in other fermentation product pathways. Alternatively, the exogenous gene may be integrated into a portion of the genome that does not correspond to a gene.

[0079] Targeted integration and/or deletion may utilize an integration construct. The term "construct" as used herein refers to a DNA sequence that is used to transform a host cell. The construct may be, for example, a circular plasmid or vector, a portion of a circular plasmid or vector (such as a restriction enzyme digestion product), a linearized plasmid or vector, or a PCR product prepared using a plasmid or genomic DNA as a template. Methods for transforming a yeast cell with an exogenous construct are described in, for example, WO99/14335, WO00/71738, WO02/42471, WO03/102201, WO03/102152, and

WO03/049525. An integration construct can be assembled using two cloned target DNA sequences from an insertion site target. The two target DNA sequences may be contiguous or non-contiguous in the native host genome. In this context, "non-contiguous" means that the DNA sequences are not immediately adjacent to one another in the native genome, but are instead are separated by a region that is to be deleted. "Contiguous" sequences as used herein are directly adjacent to one another in the native genome. Where targeted integration is to be coupled to deletion or disruption of a target gene, the integration construct may also be referred to as a deletion construct. In a deletion construct, one of the target sequences may include a region 5' to the promoter of the target gene, all or a portion of the promoter region, all or a portion of the target gene coding sequence, or some combination thereof. The other target sequence may include a region 3' to the terminator of the target gene, all or a portion of the terminator region, and/or all or a portion of the target gene coding sequence. Where targeted integration is not to be coupled to deletion or disruption of a native gene, the target sequences are selected such that insertion of an intervening sequence will not disrupt native gene expression. An integration or deletion construct is prepared such that the two target sequences are oriented in the same direction in relation to one another as they natively appear in the genome of the host cell. Where an integration or deletion construct is used to introduce an exogenous gene into a host cell, a gene expression cassette is cloned into the construct between the two target gene sequences to allow for expression of the exogenous gene. The gene expression cassette contains the exogenous gene, and may further include one or more regulatory sequences such as promoters or terminators operatively linked to the exogenous gene. Deletion constructs can also be constructed that do not contain a gene expression cassette. Such constructs are designed to delete or disrupt a gene sequence without the insertion of an exogenous gene.

[0080] An integration or deletion construct may comprise one or more selection marker cassettes cloned into the construct between the two target gene sequences. The selection marker cassette contains at least one selection marker gene that allows for selection of transformants. A "selection marker gene" is a gene that encodes a protein needed for the survival and/or growth of the transformed cell in a selective culture medium, and therefore can be used to apply selection pressure to the cell. Successful transformants will contain the selection marker gene, which imparts to the successfully transformed cell at least one characteristic that provides a basis for selection. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins (e.g., resistance to bleomycin or zeomycin (e.g., *Streptoalloteichus hindustanus* ble gene), aminoglycosides such as G418 or kanamycin (e.g., kanamycin resistance gene from transposon Tn903), or hygromycin (e.g., aminoglycoside antibiotic resistance gene from *E. coli*)), (b) complement auxotrophic deficiencies of the cell (e.g., deficiencies in leucine (e.g., *K. marxianus* LEU2 gene), uracil

(e.g., *K. marxianus*, *S. cerevisiae*, or *I. orientalis* URA3 gene), or tryptophan (e.g., *K. marxianus*, *S. cerevisiae*, or *I. orientalis* TRP gene)), (c) enable the cell to synthesize critical nutrients not available from simple media, or (d) confer the ability for the cell to grow on a particular carbon source (e.g., MEL5 gene from *S. cerevisiae*, which encodes the alpha-galactosidase (melibiase) enzyme and confers the ability to grow on melibiose as the sole carbon source). Preferred selection markers include the URA3 gene, zeocin resistance gene, G418 resistance gene, MEL5 gene, and hygromycin resistance gene. Another preferred selection marker is an L-lactate:ferricytochrome c oxidoreductase (CYB2) gene cassette, provided that the host cell either natively lacks such a gene or that its native CYB2 gene(s) are first deleted or disrupted. A selection marker gene is operatively linked to one or more promoter and/or terminator sequences that are operable in the host cell. In certain embodiments, these promoter and/or terminator sequences are exogenous promoter and/or terminator sequences that are included in the selection marker cassette. Suitable promoters and terminators are as described herein.

[0081] An integration or deletion construct is used to transform the host cell. Transformation may be accomplished using, for example, electroporation and/or chemical transformation (e.g., calcium chloride, lithium acetate-based, etc.) methods. Selection or screening based on the presence or absence of the selection marker may be performed to identify successful transformants. In successful transformants, homologous recombination events at the locus of the target site results in the disruption or the deletion of the target site sequence. Where the construct targets a native gene for deletion or disruption, all or a portion of the native target gene, its promoter, and/or its terminator may be deleted during this recombination event. The expression cassette, selection marker cassette, and any other genetic material between the target sequences in the integration construct is inserted into the host genome at the locus corresponding to the target sequences. Analysis by PCR or Southern analysis can be performed to confirm that the desired insertion/deletion has taken place.

[0082] In some embodiments, cell transformation may be performed using DNA from two or more constructs, PCR products, or a combination thereof, rather than a single construct or PCR product. In these embodiments, the 3' end of one integration fragment overlaps with the 5' end of another integration fragment. In one example, one construct will contain the first sequence from the locus of the target sequence and a non-functional part of the marker gene cassette, while the other will contain the second sequence from the locus of the target sequence and a second non-functional part of the marker gene cassette. The parts of the marker gene cassette are selected such that they can be combined to form a complete cassette. The cell is transformed with these pieces simultaneously, resulting in the formation of a complete, functional marker or structural gene cassette. Successful transformants can

be selected for on the basis of the characteristic imparted by the selection marker. In another example, the selection marker resides on one fragment but the target sequences are on separate fragments, so that the integration fragments have a high probability of integrating at the site of interest. In other embodiments, transformation from three linear DNAs can be used to integrate exogenous genetic material. In these embodiments, one fragment overlaps on the 5' end with a second fragment and on the 3' end with a third fragment.

[0083] An integration or deletion construct may be designed such that the selection marker gene and some or all of its regulatory elements can become spontaneously deleted as a result of a subsequent homologous recombination event. A convenient way of accomplishing this is to design the construct such that the selection marker gene and/or regulatory elements are flanked by repeat sequences. Repeat sequences are identical DNA sequences, native or non-native to the host cell, and oriented on the construct in the same or opposite direction with respect to one another. The repeat sequences are advantageously about 50 to 1500 bp in length, and do not have to encode for anything. Inclusion of the repeat sequences permits a homologous recombination event to occur, which results in deletion of the selection marker gene and one of the repeat sequences. Since homologous recombination occurs with relatively low frequency, it may be necessary to grow transformants for several rounds on nonselective media to allow for the spontaneous homologous recombination to occur in some of the cells. Cells in which the selection marker gene has become spontaneously deleted can be selected or screened on the basis of their loss of the selection characteristic imparted by the selection marker gene. In certain cases, expression of a recombinase enzyme may enhance recombination between the repeated sites.

[0084] An exogenous 3-HP fermentation pathway gene in the modified yeast cells provided herein may be derived from a source gene from any suitable source. For example, an exogenous gene may be derived from a yeast, fungal, bacterial, plant, insect, or mammalian source. As used herein, an exogenous gene that is "derived from" a native source gene encodes a polypeptide that 1) is identical to a polypeptide encoded by the native gene, 2) shares at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity with a polypeptide encoded by the native gene, and/or 3) has the same function in a 3-HP fermentation pathway as the polypeptide encoded by the native gene. For example, a PYC gene that is derived from a *I. orientalis* PYC gene may encode a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, a polypeptide with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 2, and/or a polypeptide that has the ability to catalyze the conversion of pyruvate to OAA. A gene derived from a

native gene may comprise a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the coding region of the native gene. In certain embodiments, a gene derived from a native gene may comprise a nucleotide sequence that is identical to the coding region of the source gene. For example, a PYC gene that is derived from a *I. orientalis* PYC gene may comprise the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence of SEQ ID NO: 1.

[0085] In certain embodiments of the modified yeast cells provided herein, the native source gene from which the exogenous 3-HP fermentation pathway gene is derived produces a polypeptide that is involved in a 3-HP fermentation pathway. In other embodiments, however, the native source gene may encode a polypeptide that is not involved in a 3-HP fermentation pathway or that catalyzes a reverse reaction in a 3-HP fermentation pathway. In these embodiments, the exogenous 3-HP pathway gene will have undergone one or more targeted or random mutations versus the native source gene that result in modified activity and/or substrate preference. For example, a native source gene may be mutated to generate a gene that encodes a polypeptide with increased activity in a desired reaction direction and/or decreased activity in a non-desired direction in a 3-HP fermentation pathway. For example, where the native source gene encodes a polypeptide capable of catalyzing both a forward and reverse reactions in a 3-HP fermentation pathway, the gene may be modified such that the resultant exogenous gene has increased activity in the forward direction and decreased activity in the reverse direction. Similarly, a native source gene may be mutated to produce a gene that encodes a polypeptide with different substrate preference than the native polypeptide. For example, a 3-HP pathway gene may be mutated to produce a polypeptide with the ability to act on a substrate that is either not preferred or not acted on at all by the native polypeptide. In these embodiments, the polypeptide encoded by the exogenous 3-HP pathway gene may catalyze a reaction that the polypeptide encoded by the native source gene is completely incapable of catalyzing. A native source gene may also be mutated such that the resultant 3-HP pathway gene exhibits decreased feedback inhibition at the DNA, RNA, or protein level in the presence of one or more downstream 3-HP pathway intermediates or side products.

[0086] In certain embodiments of the modified yeast cells provided herein, an exogenous 3-HP pathway gene may be derived from the host yeast species. For example, where the host cell is *I. orientalis*, an exogenous gene may be derived from an *I. orientalis* gene. In these embodiments, the exogenous gene may comprise a nucleotide sequence identical to the coding region of the native gene, such that incorporation of the exogenous gene into the

host cell increases the copy number of a native gene sequence and/or changes the regulation or expression level of the gene if under the control of a promoter that is different from the promoter that drives expression of the gene in a wild-type cell. In other embodiments, the exogenous 3-HP pathway gene may comprise a nucleotide sequence that differs from the coding region of a native 3-HP pathway gene, but nonetheless encodes a polypeptide that is identical to the polypeptide encoded by the native 3-HP pathway gene. In still other embodiments, the exogenous 3-HP pathway gene may comprise a nucleotide sequence that encodes a polypeptide with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to a polypeptide encoded by one or more native 3-HP pathway genes. In certain of these embodiments, the exogenous gene comprises a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to one or more native genes. In still other embodiments, the exogenous 3-HP gene may encode a polypeptide that has less than 50% sequence identity to a polypeptide encoded by a native 3-HP pathway gene but which nonetheless has the same function as the native polypeptide in a 3-HP fermentation pathway (i.e., the ability to catalyze the same reaction). A native source gene may be subjected to mutagenesis if necessary to provide a coding sequence starting with the usual eukaryotic starting codon (ATG), or for other purposes.

[0087] In other embodiments, the exogenous 3-HP pathway gene may be derived from a species that is different than that of the host yeast cell. In certain of these embodiments, the exogenous 3-HP pathway gene may be derived from a different yeast species than the host cell. For example, where the host cell is *I. orientalis*, the exogenous gene may be derived from *S. cerevisiae*. In other embodiments, the exogenous 3-HP pathway gene may be derived from a fungal, bacterial, plant, insect, or mammalian source. For example, where the host cell is *I. orientalis*, the exogenous gene may be derived from a bacterial source such as *E. coli*. In those embodiments where the exogenous 3-HP pathway gene is derived from a non-yeast source, the exogenous gene sequence may be codon-optimized for expression in a yeast host cell.

[0088] In those embodiments where the exogenous 3-HP pathway gene is derived from a species other than the host cell species, the exogenous gene may encode a polypeptide identical to a polypeptide encoded by a native 3-HP pathway gene from the source organism. In certain of these embodiments, the exogenous 3-HP pathway gene may be identical to a native 3-HP pathway gene from the source organism. In other embodiments, the exogenous gene may share at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to a native 3-HP pathway gene from the source organism. In other embodiments, the exogenous

3-HP pathway gene may encode a polypeptide that shares at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity with a polypeptide encoded by a native 3-HP pathway gene from the source organism. In certain of these embodiments, the exogenous gene may comprise a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to one or more native 3-HP pathway genes from the source organism. In still other embodiments, the exogenous 3-HP gene may encode a polypeptide that has less than 50% sequence identity to a polypeptide encoded by a native 3-HP pathway gene from the source organism, but which nonetheless has the same function as the native polypeptide from the source organism in a 3-HP fermentation pathway.

[0089] In certain embodiments, the yeast cells provided herein express one or more 3-HP pathway genes encoding enzymes selected from the group consisting of ACC (catalyzes the conversion of acetyl-CoA to malonyl-CoA), alanine 2,3 aminomutase (AAM, catalyzes the conversion of alanine to β -alanine), alanine dehydrogenase (catalyzes the conversion of pyruvate to alanine), aldehyde dehydrogenase (catalyzes the conversion of 3-HPA to 3-HP), KGD (catalyzes the conversion of OAA to malonate semialdehyde), AAT (catalyzes the conversion of OAA to aspartate), ADC (catalyzes the conversion of aspartate to β -alanine), BCKA (catalyzes the conversion of OAA to malonate semialdehyde), BAAT (catalyzes the conversion of β -alanine to malonate semialdehyde), 4-aminobutyrate aminotransferase (gabT, catalyzes the conversion of β -alanine to malonate semialdehyde), β -alanyl-CoA ammonia lyase (catalyzes the conversion of β -alanyl-CoA to acrylyl-CoA), Co-A acylating malonate semialdehyde dehydrogenase (catalyzes the conversion of malonyl-CoA to malonate semialdehyde), CoA synthetase (catalyzes the conversion of β -alanine to β -alanyl-CoA or the conversion of lactate to lactyl-CoA), CoA transferase (catalyzes the conversion of β -alanine to β -alanyl-CoA and/or the conversion of lactate to lactyl-CoA), glycerol dehydratase (catalyzes the conversion of glycerol to 3-HPA), IPDA (catalyzes the conversion of OAA to malonate semialdehyde), LDH (catalyzes the conversion of pyruvate to lactate), lactyl-CoA dehydratase (catalyzes the conversion of lactyl-CoA to acrylyl-CoA), malate decarboxylase (catalyzes the conversion of malate to 3-HP), malate dehydrogenase (catalyzes the conversion of OAA to malate), malonyl-CoA reductase (catalyzes the conversion of malonyl-CoA to malonate semialdehyde or 3-HP), OAA formatelyase (also known as pyruvate-formate lyase and ketoacid formate-lyase, catalyzes the conversion of OAA to malonyl-CoA), OAA dehydrogenase (catalyzes the conversion of OAA to malonyl CoA); PPC (catalyzes the conversion of PEP to OAA), pyruvate/alanine aminotransferase (catalyzes the conversion of pyruvate to alanine), PYC (catalyzes the conversion of pyruvate to OAA), PDH (catalyzes the conversion of pyruvate to acetyl-CoA), 2-keto acid

decarboxylase (catalyzes the conversion of OAA to malonate semialdehyde), 3-HP-CoA dehydratase (also known as acrylyl-CoA hydratase, catalyzes the conversion of acrylyl-CoA to 3-HP-CoA), 3-HPDH (catalyzes the conversion of malonate semialdehyde to 3-HP), 3-HP-CoA hydrolase (catalyzes the conversion of 3-HP-CoA to 3-HP), HIBADH (catalyzes the conversion of malonate semialdehyde to 3-HP), 3-hydroxyisobutyryl-CoA hydrolase (catalyzes the conversion of 3-HP-CoA to 3-HP), and 4-hydroxybutyrate dehydrogenase (catalyzes the conversion of malonate semialdehyde to 3-HP). For each of these enzyme activities, the reaction of interest in parentheses may be a result of native or non-native activity.

[0090] A "pyruvate carboxylase gene" or "PYC gene" as used herein refers to any gene that encodes a polypeptide with pyruvate carboxylase activity, meaning the ability to catalyze the conversion of pyruvate, CO₂, and ATP to OAA, ADP, and phosphate. In certain embodiments, a PYC gene may be derived from a yeast source. For example, the PYC gene may be derived from an *I. orientalis* PYC gene encoding the amino acid sequence set forth in SEQ ID NO: 2. In other embodiments, the gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 2. In certain embodiments, an *I. orientalis* -derived PYC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 1 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 1. In other embodiments, the PYC gene may be derived from a bacterial source. For example, the PYC gene may be derived from one of the few bacterial species that use only PYC and not PPC (see below) for anaplerosis, such as *R. sphaeroides*, or from a bacterial species that possesses both PYC and PPC, such as *R. etli*. The amino acid sequences encoded by the PYC genes of *R. sphaeroides* and *R. etli* are set forth in SEQ ID NOs: 3 and 4, respectively. A PYC gene may be derived from a gene encoding the amino acid sequence of SEQ ID NOs: 3 or 4, or from a gene encoding an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 4. Alternatively, the PYC gene may be derived from a PYC gene encoding an enzyme that does not have a dependence on acetyl-CoA for activation, such as a *P. fluorescens* PYC gene encoding the amino acid sequence set forth in SEQ ID NO: 5 (carboxytransferase subunit) or SEQ ID NO: 6 (biotin carboxylase subunit), a *C. glutamicum* PYC gene of encoding the amino acid sequence set forth in SEQ ID NO: 7, or a gene encoding an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid

sequence of SEQ ID NOs: 5, 6, or 7. A PYC gene may also be derived from a PYC gene that encodes an enzyme that is not inhibited by aspartate, such as an *S. meliloti* PYC gene encoding the amino acid sequence set forth in SEQ ID NO: 8 (Sauer FEMS Microbiol Rev 29:765 (2005), or from a gene encoding an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 8.

[0091] A "PEP carboxylase gene" or "PPC gene" as used herein refers to any gene that encodes a polypeptide with PEP carboxylase activity, meaning the ability to catalyze the conversion of PEP and CO₂ to OAA and phosphate. In certain embodiments, a PPC gene may be derived from a bacterial PPC gene. For example, the PPC gene may be derived from an *E. coli* PPC gene encoding the amino acid sequence set forth in SEQ ID NO: 10 or an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 10. In certain embodiments, an *E. coli*-derived PPC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 9 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 9. In other embodiments, a PPC gene may be derived from an "A" type PPC, found in many archaea and a limited number of bacteria, that is not activated by acetyl CoA and is less inhibited by aspartate. For example, a PPC gene may be derived from an *M. thermoautotrophicum* PPC A gene encoding the amino acid sequence set forth in SEQ ID NO: 11, a *C. perfringens* PPC A gene encoding the amino acid sequence set forth in SEQ ID NO: 12, or a gene encoding an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NOs: 11 or 12. In certain of these embodiments, the gene may have undergone one or more mutations versus the native gene in order to generate an enzyme with improved characteristics. For example, the gene may have been mutated to encode a PPC polypeptide with increased resistance to aspartate feedback versus the native polypeptide. In other embodiments, the PPC gene may be derived from a plant source.

[0092] An "aspartate aminotransferase gene" or "AAT gene" as used herein refers to any gene that encodes a polypeptide with aspartate aminotransferase activity, meaning the ability to catalyze the conversion of OAA to aspartate. Enzymes having aspartate aminotransferase activity are classified as EC 2.6.1.1. In certain embodiments, an AAT gene may be derived from a yeast source such as *I. orientalis* or *S. cerevisiae*. For example, the AAT gene may be derived from an *I. orientalis* AAT gene encoding the amino acid sequence set forth in SEQ ID NO: 14 or an *S. cerevisiae* AAT2 gene encoding the amino acid

sequence set forth in SEQ ID NO: 15. In other embodiments, the gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NOs: 14 or 15. In certain embodiments, an *I. orientalis*-derived AAT gene may comprise the nucleotide sequence set forth in SEQ ID NO: 13 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 13. In other embodiments, the AAT gene may be derived from a bacterial source. For example, the AAT gene may be derived from an *E. coli* aspC gene encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 16. In other embodiments, the gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 16.

[0093] An "aspartate decarboxylase gene" or "ADC gene" as used herein refers to any gene that encodes a polypeptide with aspartate decarboxylase activity, meaning the ability to catalyze the conversion of aspartate to β -alanine. Enzymes having aspartate decarboxylase activity are classified as EC 4.1.1.11. In certain embodiments, an ADC gene may be derived from a bacterial source. Because an active aspartate decarboxylase may require proteolytic processing of an inactive proenzyme, in these embodiments the yeast host cell should be selected to support formation of an active enzyme coded by a bacterial ADC gene.

[0094] In some embodiments, the ADC gene may be derived from an *S. avermitilis* panD gene encoding the amino acid sequence set forth in SEQ ID NO: 17. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 17. In certain embodiments, an *S. avermitilis*-derived ADC gene may comprise the nucleotide sequence set forth in any one of SEQ ID NOs: 130, 145, 146, or 147; or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in any one of SEQ ID NOs: 130, 145, 146, or 147.

[0095] In other embodiments, the ADC gene may be derived from a *C. acetobutylicum* panD gene encoding the amino acid sequence set forth in SEQ ID NO: 18. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 18. In certain

embodiments, a *C. acetobutylicum*-derived ADC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 131, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 131.

[0096] In other embodiments, the ADC gene may be derived from a *H. pylori* ADC gene encoding the amino acid sequence set forth in SEQ ID NO: 133. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 133. In certain embodiments, a *H. pylori*-derived ADC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 133, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 133.

[0097] In other embodiments, the ADC gene may be derived from a *Bacillus sp. TS25* ADC gene encoding the amino acid sequence set forth in SEQ ID NO: 135. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 135. In certain embodiments, a *Bacillus sp. TS25*-derived ADC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 134, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 134.

[0098] In other embodiments, the ADC gene may be derived from a *C. glutamicum* ADC gene encoding the amino acid sequence set forth in SEQ ID NO: 137. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 137. In certain embodiments, a *C. glutamicum*-derived ADC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 136, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 136.

[0099] In other embodiments, the ADC gene may be derived from a *B. licheniformis* ADC gene encoding the amino acid sequence set forth in SEQ ID NO: 139. In some embodiments, the ADC gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 139. In certain

embodiments, a *B. licheniformis*-derived ADC gene may comprise the nucleotide sequence set forth in any one of SEQ ID NOs: 138, 148, 149, 150, or 151; or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in any one of SEQ ID NOs: 138, 148, 149, 150, or 151.

[00100] A " β -alanine aminotransferase gene" or "BAAT gene" as used herein refers to any gene that encodes a polypeptide with β -alanine aminotransferase activity, meaning the ability to catalyze the conversion of β -alanine to malonate semialdehyde. Enzymes having β -alanine aminotransferase activity are classified as EC 2.6.1.19. In certain embodiments, a BAAT gene may be derived from a yeast source. For example, a BAAT gene may be derived from the *I. orientalis* homolog to the *pyd4* gene encoding the amino acid sequence set forth in SEQ ID NO: 20. In some embodiments, the BAAT gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 20. In certain embodiments, an *I. orientalis*-derived BAAT gene may comprise the nucleotide sequence set forth in SEQ ID NO: 19 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 19. In other embodiments, the BAAT gene may be derived from the *S. kluyveri* *pyd4* gene encoding the amino acid sequence set forth in SEQ ID NO: 21. In some embodiments, the BAAT gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 21. In certain embodiments, a *S. kluyveri*-derived BAAT gene may comprise the nucleotide sequence set forth in SEQ ID NO: 142 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 142. In other embodiments, the BAAT gene may be derived from a bacterial source. For example, a BAAT gene may be derived from an *S. avermitilis* BAAT gene encoding the amino acid sequence set forth in SEQ ID NO: 22. In some embodiments, the BAAT gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 22. In certain embodiments, a *S. avermitilis*-derived BAAT gene may comprise the nucleotide sequence set forth in SEQ ID NO: 140 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 140.

[00101] A BAAT gene may also be a "4-aminobutyrate aminotransferase" or "gabT gene" meaning that it has native activity on 4-aminobutyrate as well as β -alanine. Alternatively, a BAAT gene may be derived by random or directed engineering of a native gabT gene from a bacterial or yeast source to encode a polypeptide with BAAT activity. For example, a BAAT gene may be derived from the *S. avermitilis* gabT encoding the amino acid sequence set forth in SEQ ID NO: 23. In some embodiments, the *S. avermitilis*-derived BAAT gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 23. In other embodiments, a BAAT gene may be derived from the *S. cerevisiae* gabT gene UGA1 encoding the amino acid sequence set forth in SEQ ID NO: 24. In some embodiments, the *S. cerevisiae*-derived BAAT gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 24. In certain embodiments, an *S. cerevisiae*-derived BAAT gene may comprise the nucleotide sequence set forth in SEQ ID NO: 141 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 141.

[00102] A "3-HP dehydrogenase gene" or "3-HPDH gene" as used herein refers to any gene that encodes a polypeptide with 3-HP dehydrogenase activity, meaning the ability to catalyze the conversion of malonate semialdehyde to 3-HP. Enzymes having 3-HP dehydrogenase activity are classified as EC 1.1.1.59 if they utilize an NAD(H) cofactor, and as EC 1.1.1.298 if they utilize an NADP(H) cofactor. Enzymes classified as EC 1.1.1.298 are alternatively referred to as malonate semialdehyde reductases.

[00103] In certain embodiments, a 3-HPDH gene may be derived from a yeast source. For example, a 3-HPDH gene may be derived from the *I. orientalis* homolog to the YMR226C gene encoding the amino acid sequence set forth in SEQ ID NO: 26. In some embodiments, the 3-HPDH gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 26. In certain embodiments, an *I. orientalis*-derived 3-HPDH gene may comprise the nucleotide sequence set forth in SEQ ID NO: 25 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 25. In other embodiments, a 3-HPDH gene may be derived from the *S. cerevisiae* YMR226C gene encoding the amino acid sequence set forth in SEQ ID NO: 129. In some embodiments, the

3-HPDH gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 129. In certain embodiments, an *S. cerevisiae*-derived 3-HPDH gene may comprise the nucleotide sequence set forth in SEQ ID NO: 144 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 144.

[00104] In other embodiments, the 3-HPDH gene may be derived from a bacterial source. For example, a 3-HPDH gene may be derived from an *E. coli* ydfG gene encoding the amino acid sequence in SEQ ID NO: 27. In some embodiments, the gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 27. In certain embodiments, an *E. coli*-derived 3-HPDH gene may comprise the nucleotide sequence set forth in SEQ ID NO: 143 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 143. In other embodiments, a 3-HPDH gene may be derived from an *M. sedula* malonate semialdehyde reductase gene encoding the amino acid sequence set forth in SEQ ID NO: 29. In some embodiments, the 3-HPDH gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 29. In certain embodiments, an *M. sedula*-derived 3-HPDH gene may comprise the nucleotide sequence set forth in SEQ ID NO: 343 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 343.

[00105] A "3-hydroxyisobutyrate dehydrogenase gene" or "HIBADH gene" as used herein refers to any gene that encodes a polypeptide with 3-hydroxyisobutyrate dehydrogenase activity, meaning the ability to catalyze the conversion of 3-hydroxyisobutyrate to methylmalonate semialdehyde. Enzymes having 3-hydroxyisobutyrate dehydrogenase activity are classified as EC 1.1.1.31. Some 3-hydroxyisobutyrate dehydrogenases also have 3-HPDH activity. In certain embodiments, an HIBADH gene may be derived from a bacterial source. For example, an HIBADH gene may be derived from an *A. faecalis* M3A gene encoding the amino acid sequence set forth in SEQ ID NO: 28, a *P. putida* KT2440 or E23440 mmsB gene encoding the amino acid sequence set forth in SEQ ID NO: 30 or SEQ ID NO: 31, respectively, or a *P. aeruginosa* PAO1 mmsB gene encoding the amino acid sequence set forth in SEQ ID NO: 32. In certain embodiments, an HIBADH gene may

encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence set forth in SEQ ID NOs: 28, 30, 31, or 32.

[00106] A "4-hydroxybutyrate dehydrogenase gene" as used herein refers to any gene that encodes a polypeptide with 4-hydroxybutyrate dehydrogenase activity, meaning the ability to catalyze the conversion of 4-hydroxybutanoate to succinate semialdehyde. Enzymes having 4-hydroxybutyrate dehydrogenase activity are classified as EC 1.1.1.61. Some 4-hydroxybutyrate dehydrogenases also have 3-HPDH activity. In certain embodiments, a 4-hydroxybutyrate dehydrogenase gene may be derived from a bacterial source. For example, a 4-hydroxybutyrate dehydrogenase gene may be derived from a *R. eutropha* H16 hbd gene encoding the amino acid sequence set forth in SEQ ID NO: 33 or a *C. kluyveri* DSM 555 hbd gene encoding the amino acid sequence set forth in SEQ ID NO: 34. In other embodiments, the gene may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence set forth in SEQ ID NOs: 33 or 34.

[00107] A "PEP carboxykinase gene" or "PCK gene" as used herein refers to any gene that encodes a polypeptide with PEP carboxykinase activity, meaning the ability to catalyze the conversion of PEP, CO₂, and ADP or GDP to OAA and ATP or GTP, or vice versa. Enzymes having PEP carboxykinase activity are classified as EC 4.1.1.32 (GTP/GDP utilizing) and EC 4.1.1.49 (ATP/ADP utilizing). In certain embodiments, a PCK gene may be derived from a yeast source. In other embodiments, a PCK gene may be derived from a bacterial source, and in certain of these embodiments the gene may be derived from a bacteria in which the PCK reaction favors the production of OAA rather than the more common form of the reaction where decarboxylation is dominant. For example, a PCK gene may be derived from an *M. succiniciproducens* PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 35, an *A. succiniciproducens* PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 36, an *A. succinogenes* PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 37, or an *R. eutropha* PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 38. In other embodiments, a PCK gene has undergone one or more mutations versus the native gene from which it was derived, such that the resultant gene encodes a polypeptide that preferably catalyzes the conversion of PEP to OAA. For example, a PCK gene may be derived from an *E. coli* K12 strain PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 39, where the gene has been mutated to preferably catalyze the conversion of PEP to OAA. In other embodiments the conversion of PEP to OAA is catalyzed by a PEP carboxytransphosphorylase such as is found in propionic acid bacteria (e.g., *P. shermanii*, *A. woodii*) which use inorganic phosphate and diphosphate rather than ATP/ADP or GTP/GDP.

[00108] A "malate dehydrogenase gene" as used herein refers to any gene that encodes a polypeptide with malate dehydrogenase activity, meaning the ability to catalyze the conversion of OAA to malate. In certain embodiments, a malate dehydrogenase gene may be derived from a bacterial or yeast source.

[00109] A "malate decarboxylase gene" as used herein refers to any gene that encodes a polypeptide with malate decarboxylase activity, meaning the ability to catalyze the conversion of malate to 3-HP. Malate decarboxylase activity is not known to occur naturally. Therefore, a malate decarboxylase gene may be derived by incorporating one or more mutations into a native source gene that encodes a polypeptide with acetolactate decarboxylase activity. Polypeptides with acetolactate decarboxylase activity catalyze the conversion of 2-hydroxy-2-methyl-3-oxobutanoate to 2-acetoin, and are classified as EC 4.1.1.5. In certain embodiments, a malate decarboxylase gene may be derived from a bacterial source. For example, a malate decarboxylase gene may be derived from an *L. lactis* aldB gene encoding the amino acid sequence set forth in SEQ ID NO: 40, an *S. thermophilus* aldB gene encoding the amino acid sequence set forth in SEQ ID NO: 41, a *B. brevis* aldB gene encoding the amino acid sequence set forth in SEQ ID NO: 42, or a *E. aerogenes* budA gene encoding the amino acid sequence set forth in SEQ ID NO: 43.

[00110] An "alpha-ketoglutarate (AKG) decarboxylase gene" or "KGD gene" as used herein refers to any gene that encodes a polypeptide with alpha-ketoglutarate decarboxylase activity, meaning the ability to catalyze the conversion of alpha-ketoglutarate (2-oxoglutarate) to succinate semialdehyde. Enzymes having AKG decarboxylase activity are classified as EC 4.1.1.71. A KGD gene may be used to derive a gene encoding a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde. This activity may be found in a native KGD gene, or it may be derived by incorporating one or more mutations into a native KGD gene. In certain embodiments, a KGD gene may be derived from a bacterial source. For example, a KGD gene may be derived from a *M. tuberculosis* KGD gene encoding the amino acid sequence set forth in SEQ ID NO: 44, a *B. japonicum* KGD gene encoding the amino acid sequence set forth in SEQ ID NO: 45, or a *M. loti* (aka *Rhizobium loti*) KGD gene encoding the amino acid sequence set forth in SEQ ID NO: 46.

[00111] A "branched-chain alpha-keto acid decarboxylase gene" or "BCKA gene" as used herein refers to any gene that encodes a polypeptide with branched-chain alpha-keto acid decarboxylase activity, which can serve to decarboxylate a range of alpha-keto acids from three to six carbons in length. Enzymes having BCKA activity are classified as EC 4.1.1.72. A BCKA gene may be used to derive a gene encoding a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde. This activity may be found in a native BCKA gene, or it may be derived by incorporating one or more mutations into a native BCKA

gene. In certain embodiments, a BCKA gene may be derived from a bacterial source. For example, a BCKA gene may be derived from a *L. lactis* kdcA gene encoding the amino acid sequence set forth in SEQ ID NO: 47.

[00112] An "indolepyruvate decarboxylase gene" or "IPDA gene" as used herein refers to any gene that encodes a polypeptide with indolepyruvate decarboxylase activity, meaning the ability to catalyze the conversion of indolepyruvate to indoleacetaldehyde. Enzymes having IPDA activity are classified as EC 4.1.1.74. An IPDA gene may be used to derive a gene encoding a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde. This activity may be found in a native IPDA gene, or it may be derived by incorporating one or more mutations into a native IPDA gene. In certain embodiments, an indolepyruvate decarboxylase gene may be derived from a yeast, bacterial, or plant source.

[00113] A "pyruvate decarboxylase gene" or "PDC gene" as used herein refers to any gene that encodes a polypeptide with pyruvate decarboxylase activity, meaning the ability to catalyze the conversion of pyruvate to acetaldehyde. Enzymes having PDC activity are classified as EC 4.1.1.1. In preferred embodiments, a PDC gene that is incorporated into a modified yeast cell as provided herein has undergone one or more mutations versus the native gene from which it was derived such that the resultant gene encodes a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde. In certain embodiments, a PDC gene may be derived from a yeast source. For example, a PDC gene may be derived from an *I. orientalis* PDC gene encoding the amino acid sequence set forth in SEQ ID NO: 49, an *S. cerevisiae* PDC1 gene encoding the amino acid sequence set forth in SEQ ID NO: 50, or a *K. lactis* PDC encoding the amino acid sequence set forth in SEQ ID NO: 51. In certain embodiments, a PDC gene derived from the *I. orientalis* PDC gene may comprise the nucleotide sequence set forth in SEQ ID NO: 48 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO: 48. In other embodiments, a PDC gene may be derived from a bacterial source. For example, a PDC gene may be derived from a *Z. mobilis* PDC gene encoding the amino acid sequence set forth in SEQ ID NO: 52 or an *A. pasteurianus* PDC gene encoding the amino acid sequence set forth in SEQ ID NO: 53.

[00114] A "benzoylformate decarboxylase" gene as used herein refers to any gene that encodes a polypeptide with benzoylformate decarboxylase activity, meaning the ability to catalyze the conversion of benzoylformate to benzaldehyde. Enzymes having benzoylformate decarboxylase activity are classified as EC 4.1.1.7. In preferred embodiments, a benzoylformate decarboxylase gene that is incorporated into a modified yeast cell as provided herein has undergone one or more mutations versus the native gene

from which it was derived such that the resultant gene encodes a polypeptide capable of catalyzing the conversion of OAA to malonate semialdehyde. In certain embodiments, a benzoylformate decarboxylase gene may be derived from a bacterial source. For example, a benzoylformate decarboxylase gene may be derived from a *P. putida* mdIC gene encoding the amino acid sequence set forth in SEQ ID NO: 54, a *P. aeruginosa* mdIC gene encoding the amino acid sequence set forth in SEQ ID NO: 55, a *P. stutzeri* dpdB gene encoding the amino acid sequence set forth in SEQ ID NO: 56, or a *P. fluorescens* ilvB-1 gene encoding the amino acid sequence set forth in SEQ ID NO: 57.

[00115] An "OAA formatelyase gene" as used herein refers to any gene that encodes a polypeptide with OAA formatelyase activity, meaning the ability to catalyze the conversion of an acylate ketoacid to its corresponding CoA derivative. A polypeptide encoded by an OAA formatelyase gene may have activity on pyruvate or on another ketoacid. In certain embodiments, an OAA formatelyase gene encodes a polypeptide that converts OAA to malonyl-CoA.

[00116] A "malonyl-CoA reductase gene" as used herein refers to any gene that encodes a polypeptide with malonyl-CoA reductase activity, meaning the ability to catalyze the conversion of malonyl-CoA to malonate semialdehyde (also referred to as Co-A acylating malonate semialdehyde dehydrogenase activity). In certain embodiments, a malonyl-CoA reductase gene may be derived from a bifunctional malonyl-CoA reductase gene which also has the ability to catalyze the conversion of malonate semialdehyde to 3-HP. In certain of these embodiments, a malonyl-CoA reductase gene may be derived from a bacterial source. For example, a malonyl-CoA reductase gene may be derived from a *C. aurantiacus* malonyl-CoA reductase gene encoding the amino acid sequence set forth in SEQ ID NO: 58, an *R. castenholzii* malonyl-CoA reductase gene encoding the amino acid sequence set forth in SEQ ID NO: 59, or an *Erythrobacter* sp. *NAP1* malonyl-CoA reductase gene encoding the amino acid sequence set forth in SEQ ID NO: 60. In other embodiments, a malonyl-CoA reductase gene may be derived from a malonyl-CoA reductase gene encoding a polypeptide that only catalyzes the conversion of malonyl-CoA to malonate semialdehyde. For example, a malonyl-CoA reductase gene may be derived from an *M. sedula* Msed_0709 gene encoding the amino acid sequence set forth in SEQ ID NO: 61 or a *S. tokodaii* malonyl-CoA reductase encoding the amino acid sequence set forth in SEQ ID NO: 62.

[00117] A "pyruvate dehydrogenase gene" or "PDH gene" as used herein refers to any gene that encodes a polypeptide with pyruvate dehydrogenase activity, meaning the ability to catalyze the conversion of pyruvate to acetyl-CoA. In certain embodiments, a PDH gene may be derived from a yeast source. For example, a PDH gene may be derived from an *S. cerevisiae* LAT1, PDA1, PDB1, or LPD gene encoding the amino acid sequence set forth in

SEQ ID NOs: 63-66, respectively. In other embodiments, a PDH gene may be derived from a bacterial source. For example, a PDH gene may be derived from an *E. coli strain K12 substr. MG1655* aceE, aceF, or lpd gene encoding the amino acid sequence set forth in SEQ ID NOs: 67-69, respectively, or a *B. subtilis* pdhA, pdhB, pdhC, or pdhD gene encoding the amino acid sequence set forth in SEQ ID NOs: 70-73, respectively.

[00118] An "acetyl-CoA carboxylase gene" or "ACC gene" as used herein refers to any gene that encodes a polypeptide with acetyl-CoA carboxylase activity, meaning the ability to catalyze the conversion of acetyl-CoA to malonyl-CoA. Enzymes having acetyl-CoA carboxylase activity are classified as EC 6.4.1.2. In certain embodiments, an acetyl-CoA carboxylase gene may be derived from a yeast source. For example, an acetyl-CoA carboxylase gene may be derived from an *S. cerevisiae* ACC1 gene encoding the amino acid sequence set forth in SEQ ID NO: 74. In other embodiments, an acetyl-CoA carboxylase gene may be derived from a bacterial source. For example, an acetyl-CoA carboxylase gene may be derived from an *E. coli* accA, accB, accC, or accD gene encoding the amino acid sequence set forth in SEQ ID NOs: 75-78, respectively, or a *C. aurantiacus* accA, accB, accC, or accD gene encoding the amino acid sequence set forth in SEQ ID NOs: 79-82, respectively.

[00119] An "alanine dehydrogenase gene" as used herein refers to any gene that encodes a polypeptide with alanine dehydrogenase activity, meaning the ability to catalyze the NAD-dependent reductive amination of pyruvate to alanine. Enzymes having alanine dehydrogenase activity are classified as EC 1.4.1.1. In certain embodiments, an alanine dehydrogenase gene may be derived from a bacterial source. For example, an alanine dehydrogenase gene may be derived from an *B. subtilis* alanine dehydrogenase gene encoding the amino acid sequence set forth in SEQ ID NO: 83.

[00120] A "pyruvate/alanine aminotransferase gene" as used herein refers to any gene that encodes a polypeptide with pyruvate/alanine aminotransferase activity, meaning the ability to catalyze the conversion of pyruvate and L-glutamate to alanine and 2-oxoglutarate. In certain embodiments, a pyruvate/alanine aminotransferase gene is derived from a yeast source. For example, a pyruvate/alanine aminotransferase gene may be derived from an *S. pombe* pyruvate/alanine aminotransferase gene encoding the amino acid sequence set forth in SEQ ID NO: 84 or an *S. cerevisiae* ALT2 gene encoding the amino acid sequence set forth in SEQ ID NO: 85.

[00121] An "alanine 2,3 aminomutase gene" or "AAM gene" as used herein refers to a gene that encodes a polypeptide with alanine 2,3 aminomutase activity, meaning the ability to catalyze the conversion of alanine to β -alanine. Alanine 2,3 aminomutase activity is not known to occur naturally. Therefore, an alanine 2,3 aminomutase gene can be derived by

incorporating one or more mutations into a native source gene that encodes a polypeptide with similar activity such as lysine 2,3 aminomutase activity (see, e.g., US Patent 7,309,597). In certain embodiments, the native source gene may be a *B. subtilis* lysine 2,3 aminomutase gene encoding the amino acid sequence set forth in SEQ ID NO: 86, a *P. gingivalis* lysine 2,3 aminomutase gene encoding the amino acid sequence set forth in SEQ ID NO: 87, or a *F. nucleatum* (ATCC-10953) lysine 2,3 aminomutase gene encoding the amino acid sequence set forth in SEQ ID NO: 88.

[00122] A "CoA transferase gene" as used herein refers to any gene that encodes a polypeptide with CoA transferase activity, which in one example includes the ability to catalyze the conversion of β -alanine to β -alanyl-CoA and/or the conversion of lactate to lactyl-CoA. In certain embodiments, a CoA transferase gene may be derived from a yeast source. In other embodiments, a CoA transferase gene may be derived from a bacterial source. For example, a CoA transferase gene may be derived from an *M. elsdenii* CoA transferase gene encoding the amino acid sequence set forth in SEQ ID NO: 89.

[00123] A "CoA synthetase gene" as used herein refers to any gene that encodes a polypeptide with CoA synthetase activity. In one example this includes the ability to catalyze the conversion of β -alanine to β -alanyl-CoA. In another example, this includes the ability to catalyze the conversion of lactate to lactyl-CoA. In certain embodiments, a CoA synthetase gene may be derived from a yeast source. For example, a CoA synthetase gene may be derived from an *S. cerevisiae* CoA synthetase gene. In other embodiments, a CoA synthetase gene may be derived from a bacterial source. For example, a CoA synthetase gene may be derived from an *E. coli* CoA synthetase, *R. sphaeroides*, or *S. enterica* CoA synthetase gene.

[00124] A " β -alanyl-CoA ammonia lyase gene" as used herein refers to any gene that encodes a polypeptide with β -alanyl-CoA ammonia lyase activity, meaning the ability to catalyze the conversion of β -alanyl-CoA to acrylyl-CoA. In certain embodiments, a β -alanyl-CoA ammonia lyase gene may be derived from a bacterial source, such as a *C. propionicum* β -alanyl-CoA ammonia lyase gene encoding the amino acid sequence set forth in SEQ ID NO: 90.

[00125] A "3-HP-CoA dehydratase gene" or "acrylyl-CoA hydratase gene" as used herein refers to any gene that encodes a polypeptide with 3-HP-CoA dehydratase gene activity, meaning the ability to catalyze the conversion of acrylyl-CoA to 3-HP-CoA. Enzymes having 3-HP-CoA dehydratase activity are classified as EC 4.2.1.116. In certain embodiments, a 3-HP-CoA dehydratase gene may be derived from a yeast or fungal source, such as a *P. sojae* 3-HP-CoA dehydratase gene encoding the amino acid sequence set forth in SEQ ID NO: 91. In other embodiments, a 3-HP-CoA dehydratase gene may be derived from a bacterial

source. For example, a 3-HP-CoA dehydratase gene may be derived from a *C. aurantiacus* 3-HP-CoA dehydratase gene encoding the amino acid sequence set forth in SEQ ID NO: 92, an *R. rubrum* 3-HP-CoA dehydratase gene encoding the amino acid sequence set forth in SEQ ID NO: 93, or an *R. capsulatus* 3-HP-CoA dehydratase gene encoding the amino acid sequence set forth in SEQ ID NO: 94. In still other embodiments, a 3-HP-CoA dehydratase gene may be derived from a mammalian source. For example, a 3-HP-CoA dehydratase gene may be derived from a *H. sapiens* 3-HP-CoA dehydratase gene encoding the amino acid sequence set forth in SEQ ID NO: 95.

[00126] A "3-HP-CoA hydrolase gene" as used herein refers to any gene that encodes a polypeptide with 3-HP-CoA hydrolase activity, meaning the ability to catalyze the conversion of 3-HP-CoA to 3-HP. In certain embodiments, a 3-HP-CoA gene may be derived from a yeast or fungal source. In other embodiments, a 3-HP-CoA gene may be derived from a bacterial or mammalian source.

[00127] A "3-hydroxyisobutyryl-CoA hydrolase gene" as used herein refers to any gene that encodes a polypeptide with 3-hydroxyisobutyryl-CoA hydrolase activity, which in one example includes the ability to catalyze the conversion of 3-HP-CoA to 3-HP. In certain embodiments, a 3-hydroxyisobutyryl-CoA hydrolase gene may be derived from a bacterial source, such as a *P. fluorescens* 3-hydroxyisobutyryl-CoA hydrolase gene encoding the amino acid sequence set forth in SEQ ID NO: 96 or a *B. cereus* 3-hydroxyisobutyryl-CoA hydrolase gene encoding the amino acid sequence set forth in SEQ ID NO: 97. In other embodiments, a 3-hydroxyisobutyryl-CoA hydrolase gene may be derived from a mammalian source, such as a *H. sapiens* 3-hydroxyisobutyryl-CoA hydrolase gene encoding the amino acid sequence set forth in SEQ ID NO: 98.

[00128] A "lactate dehydrogenase gene" or "LDH gene" as used herein refers to any gene that encodes a polypeptide with lactate dehydrogenase activity, meaning the ability to catalyze the conversion of pyruvate to lactate. In certain embodiments, an LDH gene may be derived from a fungal, bacterial, or mammalian source.

[00129] A "lactyl-CoA dehydratase gene" as used herein refers to any gene that encodes a polypeptide with lactyl-CoA dehydratase activity, meaning the ability to catalyze the conversion of lactyl-CoA to acrylyl-CoA. In certain embodiments, a lactyl-CoA dehydratase gene may be derived from a bacterial source. For example, a lactyl-CoA dehydratase gene may be derived from an *M. elsdenii* lactyl-CoA dehydratase E1, EIIa, or EIIb subunit gene encoding the amino acid sequence set forth in SEQ ID NOs: 99-101.

[00130] An "aldehyde dehydrogenase gene" as used herein refers to any gene that encodes a polypeptide with aldehyde dehydrogenase activity, which in one example includes the ability to catalyze the conversion of 3-HPA to 3-HP and vice versa. In certain

embodiments, an aldehyde dehydrogenase gene may be derived from a yeast source, such as an *S. cerevisiae* aldehyde dehydrogenase gene encoding the amino acid sequence set forth in SEQ ID NO: 102 or an *I. orientalis* aldehyde dehydrogenase gene encoding the amino acid sequence set forth in SEQ ID NOs: 122, 124, or 126. In other embodiments, an aldehyde dehydrogenase may be derived from a bacterial source, such as an *E. coli* aldH gene encoding the amino acid sequence set forth in SEQ ID NO: 103 or a *K. pneumoniae* aldehyde dehydrogenase gene encoding the amino acid sequence set forth in SEQ ID NO: 104.

[00131] A "glycerol dehydratase gene" as used herein refers to any gene that encodes a polypeptide with glycerol dehydratase activity, meaning the ability to catalyze the conversion of glycerol to 3-HPA. In certain embodiments, a glycerol dehydratase gene may be derived from a bacterial source, such as a *K. pneumonia* or *C. freundii* glycerol dehydratase gene.

[00132] In certain embodiments, the genetically modified yeast cells provided herein further comprise a deletion or disruption of one or more native genes. "Deletion or disruption" with regard to a native gene means that either the entire coding region of the gene is eliminated (deletion) or the coding region of the gene, its promoter, and/or its terminator region is modified (such as by deletion, insertion, or mutation) such that the gene no longer produces an active enzyme, produces a severely reduced quantity (at least 75% reduction, preferably at least 90% reduction) of an active enzyme, or produces an enzyme with severely reduced (at least 75% reduced, preferably at least 90% reduced) activity.

[00133] In certain embodiments, deletion or disruption of one or more native genes results in a deletion or disruption of one or more native metabolic pathways. "Deletion or disruption" with regard to a metabolic pathway means that the pathway is either inoperative or else exhibits activity that is reduced by at least 75%, at least 85%, or at least 95% relative to the native pathway. In certain embodiments, deletion or disruption of a native metabolic pathway is accomplished by incorporating one or more genetic modifications that result in decreased expression of one or more native genes that reduce 3-HP production.

[00134] In certain embodiments, deletion or disruption of native gene can be accomplished by forced evolution, mutagenesis, or genetic engineering methods, followed by appropriate selection or screening to identify the desired mutants. In certain embodiments, deletion or disruption of a native host cell gene may be coupled to the incorporation of one or more exogenous genes into the host cell, i.e., the exogenous genes may be incorporated using a gene expression integration construct that is also a deletion construct. In other embodiments, deletion or disruption may be accomplished using a deletion construct that does not contain an exogenous gene or by other methods known in the art.

[00135] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme involved in ethanol fermentation, including for example pyruvate decarboxylase (PDC, converts pyruvate to acetaldehyde) and/or alcohol dehydrogenase (ADH, converts acetaldehyde to ethanol) genes. These modifications decrease the ability of the yeast cell to produce ethanol, thereby maximizing 3-HP production. However, in certain embodiments the genetically modified yeast cells provided herein may be engineered to co-produce 3-HP and ethanol. In those embodiments, native genes encoding an enzyme involved in ethanol fermentation are preferably not deleted or disrupted, and in certain embodiments the yeast cells may comprise one or more exogenous genes that increase ethanol production.

[00136] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme involved in producing alternate fermentative products such as glycerol or other byproducts such as acetate or diols. For example, the cells provided herein may comprise a deletion or disruption of one or more of glycerol 3-phosphate dehydrogenase (GPD, catalyzes reaction of dihydroxyacetone phosphate to glycerol 3-phosphate), glycerol 3-phosphatase (GPP, catalyzes conversion of glycerol-3 phosphate to glycerol), glycerol kinase (catalyzes conversion of glycerol 3-phosphate to glycerol), dihydroxyacetone kinase (catalyzes conversion of dihydroxyacetone phosphate to dihydroxyacetone), glycerol dehydrogenase (catalyzes conversion of dihydroxyacetone to glycerol), aldehyde dehydrogenase (ALD, e.g., converts acetaldehyde to acetate or 3-HP to 3-HPA), or butanediol dehydrogenase (catalyzes conversion of butanediol to acetoin and vice versa) genes.

[00137] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme that catalyzes a reverse reaction in a 3-HP fermentation pathway, including for example PEP carboxykinase (PCK), enzymes with OAA decarboxylase activity, or CYB2A or CYB2B (catalyzes the conversion of lactate to pyruvate). PCK catalyzes the conversion of PEP to OAA and vice versa, but exhibits a preference for the OAA to PEP reaction. To reduce the conversion of OAA to PEP, one or more copies of a native PCK gene may be deleted or disrupted. In certain embodiments, yeast cells in which one or more native PCK genes have been deleted or disrupted may express one or more exogenous PCK genes that have been mutated to encode a polypeptide that favors the conversion of PEP to OAA. OAA decarboxylase catalyzes the conversion of OAA to pyruvate. Enzymes with OAA decarboxylase activity have been identified, such as that coded by the *eda* gene in *E. coli* and malic enzyme (MAE) in yeast and fungi. To reduce OAA decarboxylase activity, one or more copies of a native gene encoding an enzyme with OAA decarboxylase activity may be

deleted or disrupted. In certain embodiments, yeast cells in which one or more native OAA decarboxylation genes have been deleted or disrupted may express one or more exogenous OAA decarboxylation genes that have been mutated to encode a polypeptide that catalyzes the conversion of pyruvate to OAA.

[00138] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme involved in an undesirable reaction with a 3-HP fermentation pathway product or intermediate. Examples of such genes include those encoding an enzyme that converts 3HP to an aldehyde of 3HP, which are known to be toxic to bacterial cells.

[00139] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme that has a neutral effect on a 3-HP fermentation pathway, including for example GAL6 (negative regulator of the GAL system that converts galactose to glucose). Deletion or disruption of neutral genes allows for insertion of one or more exogenous genes without affecting native fermentation pathways.

[00140] In certain embodiments, the yeast cells provided herein are 3-HP resistant yeast cells. A "3-HP-resistant yeast cell" as used herein refers to a yeast cell that exhibits an average glycolytic rate of at least 2.5 g/L/hr in media containing 75 g/L or greater 3-HP at a pH of less than 4.0. Such rates and conditions represent an economic process for producing 3-HP. In certain of these embodiments, the yeast cells may exhibit 3-HP resistance in their native form. In other embodiments, the cells may have undergone mutation and/or selection (e.g., chemostat selection or repeated serial subculturing) before, during, or after introduction of genetic modifications related to an active 3-HP fermentation pathway, such that the mutated and/or selected cells possess a higher degree of resistant to 3-HP than wild-type cells of the same species. For example, in some embodiments, the cells have undergone mutation and/or selection in the presence of 3-HP or lactic acid before being genetically modified with one or more exogenous 3-HP pathway genes. In certain embodiments, mutation and/or selection may be carried out on cells that exhibit 3-HP resistance in their native form. Cells that have undergone mutation and/or selection may be tested for sugar consumption and other characteristics in the presence of varying levels of 3-HP in order to determine their potential as industrial hosts for 3-HP production. In addition to 3-HP resistance, the yeast cells provided herein may have undergone mutation and/or selection for resistance to one or more additional organic acids (e.g., lactic acid) or to other fermentation products, byproducts, or media components.

[00141] Selection, such as selection for resistance to 3-HP or to other compounds, may be accomplished using methods well known in the art. For example, as mentioned *supra*,

selection may be chemostat selection. Chemostat selection uses a chemostat that allows for a continuous culture of microorganisms (e.g., yeast) wherein the specific growth rate and cell number can be controlled independently. A continuous culture is essentially a flow system of constant volume to which medium is added continuously and from which continuous removal of any overflow can occur. Once such a system is in equilibrium, cell number and nutrient status remain constant, and the system is in a steady state. A chemostat allows control of both the population density and the specific growth rate of a culture through dilution rate and alteration of the concentration of a limiting nutrient, such as a carbon or nitrogen source. By altering the conditions as a culture is grown (e.g., decreasing the concentration of a secondary carbon source necessary to the growth of the inoculum strain, among others), microorganisms in the population that are capable of growing faster at the altered conditions will be selected and will outgrow microorganisms that do not function as well under the new conditions. Typically such selection requires the progressive increase or decrease of at least one culture component over the course of growth of the chemostat culture. The operation of chemostats and their use in the directed evolution of microorganisms is well known in the art (see, e.g., Novick Proc Natl Acad Sci USA 36:708-719 (1950), Harder J Appl Bacteriol 43:1-24 (1977). Other methods for selection include, but are not limited to, repeated serial subculturing under the selective conditions as described in e.g., U.S. Patent No. 7,629,162. Such methods can be used in place of, or in addition to, using the glucose limited chemostat method described above.

[00142] Yeast strains exhibiting the best combinations of growth and glucose consumption in 3-HP media as disclosed in the examples below are preferred host cells for various genetic modifications relating to 3-HP fermentation pathways. Yeast genera that possess the potential for a relatively high degree of 3-HP resistance, as indicated by growth in the presence of 75 g/L 3-HP or higher at a pH of less than 4, include for example *Candida*, *Kluyveromyces*, *Issatchenkia*, *Saccharomyces*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, and *Zygosaccharomyces*. Species exhibiting 3-HP resistance included *I. orientalis* (also known as *C. krusei*), *C. lambica* (also known as *Pichia fermentans*), and *S. bulderi* (also known as *Kazachstania bulderi*). *I. orientalis* and *C. lambica* are from the *I. orientalis/P. fermentans* clade, while *S. bulderi* is from the *Saccharomyces* clade. Specific strains exhibiting 3-HP resistance included *I. orientalis* strains 24210, PTA-6658, 60585, and CD1822, *S. bulderi* strains MYA-402 and MYA-404, and *C. lambica* strain ATCC 38617.

[00143] Other wild-type yeast or fungi may be tested in a similar manner and identified to have acceptable levels of growth and glucose utilization in the presence of high levels of 3-HP as described herein. For example, Gross and Robbins (Hydrobiologia 433(103):91-109) have compiled a list of 81 fungal species identified in low pH (<4) environments that could be relevant to test as potential production hosts.

[00144] In certain embodiments, the modified yeast cells provided herein are generated by incorporating one or more genetic modifications into a Crabtree-negative host yeast cell. In certain of these embodiments the host yeast cell belongs to the genus *Issatchenkia*, *Candida*, or *Saccharomyces*, and in certain of these embodiments the host cell belongs to the *I. orientalis*/*P. fermentans* or *Saccharomyces* clade. In certain of embodiments, the host cell is *I. orientalis* or *C. lambica*, or *S. bulderi*.

[00145] The *I. orientalis*/*P. fermentans* clade is the most terminal clade that contains at least the species *I. orientalis*, *P. galeiformis*, *P. sp. YB-4149* (NRRL designation), *C. ethanolica*, *P. deserticola*, *P. membranifaciens*, and *P. fermentans*. Members of the *I. orientalis*/*P. fermentans* clade are identified by analysis of the variable D1/D2 domain of the 26S ribosomal DNA of yeast species, using the method described by Kurtzman and Robnett in "Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences," *Antonie van Leeuwenhoek* 73:331-371, 1998, incorporated herein by reference (see especially p. 349). Analysis of the variable D1/D2 domain of the 26S ribosomal DNA from hundreds of ascomycetes has revealed that the *I. orientalis*/*P. fermentans* clade contains very closely related species. Members of the *I. orientalis*/*P. fermentans* clade exhibit greater similarity in the variable D1/D2 domain of the 26S ribosomal DNA to other members of the clade than to yeast species outside of the clade. Therefore, other members of the *I. orientalis*/*P. fermentans* clade can be identified by comparison of the D1/D2 domains of their respective ribosomal DNA and comparing to that of other members of the clade and closely related species outside of the clade, using Kurtzman and Robnett's methods.

[00146] In certain embodiments, the genetically modified yeast cells provided herein belong to the genus *Issatchenkia*, and in certain of these embodiments the yeast cells are *I. orientalis*. When first characterized, the species *I. orientalis* was assigned the name *Pichia kudriavzevii*. The anamorph (asexual form) of *I. orientalis* is known as *Candida krusei*. Numerous additional synonyms for the species *I. orientalis* have been listed elsewhere (Kurtzman and Fell, *The Yeasts, a Taxonomic Study*. Section 35. *Issatchenkia Kudryavtsev*, pp 222-223 (1998)).

[00147] The ideal yeast cell for 3-HP production is ideally capable of growing at low pH levels. The ability to conduct fermentation at a low pH decreases downstream recovery costs, resulting in more economical production. Therefore, in certain embodiments the yeast host cell is capable of growing at low pH levels (e.g., at pH levels less than 7, 6, 5, 4, or 3).

[00148] A suitable host cell may possess one or more favorable characteristics in addition to 3-HP resistance and/or low pH growth capability. For example, potential host cells exhibiting 3-HP resistance may be further selected based on glycolytic rates, specific growth

rates, thermotolerance, tolerance to biomass hydrolysate inhibitors, overall process robustness, and so on. These criteria may be evaluated prior to any genetic modification relating to a 3-HP fermentation pathway, or they may be evaluated after one or more such modifications have taken place.

[00149] Because most yeast are native producers of ethanol, elimination or severe reduction in the enzyme catalyzing the first step in ethanol production from pyruvate (PDC) is required for sufficient yield of an alternate product. In Crabtree-positive yeast such as *Saccharomyces*, a deleted or disrupted PDC gene causes the host to acquire an auxotrophy for two-carbon compounds such as ethanol or acetate, and causes a lack of growth in media containing glucose. Mutants capable of overcoming these limitations can be obtained using progressive selection for acetate independence and glucose tolerance (see, e.g., van Maris Appl Environ Microbiol 70:159 (2004)). Therefore, in certain embodiments a preferred yeast host cell is a Crabtree-negative yeast cell, in which PDC deletion strains are able to grow on glucose and retain C2 prototrophy.

[00150] The level of gene expression and/or the number of exogenous genes to be utilized in a given cell will vary depending on the yeast species selected. For fully genome-sequenced yeasts, whole-genome stoichiometric models may be used to determine which enzymes should be expressed to develop a desired pathway 3-HP fermentation pathway. Whole-genome stoichiometric models are described in, for example, Hjersted et al., "Genome-scale analysis of *Saccharomyces cerevisiae* metabolism and ethanol production in fed-batch culture," *Biotechnol. Bioeng.* 2007; and Famili et al., "*Saccharomyces cerevisiae* phenotypes can be predicted by using constraint-based analysis of a genome-scale reconstructed metabolic network," *Proc. Natl. Acad. Sci.* 2003, 100(23):13134-9.

[00151] For yeasts without a known genome sequence, sequences for genes of interest (either as overexpression candidates or as insertion sites) can typically be obtained using techniques such as those described below in Example 2A. Routine experimental design can be employed to test expression of various genes and activity of various enzymes, including genes and enzymes that function in a 3-HP pathway. Experiments may be conducted wherein each enzyme is expressed in the yeast individually and in blocks of enzymes up to and including preferably all pathway enzymes, to establish which are needed (or desired) for improved 3-HP production. One illustrative experimental design tests expression of each individual enzyme as well as of each unique pair of enzymes, and further can test expression of all required enzymes, or each unique combination of enzymes. A number of approaches can be taken, as will be appreciated.

[00152] In certain embodiments, methods are provided for producing 3-HP from a genetically modified yeast cell as provided herein. In certain embodiments, these methods

comprise culturing a genetically modified yeast cell as provided herein in the presence of at least one carbon source, allowing the cell to produce 3-HP for a period of time, and then isolating 3-HP produced by the cell from culture. The carbon source may be any carbon source that can be fermented by the provided yeast. The carbon source may be a twelve carbon sugar such as sucrose, a hexose sugar such as glucose or fructose, glycan or other polymer of glucose, glucose oligomers such as maltose, maltotriose and isomaltotriose, panose, and fructose oligomers. If the cell is modified to impart an ability to ferment pentose sugars, the fermentation medium may include a pentose sugar such as xylose, xylan or other oligomer of xylose, and/or arabinose. Such pentose sugars are suitably hydrolysates of a hemicellulose-containing biomass. In the case of oligomeric sugars, it may be necessary to add enzymes to the fermentation broth in order to digest these to the corresponding monomeric sugar for fermentation by the cell. In certain embodiments, more than one type of genetically modified yeast cell may be present in the culture. Likewise, in certain embodiments one or more native yeast cells of the same or a different species than the genetically modified yeast cell may be present in the culture.

[00153] In certain embodiments, culturing of the cells provided herein to produce 3-HP may be divided up into phases. For example, the cell culture process may be divided into a cultivation phase, a production phase, and a recovery phase. One of ordinary skill in the art will recognize that the conditions used for these phases may be varied based on factors such as the species of yeast being used, the specific 3-HP fermentation pathway utilized by the yeast, the desired yield, or other factors.

[00154] The medium will typically contain nutrients as required by the particular cell, including a source of nitrogen (such as amino acids, proteins, inorganic nitrogen sources such as ammonia or ammonium salts, and the like), and various vitamins, minerals and the like. In some embodiments, the cells of the invention can be cultured in a chemically defined medium. In one example, the medium contains around 5 g/L ammonium sulfate, around 3 g/L potassium dihydrogen phosphate, around 0.5 g/L magnesium sulfate, trace elements, vitamins and around 150 g/L glucose. The pH may be allowed to range freely during cultivation, or may be buffered if necessary to prevent the pH from falling below or rising above predetermined levels. In certain embodiments, the fermentation medium is inoculated with sufficient yeast cells that are the subject of the evaluation to produce an OD₆₀₀ of about 1.0. Unless explicitly noted otherwise, OD₆₀₀ as used herein refers to an optical density measured at a wavelength of 600 nm with a 1 cm pathlength using a model DU600 spectrophotometer (Beckman Coulter). The cultivation temperature may range from around 30-40°C, and the cultivation time may be up to around 120 hours.

[00155] In one example, the concentration of cells in the fermentation medium is typically in the range of about 0.1 to 20, preferably from 0.1 to 5, even more preferably from 1 to 3 g dry cells/liter of fermentation medium during the production phase. The fermentation may be conducted aerobically, microaerobically, or anaerobically, depending on pathway requirements. If desired, oxygen uptake rate (OUR) can be varied throughout fermentation as a process control (see, e.g., WO03/102200). In some embodiments, the modified yeast cells provided herein are cultivated under microaerobic conditions characterized by an oxygen uptake rate from 2 to 45 mmol/L/hr, e.g., 2 to 25, 2 to 20, 2 to 15, 2 to 10, 10 to 45, 15 to 40, 20 to 35, or 25 to 35 mmol/L/hr. In certain embodiments, the modified yeast cells provided herein may perform especially well when cultivated under microaerobic conditions characterized by an oxygen uptake rate of from 2 to 25 mmol/L/hr. The medium may be buffered during the production phase such that the pH is maintained in a range of about 3.0 to about 7.0, or from about 4.0 to about 6.0. Suitable buffering agents are basic materials that neutralize the acid as it is formed, and include, for example, calcium hydroxide, calcium carbonate, sodium hydroxide, potassium hydroxide, potassium carbonate, sodium carbonate, ammonium carbonate, ammonia, ammonium hydroxide and the like. In general, those buffering agents that have been used in conventional fermentation processes are also suitable here.

[00156] In those embodiments where a buffered fermentation is utilized, acidic fermentation products may be neutralized to the corresponding salt as they are formed. In these embodiments, recovery of the acid involves regeneration of the free acid. This may be done by removing the cells and acidulating the fermentation broth with a strong acid such as sulfuric acid. This results in the formation of a salt by-product. For example, where a calcium salt is utilized as the neutralizing agent and sulfuric acid is utilized as the acidulating agent, gypsum is produced as a salt by-product. This by-product is separated from the broth, and the acid is recovered using techniques such as liquid-liquid extraction, distillation, absorption, and others (see, e.g., T.B. Vickroy, Vol. 3, Chapter 38 of *Comprehensive Biotechnology*, (ed. M. Moo-Young), Pergamon, Oxford, 1985; R. Datta, et al., FEMS Microbiol Rev, 1995, 16:221-231; U.S. Patent Nos. 4,275,234, 4,771,001, 5,132,456, 5,420,304, 5,510,526, 5,641,406, and 5,831,122, and WO93/00440).

[00157] In other embodiments, the pH of the fermentation medium may be permitted to drop during cultivation from a starting pH that is at or above the pKa of 3-HP, typically 4.5 or higher, to at or below the pKa of the acid fermentation product, e.g., less than 4.5 or 4.0, such as in the range of about 1.5 to about 4.5, in the range of from about 2.0 to about 4.0, or in the range from about 2.0 to about 3.5.

[00158] In still other embodiments, fermentation may be carried out to produce a product acid by adjusting the pH of the fermentation broth to at or below the pKa of the product acid prior to or at the start of the fermentation process. The pH may thereafter be maintained at or below the pKa of the product acid throughout the cultivation. In certain embodiments, the pH may be maintained at less than 4.5 or 4.0, such as in a range of about 1.5 to about 4.5, in a range of about 2.0 to about 4.0, or in a range of about 2.0 to about 3.5.

[00159] In certain embodiments of the methods provided herein, the genetically modified yeast cells produce relatively low levels of ethanol. In certain embodiments, ethanol may be produced in a yield of 10% or less, preferably in a yield of 2% or less. In certain of these embodiments, ethanol is not detectably produced. In other embodiments, however, 3-HP and ethanol may be co-produced. In these embodiments, ethanol may be produced at a yield of greater than 10%, greater than 25%, or greater than 50%.

[00160] In certain embodiments of the methods provided herein, the final yield of 3-HP on the carbon source is at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, or greater than 50% of the theoretical yield. The concentration, or titer, of 3-HP will be a function of the yield as well as the starting concentration of the carbon source. In certain embodiments, the titer may reach at least 1-3, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or greater than 50 g/L at some point during the fermentation, and preferably at the end of the fermentation. In certain embodiments, the final yield of 3-HP may be increased by altering the temperature of the fermentation medium, particularly during the production phase.

[00161] Once produced, any method known in the art can be used to isolate 3-HP from the fermentation medium. For example, common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the 3-HP from the microorganism-free broth. In addition, 3-HP can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated.

[00162] 3-HP produced using the methods disclosed herein can be chemically converted into other organic compounds. For example, 3-HP can be hydrogenated to form 1,3 propanediol, a valuable polyester monomer. Propanediol also can be created from 3-HP using polypeptides having oxidoreductase activity *in vitro* or *in vivo*. Hydrogenating an organic acid such as 3-HP can be performed using any method such as those used to hydrogenate succinic acid and/or lactic acid. For example, 3-HP can be hydrogenated using a metal catalyst. In another example, 3-HP can be dehydrated to form acrylic acid using any known method for performing dehydration reactions. For example, 3-HP can be heated in the presence of a catalyst (e.g., a metal or mineral acid catalyst) to form acrylic acid.

[00163] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention. It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the present invention. It is the intention of the inventors that such variations are included within the scope of the invention.

Examples

Media and Solutions

TE was composed of 10 mM Tris Base and 1 mM EDTA, pH 8.0.

2X YT+amp plates were composed of 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin, and 15 g/L Bacto agar.

ura selection plates were composed of 6.7 g yeast nitrogen base with ammonium sulfate, 5 g casamino acids, 100 mL 0.5 M succinic acid pH 5, 20 g Noble agar, and 855 mL deionized water. Following autoclave sterilization, 40 mL sterile 50% glucose and 2 mL 10 mg/mL chloramphenicol were added and plates poured.

ura selection media was composed of 6.7 g yeast nitrogen base with ammonium sulfate, 5 g casamino acids, 100 mL 0.5 M succinic acid pH 5, and 855 mL deionized water. Following autoclave sterilization, 40 mL sterile 50% glucose and 2 mL 10 mg/mL chloramphenicol were added.

YP+10% glucose media was composed of 500 mL YP broth and 100 mL sterile 50% glucose.

YP broth was composed of 10 g/L of yeast extract, 20 g/L of peptone.

YPD plates were composed of 10 g of yeast extract, 20 g of peptone, 20 g bacto agar, and deionized water to 960 mL. Following autoclave sterilization, 40 mL sterile 50% glucose was added and plates poured.

TAE was composed of 4.84 g/L of Tris base, 1.14 mL/L of glacial acetic acid, and 2 mL/L of 0.5 M EDTA pH 8.0.

TBE was composed of 10.8 g/L of Tris base, 5.5 g/L boric acid, and 4 mL/L of 0.5 M EDTA pH 8.0.

LiOAc/TE solution was composed of 8 parts sterile water, 1 part 1 M LiOAc, and 1 part 10X TE.

10X TE (200 mL) was composed of 2.42 g Tris Base, 4 mL 0.5M EDTA, pH 8.0. 5 M HCl was used to adjust the pH to 7.5 and the solution was sterilized by autoclave.

PEG/LiOAc/TE Solution was composed of 8 parts 50% PEG3350, 1 part 1 M LiOAc, and 1 part 10X TE.

50% PEG3350 was prepared by adding 100 g PEG3350 to 150 mL water and heating and stirring until dissolved. The volume was then brought up to 200 mL with water and the sterilized by autoclave.

ScD FOA plates were composed of 275 mL 2X-ScD 2X FOA liquid media and 275 mL 2X-ScD 2X FOA plate media, melted and cooled to 65°C.

2X-ScD 2X FOA liquid media was composed of 6.66 g yeast nitrogen base without amino acids, 1.54 g ura- DO supplement (Clontech, Mountain View, CA, USA), 20 g dextrose, 50 mg uracil, 2 mg uridine, and 2 g 5-FOA (5-fluoroorotic acid, monohydrate; Toronto Research Chemicals, North York ,ON, Canada) and water to 1 L. The resulting solution was filtered to sterilize.

2X-ScD 2X FOA plate media was composed of 11 g bacto agar and 275 mL water. The resulting solution was autoclaved to sterilize.

DM2 medium was composed of ammonium sulfate (5.0 g/L), magnesium sulfate heptahydrate (0.5 g/L), potassium phosphate monobasic (3.0 g/L), trace element solution (1 mL/L) and vitamin solution (1 mL/L). After dissolving all medium components, the pH of the medium was adjusted to the desired initial pH using an appropriate base (e.g, KOH).

Trace element solution was composed of EDTA (15.0 g/L), zinc sulfate heptahydrate (4.5 g/L), manganese chloride dehydrate (1.0 g/L), Cobalt(II)chloride hexahydrate (0.3 g/L), Copper(II)sulfate pentahydrate (0.3 g/L), disodium molybdenum dehydrate (0.4 g/L), calcium chloride dehydrate (4.5 g/L), iron sulphate heptahydrate (3 g/L), boric acid (1.0 g/L), and potassium iodide (0.1 g/L).

Vitamin solution was composed of biotin (D-; 0.05 g/L), calcium pantothenate (D+; 1 g/L), nicotinic acid (5 g/L), myo-inositol (25 g/L), pyridoxine hydrochloride (1 g/L), p-aminobenzoic acid (0.2 g/L), and thiamine hydrochloride (1 g/L).

DM1 X-α-gal plates were composed of DM1 salts, 20 g/L glucose, trace element solution, vitamin solution, 2 mL/L X-α-gal (16 mg/mL), 20 g/L agar.

DM1 salt solution was composed of 2.28 g/L urea, 3 g/L potassium phosphate monobasic, and 0.5 g/L magnesium sulfate heptahydrate.

Butterfields Phosphate Buffer was composed of 1.25 mL/L of Stock Solution (26.22 g/L Potassium Dihydrogen Phosphate and 7.78 g/L Sodium Carbonate) and 5 mL/L of a Magnesium Chloride solution (81.1 g/L MgCl₂·6H₂O). The resulting solution was autoclaved to sterilize, and pH adjusted to 7.2.

CNB1 shake flask media was composed of urea (2.3 g/L), magnesium sulfate heptahydrate (0.5 g/L), potassium phosphate monobasic (3.0 g/L), trace element solution (1 mL/L) and vitamin solution (1 mL/L), glucose (120.0 g/L), 2-(N-Morpholino)ethanesulfonic

acid (MES) (97.6 g/L). After dissolving all medium components, the pH of the medium was adjusted to an initial pH of 5.8 using an appropriate base (e.g, KOH).

Table 0: Primers sequences

Identifier	SEQ ID NO:	Sequence (5'-3')
0611166	152	TAAAACGACGGCCAGTGAATTCCGCGGCGGCCGCGAGTCCATCGGTTCTGTCA
0611167	153	CATAAGAAAATCAAAGACAGAAGGCGCGCCTTTGCTAGCATTTTTGTGTTTGCT GTGT
0611168	154	ACACAGCAAAACACAAAAATGCTAGCAAAGGCGCGCCTTCTGTCTTTGATTTTCTTATG
0611169	155	GGGGGAAAGA ACTACCAATAGGCCTCCTTTAATT CGGAGAAAATC
0611170	156	GATTTTCTCCGAATTAAGGAGGCCTATTGGTAGTTCTTTCCCCC
0611171	157	AAAATAAACTAGTAAAATAAATTAATTATCTAGAGAGGGGGTTATAT
0611172	158	ATATAACCCCTCTCTAGATAATTAATTAATTTATTTACTAGTTTATTTT
0611173	159	GACCATGATTACGCCAAGCTCCGCGGCGGCCGCCAGTCAAAACCTTCTTCTC
0611174	160	TAAAACGACGGCCAGTGAATTCCGCGGCGGCCGCCTTTGAAGGAGCTTGCCA
0611175	161	CTATTCCTTCCTCAAATTGCTGTTTAAACGCGTTGAAGATCTATTCTCC
0611176	162	GGAGAATAGATCTTCAACGCGTTTAAACAGCAATTTGAGGAAGGAATAG
0611177	163	AATGTTCATTTTACATTCAGATGTTAATTAAGGTCTAGATGTGTTTGTTGTGTG
0611178	164	CACACAAACAAACACATCTAGACCTTAATTAACATCTGAATGTAAAATGAACATT
0611179	165	GACCATGATTACGCCAAGCTCCGCGGCGGCCGCAATGCCAAGAGTTATGGGGC
0611184	166	GGCTACCCTATATATGGTGAGCG
0611185	167	GGGTCCAAGTTATCCAAGCAG
0611186	168	CCTTAATTAACCGTAAAGTTGTCTCAATG
0611189	169	CAGCAAAACACAAAAATTCTAGAAAATTTAATTAACATCTGAATGTAAAATGAAC
0611191	346	CCTTAATTAATTATCCACGGAAGATATGATG
0611195	170	GTTCATTTTACATTCAGATGTTAATTAATTTTCTAGAATTTTTGTGTTTTGCTG
0611196	171	GCTCTAGATAAAATGCCCTCTTATTCTGTGCGC
0611199	347	GCTCTAGATAAAATGTCCCAAGGTAGAAAAGC
0611225	172	GACTGGATCATTATGACTCC
0611245	272	CGAACCAATTCAAGAAAACCAAC
0611250	173	ACGCCTTGCCAAATGCGGCCGCGAGTCCATCGGTTCTGTGTCAGA
0611251	174	TCAAAGACAGAATTAATTAAGCTCTAGAATTTTTGTGTTTTGCTGTGTT
0611252	175	AACACAAAATTCTAGAGCTTAATTAATTCTGTCTTTGATTTTCTTATG
0611253	176	GTTTAAACCTTTAATTCGGAGAAAATCTGATC
0611254	177	GTTAACGGTACCGAGCTCTAAGTAGTGGTG
0611255	178	AACCGATGGACTCGCGGCCGCATTTGGCAAGGCGTATCTAT
0611256	179	GGCGCCAGCAATTTGAGGAAGGAATAGGAG
0611257	180	AAACTATTAGATTAATTAAGCTCGCGATGTGTTTGTTGTGTGTTTTGTGTG
0611258	181	CAAACAAACACATCGCGAGCTTAATTAATCTAATAGTTTAATCACAGCTTAT
0611259	182	TACATTATGGTAGCGGCCGCGTGTGACATTTTGATCCACTCG
0611260	183	TGGATCAAATGTACACGCGGCCGCTACCATAATGTATGCGTTGAG
0611261	184	GGGCCCTAAAAGTGTTGGTGTATTAGA
0611263	185	GCTAGCTCAACAACTCTTTATCAGATTTAGCA

0611264	186	GCTAGCGAGGAAAAGAAGTCTAACCTTTGT
0611266	187	TCGCGATAAAATGTCAACTGTGGAAGATCACTCCT
0611267	188	TTAATTAAGCTGCTGGCGCTTCATCTT
0611268	189	TCGCGATAAAATGTCCAGAGGCTTCTTTACTG
0611269	190	TTAATTAACATAAAGGTCTCTCACGACAGAG
0611283	191	GTTAACCCGGTTTAAACATAGCCTCATGAAATCAGCCATT
0611284	192	GGGCCCATATGGCGCCCGGGGCGTTGAAGATCTATTCTCCAGCA
0611295	193	GTTTAAACGATTGGTAGTTCTTTCCCCCTC
0611296	194	AATAAATTAAGGGCCCTTTATCGCGAGAGGGGGTTATATGTGTAAA
0611297	195	TATAACCCCTCTCGCGATAAAGGGCCCTTAATTTATTTTACTAGTTTAT
0611298	196	GTTTAAACTTTTGTAGCACCTCCTGGT
0611376	197	CAGCAAAACACAAAAAGCTAGCTAAAATGTTACGTACCATGTTCAAAA
0611377	198	GAAAATCAAAGACAGAAGGCGCGCCTTATGCTGTAACAGCCTGCGG
0611378	199	CAGCAAAACACAAAAAGCTAGC TAAA ATGTTAAGAACCATGTTCAAATC
0611379	200	GAAAATCAAAGACAGAAGGCGCGCC TTATGCAGTAACAGCTTGTGGG
0611552	273	TCTGTCCCTTGCGCACGC
0611553	274	CTTTTCAAACAGATAAGTACC
0611554	201	GCATGGTGGTGAAGCGACG
0611555	202	GGTGCTGCATTGCTGCTG
0611622	275	ATGGGCTGACCTGAAAATTC
0611631	203	TGTATACAGGATCGAAGAATAGAAG
0611632	204	GAACGTCTACAACGAGGTGAAC
0611661	205	GGCGCGCCTCGCGATAAAATG
0611662	206	AGGGCCCTTAATTAATTATGCAGTAACAGCTT
0611717	207	CGCTACGATACGCTACGATA
0611718	208	CTCCCTTCCCTGATAGAAGG
0611814	209	GGGGAGCAATTTGCCACCAGG
0611815	210	CTCCTTCATTTAACTATAACCAGACG
0611816	211	GACAGATGTAAGGACCAATAGTGTCC
0611817	212	CCATATCGAAATCTAGCCCGTCC
0611828	213	CATAAGAAAATCAAAGACAGAAGGCGCGCCTTTGCTAGCTTTTTGTGTTT TGCTGTGT
0611954	348	GCTAGCTAAAATGTTTGGTAATATTTCCCA
0611957	349	TTAATTAACATTTTATCTAATGATCCTC
0611997	350	TCTAGATAAAATGTCTATTAGTGAAAAATATTTTCCTCAAG
0611998	351	TTAATTAACTTTTAAATTTTGAAAAAGCTTGATCAATAATGG
0612055	214	ATTGGACACAACATTATAT
0612150	238	ACGCGTCGACTCGACATTTGCTGCAACGGC
0612151	239	CTAGTCTAGATGTTGTTGTTGTTGTCGT
0612271	215	GTAAAACGACGGCCAGTGAATTGTTAACATAGGCTCCAACATCTCG
0612272	216	GGAACCGATGGACTCGCGGCCGC GTGGGATATTGGAAG
0612273	217	GGAGGTGCTACAAAAGGAATTC
0612274	218	GACCATGATTACGCCAAGCTCCGCGGAGTCAAAACCTTCTTCTCTACC
0612275	219	GTAAAACGACGGCCAGTGAATTC TTTGAAGGAGCTTGCCAAGAAAC
0612276	220	CCTATTCCTTCTCAAATTGCTG
0612277	221	ATAACTCTTGGCATTGCGGCCGCAAGTTAGTTAGAGC
0612278	222	ATGACCATGATTACGCCAAGCTCCGCGGCAAGACGGTGTATTAGTGCT TG
0612356	223	CACAAAACACACAAACAAACACAGCTAGCAAAGGCGCGCCATCTAATAG TTTAATCACAGCTTA
0612357	224	GCCGTTGCAGCAAATGTGAGGCCTGTGTGACATTTTGATCCACTCG
0612358	225	CGAGTGGATCAAATGTACACAGGCCTCGACATTTGCTGCAACGGC
0612359	226	CATTTTACATTGAGATGTTAATTAATTATCTAGATGTTGTTGTTGTCG
0612360	227	CGACAACAACAACAACATCTAGATAATTAATTAACATCTGAATGTAAAATG
0612361	228	GCTCTAACTAACTTGCGCGCCGCTTTTATTATAAAATTATATATTCTT
0612366	276	GCTGAAAATATCATTGAGAGCAT
0612367	277	ACTGTTGATGTCGATGCC
0612378	229	AACACACAAACAAACACAGCTAGCTAAAATGTTAAGAACTATGTTTA

0612379	230	GATTAACTATTAGATGGCGCGCCTTATGCAGTAACTGCTTGTGGA
0612470	231	CGACGGCCAGTGAATTCGTTAACCCGTTTCGATGGGATTCCC
0612471	232	CAGGAACCGATGGACTCGCGGCCGCTCCCTTCTCTAAATGGACTGC
0612472	233	TATATAATTTTATAATAAAAGCGGCCGCACCAGGGGTTTAGTGAAGTC
0612473	234	CATGATTACGCCAAGCTCCGCGGCCATAACTGACATTTATGGTAAGG
0612579	278	TCTGAATGCAGTACGAGTTG
0612695	240	CAAAACACAGCAAAACACAAAAAGCTAGCATGTATAGAACCTTGATGAG
0612698	242	CAAAACACACAAACAAACACAGCTAGCATGTACAGAACGTTAATGTCTGC
0612724	241	CAAAGACAGAAGGCGCGCCTTATAAGATGGTTCTCGCTGG
0612725	243	GTGATTAACTATTAGATGGCGCGCCTTACAGGATGGTTCTGGCAGG
0612794	279	GAAGGGGGTCCAAGTTATCC
0612795	235	CCAACAATCTTAATTGGTGAC
0612891	236	GGCTGTTACCGCCTAATTAA
0612893	237	GTTCTTAACATTTTAGCTAGCTG
0612908	280	GATATGGGCGGTAGAGAAGA
0612909	281	GCTCCTTCAAAGGCAACACA
0612910	282	TGAACTATCACATGAACGTA
0612911	283	TCAAGGTAGGGTCACTTAAC
0613034	284	TGATTCCTTCAATCACAGGT
0613035	285	AACCGACCTATCGAATGCCT
0613178	286	ACCATGATTACGCCAAGCTTGGTACCTTGGGGTTTACGCTTACAGCGTA CT
0613179	287	TCAACGCCCCGGGGGATCTGGATCCGCGGCCGCAAGAAATTCCTTTCTT TTCCCTTTATA
0613180	288	AACCGATGGACTCCTCGAGGGATCCGCGGCCGCGCATAACAAAATTGTG CCTAACCCA
0613181	289	ACGACTCACTATAGGGCGAATTGGGCCCCGGGAAAAGGAGAGAGAAAA GGAGA
0613183	290	TATGACCATGATTACGCCAAGCTTGGTACCTCCTACAGAAGTAGCACCA GCACCA
0613184	291	GGAACCGATGGACTCCTCGAGGGATCCGCGGCCGCGAGACTACCGTGTA AATAAGAGTACC
0613185	292	TCTTCAACGCCCCGGGGGATCTGGATCCGCGGCCGCGCATTTGATATAAAC GCTTCTATAATA
0613186	293	GTAATACGACTCACTATAGGGCGAATTGGGCCCAACATCTGCTGCTGTA ATATATTCA
0613241	294	GCATGTCTGTAACTCTCAAACCAT
0613242	295	TCCATAATCCAATAGATCATCCC
0613243	296	AACACAATGGAACCAACCTAGT
0613416	297	ATTTACACGGTAGTCTGCGGCCGCCATAGCCTCATGAAATCAGCC
0613417	298	CTCTAGGTTCACTGGTTGTTTCTTGGGCTGCCTCCTTCAA
0613418	299	TTGAAGGAGGCAGCCCAAGAAACAACCAAGTGAACCTAGAG
0613419	300	TATTATAGAAGCGTTTATATCAAATGCGGCCGCGGATCCAGATCCCCCG GGGCGTT
0613550	301	AATGATCAACTTGAGAGGTA
0613551	302	CAGGTCTGTTACATAAAGCA
0613688	303	GTTTACGCTCAAATCTCCTCC
0613689	304	GGTACATGAAGCAGGCTTTGAAGG
0613695	305	GATTGTGTCCGTCAGCCTTTGCTC
0613746	306	TACCATATTTTCAGAGGATCA
0613747	307	AGGATGTTCTTGCTGCAAGT
0614233	308	GATGATATAGTTGATGCTTTCCAAAG
0614626	344	AGGGTACCTTAGTACGAAGG
0614627	345	CTATTCTTACGATGAAGGCG
CM647	262	AATGATCCATGGTCCGAGAG
oACN48	309	GGGCCCTTCATTTACGAAATAAAGTGCCGCGG
oACN49	310	GCGGCCGCAAATAAATTTAAATAAACGATATCAAAATTC
oACN50	311	CGACGCCAAAGAAGGGCTCAGCCGAAAAAG

oACN51	312	CCATTTCTTTTCGGCTGAGCCCTTCTTTGGC
oACN52	313	GCGGCCGCAATAACCTCAGGGAGAACTTTGGC
oACN53	314	GAGCTCCCAAACAAGGTCTCAGTAGGATCG
oACN54	315	CGCCATAAGGAGAGGCTGTAGATTTGTC
oACN55	316	CCAGGACATCTTGCTTGCAATGTCG
oANN1	317	GTTCCATCGGGCCCCCTAAAGGTCTCTCACGACAG
oANN2	318	CAAACACA TCGCGA TAAAATGTCCAGAGGCTTC
oANN5	319	GCAAGACCTTGATCTGAAGGG
oANN6	320	CGAACCAATTCAAGAAAACCAACAG
oANN7	321	GGGCCC GTCCCTTGCGACGCCCTGATC
oANN8	322	GCGGCCGCTATTTTTGTGTTTTGCTGTGTTTTG
oANN9	323	GCGGCCGCC ATCTGAATGTAAAATGAACATTAAATG
oANN10	324	GAGCTCCCCCAGTTGTTGTTGCAATTAAC
oANN11	325	GAAGAGACGTACAAGATCCGCC
oANN12	326	TAGGAATGGTGCATCATCCAAC
oANN13	327	TTCTTATCTGAAACTCCGAGTTCGCAAAGAAGGTTGAAG
oANN14	328	TTATTGAAATTAATCCAAGGATTCAAGTTGAACATACAATTACTG
oANN15	329	TTTCCAAAAAAGTTCGTGAGTTCGATGGTTGTATGATTATGG
oANN16	330	CCTCTACCACCACCACCAAATG
oANN20	331	GGGAAGAACTAAGAAGAAGTATG
oCA405	260	GCAACTGATGTTACGAATGCG
oCA406	261	TTGCCGTTGCAGCAAATCTC
oHJ1	332	CAATCCTTCTAGAGAGGGGGTTATATGTGTAAATATAGAGTTTG
oHJ2	333	TTCTACCCTCGAGATTGGTTCTTTCCCCCTCTCAAG
oHJJ116	334	GTGGTCAATCTAGAAAATATGACTGACAAAATCTCCCTAGGTAC
oHJJ117	335	CAATTTTGGAGCTGATTCCAAATCGTAAAC
oJLJ28	264	GCACCAAGAGCAGTTTTCCCATCTATTG
oJLJ29	265	CCATATAGTTCTTTTCTAACATCAACATCACACTTC
oJLJ30	266	GGAGAATAGATCTTCAACGCTTTAATAAAGTAGTTTG
oJLJ31	267	CGTGTTGCGCAATAAAACCAATGAC
oJLJ43	336	CAAGAGTATCCCATCTGACAGGAACCGATGG
oJLJ44	337	GCTGGAGAATAGATCTTCAACGCCCCG
oJLJ45	338	CGGAGAAGGCGTATAAAAAGGACACGGAG
oJLJ46	339	GGATAAAAGTATTACATACGTACAGGATTGTGTATTAGTGTATTTG
oJLJ57	340	CCTCCAGTGTTTTTCTCTCTGTCTCTTTGTTTTTTTTTC
oJY11	268	CCTCGAAGAGCTTGAATTTG
oJY12	269	GTAGTGAATGTCCGGATAAG
oJY13	270	GCAAGGTCATGAGGTAAAG
oJY14	271	AACACTTATGGCGTCTCCTC
oJY44	341	GGTTAATTAATTTATTTGTACATAAAAACCATATAAATGTAAAAGC
oJY45	342	GAATTCCTTTAATTCGGAGAAAATCTGATCAAGAG
WG26	263	ACGGCAGTATACCCTATCAGG

Miscellaneous sequences

Promoters: The PDC, TDH3, ENO1, and PGK1 promoters described in the Examples were derived from the *I. orientalis* sequences of SEQ ID NOs: 244, 245, 246, and 247, respectively. Terminators: The TAL, TKL, RKI, and PDC terminators described in the Examples were derived from the *I. orientalis* sequences of SEQ ID NOs: 248, 249, 250, and 251, respectively. The URA3 promoter, ORF, and terminator described in the Examples were derived from the *I. orientalis* sequence of SEQ ID NO: 252.

Example 1A: Selection of host yeast cells based on 3-HP tolerance

[00164] A set of wild-type yeast strains were tested for their ability to grow in the presence of 3-HP.

[00165] The range of 3-HP concentrations to utilize in primary screening procedures was determined by evaluating the ability of seven wild-type yeast strains (Table 1, set A) to grow on media containing varying levels of 3-HP. 3-HP used in these experiments was chemically synthesized from an aqueous acrylic acid solution (30%) and a CO₂ catalyst at 200 psi and 175°C as described in WO04/076398. Residual acrylic acid was removed using a countercurrent extraction with isopropyl ether at room temperature (see WO05/021470).

[00166] Cells were streaked onto YPD plates and grown overnight. A cell slurry with an OD₆₀₀ of around 4 was made in YPD media, pH 3.0, and this slurry was used to inoculate microtiter wells containing various concentrations of 3-HP (pH 3.9) to an OD₆₀₀ of 0.05. Plates were covered with a gas permeable membrane and incubated in a 30°C/300 RPM shaker overnight. Optical densities for each well were measured at a wavelength of 600 nm in a GENios model plate reader (Tecan), and plates were observed visually for growth. The highest 3-HP concentration that one or more of the strains grew in (125 g/L) was chosen as the upper range for primary screening procedure.

Primary Screening

[00167] For the primary screening procedure, 89 wild-type yeast strains were screened for growth on microtiter plates at 0 g/L, 75 g/L, 100 g/L, or 125 g/L 3-HP (pH 3.9) using the same protocol used for range finding. A fresh YPD plate was used for each strain, and a slurry with an OD₆₀₀ of around 4 was made in YPD media, pH 3.0. The slurry was used to inoculate each well to an OD₆₀₀ of 0.05. Plates were covered with a gas permeable membrane, and incubated in a 30°C/300 RPM shaker overnight. Optical densities for each well were measured at a wavelength of 600 nm in a GENios model plate reader, and plates were observed visually for growth. A similar protocol was run to evaluate growth at lactic acid concentrations of 0 g/L, 30 g/L, 45 g/L, and 60 g/L. Table 1 summarizes the highest concentration of 3-HP or lactic acid at which growth was observed.

[00168] Fifteen strains were identified that were capable of growing at 100 g/L 3-HP or grew well at 75 g/L 3-HP (Table 1, set B). To further narrow the strains, a second microtiter plate test was conducted. This test was similar to the first, but utilized 3-HP concentrations of 100 g/L, 112.5 g/L, and 125 g/L (pH 3.9). From this test, eleven strains were identified (Table 1, set C) that grew well at 75 g/L 3-HP or showed some growth at both 75 and 112.5 g/L 3-HP. These eleven strains were advanced to the secondary screening. Four strains that had poor growth at 75 g/L 3-HP and no growth at 112.5 g/L 3-HP were not advanced to the secondary screening. It is expected that strains not advancing to the secondary screen would exhibit

economically inferior performance in a commercial fermentation process. However, it is possible that one or more such strains could nonetheless meet the minimum requirements for a commercially viable fermentation process.

Table 1: Growth in 3-HP or lactic acid

Yeast strain	Culture collection #	Set	Primary screen: 3-HP (g/L)	Primary screen: lactic acid (g/L)
<i>Bulleromyces albus</i>	ATCC 96272		No growth at 0 g/L (NG)	NG
<i>Candida blankii</i>	ATCC 18735		0	30
<i>Candida boidinii</i>	PYCC 70-104		0	30
<i>Candida catenulata</i>	ATCC 20117		0	NG
<i>Candida etchellsii</i>	PYCC 60-8		0	30
<i>Candida famata</i>	ATCC 20284		0	0
<i>Candida fluvialilis</i>	ATCC 38623		0	0
<i>Candida geochares</i>	Cargill 1978		75	60
<i>Candida guilliermondii</i>	ATCC 20118		0	0
<i>Candida intermedia</i>	ATCC 20178		0	0
<i>Candida kefyr</i>	ATCC 44691		75	30
<i>Candida lactiscondensi</i>	ATCC 96927		0	NG
<i>Candida lambica</i>	ATCC 38617	B, C	75	45
<i>Candida methanosorbosa</i>	ATCC 20361		0	NG
<i>Candida milleri</i>	ATCC 60591		0	30
<i>Candida milleri</i>	ATCC 60592		0	45
<i>Candida naeodendra</i>	ATCC 56465		0	NG
<i>Candida parapsilosis</i>	ATCC 20179		0	0
<i>Candida pignaliae</i>	ATCC 36592		0	NG
<i>Candida pseudolambica</i>	ATCC 96309		0	0
<i>Candida rugosa</i>	ATCC 20306		0	45
<i>Candida shehatae</i>	NCYC 2389		0	0
<i>Candida sonorensis</i>	ATCC 32109	A	0	30
<i>Candida sorbophila</i>	Cargill 1973		75	45
<i>Candida sorboxylosa</i>	ATCC 24120		0	30
<i>Candida sorosivorans</i>	ATCC 38619	B	100	60
<i>Candida tenuis</i>	MUCL 47216 or MUCL 31253B		0	NG
<i>Candida valida</i>	ATCC 28525	B	75	45
<i>Candida vanderwaltii</i>	MUCL 300000		0	45
<i>Candida zemplinina</i>	PYCC 04-501	A,B,C	100	60
<i>Candida zeylanoides</i>	ATCC 20347		75	45
<i>Citeromyces matritensis</i>	ATCC 34087		0	NG

<i>Debaryomyces castellii</i>	PYCC 70-1022		0	30
<i>Debaryomyces hansenii</i>	ATCC 90624		0	NG
<i>Debaryomyces polymorphus</i>	ATCC 20280		0	0
<i>Dekkera anomala</i>	ATCC 20277		0	0
<i>Dekkera lambica</i>	ATCC 10563		0	0
<i>Hyphopichia burtonii</i>	ATCC 20030		0	0
<i>Issatchenkia orientalis</i>	ATCC 24210	B, C	100	45
<i>Issatchenkia orientalis</i>	ATCC 60585	B, C	100	45
<i>Issatchenkia orientalis</i>	ATCC PTA-6658	A,B,C	100	60
<i>Issatchenkia orientalis</i>	CD1822 (Cargill collection – see description below)	A,B,C	100	60
<i>Kluyveromyces lactis</i>	ATCC 8585	A	0	0
<i>Kluyveromyces marxianus</i>	ATCC 52486	A	75	45
<i>Kluyveromyces thermotolerans</i>	ATCC 52709		0	30
<i>Kodamaea ohmeri</i>	ATCC 20282		0	45
<i>Kluyveromyces yarrowii</i>	ATCC 36591		NG	NG
<i>Lipomyces starkeyi</i>	ATCC 12659		0	0
<i>Lipomyces tetrasporus</i>	ATCC 56306		NG	NG
<i>Metschnikowia pulcherrima</i>	ATCC 9889		0	0
<i>Myxozyma kluyveri</i>	ATCC 76214		NG	NG
<i>Nematospora coryli</i>	ATCC 20292		0	NG
<i>Pachysolen tannophilus</i>	NCYC 614		0	30
<i>Pichia anomala</i>	ATCC 2102		0	0
<i>Pichia fermentans</i>	ATCC 28526		0	30
<i>Pichia fluxuum</i>	ATCC 28778		NG	NG
<i>Pichia jadinii</i>	ATCC 9950		0	0
<i>Pichia membranifaciens</i>	NCYC 2696	B,C	125	60
<i>Pichia nakasei</i>	ATCC 24116		0	0
<i>Pichia silvicola</i>	ATCC 16768		0	0
<i>Pichia stipitis</i>	CBS 6054		0	0
<i>Pichia strasburgensis</i>	ATCC 34024		0	0
<i>Pichia tannicola</i>	ATCC 2261		0	0
<i>Pichia toletana</i>	ATCC 58362		NG	NG
<i>Saccharomyces cerevisiae</i>	ATCC 96784		75	30
<i>Saccharomyces bayanus</i>	ATCC 90739		0	45
<i>Saccharomyces bulderi</i>	MYA-402	B, C	100	60
<i>Saccharomyces bulderi</i>	MYA-404	B,C	100	45
<i>Saccharomyces capsularis</i>	ATCC 20033		0	0
<i>Saccharomyces</i>	CEN-PK 113-7D	A,B	100	45

<i>cerevisiae</i>				
<i>Saccharomyces ludwigii</i>	NCYC 734		75	45
<i>Saccharomycopsis crataegensis</i>	MUCL 44417		0	0
<i>Saccharomycopsis javensis</i>	MUCL 31237		0	45
<i>Saccharomycopsis vini</i>	NRRL Y-7290		0	0
<i>Saccharomyces uvarum</i>	ATCC 76514		0	0
<i>Schizosaccharomyces japonicus</i>	ATCC 10660		0	0
<i>Schizosaccharomyces pombe</i>	NCYC 535	B,C	100	60
<i>Torulaspora delbrueckii</i>	ATCC 52714		75	0
<i>Torulaspora pretoriensis</i>	ATCC 42479	B	75	0
<i>Wickerhamiella occidentalis</i>	CBS 8452		0	30
<i>Yamadazyma guilliermondii</i>	ATCC 90197		0	0
<i>Yamadazyma haplophila</i>	ATCC 20321		0	0
<i>Yamadazyma stipitis</i>	ATCC 201225		0	0
<i>Yarrowia lipolytica</i>	ATCC 46330		0	45
<i>Zygosaccharomyces bailii</i>	ATCC 36946		75	45
<i>Zygosaccharomyces bisporus</i>	NCYC 3134		0	45
<i>Zygosaccharomyces kombuchaensis</i>	NCYC2897		0	60
<i>Zygosaccharomyces lentus</i>	NCYC 2928	B,C	100	45
<i>Zygosaccharomyces rouxii</i>	ATCC 34890		75	30

[00169] *I. orientalis* strain CD1822 tested above was generated by evolving *I. orientalis* ATCC PTA-6658 for 91 days in a glucose limited chemostat. The system was fed with 15 g/L dextrose in a DM medium, and operated at a dilution rate of 0.06 h^{-1} at pH=3 with added lactic acid in the feed medium. The conditions were maintained with a low oxygen transfer rate of approximately $2 \text{ mmol L}^{-1}\text{h}^{-1}$, and dissolved oxygen concentration remained constant at 0% of air saturation. Single colony isolates from the final time point were characterized in two shake flask assays. In the first assay, the strains were characterized for their ability to ferment glucose to ethanol in the presence of 25 g/L total lactic acid with no pH adjustment in the DM1 defined medium. In the second assay, the growth rate of the isolates were measured in the presence of 25, 32 and 45 g/L of total lactic acid, with no pH adjustment in DM1 defined medium. Strain CD1822 was a single isolate selected based on the measured fermentation rates and growth rates. Other methods for evolving *I. orientalis* include, but are not limited to, repeated serial subculturing under the selective conditions as described in

e.g., U.S. Patent No. 7,629,162. Such methods can be used in place of, or in addition to, using the glucose limited chemostat method described above. As can be appreciated by one of skill in the art, strains could be generated using a similar evolution procedure in the presence of added 3-HP rather than lactic acid to develop improved 3-HP tolerance. Additionally, strains could be mutagenized prior to selection, as described herein (e.g., see Example 1B).

Secondary Screening

[00170] For the first part of the secondary screen, growth rates were measured at pH 3.9 in YPD media containing 0 g/L, 35 g/L, or 75 g/L 3-HP. Shake flasks were inoculated with biomass harvested from seed flasks grown overnight to an OD₆₀₀ of 6 to 10. 250 mL baffled growth rate flasks (50 mL working volume) were inoculated to an OD₆₀₀ of 0.1 and grown at 250 rpm and 30°C. Samples were taken throughout the time course of the assay and analyzed for biomass growth via OD₆₀₀. The resulting OD₆₀₀ data was plotted and growth rates were established.

Table 2: Growth rate (μ) in 3-HP

Strain	0 g/L 3-HP	35 g/L 3-HP	75 g/L 3-HP
<i>Issatchenkia orientalis</i> ATCC 60585	0.56	0.51	0.29
<i>Issatchenkia orientalis</i> CD1822	0.62	0.52	0.28
<i>Candida lambica</i> ATCC 38617	0.65	0.53	0.30
<i>Candida valida</i>	0.51	0.32	0.14
<i>Issatchenkia orientalis</i> PTA-6658	0.61	0.53	0.32
<i>Issatchenkia orientalis</i> 24210	0.58	0.51	0.26
<i>Saccharomyces bulderi</i> MYA 402	0.53	0.45	0.28
<i>Pichia membranifaciens</i>	0.41	0.39	0.32
<i>Saccharomyces bulderi</i> MYA 404	0.55	0.44	0.27
<i>Schizosaccharomyces pombe</i>	0.41	0.35	0.21
<i>Zygosaccharomyces lentus</i>	0.61	0.41	0.20

[00171] For the second part of the secondary screen, glucose consumption was measured for the same ten strains at pH 3.9 in YPD media containing 100 g/L glucose and 0 g/L, 35 g/L, or 75 g/L 3-HP. Shake flasks were inoculated with biomass harvested from seed flasks grown overnight to an OD₆₀₀ of 6 to 10. 250 mL baffled glycolytic assay flasks (50 mL working volume) were inoculated to an OD₆₀₀ of 0.1 and grown at 250 RPM and 30°C. Samples were taken throughout the time course of the assay and analyzed for glucose consumption using a 2700 Biochemistry Analyzer from Yellow Springs Instruments (YSI). The resulting data was plotted and glucose consumption rates were established.

Table 3: Glucose consumption rate (g/L/hr) in 3-HP

Strain	0 g/L 3-HP	35 g/L 3-HP	75 g/L 3-HP
<i>Issatchenkia orientalis</i> ATCC 60585	5.5	4.2	3.3
<i>Issatchenkia orientalis</i> CD1822	5.5	4.2	4.2

<i>Candida lambica</i> ATCC 38617	4.2	4.2	3.5
<i>Candida valida</i>	5.5	2.2	2.1
<i>Issatchenkia orientalis</i> PTA-6658	5.5	4.2	4.1
<i>Issatchenkia orientalis</i> 24210	4.2	4.2	3.8
<i>Saccharomyces bulderi</i> MYA 402	4.2	4.2	4.0
<i>Pichia membranifaciens</i>	0.4	2.1	1.2
<i>Saccharomyces bulderi</i> MYA 404	4.2	4.2	3.8
<i>Schizosaccharomyces pombe</i>	2.5	3.1	2.0
<i>Zygosaccharomyces lentus</i>	3.4	0.8	0.3

[00172] Four of the strains (*P. membranifaciens*, *S. pombe*, *C. valida*, and *Z. lentus*) did not achieve the 2.5 g/L/hr glucose utilization rate under the 75 g/L 3-HP (pH 3.9) conditions that would be required for an economic fermentation process.

[00173] To identify the leading strains in 3-HP, strain performance was graded in three categories. Two of these categories were based on different aspects of growth rate: 1) growth rate at highest acid concentration and 2) slope of the growth rates plotted against acid concentration. The third category was the glycolytic rate at the highest acid concentration. This grading was done on a normalized scale using the highest and lowest value for each rating as the normalized boundaries. Each strain thus received a grade between 0 and 1 for each category, with 1 being the highest possible score. The overall rating of a strain was the sum of the normalized value for the three categories. A weighted score was made in which the growth rate and glycolytic rate were equally weighted. In this case the glycolytic rate at the highest acid concentration was weighted at 50%, while the two growth rate ratings were weighted at 25% each. Normalized values per category and sum and weighted scores are summarized in Table 4.

Table 4: Strain grades in 3-HP

Strain	Growth rate @ 75 g/L 3- HP	Growth rate slope	Glycolic rate	Sum Score	Weighted score
<i>Issatchenkia orientalis</i> ATCC PTA-6658	1.00	0.37	0.97	2.34	0.83
<i>Saccharomyces</i> <i>bulderi</i> MYA 402	0.78	0.49	0.94	2.21	0.79
<i>Issatchenkia orientalis</i> CD1822	0.78	0.21	1.00	1.99	0.75
<i>Saccharomyces</i> <i>bulderi</i> MYA 404	0.72	0.42	0.90	2.04	0.74
<i>Issatchenkia orientalis</i> ATCC 60585	0.83	0.44	0.77	2.04	0.70
<i>Issatchenkia orientalis</i> 24210	0.67	0.28	0.90	1.85	0.69
<i>Candida lambica</i> ATCC 38617	0.89	0.19	0.82	1.90	0.68
<i>Pichia</i> <i>membranifaciens</i>	1.00	1.00	0.23	2.23	0.62

<i>Schizosaccharomyces pombe</i>	0.39	0.65	0.47	1.51	0.50
<i>Candida valida</i>	0	0.14	0.40	0.54	0.24
<i>Zygosaccharomyces lentus</i>	0.33	0	0	0.33	0.08

[00174] Of the strains tested, strains from the species *I. orientalis*, *C. lambica*, and *S. bulderi* showed the greatest potential as production hosts for 3-HP at low pH.

[00175] The same procedures were utilized to screen, rate, and score the original 91 wild-type yeast strains from the primary screen with media containing 0, 25, and 50 g/L lactic acid at pH 2.85 (~80% free acid). Normalized values and weighted and summed scores were derived for 13 strains that were advanced to the secondary screen.

Table 5: Strain grades in lactic acid

Strain	Growth rate @ 50 g/L lactic acid	Growth rate slope	Glycolic rate	Sum Score	Weighted score
<i>Candida lambica</i> ATCC 38617	0.92	1.00	1.00	2.92	0.98
<i>Issatchenkia orientalis</i> ATCC PTA-6658	0.94	0.95	1.00	2.89	0.97
<i>Issatchenkia orientalis</i> CD1822	1.00	0.86	1.00	2.86	0.97
<i>Issatchenkia orientalis</i> 24210	0.89	0.73	1.00	2.62	0.91
<i>Candida zemplinina</i>	0.22	0.95	1.00	2.17	0.79
<i>Saccharomyces bulderi</i> MYA 404	0.47	0.45	1.00	1.92	0.73
<i>Saccharomyces bayanus</i>	0.08	0.91	0.96	1.95	0.73
<i>Saccharomyces bulderi</i> MYA 402	0.50	0.23	1.00	1.73	0.68
<i>Candida milleri</i>	0	0.64	0.92	1.56	0.62
<i>Candida sorosivorans</i>	0.28	0.95	0.59	1.82	0.60
<i>Kodamaea ohmeri</i>	0.42	0	0.76	1.18	0.49
<i>Candida geochares</i>	0.17	0.27	0.69	1.13	0.46
<i>Saccharomyces javensis</i>	0.11	0.68	0	0.79	0.20

[00176] For lactic acid only *S. javensis* did not achieve the 2.5 g/L/hr glucose utilization rate at pH 2.85 in media with 50 g/L lactic acid. While *I. orientalis*, *C. lambica*, and *S. bulderi* showed acid tolerance for both 3-HP and lactic acids, there were a number of strains that were tolerant for only one of the acids. This can also be seen in the results of the primary screen (Table 1). For example, *C. milleri*, *C. rugosa*, *C. vanderwaltii*, *K. ohmeri*, *S. bayanus*, *S. javensis*, *Y. lipolytica*, *Z. bisporus*, and *Z. kombuchaensis* all demonstrated growth at 45-60 g/L lactic acid but no growth at even the lowest concentration of 3-HP tested (35 g/L). Thus, the tolerance of a strain to one organic acid cannot definitively be used as a predictor

of its tolerance for other acids. This is further highlighted by comparing the strains that showed 3-HP resistance above with the list of eight strains identified as preferred hosts for organic acid production in WO03/049525. While two of those strains (*C. diddensiae* and *C. entomophila*) could not be obtained for testing, the other six were included in the primary screen described above. Of these six, only *C. krusei* (tested as *I. orientalis*) was able to grow in the presence of 35 g/L 3-HP.

Example 1B: Mutagenesis and selection of mutant strains having resistance to 3-HP

[00177] Yeast cells selected in Example 1A are subjected to mutagenesis and exposed to selection pressure in order to identify mutants with high 3-HP tolerance.

[00178] For example, yeast cells from a fresh YP (yeast extract/peptone) + 20 g/L glucose plate or liquid culture (OD₆₀₀ 1-4) are resuspended in sterile water to an OD₆₀₀ of around 10. 200 µL aliquots of this cell suspension are pipetted into individual tubes and exposed to 3 µL ethane methyl sulfonate (EMS) for approximately one hour, which kills around 65% of the cells. Higher EMS concentrations can also be used to increase the kill rate. After exposure, cells are neutralized with 5% sodium thiosulfate, washed in PBS buffer, recovered in rich media for approximately four hours, and cultured on selective media. Mock samples (no EMS) are also run to ensure that the conditions are selective. Alternatively, cells can be mutagenized using UV irradiation.

[00179] To select for 3-HP resistant mutant strains, aliquots of the EMS-treated cell suspension (approximately 2×10^8 of mutagenized cells) are plated onto a potato dextrose agar (PDA) or another media containing 3-HP at a level at which the parental strain does not grow or grows very slowly. These plates are incubated for several days until colonies appear. Single colonies are purified, streaked on non-selective media to eliminate any adaptive effects of the selection, and re-tested on selective media to confirm increased resistance. Resistant strains are then tested in a shake flask format with periodic sampling for HPLC analysis of products and substrates. Alternatively, selection for 3-HP tolerance may be done by chemostat or serial shake flask evolution. Additional rounds of mutagenesis and selection can be performed. Mutagenesis can be used to increase the resistance of a host that does not natively meet 3-HP production requirements so that it has the necessary attributes for commercial 3-HP production.

Example 2A: Procedure for transformation of DNA into the host genome

[00180] DNA transformation into the yeast host genome to generate the modified yeast strains described in the following examples was conducted based on the specific procedure below.

[00181] Four mL of YP+10% glucose media was added to a 14 mL Falcon tube and the desired strain was inoculated into this media using a sterile loop. The culture was grown with shaking at 250 rpm overnight (~16 hr) at 37°C. 1 mL of the overnight culture was added to a 250 mL baffled flask containing 50 mL of liquid YP+10% glucose media. The flask was grown with shaking at 250 rpm at 37°C. Small aliquots of the culture were withdrawn at approximately hourly intervals and the OD₆₀₀ was measured. The culture was grown until the OD₆₀₀ was 0.6-1.0.

[00182] The cells were harvested by centrifugation at 2279 x g at room temperature, the pellet was resuspended in 25 mL sterile water, then centrifuged at 2279 x g at room temperature. The pellet was resuspended in 1 mL sterile water, and the resuspended cells were transferred to a 1.5 mL tube and then pelleted at 16,100 x g. The cells were resuspended in 1mL LiOAc/TE solution and then pelleted at 16,100 x g. The cell pellet was then resuspended in 500 µL LiOAc/TE solution.

[00183] The following components were added to a 1.5 mL tube: 100 µL of the above cells, 10 µL freshly boiled then iced salmon sperm DNA (Agilent Technologies, Santa Clara, CA, USA), and 10 µL of the desired, linearized transforming DNA. A control reaction with water instead of DNA was also prepared. To each transformation reaction, 600 µL of PEG/LiOAc/TE Solution was added and the reaction incubated on its side at 30°C on a 250 rpm shaker platform for 30 minutes. 40 µL DMSO was added to each reaction and then incubated in a 42°C water bath for 5 minutes. Cells were pelleted at 5,400 x g for 1 min. Cells were resuspended in water, split in two, and each half of the transformation reaction was plated to a ura selection media plate. Plates were placed at 37°C. Colonies were generally visible after 18 to 24 hr growth, depending on strain background.

[00184] A sterile loop was used to transfer a small amount of yeast from a petri dish to a 1.5 mL tube containing 300 µL Yeast Lysis Solution (EPICENTRE® Biotechnologies, Madison, WI, USA) and the genomic DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (EPICENTRE® Biotechnologies) according to the manufacturer's instructions.

[00185] Genomic DNA prepared using the MasterPure™ Yeast DNA Purification Kit (EPICENTRE® Biotechnologies) was used in PCR reactions to determine if the correct integration event had occurred in the isolated transformant. A PCR reaction (25 µL) contained 0.5 µL genomic DNA for the strain to be screened, 1X Crimson Taq™ Reaction Buffer (New England Biolabs, Ipswich, MA, USA), 25 pmol each of the sense and anti-sense primers, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 0.625 units of Crimson Taq™ DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific, Westbury, New York, USA)

programmed for one cycle at 95°C for 30 seconds followed by 30 cycles each at 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 1 minute per kbp of the largest expected product, with a final extension at 68°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE or TBE buffer and the sizes of the bands visualized and interpreted as for the specific primers sets as described.

Example 2B: Selection of insertion sites

[00186] Suitable insertion sites for incorporating exogenous genes into host yeast cells may be loci for genes that have beneficial or neutral effects on 3-HP production when deleted in the yeast host cell. Non-limiting examples of suitable insertion sites for selected yeast strains are described in the working examples herein. One skilled in the art can easily apply the teachings herein for use of these and other insertions sites, for example, loci for one or more PDC (e.g., *I. orientalis* PDC gene encoding the amino acid sequence set forth in SEQ ID NO: 49 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 48), ADH (e.g., *I. orientalis* ADH gene encoding the amino acid sequence set forth in SEQ ID NOs: 106, 108, or 110 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NOs: 105, 107, or 109), GAL6 (e.g., *I. orientalis* GAL6 gene encoding the amino acid sequence set forth in SEQ ID NO: 112 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 111), CYB2A (e.g., *I. orientalis* CYB2A gene encoding the amino acid sequence set forth in SEQ ID NO: 114 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 113), CYB2B (e.g., *I. orientalis* CYB2B gene encoding the amino acid sequence set forth in SEQ ID NO: 116 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 115), GPD (e.g., *I. orientalis* GPD gene encoding the amino acid sequence set forth in SEQ ID NO: 118 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 117), ALD (e.g., *I. orientalis* ALD homolog gene 5680 encoding the amino acid sequence set forth in SEQ ID NO: 120 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 119, *I. orientalis* ALD homolog gene 42026 encoding the amino acid sequence set forth in SEQ ID NO: 122 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 121, *I. orientalis* ALD homolog gene 42426 encoding the amino acid sequence set forth in SEQ ID NO: 124 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 123, or *I. orientalis* ALD homolog gene 42727 encoding the amino acid sequence set forth in SEQ ID NO: 126 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 125), or PCK (e.g., *I. orientalis* PCK gene encoding the amino acid sequence set forth in SEQ ID NO: 128 and/or comprising the coding region of the nucleotide sequence set forth in

SEQ ID NO: 127) genes or homologs thereof. Where sequences for these genes are unpublished, they can be obtained using standard procedure such as genome sequencing, probe hybridization of genomic or cDNA libraries, or amplification of gene fragments using degenerate primers based on known homolog sequence, followed by genome walking to obtain the full sequence. Other suitable locations for insertion sites include intergenic regions that do not contain open reading frames.

Example 2C: Techniques for insertion vectors, selection marker cassettes, gene expression cassettes, and integration constructs

[00187] Insertion site vectors are generated for integrating one or more exogenous genes into host yeast cells. The host yeast cells may be cells that have undergone a selection process as described in Example 1, or they may be cells that have not undergone mutagenesis and/or selection.

[00188] To generate insertion site vectors, a region upstream (5') and a region downstream (3') of the desired insertion site are both amplified using host genomic DNA as template. The upstream region is preferably greater than 70 bp and less than 1.5 kbp. The resultant target sequences are ligated into a cloning vector either simultaneously or sequentially to obtain a vector with one copy of each fragment so that the fragments are contiguous or nearly contiguous. A unique restriction site may be incorporated between the fragments to allow for insertion of gene expression cassettes and/or selection marker cassettes. Unique restriction enzyme sites may also be incorporated at or near the 5' end of the upstream fragment and at or near the 3' end of the downstream fragment to allow for later removal of the DNA between these sites from the cloning vector.

[00189] Selection marker cassettes for incorporation into insertion site vectors are generated using standard cloning techniques. These selection marker cassettes contain a gene for a selectable marker, and may also contain an upstream promoter and/or a downstream terminator sequence. Examples of suitable selection marker genes include the *URA3*, *TRP1*, *HIS*, *MEL5*, *CYB2A*, *LEU2*, and *G418* genes. Flanking sequences may be incorporated into the cassette on either side of the promoter/marker gene/terminator sequences to allow for future loss of the marker through recombination. These flanking sequences may include a direct or inverted repeated sequence (either functional or nonfunctional sequence) or one or more *loxP* sites.

[00190] Gene expression cassettes are generated using standard cloning techniques. These gene expression cassettes contain the gene to be over-expressed, and may also contain an upstream promoter and/or a downstream terminator sequence. In certain embodiments, two or more copies of these promoter/gene/terminator combinations may be

incorporated into a single gene-expression cassette. Heterologous genes may be codon-optimized for improved expression in the host yeast strain. A selection marker cassette as described in herein can be cloned into the gene expression cassette such that it is contiguous or nearly contiguous with the gene to be over-expressed and any associated promoter and/or terminator.

[00191] Alternatively, for replacement of native promoters with an exogenous promoter, the expression cassette may have the selection cassette upstream of the promoter to be integrated, in between targeting sequences.

[00192] Gene expression cassettes can be inserted between the two target site sequences in the insertion site vectors described herein using standard cloning techniques to generate gene expression integration constructs. One or more selection marker cassettes may also be inserted between the target sequences, either as part of the gene expression cassette or separately. In certain variations, pieces of the gene expression cassette can be cloned into different insertion site vectors so that there is an over-lapping fragment in common between the integration fragments. For example, one vector might contain an upstream insertion fragment, a promoter, a gene, and a terminator and the second vector might contain the terminator, selection marker cassette, and downstream insertion fragment. In another example, to allow simultaneous insertion of two genes, one vector could contain the upstream insertion fragment, a promoter, a gene, terminator and all or part of a selection marker cassette, and the second vector might contain all or part of the selection marker cassette, a second promoter, gene, terminator, and the downstream insertion fragment.

[00193] To generate gene knockout constructs, the insertion site vectors are made using target DNA sequences derived from the upstream and downstream flanking regions of the gene to be deleted or disrupted. The selected target sequences may include upstream and downstream flanking regions of a target gene and/or all or a portion of the target gene coding sequence or its regulatory elements (e.g., promoter or terminator). One or more selection marker cassettes may be incorporated into the insertion site vector between the two target sequences. Where the knockout is to be coupled with expression of an exogenous gene, one or more gene expression cassettes are also incorporated into the insertion site vector.

[00194] DNA fragments to be integrated into a host yeast genome can be linearized by restriction enzyme digest of the fragment from a cloning vector, or of overlapping fragments from multiple vectors. Alternatively, linear integration fragments can be generated using PCR, or a combination of PCR and restriction enzyme digest. The insertion site flanking regions can be incorporated into the integration fragment either by their presence in the

vector template or by incorporation into the amplification primers. In the latter case, a minimum of 70 nucleotides of a flanking region is preferably incorporated into a primer.

[00195] Non-limiting examples of suitable insertion vectors, selection marker cassettes, gene expression cassettes, and integration constructs for selected yeast strains are described in the following working examples. One skilled in the art can easily apply the teachings from these examples and the preceding specification to generate alternative modified yeast strains that produce 3-HP.

Example 2D: Construction of insertion vector for expressing an exogenous gene at the *adh1202* locus

[00196] The plasmid pMiBa107 was created to allow integration of a single gene at the *I. orientalis adh1202* locus under the control of the PDC promoter and terminator using URA3 as a selectable marker. The PDC promoter and terminator with the *ura* selectable marker were PCR amplified and cloned into pCR4™4BLUNT TOPO® (Invitrogen, La Jolla, CA, USA) as described below. The PCR fragment containing the PDC promoter, terminator and URA3 selectable marker was constructed by SOE PCR. The PDC promoter was amplified with a primer that contains homology to the PDC terminator on the 3' end of the PCR product and the PDC terminator and URA3 selectable marker were amplified using a primer with homology to the PDC promoter on the 5' end of the product. These two fragments were then put together via SOE PCR.

[00197] The PDC promoter was amplified from pACN5 (Figure 19) using primers 0611184 and 0611195. Primer 0611184 introduces a *NotI* restriction site to the 5' end of the PCR product. Primer 0611195 introduces an *XbaI* restriction site after the PDC promoter and introduces homology to the PDC terminator on the 3' end of the PCR product.

[00198] The amplification reactions were performed using Platinum® *Pfx* DNA polymerase (Invitrogen) according to manufacturer's instructions. Each PCR reaction contained 0.5 µL of diluted pACN5 (Figure 19), 25 pM each of primers 0611184 and 0611195, 1X *Pfx* amplification buffer (Invitrogen), 2 mM MgSO₄, 0.2 mM dNTP mix, 1.25 Units Platinum® *Pfx* DNA polymerase (Invitrogen) in a final volume of 50 µL. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes; with a final extension at 72°C for 3 minutes.

[00199] The PCR product was purified by 1% agarose gel electrophoresis using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. A fragment of approximately 700 bp was excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen, Valencia, CA, USA).

[00200] The PDC terminator and URA3 selectable marker were amplified from pHJJ76 (Figure 24) using primers 0611189 and 0611185. Primer 0611189 introduces homology to the PDC promoter on the 5' end of the PCR product and a *PacI* restriction site directly in front of the PDC terminator. Primer 0611185 introduces a *NotI* restriction site to the 3' end of the PCR product. The amplification reactions were performed using Platinum® *Pfx* DNA polymerase (Invitrogen) according to manufacturer's instructions. Each PCR reaction contained 0.5 µL of diluted pHJJ76, 25 pM each of primers 0611189 and 0611185, 1X *Pfx* amplification buffer (Invitrogen), 2 mM MgSO₄, 0.2 mM dNTP mix, 1.25 Units Platinum® *Pfx* DNA polymerase (Invitrogen) in a final volume of 50 µL. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes; with a final extension at 72°C for 3 minutes.

[00201] The PCR product was purified by 1% agarose gel electrophoresis using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. A fragment of approximately 2000 bp was excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen).

[00202] The 2000 bp PDC terminator and URA3 selectable marker PCR product and the 700 bp PDC promoter PCR product were fused using SOE-PCR. The amplification reactions were performed using Platinum® *Pfx* DNA polymerase (Invitrogen) according to manufacturer's instructions. Each PCR reaction contained 8 ng of 2000 bp PDC terminator and URA3 selectable marker PCR product, 24 ng of the 700 bp PDC promoter PCR product, 50 pM each of primers 0611184 and 0611185, 1X *Pfx* amplification buffer (Invitrogen), 2 mM MgSO₄, 0.2 mM dNTP mix, 2.5 Units Platinum® *Pfx* DNA polymerase (Invitrogen) in a final volume of 100 µL. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 3 minutes; and 1 cycle at 68°C for 3 minutes.

[00203] The 2700 bp PCR product was purified by 1% agarose gel electrophoresis using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. A fragment of approximately 2700 bp was excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen).

[00204] The 2700 bp PCR product was cloned into pCR™4BLUNT TOPO® (Invitrogen) vector using the Zero Blunt® TOPO® PCR cloning kit for sequencing (Invitrogen) according to the manufacturer's instructions. In a total reaction volume of 6 µL either 1 or 4 µL of the 2700 bp PCR product, 1 µL salt solution (Invitrogen) and 1 µL pCR™4BLUNT TOPO® (Invitrogen) were incubated together at room temperature for 15 minutes. 2 µL of each

cloning reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) cells according to manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR product by *NotI* digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMIBa100.

[00205] The plasmid pHJJ76 (Figure 24) contains homology to allow gene integration at the *adh1202* locus. Plasmid pHJJ76 was digested with *NotI* to remove the URA3 selectable marker present inside of the *adh1202* homology sequences. The digested pHJJ76 was purified by 1% agarose gel electrophoresis using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. A 5.2 kbp fragment was extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen), and then ligated back together using T4 DNA ligase. The ligation products were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) cells according to manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several resulting transformants were screened by *Apal* and *SacI* digestion. A clone yielding the desired digestion products was designated pHJJ76-no ura.

[00206] The PDC promoter and terminator and URA3 selectable marker from pMIBa100 (*supra*) was cloned into pHJJ76-no ura to create a plasmid where a gene could be placed under the control of the PDC promoter and terminator for integration at *adh1202*. pHJJ76-no ura was digested with *NotI* followed by treatment with CIP. The linear 5.2 kbp fragment was purified using a QIAQUICK® PCR Purification Kit (Qiagen). pMIBa100 was digested with *NotI* and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. A 2742 bp fragment was excised from the gel, extracted using a QIAQUICK® Gel Extraction Kit (Qiagen), and then ligated into the 5.2 kbp fragment of pHJJ76-no ura using T4 DNA Ligase. The ligation products were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) cells according to manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by *KpnI* and *XbaI* digestion. A clone yielding the desired digestion products was designated pMIBa107 (Figure 2).

Example 2E: Construction of insertion vector fragments for expressing multiple exogenous genes at the PDC locus

[00207] The following insertion vector fragments can be used to generate a designed DNA construct that replaces an endogenous *I. orientalis* PDC gene with a cassette that expresses multiple genes, e.g., three genes described herein expressed from the PDC, ENO1, and

TDH3 promoters. Homologous recombination between the left construct (pMhCt068 and derivatives) and the right construct (pMhCt069 and derivatives) results in expression of the URA3 protein, resulting in conversion of the strain from uracil auxotrophy to uracil prototrophy, allowing for selection of desired integrants. The 5' end of each left-hand construct is homologous to the DNA upstream of the PDC locus, while the 3' end of each right-hand construct is homologous to the DNA downstream of the PDC locus. These homologous regions serve to target the expression cassette to the PDC locus. This targeting approach is depicted schematically in Figure 3, and can be modified to use any combination of multiple genes described herein to target any suitable locus, e.g., any locus described above, such as an ADH locus (see example below) or an ALD locus.

Construction of a left-hand fragment

[00208] An empty vector left-hand construct, pMhCt068, was cloned in multiple steps as described below.

[00209] A PCR fragment containing the PDC promoter region and desired additional restriction sites and flanking DNA was amplified from genomic *I. orientalis* DNA using primers 0611166 and 0611167.

[00210] The PCR reaction (50 μ L) contained 100 ng of genomic *I. orientalis* DNA (preparable, e.g., using a MasterPure™ Yeast DNA Purification Kit from EPICENTRE® Biotechnologies), 1X ThermoPol Reaction buffer (New England Biolabs), 100 pmol each of primers 0611166 and 0611167, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 2 μ L 100 mM MgSO₄, and 2 units of Vent_R® (exo-) DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 94°C for 2 minutes followed by 34 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 780 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel, Bethlehem, PA, USA) according to the manufacturer's instructions.

[00211] A PCR fragment containing the TAL terminator region and desired additional restriction sites and flanking DNA was amplified from pACN5 (Figure 19) using primers 0611168 and 0611169. The PCR reaction (50 μ L) contained 1 μ L of pACN5 mini-prep plasmid DNA, 1X iProof™ HF buffer (Bio-Rad Laboratories, Hercules, CA, USA), 100 pmol each of primers 0611168 and 0611169, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L DMSO and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10

seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 460 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00212] PCR then was used to create a single amplification product fusing both of the products above. The PCR reaction (50 µL) contained 125 ng of the PDC promoter containing PCR product, 76 ng of the TAL terminator containing PCR product, 1X ThermoPol Reaction buffer (New England Biolabs), 100 pmol each of primers 0611166 and 0611169, 200 µM each of dATP, dCTP, dGTP, and dTTP, 2 µL 100 mM MgSO₄, and 2 units of Vent_R® (exo-) DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 94°C for 2 minutes followed by 34 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1,250 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00213] A PCR fragment containing the ENO1 promoter region and desired additional restriction sites and flanking DNA was amplified from pACN43 (Figure 22) using primers 0611170 and 0611171. The PCR reaction (50 µL) contained 1 µL of pACN43 mini-prep plasmid DNA, 1X Phusion HF buffer (New England Biolabs), 100 pmol each of primers 0611170 and 0611171, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1050 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00214] A PCR fragment containing the RKI terminator region followed by the URA3 promoter region and the 5' end of the URA3 ORF, along with desired additional restriction sites and flanking DNA, was amplified from pACN43 (Figure 22) using primers 0611172 and 0611173. The PCR reaction (50 µL) contained 1 µL of pACN43 mini-prep plasmid DNA, 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0611172 and 0611173,

200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L DMSO and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1400 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00215] PCR was used to create a single amplification product fusing both of the products above. The PCR reaction (50 μ L) contained 93 ng of the ENO1 promoter containing PCR product (*supra*); 125 ng of the Rkl terminator, URA3 promoter and partial ORF containing PCR product (*supra*); 1X Phusion HF buffer (New England Biolabs); 100 pmol each of primers 0611170 and 0611173; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 1.5 μ L DMSO; and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 56°C for 20 seconds, and 72°C for 2 minutes and 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 2460 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00216] To create a recipient vector for the PCR products, the plasmid pMhCt017 (the standard cloning vector pUC19 with an irrelevant insert) was digested with *Hind* III and *Eco*RI, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 2.6 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The resulting *Hind* III to *Eco*RI purified fragment was identical to that found in pUC18 (Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, 33, 103-119).

[00217] The purified 1250 bp and 2460 bp PCR products from above were then inserted into the digested pMhCt017 fragment using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 μ L composed of 125 ng pMhCt017 *Hind* III to *Eco*RI vector fragment, 92 ng of the PDC promoter and TAL terminator PCR product, 165 ng of the ENO1 promoter and URA3 promoter and partial ORF containing PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 μ L of IN-FUSION™ enzyme (Clontech). The

reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction then was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Apa* LI digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt068.

[00218] The plasmid pMhCt068 contains the PDC promoter region followed by *Nhe*I and *Asc*I restriction sites for addition of an ectopic gene of interest described herein, the TAL terminator, the ENO1 promoter region followed by *Xba*I and *Pac*I restriction sites for cloning of a second ectopic gene of interest described herein, the RKL terminator, the *I. orientalis* URA3 promoter and the 5' end of the *I. orientalis* URA3 ORF. Plasmid pMhCt068 was found to have an A to T nucleotide change at about 200 bp into the PDC promoter, a G to T change at about 2/3 of the way into the PDC promoter, and a premature start codon (ATG) present on the 5' side of the *Nhe*I restriction site. Accordingly, a corrected version of pMhCt068 was constructed as described below.

[00219] The PDC promoter region was PCR amplified from pACN5 (Figure 19) with primer 0611166 and 0611828, which do not introduce the undesired start codon above. The PCR reaction (50 µL) contained 1 µL of a 1 to 50 dilution of a mini-prep of pACN5, 1X ThermoPol Reaction buffer (New England Biolabs), 100 pmol each of primers 0611166 and 0611828, 200 µM each of dATP, dCTP, dGTP, and dTTP, 2 µL 100 mM MgSO₄, and 2 units of Vent_R® (exo-) DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 94°C for 2 minutes followed by 34 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 780 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00220] The PDC promoter containing PCR product was then fused to the TAL terminator containing PCR product as described above. Since the TAL terminator PCR fragment was made with the 0611168 primer, the resulting PCR fusion products should be a mixture, with products that lack the premature start codon and include the undesired start codon. The resulting ~1250 bp PCR product was purified and combined via IN-FUSION™ Advantage PCR Cloning Kit (Clontech) with the the RKL terminator, URA3 promoter and partial ORF containing fusion PCR product and pUC18 as described above. A clone yielding the

expected ApaI digestion pattern was shown to be correct by DNA sequencing, including the desired absence of mutations in the PDC promoter and lack of premature ATG 5' of the *NheI* restriction site, and designated pMhCt082.

Construction of a right-hand fragment

[00221] The empty vector right-hand construct, pMhCt069, was cloned in multiple steps as described below.

[00222] A PCR fragment containing the 3' end of the *I. orientalis* URA3 ORF, the URA3 terminator (the 275 bp downstream of the URA3 stop codon), the URA3 promoter (to serve as a repeat region for looping out of the marker after integration into the yeast host) and desired additional restriction sites and flanking DNA was amplified from pACN43 (Figure 22) using primers 0611174 and 0611175. The PCR reaction (50 μ L) contained 1 μ L of pACN43 mini-prep plasmid DNA, 1X Phusion HF buffer (New England Biolabs), 100 pmol each of primers 0611174 and 0611175, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1210 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00223] A PCR fragment containing the TDH3 promoter region and desired additional restriction sites and flanking DNA was amplified from pACN23 (Figure 20) using primers 0611176 and 0611177. The PCR reaction (50 μ L) contained 1 μ L of pACN23 mini-prep plasmid DNA, 1X Phusion HF buffer (New England Biolabs), 100 pmol each of primers 0611176 and 0611177, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1028 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00224] A PCR fragment containing the region 3' of the stop codon of the *I. orientalis* PDC gene region (PDC terminator region) and desired additional restriction sites and flanking

DNA was amplified from *I. orientalis* genomic DNA using primers 0611178 and 0611179. The PCR reaction (50 μ L) contained 100 ng of *I. orientalis* genomic DNA, 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0611178 and 0611179, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L DMSO and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 938 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00225] PCR was used to create a single amplification product fusing both of the last two PCR products described above. The PCR reaction (50 μ L) contained 125 ng of the TDH3 promoter containing PCR product, 114 ng of the PDC terminator region containing PCR product, 1X Phusion HF buffer (New England Biolabs), 100 pmol each of primers 0611176 and 0611179, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 56°C for 20 seconds, and 72°C for 2 minutes and 30 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1966 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00226] The purified 1210 bp PCR product and the 1966 bp PCR fusion product from above were then inserted into the *Hind* III and *Eco*RI digested pMhCt017 fragment as described above using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 μ L composed of 125 ng pMhCt017 *Hind* III to *Eco*RI vector fragment, 54 ng of PCR product containing the 3' end of the URA3 ORF followed by the URA3 terminator, 200 ng of the TDH3 promoter and PDC terminator fusion PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 μ L of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 μ L of TE buffer and 2.5 μ L was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR

products by *Apa* LI digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt069.

[00227] Plasmid pMhCt069 contains the 3' end of the *I. orientalis* URA3 marker, the corresponding URA3 terminator, the URA3 promoter (for later looping out of the URA3 marker), the TDH3 promoter, *Xba*I and *Pac*I restriction sites for subcloning of desired genes for ectopic expression, and the 3' flanking region of the PDC locus.

Example 2F: Construction of insertion vector fragments for expressing multiple exogenous genes at the adh9091 locus:

[00228] The following insertion vector fragments were designed using a similar approach to that described in Example 2E in order to replace an endogenous *I. orientalis* adh9091 gene with a cassette that expresses multiple genes of interest described herein.

Construction of a left-hand fragment

[00229] An empty vector left-hand construct, pGREr125, was cloned in multiple steps as described below.

[00230] A construct comprising the 5' flank needed for homologous recombination at the *I. orientalis* adh9091 locus and the empty expression cassette PDC promoter / TAL terminator was PCR cloned into vector plasmid pCR2.1-TOPO (Invitrogen). The PDC promoter fragment was PCR amplified from plasmid pACN5 (Figure 19) using primers 0611250 and 0611251. The PCR reaction (50 μ L) contained 15 ng of plasmid pACN5 DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611250 and 0611251, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 900 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 753 bp with a *Nof*I restriction site at the 5' end of the fragment and a *Pac*I and an *Xba*I restriction site at the 3' end of the fragment.

[00231] A second PCR fragment containing 5' homology to the PCR product above including the *Xba*I and *Pac*I restriction sites was generated to amplify the TAL terminator region from plasmid pACN5 (Figure 19) using primers 0611252 and 0611253. The PCR reaction (50 μ L) contained 15 ng of plasmid pACN5 DNA (*supra*), 1X Phusion HF buffer

(New England Biolabs), 50 pmol each of primers 0611252 and 0611253, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 900 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was about 435 bp with *Xba*I and *Pac*I restriction sites at the 5' end of the fragment and a *Pme*I restriction site at the 3' end.

[00232] The 753 bp fragment and the 435 bp fragment were fused together by PCR using the primers 0611250 and 0611253, leading to a resulting 1149 bp fragment in which the PDC promoter is upstream of the TAL terminator. The PCR reaction (50 μ L) contained 125 ng of the 753 bp fragment, 75 ng of the 435 bp fragment, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611250 and 0611253, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction product was separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1149 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00233] A PCR fragment containing 3' homology to the 1149 bp PCR product above including the *Not*I restriction site, was generated to amplify the 5' flank for the *I. orientalis* adh9091 locus using primers 0611254 and 0611255. The PCR reaction (50 μ L) contained 15 ng of plasmid pHJJ27 (Figure 21) as template DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611254 and 0611255, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 900 bp PCR product was excised

from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment is approximately 891 bp with an *HpaI* restriction site at the 5' end of the fragment and a *NotI* restriction site at the 3' end.

[00234] The 891 bp fragment then was fused upstream of the 1149 bp PDC promoter/TAL terminator fragment by PCR using the primers 0611254 and 0611253 generating an approximately 2005 bp fragment. The PCR reaction (50 µL) contained 125 ng of the 1149 bp fragment, 95 ng of the 891 bp fragment, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611254 and 0611253, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2005 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00235] The resulting 2005 bp fragment, comprising the 5' flank for integration at the *adh9091* locus, the PDC promoter and the TAL terminator, was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E.coli* cells using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *AvaI* digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr112. Plasmid pGMEr112 comprises the 5' flank for homologous recombination at the *adh9091* locus followed the empty expression cassette PDC promoter/TAL terminator.

[00236] The truncated 5' URA3 marker gene driven by the URA3 promoter fragment was PCR amplified from plasmid pHJJ27 (Figure 21) using primers 0611283 and 0611263. The PCR reaction (50 µL) contained 15 ng of plasmid pHJJ27, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611263 and 0611283, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel

electrophoresis in TBE buffer where an approximately 900 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 885 bp with *HpaI* and *PmeI* restriction sites at the 5' end of the fragment and a *NheI* restriction site at its 3' end.

[00237] The resulting 885 bp fragment was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E. coli* cells using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *BglI* digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr108. Plasmid pGMEr108 comprises the URA3 promoter followed by the truncated 5' segment of the URA3 gene, such fragment is flanked by *HpaI* and *PmeI* restriction sites at the 5' end and by the *NotI* restriction site at the 3' end.

[00238] A 1998 bp *HpaI* and *PmeI* restriction fragment from plasmid pGMEr112 (*supra*), comprising the 5' *adh9091* flank followed by the construct PDC promoter/TAL terminator, was ligated to the 4806 bp vector from pGMEr108 (*supra*) linearized by *HpaI* and *PmeI*. The double restriction reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where the 1998 bp insert fragment and the 4806 bp vector fragment were excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The ligation reaction was performed using a 1:3 vector: insert ratio; in particular the reaction was set up with 2 µL of the 4806 bp linearized vector, 6 µL of the 1998 bp insert fragment, 9 µL of 2X Quick Ligation reaction Buffer and 1 µL Quick T4 DNA Ligase (New England Biolabs), and performed according to the manufacturer's instructions.

[00239] Five µL of the ligation product was transformed into *E. coli* XL10-Gold® Ultracompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Hind III* digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr117.

[00240] Plasmid pGMEr117 comprises the 5' *adh9091* flank, followed by the empty expression cassette PDC promoter/TAL terminator and by the truncated 5' URA3 gene driven by the URA3 promoter. Additionally, plasmid pGMEr117 bears two different *XbaI* restriction sites: a first restriction site between the PDC promoter and the TAL terminator (and adjacent to restriction site *Pac I*) which can be used to insert the gene of interest, and a

second *Xba*I restriction site that was inherited from the original pCR2.1-TOPO back bone. In order to eliminate this second *Xba*I restriction site, plasmid pGMEr117 was digested with restriction enzyme *Apa*I, and the linearized plasmid was then treated with the enzyme DNA polymerase I, large (Klenow) fragment (New England Biolabs) according to the manufacturer's instructions. The resulting linear vector (containing blunt ends) was digested with restriction enzyme *Eco* RV, which cut a 43 bp fragment from the vector comprising the *Xba*I restriction site. The restriction reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer and the 6761 bp vector fragment was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The self ligation reaction was set up with 3 µL of the linearized vector, 6 µL of sterile double-distilled water, 10 µL of 2 X Quick Ligation reaction Buffer and 1 µL Quick T4 DNA Ligase (New England Biolabs) and performed according to the manufacturer's instructions.

[00241] Five µL of the ligation product was transformed into *E. coli* XL10-Gold® Ultracompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Xba*I digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr122.

[00242] The ENO1 promoter fragment was PCR amplified from plasmid pACN43 (Figure 22) using the primers 0611295 and 0611296. The PCR reaction (50 µL) contained 15 ng of plasmid pACN43, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611295 and 0611296, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1009 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 1009 bp with a *Pme*I restriction site at the 5' end of the fragment and *Apa*I and *Nru*I restriction sites at the 3' end.

[00243] A second PCR fragment containing 5' homology to the PCR product above, including the *Nru*I and the *Apa*I restriction sites, was generated to amplify the RKL terminator region from plasmid pACN43 (Figure 22) using the primers 0611297 and 0611298. The PCR

reaction (50 μ L) contained 15 ng of plasmid pACN43 DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611297 and 0611298, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 438 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was about 438 bp with *Nru*I and *Apa*I restriction sites at the 5' end of the fragment and a *Pme*I restriction site at the 3' end of the fragment.

[00244] The 1009 bp promoter fragment and the 438 bp terminator fragment were fused together by PCR using primers 0611295 and 0611298, leading to an approximately 1447 bp fragment in which the ENO1 promoter is upstream of the RKI terminator. The PCR reaction (50 μ L) contained 125 ng of the 1009 bp fragment, 65 ng of the 438 bp fragment, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611295 and 0611298, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minute, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction product was separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1447 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00245] The resulting 1447 bp fragment, comprising the ENO1 promoter/RKI terminator construct, was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E.coli* cells using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *Bam* HI digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr114, comprising the empty expression cassette ENO1 promoter/RKI terminator.

[00246] Plasmids pGMEr122 and pGMEr114 were digested with restriction enzyme *PmeI* at 37°C for 3 hours. Approximately one hour before stopping each digestion reaction, 1 µL of Calf Intestinal Alkaline Phosphatase (New England Biolabs) was added to each digestion tube in order to de-phosphorylate the ends and prevent self-ligation. The resulting 6761 bp vector fragment from plasmid pGMEr122, and the resulting insert fragment comprising the construct ENO1 promoter/TAL terminator (1439 bp) from plasmid pGMEr114, were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel, and purified using the QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00247] A subsequent ligation reaction was then prepared comprising 3 µL of the vector fragment from plasmid pGMEr122, 4 µL of the insert fragment from plasmid pGMEr114, 2 µL of sterile dd water, 10 µL of 2X Quick Ligase Buffer and 1 µL of Quick T4 Ligase (New England Biolabs) and performed according to the manufacturer's instructions. A 5 µL aliquot of the ligation reaction above was transformed into XL10-Gold® Ultracompetent *E. coli* cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by digestion using *XbaI* and *Bst* BI. A clone yielding the desired band sizes was confirmed by sequencing and designated pGMEr125. The ENO1 promoter/RKI terminator construct was inserted in opposite orientations, resulting in two versions of plasmid pGMEr125 designated (a) and (b) (Figures 4 and 5).

[00248] The plasmids pGMEr125a and pGMEr125b contain the PDC promoter region, the TAL terminator, the ENO1 promoter region, the RKI terminator, the *I. orientalis* URA3 promoter, the 5' end of the corresponding URA3 marker and the 5' flanking region of the *I. orientalis* adh9091 locus.

Construction of a right-hand fragment

[00249] An empty vector right-hand construct, pGREr121, was cloned in multiple steps as described below.

[00250] The TDH3 promoter fragment was PCR amplified from plasmid pACN23 (Figure 20) using primers 0611256 and 0611257. The PCR reaction (50 µL) contained 15 ng of plasmid pACN23 DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611256 and 0611257, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds,

with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 994 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 994 bp with a *SfoI* restriction site at the 5' end of the fragment and *PacI* and *NruI* restriction sites at the 3' end.

[00251] A second PCR fragment containing 5' homology to the PCR product above, including the *NruI* and *PacI* restriction sites, was generated to amplify the TKL terminator region from plasmid pACN23 (Figure 20) using primers 0611258 and 0611259. The PCR reaction (50 µL) contained 15 ng of plasmid pACN23 DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611258 and 0611259, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 469 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was about 469 bp with *NruI* and *PacI* restriction sites at the 5' end of the fragment and a *NotI* restriction site at the 3' end.

[00252] The 994 bp and 469 bp fragments above were fused together by PCR using primers 0611256 and 0611259, leading to an approximately 1433 bp fragment in which the TDH3 promoter is upstream of the TKL terminator. The PCR reaction (50 µL) contained 125 ng of the 994 bp fragment, 60 ng of the 469 bp fragment, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611256 and 0611259, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction product was separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1433 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00253] A PCR fragment containing 5' homology to the 3' end of the 1433 bp PCR product above which includes the *NotI* restriction site was generated to amplify the 3' flank for the *adh9091* locus using primers 0611260 and 0611261. The PCR reaction (50 μ L) contained 15 ng of plasmid pHJJ27 DNA (Figure 21) as template DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611260 and 0611261, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1019 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment is approximately 1019 bp with a *NotI* restriction site at the 5' end of the fragment and an *Apal* restriction site at the 3' end.

[00254] The 1019 bp fragment was then fused downstream of the 1433 bp TDH3 promoter/TKL terminator fragment by PCR using primers 0611256 and 0611261 generating an approximately 2405 bp fragment. The PCR reaction (50 μ L) contained 125 ng of the 1433 bp fragment, 90 ng of the 1019 bp fragment, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611256 and 0611261, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 2405 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00255] The resulting 2405 bp fragment comprising the 3' flank for integration at the *adh9091* locus downstream of the TDH3 promoter/TAL terminator construct was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E.coli* cells using a TOPO TA Cloning kit, (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *AvaI* digestion. A clone yielding the desired band sizes was confirmed and designated pGMER113. Plasmid

pGMEr113 comprises the 3' flank for homologous recombination at the *I. orientalis* adh9091 locus preceded by the empty expression cassette TDH3 promoter/TKL terminator.

[00256] PCR was used to amplify the truncated 3' fragment of the URA3 ORF, the URA3 terminator, and the URA3 promoter (to serve as a repeat region for looping out of the marker after integration into the yeast host as described above) from plasmid pHJJ27 (Figure 21) using primers 0611264 and 0611284. The PCR reaction (50 μ L) contained 15 ng of plasmid pHJJ27 DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611264 and 0611284, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1324 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 1324 bp with a *NheI* restriction site at the 5' end of the fragment and *Apal* and *SfoI* restriction sites at the 3' end.

[00257] The gel-purified 1324 bp fragment above was cloned into the pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E. coli* cells using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Hind* III digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr109. Plasmid pGMEr109 comprises the 3' fragment of the URA3 ORF and the URA3 terminator, followed by the URA3 promoter. The upstream portion of the 3' fragment of the URA3 gene in plasmid pGMEr109 bears a 460 bp homology with the extremity of the truncated 5' URA3 fragment cloned into plasmid pGMEr108. The region of homology allows recombination between the two portions of the gene creating a functional selection marker upon co-transformation of the host organism with the construct containing both segments.

[00258] Plasmid pGMEr109 was digested with *KpnI*, and treated with DNA polymerase I, large (Klenow) fragment (New England Biolabs) according to the manufacturer's instructions. The linearized pGMEr109 plasmid (containing blunt ends) was digested with *Bam* HI. The products were separated by 0.8% agarose gel electrophoresis in TBE buffer and the 5247 bp vector fragment was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00259] Plasmid pGMEr113 was digested with *Bam* HI and *Eco* RV resulting in a 2466 bp fragment bearing the construct TDH3 promoter/TKL terminator followed by the truncated 3' fragment of the URA3 ORF with the URA3 terminator, followed by the URA3 promoter. The double restriction reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer and the approximately 2466 bp vector fragment was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The 2466 bp *Bam* HI/ *Eco* RV digested fragment then was ligated to the 5247 bp vector fragment from plasmid pGMEr109. The ligation reaction was set up with 3 µL of the 5247 bp linearized vector, 3 µL of the 2466 bp insert fragment, 3 µL of sterile dd water, 10 µL of 2X Quick Ligation reaction Buffer and 1 µL Quick T4 DNA Ligase (New England Biolabs), and performed according to the manufacturer's instructions.

[00260] Five µL of the ligation product was transformed into *E. coli* XL10-Gold® Ultracompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by digestion with *Xba*I and *Pac* I. A clone yielding the desired band sizes was confirmed and designated pGMEr121 (Figure 6).

[00261] Plasmid pGMEr121 contains the the 3' end of the *I. orientalis* URA3 marker followed by the corresponding URA3 promoter, the TDH3 promoter, the TKL terminator and the 3' flanking region of the *adh9091* locus.

Example 2G: Construction of *I. orientalis* CNB1

[00262] *I. orientalis* CNB1 was constructed from *I. orientalis* CD1822 as described below (see Example 1A for generation of *I. orientalis* CD1822 from *I. orientalis* ATCC PTA-6658). Both copies of the URA3 gene contained in strain CD1822 were deleted to allow use of this gene as a selection marker for genetic engineering. URA3 is a versatile marker for yeast genetics due to the selection available for both the presence (by growth in uracil deficient media) and absence (by growth in the presence of 5-fluorotic acid) of the gene. Disruption of one of the URA3 genes was done by replacement with a selection cassette containing the MEL5 selection marker flanked by repeated DNA sequences. Strains testing positive for the MEL5 selection cassette were then screened for the loss of MEL5 marker gene. Loss of the second URA3 gene was then selected for by growth in the presence of 5-fluorotic acid.

[00263] CD1822 was transformed with 2.8µg of *Sac* I/*Psp*OMI digested DNA of vector pMI458 (Figure 27). Plasmid pMI458 contains the *S. cerevisiae* MEL5 gene (SEQ ID NO: 255) under control of the *I. orientalis* PGK promoter (P-loPGK, SEQ ID NO: 247), flanked by DNA fragments homologous to sequence upstream (P-loURA3, SEQ ID NO: 253) and

downstream (T-loURA3, SEQ ID NO: 254) of the *I. orientalis* URA3 gene. The P-loURA3 and T-loURA3 fragments are in the same relative orientation as in the *I. orientalis* genome. Roughly 500 Mel⁺ colonies were obtained after five days at 30°C. Ten colonies were single colony isolated by inoculating a 10mL BFP (Butterfields Phosphate buffer) tube and plating 25 µL onto DM1 X-α-gal plates. A single blue colony from each of the initial isolates was then picked onto YPD for further analysis.

[00264] PCR was used to screen transformants for the desired genetic events. To obtain genomic DNA for use as template in PCR screenings, cells from 1.5 mL overnight cultures were spun down in a screw-cap microcentrifuge tube and resuspended in 0.2 ml of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM Na₂EDTA pH 8.0 solution. 0.2 mL of a phenol:chloroform:isoamyl alcohol (25:24:1) mixture equilibrated with 10 mM Tris pH 8.0 / 1 mM EDTA (Sigma) and 0.3 g of glass beads were added. The tube was shaken for 2 minutes at full speed with a Mini-BeadBeater-8 (BioSpec). 0.2 mL of TE was added and the tube was vortexed briefly. The aqueous phase was separated by centrifugation at 16,100 x g for 5 minutes. The supernatant was removed to a new tube and 1 mL of 100% ethanol added. The tube was placed at 20°C for 30 minutes, centrifuged at 16,100 x g for 5 minutes, and the liquid decanted off. The DNA was air dried and resuspended in 500 µL TE.

[00265] The PCR screen for the desired 5' cross-over was done using primers oCA405 and oCA406, which produce a 1.5 kbp product. The screen for the desired 3' cross-over was done using primers WG26 and CM647, which produce a 1.6 kbp product. Primers outside (farther upstream or downstream) the URA3 regions used to create pMI458 are oCA405 and CM647, which produce a 3.2 kbp product for the wild type, 5.0 kbp product for a pMI458 disrupted allele, and 2.2 kbp product when the selection marker has been looped out. These PCR reactions were done using a 55°C annealing temperature. PCR was also used to screen for the loss of URA3 open reading frame using a four-primer approach. Primers pJLJ28 and pJLJ29 amplify an 800 bp fragment of the actin gene and primers pJLJ30 and pJLJ31 amplify a 600 bp fragment of the URA3 gene. Use of all the primers in one reaction provides a positive internal control (the actin fragment). Taq DNA polymerase from Roche was used as per manufacturer's protocol, with an annealing temperature of 61°C. Strains 1822ura het MEL-1 and 1822ura het MEL-2 were confirmed as having integrated the MEL5 selection cassette in the URA3 locus.

[00266] The MEL5 marker was then removed from the genomes of 1822ura het MEL-1 and 1822ura het MEL-2 by allowing recombination between the KtSEQ1a (SEQ ID NO: 256) and KtSEQ1b (SEQ ID NO: 257) sequences. The MEL⁺ strains were grown overnight in YPD media to an OD₆₀₀ of roughly 0.5 to 2.0. The cultures were then diluted back to an OD₆₀₀ of

approximately 0.00001 in YPD medium. 200 μ L of culture dilution was transferred into each well of a 96 well microtiter plate. The plates were covered with an adhesive cover and incubated in a 30°C incubator, with maximum agitation for 6-7 hours (roughly 6 cell divisions, depending on growth rate of the strain). 100 μ L from each well (approximately ~1000 cfu per plate) was plated onto DM1 + X- α -gal medium. Plates were incubated at 30°C overnight or at room temperature for 2 days to observe color differentiation. White colonies (putative mel-) were streaked onto similar media, and screened by PCR as described above. Two independent loop-outs were found, one from 1822ura het MEL-1, saved as 1822ura het mel-1 and the other from 1822ura het MEL-2, saved as 1822ura het mel-2. Oddly, the vast majority of white colonies obtained did not give the expected band of 2.2 kbp.

[00267] To obtain ura- derivatives, 1822ura het mel-1 and 1822ura het mel-2 were grown overnight in YP5D media (YP + 100 g/L Dextrose) and aliquots (0.5, 5 and 50 μ L) of the overnight culture were plated on ScD-2X FOA plates. FOA-resistant colonies were streaked for single colonies and verified for the ura- phenotype by plating on ScD-ura plates. Two colonies from 1822ura het mel-2 and six colonies from 1822ura het mel-1 were picked for further analysis. These colonies were grown overnight in YPD and genomic DNA was extracted using the above phenol/chloroform method.

[00268] The presence of the URA3 open reading frame was screened with PCR; none of the eight strains contained the URA3 gene. Two ura- descendants of 1822ura het mel-1 were named yJLJ3 (CNB1) and yJLJ4. Based genomic sequencing, yJLJ3 (CNB1) and yJLJ4 were determined to contain a deletion of both the URA3 gene and a nearby permease gene; preferably only the URA3 gene would be deleted. To create a ura3 auxotroph of CD1822 without disruption of this permease gene, CD1822 was transformed with 1 μ g of Sac I/ApaI digested pCM208 (Figure 28). Plasmid pCM208 contains DNA sequence homologous to the upstream (5' URA flank (near), SEQ ID NO: 258) and downstream (3' URA3 flank (near), SEQ ID NO: 259) flanking regions of the *I. orientalis* URA3 gene. Roughly 200 Mel+ colonies were obtained after five days at 30°C. Eight blue colonies were isolated by streaking on ScD X- α -gal plates. PCR was used to screen transformants for the desired genetic events. The 5' cross-over screen was done using primers oJY11 and oJY12, which produce a 0.9 kbp product in desired transformants. The 3' cross-over screen was done using primers oJY13 and oJY14, which produce a 1.0 kbp product in desired transformants. Three of eight colonies showed the desired PCR products. The MEL markers for these colonies can be looped out and the second URA3 gene deleted as described above. Alternatively, the URA3 and permease gene deletions in strains derived from yJLJ3 or yJLJ4 can be fixed in a one-step transformation, as described in Example 2H.

Example 2H: Construction of MBin500 control strain containing the URA3 selection marker

[00269] As described *supra*, the *I. orientalis* strain designated CNB1 used herein was a uridine auxotroph due to the homozygous deletion of the URA3 gene. A heterozygous repair of the URA3 locus was made using the ura fix vector, pJLJ62 (Figure 26) which contains a ura fix cassette comprised of the URA3 gene with 691 bp of 5' flanking DNA, and 1500 bp of 3' flanking DNA. The ura fix cassette is flanked by a 5' *NotI* restriction site and a 3' *Apal* restriction site. A restriction digest using *NotI* and *Apal* was performed to remove the 2796 bp ura fix cassette from the vector backbone. The digest was purified using a QIAquick PCR Purification kit (Qiagen) as specified by the manufacturer. The DNA was eluted in glass distilled water and 1 µg was used to transform *I. orientalis* CNB1. Transformants were selected on ura selection plates, and a single colony that does not require uridine supplementation was designated MBin500.

Example 2I: Removal of the URA3 selection marker

[00270] In order to isolate strains in which the URA3 selection marker gene was removed via recombination of the two URA3 promoter regions present in the integration cassettes, the ura⁺ strain of interest was inoculated into 3 mL of YP+10% glucose media and grown with shaking at 250 rpm at 37°C for at least four hours and up to overnight. 50-100 µL of the culture was plated onto ScD FOA plates and grown at 37°C for 48-60 hours until colonies appeared. Growth on FOA selected for the removal of the URA3 marker since FOA is converted to a toxic compound by the URA3 protein, resulting in the death of ura⁺ cells. Several FOA-resistant colonies were purified twice by growing on YPD plates 37°C. These purified isolates were then screened for appropriate URA3 loop-out via PCR as described herein.

Example 2J: Procedure for shake flask growth of modified yeast strains for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and enzyme assays

[00271] Four mL of ura selection media was added to a 14 mL Falcon tube and the desired strain was inoculated into this media using a sterile loop. The culture was grown with shaking at 250 rpm overnight (~16 hrs) at 37°C. For strains that have at least one wild-type copy of the *I. orientalis* locus, 500 µL of the overnight culture was added to a 125 mL baffled flask containing 25 mL of YP+10% glucose media. For *pdΔ/pdcΔ* strains, 1 mL of the overnight culture was added to a 125 mL baffled flask containing 25 mL of liquid YP+100 g/L dextrose media. The flask was grown with shaking at 250 rpm at 37°C. Small aliquots of the culture were withdrawn at approximately hourly intervals and the OD₆₀₀ was measured. The culture was grown until the OD₆₀₀ was between 4 and 6.

[00272] In order to prepare a small sample of cells for SDS-PAGE analysis, a volume of culture corresponding to 2.5 OD units was taken for the culture and placed in a 1.5 mL tube. The cells were pelleted at 16,100 x g, the supernatant removed, and the cell pellet stored at -20°C until use.

[00273] The remaining cells in the growth flask were harvested by centrifugation at 2279 x g at room temperature, the pellet was resuspended in 12.5 mL 0.85 M NaCl, then centrifuged at 2279 x g at room temperature. The pellet was resuspended in 1 mL 0.85 M NaCl, and the resuspended cells were transferred to a 2.0 mL tube and then pelleted at 16,100 x g. The supernatant was then removed and the pellet stored at -20°C if they would be used for enzymatic assays within one week, or at -80°C for longer term storage.

[00274] For SDS-PAGE analysis of the cell pellet corresponding to 2.5 OD units, the cells were resuspended in 100 dH₂O, then 100 µL 0.2 M NaOH was added. The sample was incubated at room temperature for 5 minutes, then the cells were pelleted by centrifugation at 16,100 x g and resuspended in 100 µL SDS sample buffer (Bio-Rad Laboratories). The sample was heated at 95°C for 5 minutes and cells were pelleted by brief centrifugation. 1 to 5 µL of the supernatant was analyzed on a Criterion 8-16% Pre-Cast gel (Bio-Rad Laboratories) according to the manufacturer's instructions. Bands were visualized using InstantBlue™ Coomassie-Based Staining Solution (Expedeon Protein Solutions, San Diego, CA, USA).

Example 2K: Procedure for shake flask growth of modified yeast strains for product analysis

[00275] Strains were streaked out for single colonies on Ura Selection Plates and incubated at 30°C for 1-2 days. Seed cultures were prepared in 250 ml baffled flasks containing 50 mL CNB1 shake flask media inoculated with 1-2 colonies from the Ura Selection Plate. Seed cultures were grown for approximately 18 hours at 30°C with shaking at 200 rpm. Small aliquots of the culture were then withdrawn to measure the OD₆₀₀ until reaching an OD₆₀₀ of 4-6. The residual glucose present was measured using an Uristix® Reagent Strip (Bayer, Elkhart, IN, USA). The seed flask cultivation was used to inoculate 125 ml baffled flasks containing 50 mL CNB1 shake flask media to an OD₆₀₀=0.2. Cultures were incubated at 30°C with shaking at 140 rpm for 20 hr. Samples of the broth were removed for analysis as described below. An aliquot of the sample was used to measure the optical density (OD) of the culture and residual glucose present was measured using a Uristix® Reagent Strip. The rest of the sample was then centrifuged and the supernatant used for product analysis.

Example 2L: Procedure for fermentation of modified yeast strains for product analysis

[00276] Strains described herein are cultivated using a seed propagation stage and followed by a single stage fermentation in a 2 L bioreactor (Applikon, Foster City, CA, USA).

[00277] For seed stage preparation 25 mL of 1X DM2 medium (adjusted to the desired pH with KOH) was added to a 125 mL baffled flask, followed by inoculation with the strain of interest using a sterile loop. The culture was grown with shaking at 250 rpm at the desired temperature overnight for approximately 16 hr. Small aliquots of the culture were then withdrawn at approximately hourly intervals to measure the OD₆₀₀ until reaching an OD₆₀₀ of 4-6.

[00278] The residual glucose present was measured using a Uristix® Reagent Strip (Bayer, Elkhart, IN, USA). 12 mL of the culture was then added to 4 mL of sterile chilled 75% glycerol, mix thoroughly, and incubated on ice for ten minutes. The culture and glycerol mixture was then remixed and 1.0 mL was aliquoted to each of 10 sterile 1.8 mL cryovials (Thermo Scientific, Rochester, NY, USA) and stored at -80°C.

[00279] 25 mL of the seed flasks cultivation was used to inoculate the 2 L bioreactor containing 1.5 L of DM2 medium. The fermentation in the bioreactor was performed at a temperature of about 30°C - 40°C, with the pH controlled in the range of about 2.0-7.0 and under agitation and aeration conditions that lead to an oxygen uptake rate (OUR) in the range of 2-45 mmol/L/hr. In the examples presented herein, the temperature, pH and OUR for the culture in the bioreactor were 30°C, 4.0 and 25-30, respectively.

[00280] Samples of the fermentation broth were removed periodically for analysis. Briefly, an aliquot of the sample was used to measure the optical density (OD) of the culture, the glucose concentration and pH. The rest of the sample was then centrifuged. The pellet was stored at -80°C for enzyme assays, and the supernatant was used for analysis of 3-HP and other extracellular compounds. All 3-HP production values reported herein are for the 48-hour time point in the fermentation unless specified otherwise. Carbon dioxide production and oxygen consumption during the fermentation process were measured by determining the carbon dioxide content and oxygen content of the gasses vented from the bioreactor.

Example 2M: Procedure for analysis of 3-HP and β-alanine produced by modified yeast strains:

[00281] Culture samples were acidified by 10X dilution into 1% formic acid and filtered through a 0.46 μm 96-well filter plate. Further dilution was made in water depending on analyte concentration in the sample. A further 10X dilution was made in a sample buffer of 1 mM NH₄Ac, 0.1% NH₃ and 5 mg/L of ¹³C uniformly labeled 3-HP (as internal standard for 3-HP), or 1% formic acid and 3 mg/L of ¹³C uniformly labeled β-alanine (as internal standard

for β -alanine). The total dilution factor was approximately 100 to 1000 was used depending on the concentrations of β -alanine or 3-HP.

[00282] A 2 μ L sample was injected into an Agilent 1200 HPLC (Agilent) controlled by MassHunter program with an Agilent 6410 Triple Quad MS/MS detector using the instrument settings and columns listed in Table 6. The ratio of the quantifying ion fragment peak area to its stable isotope counterpart (from internal standard) was used for quantification to eliminate ion suppression effect and instrument drifting. Standard deviation was below 5% from day to day assays.

Table 6: LC/MS/MS Settings for β -Alanine and 3-HP analysis

	3-HP (^{13}C 3-HP)	β -Alanine (^{13}C β-Alanine)
<i>Column</i>	Xbridge HILIC Silica 3.5 μm , 2.1 x 150 mm	Atlantis HILIC Silica 3 μm 2.1 x 150 mm
<i>Elution buffer</i>	62% acetonitrile, 0.35mM NH_4Ac	38% acetonitrile, 0.6% formic acid
<i>Flow rate (mL/min)</i>	0.30	0.30
<i>Column temperature</i>	50°C	45°C
<i>Retention time (min)</i>	1.07	1.64
<i>Run time (min)</i>	3	3
<i>Pre-cursor ion</i>	89 (92)	90 (93)
<i>Product ion as quantifier</i>	59 (61)	72 (75)
<i>Product ion as qualifier</i>	41 (43)	30 (31)
<i>Fragmentor Voltage</i>	50	70
<i>Collision energy</i>	5 for quantifier; 21 for qualifier	3 for quantifier; 7 for qualifier
<i>Polarity</i>	Negative	Positive
<i>Nebulizer N_2 pressure(psi)</i>	10	11
<i>N_2 flow (L/min)</i>	32	35
<i>N_2 temperature</i>	300°C	340°C
<i>Capillary (V)</i>	4000	4000
<i>Delta EMV</i>	450	400

Example 3: Modified yeast strains expressing 3-HP fermentation pathway genes

[00283] One or more genes encoding enzymes involved in various 3-HP fermentation pathways can be expressed, either alone or in combination, in yeast host cells. The 3-HP pathway enzymes may be expressed from exogenous genes, endogenous genes, or some combination thereof. Exogenous genes to be expressed may be introduced into the yeast cell using gene expression constructs, e.g., expression constructs described in Example 2. Exogenous genes may be integrated into the host yeast genome at a single site or at multiple locations, and integration of the exogenous gene may be coupled with deletion or disruption of a target gene at the insertion site as described below.

Example 3A: Modified yeast strains expressing aspartate/malonate semialdehyde pathway genes

[00284] Yeast cells that produce 3-HP via a pathway that utilizes PEP and/or pyruvate, OAA, aspartate, β -alanine, and malonate semialdehyde intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of a PPC, PYC, AAT, ADC, BAAT, gabT, 3-HPDH (including malonate semialdehyde reductase), HIBADH, or 4-hydroxybutyrate dehydrogenase gene.

[00285] The expressed genes may be derived from a gene that is native to the host cell. For example, where the yeast host cell is *I. orientalis*, expressed genes may be derived from an *I. orientalis* PYC (e.g., *I. orientalis* PYC gene encoding the amino acid sequence set forth in SEQ ID NO: 2 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 1), AAT (e.g., *I. orientalis* AAT gene encoding the amino acid sequence set forth in SEQ ID NO: 14 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 13), BAAT (e.g., *I. orientalis* pyd4 homolog gene encoding the amino acid sequence set forth in SEQ ID NO: 20 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 19), or 3-HPDH (e.g., *I. orientalis* homolog to the YMR226C gene encoding the amino acid sequence set forth in SEQ ID NO: 26 and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 25) gene. Where the yeast host cell is another 3-HP tolerant yeast strain, gene sequences can be obtained using techniques known in the art and the native homologs for pathways genes can be expressed exogenously or in conjunction with exogenous regulatory elements. Native pathway genes may include one or more PPC, PYC, AAT, BAAT, and/or 3-HPDH genes.

[00286] Alternatively, one or more of the expressed 3-HP genes may be derived from a source gene that is non-native to the host cell. For example, where the yeast host cell is *I. orientalis*, the cell may be engineered to express one or more non-native PYC genes such as an *R. sphaeroides* PYC gene encoding the amino acid sequence of SEQ ID NO: 3, an *R. etli* PYC gene encoding the amino acid sequence of SEQ ID NO: 4, a *P. fluorescens* PYC gene encoding the amino acid sequence of SEQ ID NOs: 5 or 6, a *C. glutamicum* PYC gene encoding the amino acid sequence of SEQ ID NO: 7, or an *S. melliloti* PYC gene encoding the amino acid sequence of SEQ ID NO: 8; one or more non-native PPC genes such as an *E. coli* PPC gene encoding the amino acid sequence of SEQ ID NO: 10, an *M. thermoautotrophicum* PPC gene encoding the amino acid sequence of SEQ ID NO: 11, or a *C. perfringens* PPC gene encoding the amino acid sequence of SEQ ID NO: 12; one or more non-native AAT genes such as an *E. coli* aspC gene encoding the amino acid sequence of SEQ ID NO: 16 or an *S. cerevisiae* AAT2 gene encoding the amino acid sequence of SEQ

ID NO: 15; one or more non-native ADC genes such as an *S. avermitilis* panD gene encoding the amino acid sequence of SEQ ID NO: 17 (and/or comprising the coding region of the nucleotide sequence set forth in any one of SEQ ID NOs: 130, 145, 146, or 147), a *C. acetobutylicum* panD gene encoding the amino acid sequence of SEQ ID NO: 18 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 131), an *H. pylori* ADC gene encoding the amino acid sequence of SEQ ID NO: 133 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 132), a *Bacillus sp.* TS25 ADC gene encoding the amino acid sequence of SEQ ID NO: 135 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 134), a *C. glutamicum* ADC gene encoding the amino acid sequence of SEQ ID NO: 137 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 136), or a *B. licheniformis* ADC gene encoding the amino acid sequence of SEQ ID NO: 139 (and/or comprising the coding region of the nucleotide sequence set forth in any one of SEQ ID NOs: 138, 148, 149, 150, or 151); one or more non-native BAAT or gabT genes such as an *S. kluyveri* pyd4 gene encoding the amino acid sequence of SEQ ID NO: 21 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 142), an *S. avermitilis* BAAT gene encoding the amino acid sequence of SEQ ID NO: 22 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 140), an *S. avermitilis* gabT gene encoding the amino acid sequence set forth in SEQ ID NO: 23, or an *S. cerevisiae* UGA1 gene encoding the amino acid sequence set forth in SEQ ID NO: 24 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 141); one or more non-native 3-HPDH genes such as an *E. coli* ydfG gene encoding the amino acid sequence of SEQ ID NO: 27 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 143) or an *S. cerevisiae* YMR226C gene encoding the amino acid sequence of SEQ ID NO: 129 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 144); one or more non-native malonate semialdehyde reductase genes such as an *M. sedula* malonate semialdehyde reductase gene encoding the amino acid sequence set forth in SEQ ID NO: 29 (and/or comprising the coding region of the nucleotide sequence set forth in SEQ ID NO: 343); one or more non-native HIBADH genes such as an *A. faecalis* M3A gene encoding the amino acid sequence set forth in SEQ ID NO: 28, a *P. putida* KT2440 or E23440 mmsB gene encoding the amino acid sequence set forth in SEQ ID NO: 30 or SEQ ID NO: 31, respectively, or a *P. aeruginosa* PAO1 mmsB gene encoding the amino acid sequence set forth in SEQ ID NO: 32; and/or one or more non-native 4-hydroxybutyrate dehydrogenase genes such as an *R. eutropha* H16 4hbd gene encoding the amino acid sequence set forth in SEQ ID NO: 33 or a *C. kluyveri* DSM 555 hbd gene encoding the amino acid sequence set forth in SEQ ID NO: 34.

Example 3A-0: Enzymatic activity assays for modified yeast strains expressing aspartate/malonate semialdehyde pathway genes

Preparation of crude cell-free extracts (CFE) for enzyme assays:

[00287] The indicated cells herein from shake flask or bioreactor cultures were collected by centrifugation, the supernatant discarded, and the cell pellet stored at -80°C as described above. For preparation of CFE, the cells pellets were thawed, washed with phosphate-buffered saline (PBS) and again collected by centrifugation. The supernatant was discarded and the cell pellet was resuspended in an approximately equal volume of lysis buffer containing 1% Protease Inhibitor Cocktail, P8215 from Sigma) in 2.0 mL microcentrifuge tubes. Approximately 300 µL of 0.5 mm zirconia beads (BioSpec) were added, and cell lysis was performed on FastPrep®-24 disruptor (MP Biomedicals) for 3 rounds at setting 6 / 20 seconds. Sample tubes were cooled on ice for 5 minutes between each round. After lysis, the samples were centrifuged at maximum speed in a microcentrifuge for 15 minutes at 4°C. The supernatants were transferred to fresh 1.5 mL tubes and kept on ice. Total protein concentrations in the lysates were determined using the Bio-Rad protein assay reagent (Bradford assay) and bovine serum albumin as the standard, according to the instructions provided by the manufacturer.

Pyruvate Carboxylase (PYC) activity:

[00288] Pyruvate carboxylase activity in CFE of the indicated cells herein was determined as follows. A stock reaction mix solution was prepared that, when combined with CFE in the assay reaction mixture, provides the following final concentration of components: Tris (pH 8.0), 100 mM; NaHCO₃, 10 mM; MgCl₂, 5 mM; NADH, 0.2 mM; ATP, 1 mM; acetyl CoA, 1 mM; pyruvate, 1 mM; biotin (if required by the PYC enzyme being assayed), 5 µM; bovine heart malate dehydrogenase, 0.02 units/mL. 270 µL of this mixture was added to the wells of a 96-well microtiter plate and 30 µL of an appropriately diluted CFE was added to start the reaction. Consumption of NADH was monitored at 340 nm using a SpectraMax 340 PC plate reader. Pyruvate carboxylase activity is expressed as nmoles NADH consumed/sec/mg protein.

Phosphoenolpyruvate Carboxylase (PPC) activity:

[00289] Phosphoenolpyruvate Carboxylase (PPC) activity in CFE may be determined as follows. A stock reaction mix solution is prepared that, when combined with CFE in the assay reaction mixture, provides the following final concentration of components: Tris (pH 8.0), 100 mM; NaHCO₃, 10 mM; MgCl₂, 5 mM; NADH, 0.1 mM; acetyl CoA, 0.5 mM; phosphoenolpyruvate, 3.3 mM; bovine heart (or porcine heart) malate dehydrogenase, 0.02 units/mL. 270 µL of this mixture is added to the wells of a 96-well microtiter plate and 30 µL

of an appropriately diluted CFE is added to start the reaction. Consumption of NADH is monitored at 340 nm using a SpectraMax 340 PC plate reader.

Aspartate Aminotransferase (AAT) activity:

[00290] Aspartate aminotransferase activity in CFE of the indicated cells herein was determined as follows. A stock reaction mix solution was prepared that, when combined with CFE in the assay reaction mixture, provides the following final concentration of components: 100 mM Tris (pH 8.0), 100 mM; NaHCO₃, 10 mM; MgCl₂, 5 mM; NADH, 0.1 mM; aspartate, 1mM; α -ketoglutarate, 1 mM; and malate dehydrogenase, 0.02 units/mL. In some assays, the stock reaction mixture also contained pyridoxal 5'-phosphate (0.1 mM). 270 μ L of this mixture was added to the wells of a 96-well microtiter plate and 30 μ L of an appropriately diluted CFE was added to start the reaction. Consumption of NADH was monitored at 340 nm using a SpectraMax 340 PC plate reader. Aspartate aminotransferase activity is expressed as nmoles NADH consumed/sec/mg protein.

Aspartate Decarboxylase (ADC) activity:

[00291] Aspartate Decarboxylase activity in CFE of the indicated cells herein was determined as follows. 165 μ L of 100 mM NH₄Ac buffer (pH 6.8), and 25 μ L of 80 mM aspartate were added to each well of a 96-well microtiter plate thermostatted at 37°C. The reaction was initiated by adding 10 μ L of CFE. At different time intervals (5, 10, 15, 20, 25, 30, 40, 60 minutes), 20 μ L of sample was withdrawn from the reaction mixture and added to 180 μ L of quenching buffer (2% formic acid plus 2mg/L of ¹³C labeled β -Alanine as internal standard). After filtration, β -Alanine in the sample was analyzed by LC/MS/MS. Slopes were obtained from β -Alanine vs time plots. Activity was calculated by dividing the slope by total cellular protein concentration in the reaction. ADC activity is expressed as μ moles β -alanine formed/sec/g protein.

[00292] A modified ADC assay was used in some experiments. In these cases, ADC activity in CFE of the indicated cells herein was determined as follows. 110 μ L of 100 mM NH₄Ac buffer (pH 7.6), and 80 μ L of 25 mM aspartate (after neutralizing with NaOH) were added to each well of a 96-well microtiter plate thermostatted at 40°C. The reaction was initiated by adding 10 μ L of CFE. At different time intervals (2, 4, 6, 8, 10 minutes), 20 μ L of sample was withdrawn from the reaction mixture and added to 180 μ L of quenching buffer (2% formic acid with 2 mg/L of ¹³C labeled β -Alanine as internal standard or quenched in 2% formic acid and then transferred 1:10 into 20% methanol/80% water with 2mg/L of ¹³C labeled β -Alanine as internal standard). After filtration, β -Alanine in the sample was analyzed by LC/MS/MS. Slopes were obtained from β -Alanine vs time plots. Activity was calculated by dividing the slope by total cellular protein concentration in the reaction. ADC activity is expressed as μ moles β -alanine formed/sec/g protein.

β -alanine aminotransferase (BAAT) activity:

[00293] β -Alanine aminotransferase (BAAT) activity in CFE was determined as follows. 190 μ L of a reaction mixture containing 100 mM of NH_4HCO_3 (pH 7.6), 8 mM α -ketoglutarate, 0.5 mM acetyl-CoA, 0.1 mM pyridoxal-5'-phosphate, and 200 mM β -alanine was added to a 96 well microtiter plate at room temperature. The reaction was initiated by adding 10 μ L of CFE. Samples of 20 μ L each were taken at 2, 4, 6, 8, 10, 12, 15, and 20 minutes and added to 75 μ L of quenching buffer (2.5% formic acid). Samples were neutralized and pH controlled by adding 5 μ L 10 M NaOH and 50 μ L 100 mM NaCO_3 (pH 10). Filtered samples were derivatized by mixing, at injection, with OPA (*o*-phthaldialdehyde) reagent, 10 mg/mL (Agilent Technologies 5061-3335). Glutamate derivatized with OPA was quantified after HPLC separation by fluorescence detection (excitation at 340 nm; emission at 460 nm). Samples of 15 μ L were injected onto an analytical reverse phase Gemini C18 column with 5 μ m packing (Phenomenex 150 x 4.6 mm). The column was equilibrated in 62.5% 20 mM phosphate buffer (pH 7.8) (A) and 37.5% methanol (B). Linear gradients were as follows: ramp to 40% B, 0-0.3 min; 40% B, 0.3-1 min; ramp to 85% B, 1-1.75 min; 85% B, 1.75-2.25 min; ramp to 37.5%, 2.25-3 min; 37.5% B, 3-4 min. The flow rate was 2 mL/min. Standard curves of glutamate in reaction buffer were used to determine the concentration of the samples. Slopes were obtained from [glutamate] vs time plots. Activity was calculated by dividing the slope with total cellular protein concentration in the reaction.

3-HP dehydrogenase (3-HPDH) activity:

[00294] 3-HP dehydrogenase activity in CFE of the indicated cells herein was determined as follows. 190 μ L of diluted (typically a 100X dilution) CFE in 100 mM of NH_4HCO_3 (pH 7.6) and NADPH were added to each well of a 96-well microtiter plate thermostatted at 37°C. The reaction was initiated by adding 10 μ L of 60mM malonate semialdehyde (MSA, freshly prepared in 10 mM H_2SO_4 from 200 mM MSA stock solution in 10 mM H_2SO_4). Samples of 20 μ L each were taken at 1, 2, 4, 6, 8, 10, and 12 minutes, and quenched in 80 μ L of boiling water. After cooling, mix 75 μ L of quenched mixture with 75 μ L of buffer containing 2 mM NH_4Ac (pH 6.8) and 3 mg/L of ^{13}C labeled 3-HP. After filtration, 3-HP in the sample was quantified by LC/MS/MS. Slopes were obtained from 3-HP vs time plots. Activity was calculated by dividing the slope by total cellular protein concentration in the reaction. 3-HP dehydrogenase activity is expressed as nmoles NADPH formed/sec/mg protein.

[00295] A modified 3-HPDH assay was used in some experiments. In these cases, 3-HPDH activity in CFE of the indicated cells herein was determined as follows. Malonate semi-aldehyde reduction was measured by following the disappearance of the NADPH over time at 340 nm. Malonate semi-aldehyde was synthesized in-house according to the protocol developed by Yamada and Jacoby (Yamada, E.W., Jacoby, W.B., 1960, Direct conversion of

malonic semialdehyde to acetyl-coenzyme A, Journal of Biological Chemistry, Volume 235, Number 3, pp. 589-594). The assay was conducted in a 96 well micro-plate, and the final volume was 200 μ L. The reaction was started by adding 30 μ L of CCE into 170 μ L of assay buffer (2 mM malonate semi-aldehyde, 100 mM Tris pH 8.0 and 0.5 mM NADPH). Absorbance at 340 nm was followed on a micro-plate reader (Spectra Max 340PC, Molecular Devices LLC, Sunnyvale, CA) for 10 minutes at room temperature ($\sim 25^{\circ}\text{C}$). One unit of 3-HPDH activity is defined as the amount of enzyme necessary to oxidize 1 μ mol of NADPH in one minute in the presence of malonate semi-aldehyde.

Example 3A-1: Insertion vectors for expressing aspartate decarboxylase (ADC) at the adh1202 locus

[00296] Several aspartate decarboxylase genes were codon-optimized for expression in *I. orientalis* and synthesized by GeneArt® (Burlingame, CA, USA) resulting in the plasmids listed in the Table 7. The synthetic genes arrived in the vector pMA-T and can be elicited from the vector via *Xba*I and *Pac*I restriction digest. The restriction fragment can then be cloned into the same sites in pMIBa107 placing the gene under the control of the PDC promoter and terminator, and allowing integration to occur at the *I. orientalis* adh1202 locus.

Table 7: Transformant constructs

Construction Plasmid	Gene Source	Gene Number	Gene SEQ ID NO	Integration construct	Transformant
1051387	<i>Helicobacter pylori</i>	P56065	132	pWTY10-0033-1	yWTY1-1 yWTY1-2
1051391	<i>Bacillus sp. TS25</i>	ZY440006.gene3	134	pWTY10-0033-2	yWTY1-5 yWTY1-6
1051389	<i>Corynebacterium glutamicum</i>	Q9X4N0	136	pWTY10-0033-3	yWTY1-9 yWTY1-10
1051388	<i>Clostridium acetobutylicum</i>	P58285	131	pWTY10-0033-4	yWTY1-13 yWTY1-14
1051390	<i>Bacillus licheniformis</i>	Q65158	138	pWTY10-0033-5	yWTY1-17 yWTY1-18
1045172	<i>Streptomyces avermitilis</i>		130	pWTY10-0033-7	yWTY1-25 yWTY1-26

[00297] Plasmids 1045172, 105387, 105388, 105389, 105390, and 105391 were digested with *Xba*I and *Pac*I and run on a 1.3% agarose gel using TBE buffer. Fragments of 400-500 bp from each digest corresponding to the ADC (panD) gene were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen).

[00298] Plasmid pMIBa107 was digested with *Xba*I and *Pac*I, treated with calf intestinal phosphatase (New England Biolabs) and the vector band purified after agarose gel electrophoresis in TBE buffer. The *Xba*I and *Pac*I digested panD fragments were ligated into this purified pMIBa107 vector using T4 DNA ligase and a Quick ligation kit (New England

Biolabs) according to the manufacturer's instructions. The ligation products were transformed into XL10-GOLD ULTRA cells (Agilent Technologies) according to manufacturer's instructions. Transformants were plated onto 2X YT+ amp plates and incubated at 37°C overnight. Twenty-four transformants from each reaction were picked to 2X YT + amp plates. Mini-prep DNA from four each of the resulting transformants was screened by *Apal*, *NcoI* and *SacI* digestion. Clones yielding the desired band sizes were confirmed to be correct by DNA sequencing and were designated as shown in Table 7. The resulting plasmids allow integration of each ADC gene at the *adh1202* locus with the expression cassette oriented in the forward direction.

[00299] Approximately 10 µg each of each integration construct was digested with *Apal* and *KpnI* and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Fragments of approximately 4450 bp for each plasmid were excised from the gel and extracted using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The concentration of the purified products was found to be between 39-138 ng/ul. 0.39-1.4 µg of the fragments from the integration constructs (digested with *Apal* and *KpnI*) were transformed into *I. orientalis* CNB1 as described above. Transformants were plated onto ura selection media and incubated at 37°C, re-streaked onto ura selection media, and incubated at 37°C overnight. Genomic DNA was prepared from the URA3+ colonies and checked by PCR to confirm integration. Primers 0611718 and 0611632 were used to amplify a 2.5 kbp fragment to confirm integration. Each PCR reaction contained 2.5 µL of genomic DNA, 12.5 pM each of primers 0611718 and 0611632, 1X Crimson *Taq*[™] Reaction Buffer (New England Biolabs), 0.2 mM dNTP mix, 0.625 Units Crimson *Taq*[™] DNA polymerase (New England Biolabs) in a final volume of 25 µL. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 30 seconds; 30 cycles each at 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 3 minutes; and 1 cycle at 68°C for 10 minutes.

[00300] Two URA3+ confirmed transformants for each construct were designated as shown in Table 7. These strains are heterozygous at the *adh1202* for the indicated ADC gene with expression driven by the PDC promoter and terminator from *I. orientalis*.

[00301] PanD expression and enzyme activity from strains listed in Table 7 (and strain MBin500, *supra*, as negative control) was tested. Overnight cultures of each strain were grown overnight in YPD ON at 37°C, diluted 1:50 into 25 mL of fresh YPD in 125 mL baffled flask at 37°C, and grown to an OD₆₀₀ ~2-8. The cell pellets were then used to prepare CFE, which was then assayed for ADC activity as described *supra*. Representative results are shown in Table 8.

Table 8: Transformant enzyme activity data

Strain	Source of ADC Gene	Gene SEQ ID NO	ADC activity
MBin500 (control)	N/A	N/A	0
yWTY1-1 yWTY1-2	<i>Helicobacter pylori</i>	132	0
yWTY2-5 yWTY2-6	<i>Bacillus sp. TS25</i>	134	0.31 0.15
yWTY3-9 yWTY3-10	<i>Corynebacterium glutamicum</i>	136	0.31 0.19
yWTY4-13 yWTY4-14	<i>Clostridium acetobutylicum</i>	131	0.37 0.23
yWTY5-17 yWTY5-18	<i>Bacillus licheniformis</i>	138	0.56 0.61
yWTY7-25 yWTY7-26	<i>Streptomyces avermitilis</i>	130	0.23 0.30

[00302] Next, homozygous versions of yWTY5-17 and yWTY7-25 were created. First, ura- derivatives yWTY5-17 and yWTY7-25 were isolated as described above. Genomic DNA was prepared from the FOA-resistant colonies and checked by PCR as describe above to confirm loss of the URA3 selectable marker. Primers 0611718 and 0611632 were used to amplify a 2.4 kbp fragment for integration with the ura marker present and 1100 bp fragment in the absence of the ura marker. Ura- strains of yWTY5-17 and yWTY7-25 that yielded a PCR fragment of 1100 bp with primers 0611718 and 0611632 were designated MIBa331 and MIBa332, respectively.

[00303] 10 µg each of pWTY10-0033-5 and pWTY10-0033-7 were digested with *Apal*, *KpnI*, and *NcoI* and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Fragments of approximately 4450 bp for each plasmid were excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit (Qiagen). The purified fragments from pWTY10-0033-5 and pWTY10-0033-7 were transformed into MIBa331 and MIBa332, respectively as described above. Transformants were plated onto ura selection media and incubated overnight at 37°C, and then re-streaked onto ura selection media and incubated at 37°C overnight. Genomic DNA was prepared and Crimson Taq™ PCRs were run to confirm integration as described above. Primers 0611718 and 0611632 amplify a 2.4 kbp fragment for integration with the ura marker present, and amplify a 1100 bp fragment in the absence of the ura marker. Transformants of MIBa331 and MIBa332 that yielded PCR fragments of 1100 bp and 2.4 kbp with primers 0611718 and 0611632 were saved and designated MIBa338 and MIBa337, respectively. MIBa337 is homozygous for ADC gene from *S. avermitilis* at the adh1202 loci and MIBa338 is homozygous for the ADC gene from *B. licheniformis* at the adh1202 loci. Both strains have

the control of the respective ADC gene under the PDC promoter and terminator from *I. orientalis*.

[00304] ADC expression and enzyme activity from the panD homozygous strains MIBa337 and MIBa338 were compared to the heterozygous panD strains yWTY5-17 and yWTY7-25. Cultures were grown in YPD overnight at 37°C, and then diluted 1:50 into 25 mL of fresh YPD in 125 mL baffled flasks at 37°C and grown to an OD₆₀₀ ~2-8. The cell pellets were used to prepare CFE, which was then assayed for ADC activity as described above. Representative results for two independent experiments are shown in Table 9A.

Table 9A: Transformant enzyme activity data

Strain	Source of panD Gene	Allele Type	Gene SEQ ID NO	ADC activity (Exp. 1, Exp. 2)
MBin500 (control)	N/A	N/A	N/A	0, 0
yWTY5-17	<i>Bacillus licheniformis</i>	heterozygous	138	0.6, 0.29
MIBa338	<i>Bacillus licheniformis</i>	homozygous	138	0, 0.22
yWTY7-25	<i>Streptomyces avermitilis</i>	heterozygous	130	0.19, 0.13
MIBa337	<i>Streptomyces avermitilis</i>	homozygous	130	0.28, 0.19

[00305] The results of a third independent experiment to compare ADC activity in CFE prepared from strains MIBa337 and MIBa338 are shown in Table 9B.

Table 9B: Transformant enzyme activity data

Strain	Source of panD gene	Allele type	Gene SEQ ID NO	ADC activity
MBin500 (control)	N/A	N/A	N/A	0
yWTY5-17	<i>Bacillus licheniformis</i>	heterozygous	138	0.218
MIBa338	<i>Bacillus licheniformis</i>	homozygous	138	0.453
yWTY7-25	<i>Streptomyces avermitilis</i>	heterozygous	130	0.087
MIBa337	<i>Streptomyces avermitilis</i>	homozygous	130	0.188

[00306] SDS-PAGE analysis of the samples above indicated that panD expression from MIBa338 was the highest among these strains.

[00307] Strains MBin500, MIBa337 and MIBa338 were evaluated in bioreactors for 3-HP production, using the method described herein. Control strain MBin500 produced no detectable 3-HP (average of two independent fermentations). Strain MIBa337 produced 1.33 g/L 3-HP (one fermentation performed) and strain MIBa338 produced 3.15 g/L 3-HP (average of three independent fermentations). Individual fermentations of strains MIBa337 and MIBa338 were further compared with respect to their 3-HP production performance and

ADC activity (Table 10). In order to account for differences in cell mass in these fermentations, the 3-HP production performance is reported in Table 10 as 3-HP concentration per unit of cell mass (expressed as [g/L 3-HP]/[g/L dry cell weight]). The results show the improved ADC activity and 3-HP production performance when using the *Bacillus licheniformis* panD gene (strain MIBa338) vs. the *Streptomyces avermitilis* panD gene (strain MIBa337).

Table 10: 3-HP production performance and ADC activity in strains MIBa337 and MIBa338

Fermentation time (hr)	MIBa337		MIBa338	
	ADC Activity (mmol/min/g prot)	3HP/DCW	Activity (mmol/min/g prot)	3HP/DCW
11	0.005	0.00	0.021	0.024
22	0.011	0.05	0.055	0.131
31	0.003	0.04	0.029	0.159
48	0.001	0.05	0.018	0.142

Example 3A-2: Insertion vectors for expressing β -alanine aminotransferase (BAAT) or 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the adh1202 locus

[00308] *I. orientalis* codon-optimized versions of BAAT from *S. avermitilis*, UGA1 from *S. cerevisiae*, PYD4 from *S. kluyveri*, YMR226c from *S. cerevisiae*, and *ydfG* from *E. coli* were synthesized by GeneArt® resulting in the plasmids listed below. The synthetic genes arrived in the vector pMA-T and can be elicited from the vector via digest using *Xba*I and *Pac*I. The digested fragment can then be cloned into the same sites in pMIBa107, placing the gene under the control of the PDC promoter and terminator and allowing integration to occur at the adh1202 locus.

Table 11: Transformant constructs

Construction Plasmid	Gene	Gene Source	SEQ ID NO	Integration construct	Transformant
1045169	gabT (UGA1)	<i>S. cerevisiae</i>	141	pMIBa122	MIBa310
1045170	BAAT	<i>S. avermitilis</i>	140	pMIBa121	MIBa309
1045171	BAAT (PYD4)	<i>S. kluyveri</i>	142	pMIBa124	MIBa312
1045173	3-HPDH (YMR226c)	<i>S. cerevisiae</i>	144	pMIBa123	MIBa311
1045168	3-HPDH (<i>ydfG</i>)	<i>E. coli</i>	143	pMIBa120	MIBa308

[00309] Plasmids 1054168, 1054169, 1054170, 1054171, 1054172, and 1054173 were digested with *Xba*I and *Pac*I and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Fragments of 761 (*ydfG*) bp from 1045168, 1430 (UGA1) bp from 1045169, 1370 (BAAT) bp from 1045170, 1442 (PYD4) bp from

1045171, or 814 (YMR226c) bp from 1045173 were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). Plasmid pMIBa107 was digested with *Xba*I and *Pac*I followed by treatment with CIP resulting in a 7.9 kbp linear fragment. The digest was purified using a QIAQUICK® PCR Purification Kit (Qiagen). The digested fragments of ydfG, UGA1, BAAT, PYD4, or YMR226c were then ligated into pMIBa107 (digested with *Xba*I and *Pac*I and treated with CIP) using T4 DNA ligase as described herein. The ligation products were transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated onto 2X YT+ amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by digestion with *Xba*I and *Pac*I. Clones yielding the desired band sizes were confirmed to be correct by DNA sequencing and designated pMIBa120, pMIBa121, pMIBa122, pMIBa123, and pMIBa124 for ydfG, BAAT, UGA1, YMR226c, or PYD4, respectively. The resulting plasmids allow integration of the desired gene at the adh1202 locus with the expression cassette oriented in the forward direction.

[00310] The integration constructs in Table 11 were used to integrate the genes of interest codon-optimized for expression in *I. orientalis* into the adh1202 locus under the control of the PDC promoter and terminator. The expression cassette also contains a URA3 selectable marker to allow selection of transformants within a ura⁻ host as described herein. The expression cassettes and adh1202 homology regions are flanked by *Apal* and *Kpn*I restriction sites to allow release of the fragment from the plasmid.

[00311] 15 µg each of integration constructs in Table 11 were digested with *Apal*, *Kpn*I, and *Nco*I and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Digestion with *Nco*I breaks up the vector backbone and makes it easier to extract the fragment of interest from the agarose gel. Fragments of 4884 bp, 5493 bp, 5553 bp, 4937 bp, and 5565 bp from pMIBa120, pMIBa121, pMIBa122, pMIBa123, and pMIBa124, respectively, were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). The concentration of the purified products was found to be between 80-120 ng/µL. 0.8-1.2 µg of the restriction fragments from pMIBa120-4 were transformed into *I. orientalis* CNB1 (ura⁻) as described herein. The transformants then were plated onto ura selection media and grown at room temperature for 60 hours. Transformants were re-streaked onto ura selection media and incubated at 37°C overnight.

[00312] Several transformants of each were checked by colony PCR to confirm integration. Correct integration was confirmed by using primer pairs that check the 5' and 3' ends of the integrations and are listed below. The primer 0611717 anneals in the PDC promoter in the reverse direction, while primer 0611225 anneals in the URA3 selectable marker in the

forward direction. Primers 0611631 and 0611632 anneal outside of the site of integration going in the forward and reverse directions, respectively; primers 0611717 and 0611631 amplify a 976 bp fragment in correct integrants; primers 0611225 and 0611632 amplify a 1.4 kbp fragment in correct integrants; and primers 0611631 and 0611632 amplify a 2.7 kbp fragment indicating a wildtype chromosome and will amplify fragments ~5 kbp for integrations. To create genomic DNA, one colony of each transformant was incubated in 50 μ L of 0.05 U/ μ L lyticase (Sigma, St. Louis, MO, USA) in TE at 37°C for 30 minutes followed incubation at 95°C for 10 minutes. PCRs were run as described herein to confirm integration. One transformant of each heterozygous integrant that yielded PCR fragments of 976 bp with 0611717 and 0611631, 1.4 kbp with 0611225 and 0611632, and 2.7 kbp with 0611631 and 0611632 was saved and designated MIBa308, MIBa309, MIBa310, MIBa311, and MIBa312 as shown in Table 11.

[00313] Cultures of the transformants MIBa308, MIBa309, MIBa310, MIBa311, and MIBa312 were grown overnight in YPD at 37°C. Cultures were then diluted 1:50 into 25 mL of fresh YPD in 125 mL baffled flask at 37°C and grown to an OD₆₀₀ ~4-10. Samples of the cells were analyzed for protein expression by SDS-PAGE using the methods described herein. CFE were also prepared from cell pellets from the cultures, and 3HP dehydrogenase activity was measured in the CFE using the method described herein. Expression of UGA1 and PYD4 from strains MIBa310 and MIBa312, respectively, was detected by SDS-PAGE by the appearance of a ~53 KDa band that was absent in strains not integrated for either gene. Expression of BAAT in MIBa309 was not detected by SDS-PAGE under these conditions. Table 12A shows the 3-HP dehydrogenase (3-HPDH) activity in the CFE of the strains.

Table 12A: Transformant enzyme activity data

Strain	Gene Overexpressed	Gene Source	Gene SEQ ID NO	3-HPDH activity
MBin500	N/A	N/A	N/A	0.28, 0.24
MIBa310	gabT (UGA1)	<i>S. cerevisiae</i>	141	0.39
MIBa309	BAAT	<i>S. avermitilis</i>	140	0.39
MIBa312	BAAT (PYD4)	<i>S. kluyveri</i>	142	0.45
MIBa311	3-HPDH (YMR226c)	<i>S. cerevisiae</i>	144	1.1
MIBa308	3-HPDH (<i>ydfG</i>)	<i>E. coli</i>	143	0.67

[00314] In an independent experiment using improved assay conditions, the BAAT activity in CFE prepared from strains MBin500 (control), MIBa310, MIBa309 and MIBa312 was compared. The results of this experiment are shown in Table 12B.

Table 12B: Transformant enzyme activity data.

Strain	Gene Overexpressed	Gene Source	Gene SEQ ID NO	BAAT activity
MBin500	N/A	N/A	N/A	0.67
MIBa310	gabT (UGA1)	<i>S. cerevisiae</i>	141	9.05
MIBa309	BAAT	<i>S. avermitilis</i>	140	0.42
MIBa312	BAAT (PYD4)	<i>S. kluyveri</i>	142	105.85

[00315] The plasmids pMIBa120-4 (*supra*) contain *NotI* restriction sites that flank the expression cassette as follows: PDC promoter, gene of interest (BAAT or 3-HPDH), PDC terminator, and the URA3 selection marker. The homology for integration at the *adh1202* locus is outside of the *NotI* restriction sites. These plasmids all have the expression cassette in forward orientation.

[00316] New plasmids were constructed with the expression cassette oriented in the reverse direction to allow ease of screening homozygous integration strains. Plasmids pMIBa120-4 were digested with *NotI* and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Fragments of 3.5 kbp (pMIBa120), 4.2 kbp (pMIBa121), 4.2 kbp (pMIBa122), 3.5 kbp (pMIBa123), and 4.2 kbp (pMIBa124) were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). Each of these fragments was ligated into the 5.2 kbp linear *NotI*/CIP treated pHJJ76-no ura using T4 DNA ligase as described herein. The ligation products were transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated onto 2X YT+ amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by *XbaI* and *KpnI* digestion. Clones yielding the desired band sizes were designated pMIBa131, pMIBa132, pMIBa133, pMIBa134, and pMIBa135 for UGA1, YMR226c, PYD4, ydfG, and BAAT, respectively. The resulting plasmids allow integration of the desired gene at the *adh1202* locus with the expression cassette oriented in the reverse direction.

[00317] Ura⁻ derivatives of MIBa308-12 were isolated as described herein. Several FOA-resistant colonies for MIBa308-12 were colony purified twice by growing on YPD plates 37°C. Genomic DNA was prepared from the FOA-resistant colonies and checked by PCR to confirm loss of URA3 selectable marker as described herein. Primers 0611631 and 0611632 anneal outside of the site of integration going in the forward and reverse directions, respectively. Primer 0611718 anneals in the PDC terminator upstream of the ura selectable marker; primers 0611632 and 0611631 amplify a 2.7 kbp fragment for a wildtype chromosome; and primers 0611718 and 0611632 amplify a 2.4 kbp fragment for an integration with the ura marker present and 1100 bp fragment in the absence of the ura

marker. One *ura⁻* strain of MIBa308-12 that yielded the PCR fragments of 1100 bp with 0611718 and 0611632, and 2.7 kbp with 0611631 and 0611632 was saved and designated MIBa314 (*ura⁻* strain of MIBa310), MIBa315 (*ura⁻* strain of MIBa312), MIBa316 (*ura⁻* strain of MIBa311), MIBa326 (*ura⁻* strain of MIBa308), and MIBa328 (*ura⁻* strain of MIBa309).

[00318] 10-15 µg each of pMIBa131, pMIBa132, pMIBa133, and pMIBa135 were digested with *Apal*, *KpnI*, and *NcoI* and run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. Digestion with *NcoI* facilitates extraction of the fragment of interest from the agarose gel. Fragments of 5553 bp, 4937 bp, 5565 bp, 5493 bp from pMIBa131-3, pMIBa135, respectively, were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). The concentration of the purified products was found to be between 67-80 ng/µL. 0.67-0.8 µg of the restricted fragments from pMIBa131-3, and pMIBa135 were transformed into MIBa314, MIBa316, MIBa315, or MIBa328 as described herein. Transformants were plated onto *ura* selection media and incubated overnight at 37°C, and then re-streaked onto *ura* selection media and incubated overnight at 37°C overnight. Genomic DNA was prepared from the URA3⁺ colonies and checked by PCR as described herein to confirm integration of the second expression cassette, making the strain homozygous for the gene of interest. Primers 0611718 and 0611632 amplify a 1100bp fragment for the first integration as described above, and primers 0611632 and 0611717 amplify a 814 bp fragment for the second integration in the reverse orientation. URA3⁺ transformants of each lineage that amplified a 1100 bp fragment with 0611718 and 0611632 and a 814 bp fragment with 0611717 and 0611632 were designated MIBa317, MIBa318, MIBa319, and MIBa329 (see Table 13).

Table 13: Transformant genotypes

Strain	Genotype
MIBa317	<i>adh1202Δ::</i> (PDC _{promo} -Opt.ScYMR226c, URA3-Scar)/ <i>adh1202Δ::</i> (PDC _{promo} -Opt.ScYMR226c, URA3) <i>ura3-/ura3-</i>
MIBa318	<i>adh1202Δ::</i> (PDC _{promo} -Opt.ScUGA1, URA3-Scar)/ <i>adh1202Δ::</i> (PDC _{promo} -Opt.ScUGA1, URA3) <i>ura3-/ura3-</i>
MIBa319	<i>adh1202Δ::</i> (PDC _{promo} -Opt.SkPYD4, URA3-Scar)/ <i>adh1202Δ::</i> (PDC _{promo} -Opt.SkPYD4, URA3) <i>ura3-/ura3-</i>
MIBa329	<i>adh1202Δ::</i> (PDC _{promo} -Opt.SaBAAT, URA3-Scar)/ <i>adh1202Δ::</i> (PDC _{promo} -Opt.SaBAAT, URA3) <i>ura3-/ura3-</i>

[00319] The expression and enzyme activities from strains homozygous or heterozygous for YMR226c, UGA1, PYD4, and BAAT were determined. Overnight cultures of MIBa309-12, MIBa317-9 and MIBa329 were grown in YPD ON at 37°C, and then diluted 1:50 into 25 mL of fresh YPD in 125 mL baffled flask at 37°C and grown to an OD₆₀₀ ~4-10. Samples of the cells were analyzed for protein expression by SDS-PAGE using the method described herein. CFE were also prepared from cell pellets from the cultures, and 3HP dehydrogenase

activity was measured in the CFE using the method described herein. Based on SDS-PAGE results, strains MIBa310, MIBa318, MIBa312 and MIBa319 contained a protein with a mass of ~53 KDa (the expected size of the proteins encoded by UGA1 or PYD4 genes). The band corresponding to this protein was not observed in the SDS-PAGE analysis of strain MBin500. In addition, expression of UGA1 and PYD4 from homozygous strains MIBa318 and MIBa319, respectively, was greater than the corresponding heterozygous strains MIBa310 or MIBa312 (as judged by the SDS-PAGE analysis). BAAT expression was not detected (by SDS-PAGE) in strains MIBa309 or MIBa329 under these conditions. Table 14A shows the 3-HP dehydrogenase ("3-HPDH") activity in CFE of strains MBin500, MIBa311 and MIBa317.

Table 14A: Transformant enzyme activity data

Strain	Gene Overexpressed	Gene SEQ ID NO	Source	Allele Type	3-HPDH activity
MBin500 (control)	N/A	N/A	N/A	N/A	0.13
MIBa311	3-HPDH (YMR226c)	144	<i>S. cerevisiae</i>	heterozygous	1.49
MIBa317	3-HPDH (YMR226c)	144	<i>S. cerevisiae</i>	homozygous	2.85

[00320] In an independent experiment using improved assay conditions, the BAAT activity in CFE prepared from strains MBin500 (control), MIBa319 and MIBa329 was compared. The results of this experiment are shown in Table 14B.

Table 14B: Transformant enzyme activity data

Strain	Gene Overexpressed	Gene SEQ ID NO	Source	BAAT activity
MBin500 (control)	N/A	N/A	N/A	0.67
MIBa319	BAAT (PYD4)	142	<i>S. kluyveri</i>	228.01
MIBa329	BAAT	140	<i>S. avermitilis</i>	0.38

[00321] Ura- derivatives of strains MIBa317, MIBa318, and MIBa319 were isolated as described herein. Several FOA-resistant colonies for MIBa317, MIBa18, and MIBa19 were colony purified by growing on YPD plates at 37°C. Genomic DNA was prepared from the FOA-resistant colonies and checked by PCR as described herein to confirm loss of URA3 selectable marker. Primers 0611718 and 0611632 amplify a 1100 bp fragment indicating the first integration as described above, and primers 0611632 and 0611717 amplify a 814 bp fragment indicating the presence of the second integration in the reverse orientation. Primers 0611718 and 0611631 amplify a 2.6 kbp fragment indicating the second integration with the

ura marker and a 1200 bp fragment without the ura marker. Ura⁻ strains of MIBa317 and MIBa318 that yielded PCR fragments of 1100 bp with 0611718 and 0611632, 814 bp with 0611632 and 0611717, or 1200 bp with 0611718 and 0611631 were saved and designated MIBa320 and MIBa321, respectively. When the ura marker was removed from MIBa319 a possible gene conversion event occurred resulting in MIBa322 as indicated by PCR (no 2.7 kbp fragment with 0611632 and 0611631 primers or 814 bp fragment with 0611632 and 0611717, but amplified 1100bp fragment with 0611718 and 0611632) so that both expression cassettes were oriented in the forward direction.

Example 3A-3: Construction of left-hand fragments of insertion vectors for expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the *pdh* locus

Left-hand fragment containing S. avermitilis ADC (SEQ ID NO: 130) and S. avermitilis BAAT (SEQ ID NO: 140)

[00322] To allow insertion of a gene for expression between the ENO1 promoter and PDC terminator regions, the pMhCt068 vector was digested with *Xba*I and *Pac*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 6.1 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00323] The *Saccharomyces kluyveri* BAAT (*pyd4*) gene (SEQ ID NO: 142) was then amplified with primers 0611196 and 0611186 which contain restriction sites for subsequent subcloning. The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of a mini-prep of plasmid containing the *S. kluyveri* *pyd4* gene, 1X *Pfx* Amplification Buffer (Invitrogen), 100 pmol each of primers 0611196 and 0611186, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L DMSO and 2.5 units of PlatinumR *Pfx* DNA Polymerase (Invitrogen). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 95°C for 2 minutes followed by 34 cycles each at 95°C for 30 seconds, 40.8°C for 30 seconds, and 72°C for 1 minute 30 seconds, with a final extension at 72°C for 5 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1428 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00324] The *pyd4* PCR product generated above was digested with *Xba*I and *Pac*I and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 1.4 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit

(Macherey-Nagel) according to the manufacturer's instructions. This purified DNA was cloned into the *Xba*I and *Pac*I restricted pMhCt068 vector described above in a ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 100 ng *Xba*I/*Pac*I pMhCt068 vector, 70.5 ng *Xba*I/*Pac*I pyd4 insert, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then cooled on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by digestion with *Xba*I and *Pac*I as described herein, with one verified isolate designated "left+pyd4#1".

[00325] A polynucleotide encoding the *S. avermitilis* ADC of SEQ ID NO: 17 and codon-optimized for expression in *E. coli* was amplified with the primers 0611376 and 0611377 (note that primer 0611376 results in the removal of the "T" base on the 5' end of the *Nhe*I restriction site following insertion via In-Fusion into pMhCt068, which removes an unwanted ATG start codon present in the initial pMhCt068 clone). The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of a mini-prep of plasmid containing the *S. avermitilis* ADC gene optimized for *E. coli*, 1X ThermoPol Reaction buffer (New England Biolabs), 100 pmol each of primers 0611376 and 0611377, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 2 μ L 100 mM MgSO₄, and 2 units of Vent_R® (exo-) DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 94°C for 2 minutes followed by 34 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 420 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00326] The "left+pyd4#1" plasmid then was digested with *Nhe*I and *As*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 7.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The purified PCR fragment above containing the *S. avermitilis* ADC gene optimized for *E. coli* was digested with *Nhe*I and *As*I and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 420 bp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The resulting fragment then was ligated into the linearized "left+pyd4#1" vector in a ligation reaction (20 μ L) containing

1X Quick ligation buffer (New England Biolabs), 100 ng *NheI*/*Ascl* “left+pyd4#1” vector, 31 ng of the *NheI* and *Ascl* digested panD insert, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature and then placed on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer’s instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the *S. avermitilis* ADC gene optimized for *E. coli* by digestion with *Ascl* and *Pvu* II as described herein. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt070.

[00327] The plasmid pMhCt070 served as the base vector for the addition of ADC and BAAT homologs that had been codon-optimized for expression in the yeast host. pMhCt070 was digested with *XbaI* and *PacI*, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 6.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer’s instructions. An *XbaI* and *PacI* digested fragment described above containing a polynucleotide that encodes the *S. avermitilis* BAAT (SEQ ID NO: 140) and codon-optimized for expression in *I. orientalis* was ligated into the pMhCt070 cut vector as follows: A ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 42 ng of the *XbaI* and *PacI* digested pMhCt070 vector, 4 μ L of the codon-optimized *S. avermitilis* BAAT *XbaI* and *PacI* digested insert, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then placed on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer’s instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired BAAT ORF by *XbaI*, *PacI*, and *Eco* RV digestion as described herein. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt072.

[00328] The *S. avermitilis* ADC gene codon-optimized for expression in *E. coli* in pMhCt072 then was replaced with a version codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130). The *I. orientalis* codon-optimized ADC gene (SEQ ID NO: 130) and desired additional restriction sites and flanking DNA were amplified with primers 0611378 and 0611379. The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of mini-prep of the plasmid containing the codon-optimized *S. avermitilis* panD (GeneArt®), 1X ThermoPol Reaction buffer (New England Biolabs), 100 pmol each of primers 0611378 and 0611379, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 2 μ L 100 mM MgSO₄, and 2 units of Vent_R® (exo-)

DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 94°C for 2 minutes followed by 34 cycles each at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 420 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00329] 5 µL of a mini-prep of pMhCt072 was digested with *Xba*I and *Pac*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 1% agarose gel electrophoresis in TAE buffer, and an approximately 7.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The isolated PCR product containing the codon-optimized *S. avermitilis* ADC gene from above was added to the vector in the following IN-FUSION™ Advantage PCR Cloning Kit (Clontech) reaction: the 10 µL reaction volume was composed of 6 µL of the pMhCt072 digested and purified vector, 1 µL of the purified codon-optimized panD PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Nhe*I, *Asc*I, and *Cl*aI digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt074 (Figure 7).

[00330] pMhCt074 is a left-hand PDC targeting construct containing the PDC promoter driving expression of the codon-optimized *S. avermitilis* ADC (panD, SEQ ID NO: 130), the TAL terminator, the ENO1 promoter driving expression of the codon-optimized *S. avermitilis* BAAT (SEQ ID NO: 140), the RKI terminator, the *I. orientalis* URA3 promoter and the 5' fragment of the *I. orientalis* URA3 ORF.

Left-hand fragment containing S. avermitilis ADC (SEQ ID NO: 130) and *S. kluyveri* BAAT (SEQ ID NO: 142)

[00331] To create a left-hand DNA construct that expresses the *S. kluyveri* BAAT (pyd4), a fragment from *Xba*I and *Pac*I digestion containing the *S. kluyveri* BAAT sequence codon-optimized for expression in *I. orientalis* (SEQ ID NO: 142, *supra*) was ligated into the pMhCt070 digested vector above as follows: A ligation reaction (20 µL) containing 1X Quick

ligation buffer (New England Biolabs), 42 ng of the *Xba*I and *Pac*I digested pMhCt070 vector, 4 µL of the codon-optimized *S. kluyveri* pyd4 insert digested with *Xba*I and *Pac*I, and 1 µL Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 minutes at room temperature, and then placed on ice. 5 µL of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired pyd4 ORF by *Xba*I, *Pac*I, and *Eco* RV digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt073.

[00332] The plasmid pMhCt073 contains the desired *S. kluyveri* BAAT (pyd4) sequence codon-optimized for expression in *I. orientalis* but does not contain the desired *S. avermitilis* ADC (panD) sequence codon-optimized for expression in *I. orientalis*. To move in this ORF, 5 µL of a mini-prep of pMhCt073 was digested with *Xba*I and *Pac*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 1% agarose gel electrophoresis in TAE buffer. An approximately 7.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The isolated PCR product containing the codon-optimized *S. avermitilis* panD (*supra*) was added to the vector in the following IN-FUSION™ Advantage PCR Cloning Kit (Clontech) reaction: the 10 µL reaction volume was composed of 6 µL of the pMhCt073 digested and purified vector, 1 µL of the purified codon-optimized panD PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Nhe*I, *Asc*I, and *Cla*I digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt076.

[00333] Plasmid pMhCt076 is a left-hand PDC targeting construct containing the PDC promoter driving expression of the *S. avermitilis* ADC codon-optimized for expression in *I. orientalis* (panD, SEQ ID NO: 130), the TAL terminator, the ENO1 promoter driving expression of the *S. kluyveri* BAAT codon-optimized for expression in *I. orientalis* (pyd4, SEQ ID NO: 142), the RKL terminator, the *I. orientalis* URA3 promoter and the 5' fragment of the *I. orientalis* URA3 ORF.

[00334] Sequencing determined that plasmid pMhCt076 contains an A to T nucleotide change at about 200 bp into the PDC promoter, and a G to T nucleotide change ~2/3 of the way thru the PDC promoter that are present in the pMhCt068 parent vector (*supra*). To address any concern about potential alteration in gene expression, a construct similar to pMhCt076 but containing the corrected PDC promoter was cloned as described below.

[00335] 5 μ L of a mini-prep of pMhCt082 was digested with Nhe and PaeI, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 4.7 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. 4 μ L of a mini-prep of pMhCt076 was digested with NheI and PaeI and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 3.3 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The purified 4.7 kbp vector and 3.3 kbp insert were then ligated together in a ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 3 μ L pMhCt082 vector, 6 μ L pMhCt076 insert, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then placed on ice. 5 μ L of this reaction was used to transform ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired PCR products by digestion with *Stu*I and *Not* I. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt083 (Figure 8).

[00336] Plasmid pMhCt083 is identical to pMhCt076 except at the former contains the correct PDC promoter sequence, while the latter has one A to T nucleotide change and one G to T nucleotide change described above. Testing showed no difference in panD enzymatic activity from strains expressing *S. avermitilis* panD from integration of pMhCt076 and pMhCt077 as compared to pMhCt083 and pMhCt077.

Left-hand fragment containing S. avermitilis ADC (SEQ ID NO: 130) and Saccharomyces cerevisiae gabT (SEQ ID NO: 141)

[00337] 4 μ L of a mini-prep of pMhCt083 was digested with XbaI and PaeI, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 6.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. A fragment digested with XbaI and PaeI containing the *Saccharomyces cerevisiae* gabT codon-optimized for expression in *I.*

orientalis (UGA1, SEQ ID NO: 141) was ligated into the pMhCt083 cut vector as follows: A ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 1 μ L of the purified pMhCt083 vector digested with *Xba*I and *Pac*I, 3 μ L codon-optimized *S. cerevisiae* UGA1 *Xba*I and *Pac*I digested insert, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature and then the tube was placed on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired BAAT ORF by *Xba*I and *Bgl*II digestion. A clone yielding the desired band sizes was designated pMhCt087 (Figure 9).

[00338] Plasmid pMhCt087 is a left-hand PDC targeting construct containing the PDC promoter driving expression of the *S. avermitilis* ADC codon-optimized for expression in *I. orientalis* (panD, SEQ ID NO: 130), the TAL terminator, the ENO1 promoter driving expression of the *S. cerevisiae* gabT codon-optimized for expression in *I. orientalis* (UGA1, SEQ ID NO: 141), the RKL terminator, the *I. orientalis* URA3 promoter and the 5' fragment of the *I. orientalis* URA3 ORF.

Example 3A-4: Construction of right-hand fragments of insertion vectors for expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the *pd*c locus

Right-hand fragment containing E. coli 3-HPDH (SEQ ID NO: 143)

[00339] 2 μ g of a mini-prep of pMhCt069 (*supra*) was digested with *Xba*I and *Pac*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 2.2 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. A fragment digested with *Xba*I and *Pac*I containing *E. coli* 3-HPDH gene codon-optimized for expression in *I. orientalis* (ydfG, SEQ ID NO: 143) was ligated into the pMhCt069 cut vector as follows: A ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 2 μ L of the purified pMhCt069 vector digested with *Xba*I and *Pac*I, 4 μ L of the codon-optimized *E. coli* ydfG insert digested with *Xba*I and *Pac*I, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then placed on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of

the resulting transformants were screened for proper insertion of the desired ydfG ORF by *Xba*I, *Pac*I, and *Eco* RV digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing as described herein and designated pMhCt075 (Figure 10).

[00340] Plasmid pMhCt075 contains the 3' fragment of the *I. orientalis* URA3 ORF, the URA3 terminator from *I. orientalis* followed by the URA3 promoter (for later looping out of the URA3 marker), *E. coli* 3-HPDH gene codon-optimized for expression in *I. orientalis* (ydfG, SEQ ID NO: 143) driven by the *I. orientalis* TDH3 promoter, and the *I. orientalis* PDC terminator regions.

Right-hand fragment containing Saccharomyces cerevisiae 3-HPDH (SEQ ID NO: 144)

[00341] A fragment digested with *Xba*I and *Pac*I containing the *S. cerevisiae* YMR226C gene codon-optimized for expression in *I. orientalis* (*supra*) was ligated into the pMhCt069 cut vector as follows: A ligation reaction (20 μ L) containing 1X Quick ligation buffer (New England Biolabs), 2 μ L of the purified pMhCt069 vector digested with *Xba*I and *Pac*I, 4 μ L of the codon-optimized *S. cerevisiae* 3-HPDH (YMR226C, SEQ ID NO: 144) insert digested with *Xba*I and *Pac*I, and 1 μ L Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then placed on ice. 5 μ L of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired YMR226C ORF by *Xba*I, *Pac*I, and *Eco* RV digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and was designated pMhCt077 (Figure 11).

[00342] Plasmid pMhCt077 contains the 3' fragment of the *I. orientalis* URA3 ORF, the URA3 terminator from *I. orientalis* followed by the URA3 promoter (for later looping out of the URA3 marker), the *S. cerevisiae* 3-HPDH gene codon-optimized for expression in *I. orientalis* (YMR226C, SEQ ID NO: 144) driven by the *I. orientalis* TDH3 promoter, and the *I. orientalis* PDC terminator regions.

Example 3A-5: Heterozygous and homozygous yeast strains expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the PDC locus

[00343] Examples 3A-3 and 3A-4 above describe the construction of various left-hand or right-hand constructs for targeting expression of three ectopic genes simultaneously to the *I. orientalis* PDC locus. Prior to transformation, approximately 10 μ g of each construct (one desired left-hand construct and one desired right-hand construct) was digested with *Nof*I to release the desired transforming DNA from the pUC18 backbone vector; for most digestions,

the restriction enzyme *PvuI* was also included with the *NotI* digestion. Restriction enzyme *PvuI* digests the pUC18 vector fragment approximately in half, making separation of the larger, desired DNA fragment more facile by gel electrophoresis. The larger, expression cassette containing band was separated from the pUC18 backbone DNA by gel electrophoresis, excised from the gel, and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. 30 μ L elution buffer was used for the elution step. An equimolar ratio of one left-hand and one right-hand construct totaling 10 μ L were used to transform the *I. orientalis* strain CNB1 or appropriate derivative. Transformants were selected on ura selection plates and placed at 37°C for growth. The next day, approximately twelve transformants were picked and restreaked for single colonies to ura selection plates and grown at 37°C. The following day, a single colony was picked from each of the streaks generated by each initial transformant and restreaked to ura selection plates for single colonies. After another night of growth at 37°C, a final single colony was picked from each streak and restreaked to a ura selection plate and grown overnight at 37°C. After this second round of single colony purification and outgrowth, genomic DNA was prepared for use in PCR to verify the desired targeted integration occurred as described above. For targeting to PDC using the left- and right-hand constructs, verification of the desired integration event was determined using primers 0611814, 0611554, and 0611555. Primer 0611554 binds in the *I. orientalis* genomic DNA just 3' of the PDC terminator region present in the right-hand PDC targeting constructs; primer 0611555 binds in PDC ORF and amplifies toward stop; and primer 0611814 binds near the 3' end of the TDH3 promoter region present in the right-hand constructs and amplifies in the 3' direction. Generation of an approximately 1.9 kbp band from PCRs that contained primers 0611814 and 0611554 indicated the occurrence of the desired integration event at the PDC locus. Generation of an approximately 1.4 kbp band from PCRs that contained primers 0611555 and 0611554 indicated the presence of a wild-type PDC locus. Since this integration event is the first targeting event in the diploid *I. orientalis* CNB1, the desired integrants will show both a 1.9 kbp band for primers 0611814 and 0611554 and a 1.4 kbp band from primers 0611555 and 0611554. Two independent transformants giving the desired band pattern for each plasmid were designated as shown in Table 15.

Table 15: Transformant genotypes

Strain	Plasmid w/ left-hand fragment	Plasmid w/ right-hand fragment	Genotype
yMhCt002	pMhCt074	pMhCt075	pdc Δ ::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SaBAAT, URA3, TDH3 _{promo} -Opt.EcYdfG)/PDC ura3-/ura3-
74/75 #1			

yMhCt004	pMhCt083	pMhCt077	pdcΔ::(PDC_{promo} -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.SkPyd4, URA3, $TDH3_{\text{promo}}$ -Opt.ScYMR226C)/PDC ura3-/ura3-
83/77 #2			
yMhCt005	pMhCt087	pMhCt077	pdcΔ::(PDC_{promo} -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.ScUGA1, URA3, $TDH3_{\text{promo}}$ -Opt.ScYMR226C)/PDC ura3-/ura3-
87/77 #2			

[00344] Next, a ura⁻ derivative of yMhCt004 or yMhCt005 was isolated as described above. Genomic DNAs from several FOA resistant colonies of each parent strain were screened by PCR for the desired loop-out event with primers 0611815 and 0611817. Primer 0611815 anneals in the RKL terminator of the left-hand construct and amplifies toward the URA3 promoter. Primer 0611817 anneals in $TDH3$ promoter and amplifies back toward the URA3 cassette. The presence of an 828 bp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.2 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions with Crimson Taq™ DNA polymerase (New England Biolabs) were carried out as described above. One FOA resistant colony from parent strain yMhCt004, designated yMhCt012, and one FOA resistant colony from parent strain yMhCt005, designated yMhCt007, gave the desired 828 bp band.

[00345] Strains yMhCt012 and yMhCt007 were next transformed to create homozygous strains with the PDC gene deleted and replaced with expression cassettes for panD, pyd4, and YMR226C or panD, UGA1, and YMR226C, respectively. Strain yMhCt012 was transformed with linear DNA from pMhCt083 and pMhCt077, while yMhCt007 was transformed with linear DNA from pMhCt087 and pMhCt077. After two rounds of single colony purification, genomic DNAs from several transformants from each parent strain were screened by PCR with primers 0611815 and 0611817 as described above. Two independently isolated integrants from each parent strain that had both the 828 bp band (from amplification of the ura3 scar region from the originally targeted PDC locus) and the 2.2 kbp band (from integration of the URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette from the second integration event on the other chromosome) were designated as shown in Table 16.

Table 16: Transformant genotypes

Strain designation	Parent strain	Genotype
yMhCt013	yMhCt012	pdcΔ::(PDC_{promo} -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.SkPyd4, URA3-Scar, $TDH3_{\text{promo}}$ -Opt.ScYMR226C) /pdcΔ::(PDC_{promo} -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.SkPyd4-URA3, $TDH3_{\text{promo}}$ -Opt.ScYMR226C) ura3-/ura3-
yMhCt014		

yMhCt008	yMhCt007	pdcΔ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD, $ENO1_{\text{promo}}^{-}$ -Opt.ScUGA1, URA3-Scar, $TDH3_{\text{promo}}^{-}$ -Opt.ScYMR226C) /pdcΔ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD, $ENO1_{\text{promo}}^{-}$ -Opt.ScUGA1, URA3, $TDH3_{\text{promo}}^{-}$ -Opt.ScYMR226C) ura3-/ura3-
yMhCt009		

[00346] A ura⁻ derivative of yMhCt008 was isolated as described above. Genomic DNAs from several FOA resistant colonies from the yMhCt008 parent strain were screened by PCR for the desired loop-out event with the primers 0611815 and 0611817 as described herein. The presence of an 828 bp band indicated the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired. Isolates that showed only the 828 bp band were further screened using primers 0611555 and 0611554 as described herein. Generation of an approximately 1.4 kbp band from PCRs that contained primers 0611555 and 0611554 indicated the presence of a wild-type PDC locus. An isolate lacking this band, indicating that the PDC locus on both chromosomes had been lost, was designated yMhCt010.

[00347] Strains were grown in shake flasks and CFE were prepared and assayed for aspartate decarboxylase (ADC) activity and 3-HP dehydrogenase (3-HPDH) activity as described in herein. The experimental results for several assay sets (denoted as Trials 1-4) are shown in Table 17. The strains from Trial 1 of Table 17 were also analyzed by SDS-PAGE as described herein. Strain 74/75 #1 and strain yMhCt002 of Trial 1 gave a band in SDS-PAGE analysis at 27 kD that was not present in the control strain of Trial 1 (MBin500). The size of this protein band is consistent with its identity as the protein encoded by the ydfG gene.

Table 17: Transformant enzyme activity data

Trial	Strain	Gene Overexpressed	Allele Type	ADC activity	3-HPDH activity
1	MBin500 (control)	N/A	N/A	Not tested	0.25
2				0.00	0.12
3				0.00	0.23
4				0.00	0.45
4				0.00	0.54
1	74/75 #1	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 140)	heterozygous	Not tested	0.68
1	yMhCt002	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 143) BAAT (SEQ ID NO: 140)	heterozygous	Not tested	0.93
2	yMhCt004	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) BAAT (SEQ ID NO: 142)	heterozygous	0.26	1.81
4				0.30	2.20
4				0.24	1.81
2	83/77 #2	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) BAAT (SEQ ID NO: 142)	heterozygous	0.25	1.80
2	yMhCt005	ADC (SEQ ID NO: 130)	heterozygous	0.17	1.51

4		3-HPDH (SEQ ID NO: 144)		0.17	1.26
4		gabT (SEQ ID NO: 141)		0.14	1.24
2	87/77 #2	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	heterozygous	0.20	1.68
3	yMhCt005	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	heterozygous	0.16	1.27
3	yMhCt008	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	homozygous	0.80	2.29
4				0.25	0.85
4				0.42	1.37
3	yMhCt009	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) gabT (SEQ ID NO: 141)	homozygous	.045	1.33
4				0.22	0.55
4				0.43	1.15
4	yMhCt013	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) BAAT (SEQ ID NO: 142)	homozygous	0.47	0.65
4				0.50	0.82
4	yMhCt014	ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144) BAAT (SEQ ID NO: 142)	homozygous	0.34	0.51
4				0.54	1.06

[00348] The experimental results for another assay set are shown in Table 17 (Trial 2). The strains from Trial 2 of Table 17 were also analyzed by SDS-PAGE as described herein. All strains of Trial 2 except MBin500 gave a band at 29 kD in the SDS-PAGE analysis. The size of this protein band is consistent with its identity as the protein encoded by the YMR226c gene. Strains yMhCt005 and 87/77 #2 for Trial 2 gave a band at 53 kD that was not present in the three other samples for this trial. The size of this protein band is consistent with its identity as the protein encoded by the UGA1 gene.

[00349] The experimental results for another assay set are shown in Table 17 (Trial 3). The strains from Trial 3 of Table 17 were also analyzed by SDS-PAGE as described herein. All strains of Trial 3 except MBin500 gave a band at 53 kD and a band at 29 kD in the SDS-PAGE analysis. The sizes of these protein bands are consistent with the proteins encoded by the UGA1 and YMR226c genes, respectively. Strains MBin500 and yMhCt005 of Trial 3 showed a band at 64 kD in the SDS-PAGE analysis that was absent in yMhCt008 and yMhCt009 for this trial. The size of this protein band is consistent with its identity as the protein encoded by the native pyruvate decarboxylase (PDC) gene in *I. orientalis* CNB1.

[00350] The experimental results for another assay set are shown in Table 17 (Trial 4). The strains from Trial 4 of Table 17 were also analyzed by SDS-PAGE as described herein. All strains of Trial 4 except MBin500 gave a band at 29 kD. The size of this protein band is consistent with its identity as the protein encoded by the YMR226c gene. Strains yMhCt005, yMhCt008, and yMhCt009 of Trial 4 showed a band at 53 kD. The size of this band is consistent with the protein encoded by the UGA1 gene. Strains yMhCt013, and yMhCt014 of Trial 4 showed a faint band at 53 kD. The size of this band is consistent with the protein

encoded by the PYD4 gene. Strains MBin500, yMhCt004, and yMhCt005 of Trial 4 showed a band at 64 kD that was absent in strains yMhCt008, yMhCt009, yMhCt013, and yMhCt014. The size of this protein band is consistent with its identity as the protein encoded by the native pyruvate decarboxylase (PDC) gene in *I. orientalis* CNB1.

[00351] Strains MBin500 and yMhCt008 were tested evaluated in bioreactors for 3-HP production, using the method described herein. Control strain MBin500 produced no detectable 3-HP (average of two independent fermentations). Strain yMhCt008 produced 2.45 g/L 3-HP (average of twelve independent fermentations).

Example 3A-6: Yeast strains expressing β -alanine aminotransferase (BAAT) or 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the adh1202 locus, and expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the pdc locus

[00352] 20 μ g of pMhCt077, pMhCt083, and pMhCt087 (*supra*) were digested with *NotI* and *PvuI* and then run on a 1% agarose gel using 89 mM Tris base-89 mM Boric Acid-2 mM disodium EDTA (TBE) buffer. *NotI* digested fragments of 3815 bp, 5332 bp, or 5320 bp from pMhCt077, pMhCt083, and pMhCt087, respectively, were excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). 560 ng of *NotI* digested pMhCt077 and 560 ng of *NotI* digested pMhCt083 or pMhCt087 were transformed into strains MIBa320, MIBa321, and MIBa322. MIBa320 was transformed with pMhCt077/83 and pMhCt077/87 combinations. MIBa321 was transformed with pMhCt077/87 and MIBa322 was transformed with pMhCt077/83 as described herein. Transformants were plated onto ura selection media and incubated for approximately 60 hours at room temperature. Transformants were re-streaked onto ura selection media and incubated at 37°C overnight. Genomic DNA was prepared from the URA3+ colonies and checked by PCR as described herein to confirm integration of the expression cassette. The primer pair 611814 and 611554 amplify a 1.9 kbp fragment indicating integration. The primer pair 611555 and 611554 amplify a 1.4 kbp fragment indicating a wildtype locus. One URA3+ transformant of each lineage that amplified PCR fragments of 1.9 kbp with 611554 and 611814 and 1.4 kbp with 611555 and 611554 was saved; these were designated MIBa323, MIBa324, MIBa325, and MIBa327 (see Table 18 for genotypes). Promoters and terminators were derived from *I. orientalis* genes.

Table 18: Transformant genotypes

Strain designation	Genotype
MIBa323	adh1202 Δ ::(PDC _{promo} -Opt.ScYMR226c, URA3-Scar)/ adh1202 Δ ::(PDC _{promo} -Opt.ScYMR226c, URA3-Scar)

	<p>pdΔ::(PDC_{promo}-Opt.SaPanD, ENO1_{promo}-Opt.ScUGA1, URA3, TDH3_{promo}-Opt.ScYMR226c)/PDC ura3-/ura3-</p>
MIBa324	<p>adh1202Δ::(PDC_{promo}-Opt.ScYMR226c, URA3-Scar)/ adh1202Δ::(PDC_{promo}-Opt.ScYMR226c, URA3-Scar) pdΔ::(PDC_{promo}-Opt.SaPanD, ENO1_{promo}-Opt.SkPyd4, URA3, TDH3_{promo}-Opt.ScYMR226c)/PDC ura3-/ura3-</p>
MIBa325	<p>adh1202Δ::(PDC_{promo}-Opt.ScUGA1, URA3-Scar)/adh1202Δ::(PDC_{promo}-Opt.ScUGA1, URA3-Scar) pdΔ::(PDC_{promo}-Opt.SaPanD, ENO1_{promo}-Opt.ScUGA1, URA3, TDH3_{promo}-Opt.ScYMR226c)/PDC ura3-/ura3-</p>
MIBa327	<p>adh1202Δ::(PDC_{promo}-Opt.SkPyd4, URA3-Scar)/ adh1202Δ::(PDC_{promo}-Opt.SkPyd4, URA3-Scar) pdΔ::(PDC_{promo}-Opt.SaPanD, ENO1_{promo}-Opt.SkPyd4, URA3, TDH3_{promo}-Opt.ScYMR226c)/PDC ura3-/ura3-</p>

[00353] Strains were grown in shake flasks and CFE were prepared and assayed for 3-HP dehydrogenase (3-HPDH) activity as described herein. The results are shown in Table 19.

Table 19: Transformant enzyme activity data

Strain	Gene Overexpressed	3-HPDH activity
MBin500 (control)	N/A	0.14
MIBa314	gabT (SEQ ID NO: 141)	0.09
MIBa318	gabT (SEQ ID NO: 141)	0.41
MIBa321	gabT (SEQ ID NO: 141)	0.08
MIBa325	gabT (SEQ ID NO: 141) ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144)	1.15
MIBa315	BAAT (SEQ ID NO: 142)	0.12
MIBa319	BAAT (SEQ ID NO: 142)	0.17
MIBa322	BAAT (SEQ ID NO: 142)	0.09
MIBa327	BAAT (SEQ ID NO: 142) ADC (SEQ ID NO: 130) 3-HPDH (SEQ ID NO: 144)	0.98
MIBa316	3-HPDH (SEQ ID NO: 144)	0.48
MIBa317	3-HPDH (SEQ ID NO: 144)	2.15
MIBa320	3-HPDH (SEQ ID NO: 144)	1.07
MIBa323	3-HPDH (SEQ ID NO: 144) ADC (SEQ ID NO: 130) gabT (SEQ ID NO: 141)	2.66
MIBa324	3-HPDH (SEQ ID NO: 144) ADC (SEQ ID NO: 130) BAAT (SEQ ID NO: 142)	2.12

[00354] Ura⁻ derivatives of MIBa323, MIBa324, MIBa325 and MIBa327 were isolated as described herein. Genomic DNA was prepared from the FOA-resistant colonies and checked by PCR as described herein to confirm loss of URA3 selectable marker. Primer 0611815 anneals in the RKL terminator of the left-hand construct and amplifies toward the URA3 promoter, and primer 0611817 anneals in TDH3 promoter and amplifies back toward the

URA3 cassette. The presence of an 828 bp band indicates the presence of only the URA3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.2 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. Ura⁻ strains of MIBa323, MIBa324, MIBa325, and MIBa327, that yielded PCR fragments of 828 bp with primers 0611815 and 0611817 were saved and designated MIBa335, MIBa333, MIBa334, and MIBa336, respectively.

[00355] Strains MIBa333 and MIBa334 were transformed with the fragments from pMhCt077 and pMhCt087, and strains MIBa335 and MIBa336 were transformed with the fragments from pMhCt077 and pMhCt083 as described above in the section on MIBa320-2 transformations. Transformants were selected for by growth on ura selection media as described herein. Genomic DNA was prepared from the URA3⁺ colonies and checked by PCR as described herein to confirm integration of the expression cassette. Primer 0611815 anneals in the RKI terminator of the left-hand construct and amplifies toward the URA3 promoter. Primer 0611817 anneals in TDH3 promoter and amplifies back toward the URA3 cassette. The presence of an 828 bp band indicates the presence of only the URA3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired for the first integration, and a band of approximately 2.2 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette for the second integration. Primer pair 0611815 and 0611816 amplifies a 625 bp fragment when the ura marker is present. Primers 0611555 and 0611554 amplify a 1.4 kbp fragment when the PDC locus is present. Homozygous integrants should not amplify a fragment with these primers. One URA3⁺ transformant of each lineage that amplified PCR fragments of 828 bp and 2.2 kbp with primers 0611815 and 0611817, 625 bp with primers 0611815 and 0611816 and no fragment with primers 0611555 and 0611554 was saved; these were designated MIBa340, MIBa341, MIBa345, and MIBa348 (see Table 20A). Promoters and terminators were derived from *I. orientalis* genes.

Table 20A: Transformant genotypes

Strain Designation	Genotype
MIBa345	adh1202Δ::(PDC _{promo} -Opt.ScYMR226c URA3-Scar)/adh1202Δ::(PDC _{promo} -Opt.ScYMR226c URA3-Scar) pdcΔ::(PDC _{promo} -Opt.SaPanD ENO1 _{promo} -Opt.ScUGA1, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c)/ pdcΔ::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa348	adh1202Δ::(PDC _{promo} -Opt.ScYMR226c URA3-Scar)/adh1202Δ::(PDC _{promo} -Opt.ScYMR226c URA3-Scar) pdcΔ::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c) / pdcΔ::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -

	Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa340	adh1202Δ:::(PDC _{promo} -Opt.ScUGA1 URA3-Scar)/ adh1202Δ:::(PDC _{promo} -Opt.ScUGA1 URA3-Scar) pdcΔ:::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.ScUGA1, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c)/ pdcΔ:::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} - Opt.ScUGA1, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-
MIBa341	adh1202Δ:::(PDC _{promo} -Opt.SkPyd4 URA3-Scar)/ adh1202Δ:::(PDC _{promo} -Opt.SkPyd4 URA3-Scar) pdcΔ:::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} -Opt.SkPyd4, URA3-Scar, TDH3 _{promo} -Opt.ScYMR226c) / pdcΔ:::(PDC _{promo} -Opt.SaPanD, ENO1 _{promo} - Opt.SkPyd4, URA3, TDH3 _{promo} -Opt.ScYMR226c) ura3-/ura3-

[00356] The aspartate 1-decarboxylase (ADC), beta-alanine aminotransferase (BAAT) and 3-HP dehydrogenase (3-HPDH) activities in CFE prepared from strains MBin500 (control), MIBa345, MIBa348, MIBa340 and MIBa341 were compared. The results of this experiment are shown in Table 20B.

Table 20B: Transformant enzyme activity data

Strain	Genes Overexpressed	Gene Sources	ADC Activity	BAAT Activity	3-HPDH Activity
MBin500 (control)	N/A	N/A	0.002	0.61	0.4
MIBa345	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.194	14.37	79.5
	ADC (SEQ ID NO: 130)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			
MIBa348	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.169	173.67	76.7
	ADC (SEQ ID NO: 130)	<i>S. avermitilis</i>			
	PYD4 (SEQ ID NO: 142)	<i>S. kluyveri</i>			
MIBa340	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.239	19.51	64.8
	ADC (SEQ ID NO: 130)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			
MIBa341	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.22	386.92	65.5
	ADC (SEQ ID NO: 130)	<i>S. avermitilis</i>			
	PYD4 (SEQ ID NO: 142)	<i>S. kluyveri</i>			

Example 3A-7: Left-hand fragments of insertion vectors with multiple nucleotide sequences for expressing aspartate 1-decarboxylase (ADC) at the adh1202 locus

[00357] Constructs were designed to incorporate four copies of nucleotides encoding an ADC (SEQ ID NO: 17) at the *I. orientalis* adh1202 locus. In a similar approach to that described herein for the PDC locus, a left-hand and a right-hand construct were designed to allow homologous recombination. The general design of the integration vectors and desired recombination event is shown in Figure 3. This approach was also used for expression of an alternative ADC (SEQ ID NO: 139) as described in the examples below.

[00358] To prevent recombination from occurring between the multiple copies of the nucleotide sequences encoding the same ADC sequence, four distinct nucleotide sequences codon-optimized for expression in *I. orientalis* (SEQ ID NOs: 130, 145, 146, and 147) were

designed to encode the same ADC sequence of SEQ ID NO: 17. Additionally, since the initial set of constructs was designed to target the ald5680 locus of *I. orientalis*, the adh1202 targeting sequences were incorporated into these vectors at a late step in the cloning. The ald5680 constructs can be used to target a second locus in an *I. orientalis* CNB1 strain already homozygous for ectopic four copies of panD at adh1202 with four additional copies of panD at ald5680.

[00359] The left-hand ald5680 targeting vector was constructed as follows. A PCR product containing the sequence just 5' of the ald5680 ORF, along with the desired additional restriction sites and flanking DNA for cloning was amplified with primers 0612271 and 0612272. The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of pHJJ75 mini-prep plasmid DNA (Figure 23), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612271 and 0612272, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 930 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00360] In order to allow purification of a greater quantity of DNA, the PCR reaction described above was repeated using the purified 930 bp PCR product as the template DNA. Five 50 μ L reactions were set up and amplified with the conditions described above except that 1 μ L of the purified 930 bp PCR product replaced the pHJJ75 plasmid (*supra*) as template DNA. Following thermocycling, the amplified 930 bp product was purified as above.

[00361] A fragment containing PDC promo-optPanD-ENO1-UGA1 (which contains the *I. orientalis* codon-optimized *S. avermitilis* ADC encoding sequence of SEQ ID NO: 130) was excised from pMhCt087 (*supra*) via *NotI* and *EcoRI* digestion. 10 μ g of a midi-prep of pMhCt087 was digested with *NotI* and *EcoRI* and then purified by 0.9% agarose gel electrophoresis in TAE buffer. An approximately 4.4 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00362] A PCR product containing the 5' half of the URA3 split marker with the desired additional restriction sites and flanking DNA for cloning was amplified using the primers 0612273 and 0612274. The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of pMhCt082 mini-prep plasmid DNA (*supra*), 1X iProof™ HF buffer (Bio-Rad Laboratories),

100 pmol each of primers 0612273 and 0612274, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 960 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00363] To create a recipient vector for the above DNA fragments, the plasmid pUC19 (Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, 33, 103-119) was digested with *Hind* III and *Eco*RI, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 2.6 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00364] The purified 930 bp, 4.4 kbp, and 960 bp DNA fragments from above were then inserted into the digested pUC19 fragment using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 μ L composed of 150 ng of the pUC19 vector digested with *Hind* III and *Eco*RI, 56 ng of the 930 bp DNA containing the ald5680 flanking DNA, 250 ng of the PDC promo-optPanD-ENO1-UGA1 fragment from pMhCt087 digested with *Not*I and *Eco*RI, 55 ng of the 960 bp PCR product 5' containing the 5' half of the URA3 split marker, 1X In-Fusion reaction buffer (Clontech) and 1 μ L of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction then was diluted with 40 μ L of TE buffer and 2.5 μ L was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Sal*I and *Hpa*I digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt089.

[00365] Next, the UGA1 ORF in pMhCt089 was replaced with the *S. avermitilis* panD gene codon-optimized for expression in *I. orientalis* which encodes the ADC of SEQ ID NO: 17. *S. avermitilis* panD version r1 (SEQ ID NO: 145) was synthesized by GeneArt® in the vector pMA-T. The plasmid pMA-T was digested with *Xba*I and *Pac*I and the resulting fragments were separated by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately

434 bp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. Plasmid pMhCt089 was digested with *Xba*I and *Pac*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and the resulting fragments were separated by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 4.5 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The pMhCt089 vector and *S. avermitilis* panD version r1 were joined in a ligation reaction (20 µL) containing 1X Quick ligation buffer (New England Biolabs), 2 µL *Xba*I/*Pac*I pMhCt089 vector, 2 µL *Xba*I/*Pac*I *S. avermitilis* panD version r1 insert, and 1 µL Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then placed on ice. 5 µL of this reaction was used to transform ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Xba*I and *Pac*I digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt092.

[00366] The final cloning step for the left-hand construct was to replace the ald5680 5' homology region present in pMhCt092 with the adh1202 5' homology region. A PCR product containing the sequence 5' of the adh1202 ORF, along with the desired additional restriction sites and flanking DNA for cloning was amplified with the primers 0612470 and 0612471. The PCR reaction (50 µL) contained 1 µL of a 1 to 50 dilution of pGMEr140 mini-prep plasmid DNA (a derivative of pMIBa107 described herein wherein the PCR amplified region is identical), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612470 and 0612471, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 790 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00367] To create a recipient vector for the above PCR product, the plasmid pMhCt092 was digested with *Hpa*I and *Not*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 7.0 kbp band was excised from the gel and purified using a

NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The PCR product and linear vector were joined using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 µL composed of 120 ng pMhCt092 vector digested with *HpaI* and *NotI*, 30 ng of the adh1202 5' homology containing PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by digestion with *BamHI* and *PstI*. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt095 (Figure 12).

[00368] Plasmid pMhCt095 is a left-hand *I. orientalis* adh1202 targeting construct containing the PDC promoter driving expression of a *S. avermitilis* ADC gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130), the TAL terminator, the ENO1 promoter driving expression of a second *S. avermitilis* ADC gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 145), the *I. orientalis* RKI terminator, the *I. orientalis* URA3 promoter and the 5' fragment of the *I. orientalis* URA3 ORF.

Example 3A-8: Right-hand fragments of insertion vectors with multiple nucleotide sequences for expressing aspartate 1-decarboxylase (ADC) at the adh1202 locus

[00369] A PCR product containing the 3' fragment of the *I. orientalis* URA3 ORF, along with the desired additional restriction sites and flanking DNA for cloning was amplified with primers 0612275 and 0612276. The PCR reaction (50 µL) contained 1 µL of a 1 to 50 dilution of pMhCt069 mini-prep plasmid DNA (*supra*), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612275 and 0612276, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 1155 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00370] A fragment containing the TDH3 promoter, *Xba*I and *Pac*I restriction sites for insertion of an ectopic gene, and the PDC terminator was excised from pMhCt069 via digestion with *Not*I and *Pme*I. 10 µg of a midi-prep of pMhCt069 was digested with *Not*I and *Pme*I and then purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 1.85 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00371] A PCR product containing the sequence 3' of the ald5680 ORF, along with the desired additional restriction sites and flanking DNA for cloning was amplified with the primers 0612277 and 0612278. The PCR reaction (50 µL) contained 1 µL of a 1 to 50 dilution of pHJJ75 mini-prep plasmid DNA (Figure 23), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612277 and 0612278, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 844 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00372] The purified 1155 bp, 1.85 kbp, and 844 bp DNA fragments from above were then inserted into pUC19 digested with *Eco*RI and *Hind*III using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 µL composed of 150 ng of the fragment from the pUC19 vector digested with *Hind*III and *Eco*RI; 66 ng of the 1155 bp DNA containing the 3' portion of the URA3 split marker; 106 ng of the 1.85 kbp fragment digested with *Pme*I and *Not*I and containing the TDH3 promoter, *Xba*I and *Pac*I restriction sites for insertion of an ectopic gene, and PDC terminator from pMhCt069; 48 ng of the 844 bp PCR product containing the 3' ald5680 flanking DNA; 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Sal*I and *Hpa*I digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated ald5680 right #20.

[00373] The TKL terminator, the PGK1 promoter, *Xba*I and *Pac*I restriction sites, and a shorter version of the PDC terminator region was added between the TDH3 promoter and 3' ald5680 flanking DNA of ald5680 right #20 as follows. The TKL terminator along with the desired additional restriction sites and flanking DNA for cloning was amplified with the primers 0612356 and 0612357. The desired PCR product was amplified using a temperature gradient for the annealing temperature and DMSO in some reactions. Four identical PCR reactions were prepared, with each PCR reaction (50 μ L) containing 1 μ L of a 1 to 50 dilution of pACN23 mini-prep plasmid DNA (Figure 20), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612356 and 0612357, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). A second set of four tubes was set up as above except that the reactions each included the addition of 1.5 μ L of DMSO. The PCRs were performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, X°C for 20 seconds, where X = 47.6°C, 51.8°C, 57.1°C, or 62.1°C, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. A PCR with and without DMSO was run for each annealing temperature shown. Following thermocycling, 10 μ L of each PCR reaction was separated by 1% agarose gel electrophoresis in TAE buffer. Visualization of this gel revealed that PCR reactions performed with DMSO at the two highest annealing temperatures and without DMSO at the two lowest annealing temperature gave the highest yield of the desired 844 bp product. These four PCRs were combined, separated by 1% agarose gel electrophoresis in TAE buffer, where the approximately 844 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00374] PCR amplification of the desired PGK1 promoter region was done as a two step process. First, a PCR product containing the PGK1 promoter DNA was cloned following amplification with the following primers 0612150 and 0612151. The PCR reaction (50 μ L) contained 3 μ L of pJLJ49 mini-prep DNA (Figure 25), 1X *Pfx* amplification buffer (Invitrogen), 2 mM MgSO₄, 100 pmol each of primers 0612150 and 0612151, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1.25 Units Platinum® *Pfx* DNA polymerase (Invitrogen). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 95°C for 2 minutes followed by 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 630 bp PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00375] The approximately 630 bp PCR product was cloned into pCR4™BLUNT TOPO® (Invitrogen) vector using the Zero Blunt® TOPO® PCR cloning kit for sequencing (Invitrogen) according to the manufacturer's instructions. In a total reaction volume of 6 µL either 0.5 or 4 µL of the 630 bp PCR product, 1 µL salt solution (Invitrogen) and 1 µL pCR4™BLUNT TOPO® (Invitrogen) were incubated together at room temperature for 15 minutes. 2 µL of each cloning reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) cells according to manufacturer's instructions. Transformants were plated onto LB+kan plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR product by *EcoRI* digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated PGK1_in_TOPO.

[00376] The PGK1 promoter from PGK1_in_TOPO was isolated and purified prior to use as a PCR template as follows. 25 µL of a midi-prep of PGK1_in_TOPO was digested with *XbaI* and *PacI* and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 640 bp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00377] The PGK1 promoter along with the desired additional restriction sites and flanking DNA for cloning was amplified with the primers 0612358 and 0612359 using a temperature gradient. Eight identical PCR reactions were set up, each PCR reaction (50 µL) contained 20 ng of purified PGK1 promoter DNA via *XbaI* and *PacI* digestion of PGK1_in_TOPO, 1X Herculase reaction buffer (Agilent Technologies), 100 pmol each of primers 0612358 and 0612359, 200 µM each of dATP, dCTP, dGTP, and dTTP, and 2.5 units of Herculase HotStart DNA Polymerase (Agilent Technologies). The PCRs were performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, X°C for 20 seconds, where X = 53.7°C, 55.4°C, 57.6°C, 60.0°C, 62.4°C, 64.8°C, 66.9°C, 68.6°C, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, 10 µL of each PCR reaction was separated by 1% agarose gel electrophoresis in TAE buffer. Visualization of this gel revealed that four PCR reactions performed with the highest annealing temperature gave the highest yield of the desired approximately 700 bp product. These four PCRs were combined, separated by 1% agarose gel electrophoresis in TAE buffer, where the approximately 700 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00378] Plasmid ald5680_right #20 contains approximately 870 bp of the region downstream from the *I. orientalis* PDC ORF as the PDC terminator region. However, this

region is likely larger than necessary for proper function as a terminator and if maintained in its current size might serve as a catalyst for unwanted homologous recombination to the PDC locus. Therefore, a PCR product to replace the PDC terminator in ald5680_right #20 with a smaller version was amplified with the primers 0612360 and 0612361. The desired PCR product was amplified using a temperature gradient for the annealing temperature and DMSO in some reactions. Four identical PCR reactions were set up, each PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of pJLJ49 mini-prep plasmid DNA (Figure 25), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612360 and 0612361, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). A second set of four tubes was set up as above except that the reactions each included the addition of 1.5 μ L of DMSO. The PCRs were performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 32 cycles each at 98°C for 10 seconds, X°C for 20 seconds, where X = 47.6°C, 51.8°C, 57.1°C, or 62.1°C, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. A PCR with and without DMSO was run for each annealing temperature shown. Following thermocycling, 10 μ L of each PCR reaction was separated by 1% agarose gel electrophoresis in TAE buffer. Visualization of this gel revealed that the four PCR reactions performed with DMSO, regardless of annealing temperature, gave the highest yield of the desired 338 bp product. These four PCRs were combined, separated by 1% agarose gel electrophoresis in TAE buffer, where the approximately 338 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00379] PCR was used to create a single amplification product fusing approximately 700 bp PGK1 containing PCR product to the 338 bp PDC terminator product. The PCR reaction (50 μ L) contained 107 ng of the PGK1 containing PCR product, 56 ng of the PDC terminator containing PCR product, 1X Phusion HF buffer (New England Biolabs), 100 pmol each of primers 0612358 and 0612361, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 56°C for 20 seconds, and 72°C for 2 minutes and 45 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 1020 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00380] The plasmid ald5680_right #20 was digested with *Xba*I and *Not*I, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified

by 0.9% agarose gel electrophoresis in TAE buffer. A band of approximately 5.6 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00381] The purified 487 bp and 1020 bp PCR products from above were then inserted into the digested ald5680_right#20 fragment using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 µL composed of 150 ng of the ald5680_right #20 vector digested with *Xba*I and *Not*I, 13 ng of the TKL terminator PCR product, 28 ng of the PGK1 promoter-PDC terminator PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *Acc*I digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt091.

[00382] Plasmid pMhCt091 is an empty right-hand *I. orientalis* ald5680 targeting construct containing the 3' fragment of the *I. orientalis* URA3 ORF, the *I. orientalis* TDH3 promoter followed by *Nhe*I and *As*cI restriction sites for addition of a gene of interest, the *I. orientalis* TKL terminator, the *I. orientalis* PGK1 promoter followed by *Xba*I and *Pac*I restriction sites for addition of a second gene of interest, the *I. orientalis* PDC terminator, and flanking DNA to target homologous recombination to the 3' ald5680 locus.

[00383] *S. avermitilis* panD version r5 (SEQ ID NO: 146) was synthesized in the vector 1075328_SaPanD_r5 by GeneArt®. The *Xba*I and *Pac*I restriction sites were changed to the desired *Nhe*I and *As*cI sites for cloning into the 5' cloning site of pMhCt091 by PCR. The PCR reaction (50 µL) contained 1 µL of a 1 to 50 dilution of 1075328_SaPanD_r5 mini-prep plasmid DNA (GeneArt®), 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612378 and 0612379, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL DMSO and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 0.9% agarose gel electrophoresis in TAE buffer where an approximately 471 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00384] The plasmid pMhCt091 (*supra*) was digested with *NheI* and *Ascl*, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer. A band of approximately 6.9 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The purified panD r5 containing PCR product from above was then inserted into the digested pMhCt091 fragment using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 µL composed of 150 ng of the pMhCt091 vector digested from *NheI* and *Ascl*, 19 ng of the panD r5 PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 µL of TE buffer and 2.5 µL was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by *SmaI* digestion. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt093.

[00385] The plasmid pMhCt093 was digested with *XbaI* and *PacI*, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 7.4 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. *S. avermitilis* panD version r2 (SEQ ID NO: 147) was codon-optimized for expression in *I. orientalis* and synthesized in the vector pMA-T by GeneArt®. The plasmid was digested with *XbaI* and *PacI* and the resulting fragments were separated by 0.9% agarose gel electrophoresis in TAE buffer. A band of approximately 434 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00386] The purified ~434 bp fragment above was cloned into the pMhCt093 vector digested with *XbaI* and *PacI* in a ligation reaction (20 µL) containing 1X Quick ligation buffer (New England Biolabs), 2 µL of the pMhCt093 vector digested with *XbaI* and *PacI*, 2 µL of the ~434 bp fragment above, and 1 µL Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then the tube placed on ice. 5 µL of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at room temperature for three days. Several of the resulting transformants were screened for proper insertion of the desired PCR products by digestion with *XbaI* and *PacI*. Isolate pMhCt094 was chosen for future work.

[00387] The final cloning step for the right-hand construct was to replace the ald5680 3' homology region present in pMhCt094 with the adh1202 3' homology region. A PCR product containing the sequence just 3' of the adh1202 ORF, along with the desired additional restriction sites and flanking DNA for cloning was amplified with the primers 612472 and 612473. The PCR reaction (50 μ L) contained 1 μ L of a 1 to 50 dilution of pGMEr140 (*supra*) mini-prep plasmid DNA, 1X iProof™ HF buffer (Bio-Rad Laboratories), 100 pmol each of primers 0612472 and 0612473, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 1 unit of iProof™ High Fidelity DNA polymerase (Bio-Rad Laboratories). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 30 seconds followed by 34 cycles each at 98°C for 10 seconds, 59°C for 20 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer where an approximately 620 base pair PCR product was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00388] To create a recipient vector for the above PCR product, the plasmid pMhCt094 was digested with *SacII* and *NotI*, treated with 10 units calf intestinal phosphatase (New England Biolabs) at 37°C for 30 minutes, and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 7.0 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The PCR product and linear vector were joined using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a total reaction volume of 10 μ L composed of 191 ng of the pMhCt094 vector digested with *SacII* and *NotI*, 36 ng of the adh1202 3' homology containing PCR product, 1X In-Fusion reaction buffer (Clontech) and 1 μ L of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. The reaction was diluted with 40 μ L of TE buffer and 2.5 μ L was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired PCR products by digestion with *NsiI* and *PvuI*. A clone yielding the desired band sizes was confirmed to be correct by DNA sequencing and designated pMhCt096 (Figure 13).

[00389] Plasmid pMhCt096 is a right-hand *I. orientalis* adh1202 targeting construct containing the 3' fragment of the *I. orientalis* URA3 ORF, the *I. orientalis* TDH3 promoter driving expression of a third *S. avermitilis* ADC gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 146), the *I. orientalis* TKL terminator, the *I. orientalis* PGK1 promoter

driving expression of a forth *S. avermitilis* ADC gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 147), the *I. orientalis* PDC terminator, and flanking DNA to target homologous recombination to the 3' adh1202 locus.

Example 3A-9: Yeast strains expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the pdc locus; and aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus

[00390] Examples 3A-7 and 3A-8 above describe creation of left-hand and right-hand constructs for targeting expression of four nucleotide variants of the *S. avermitilis* ADC gene codon-optimized for expression in *I. orientalis* at the adh1202 locus. Prior to transformation into *I. orientalis* CNB1, 10 μ g of pMhCt095 was digested with *Hpa*I and *Sac*II to release the desired transforming DNA from the pUC19 backbone vector. Likewise, 10 μ g of pMhCt096 was digested with *Eco*RI and *Sac*II to release the desired transforming DNA from the pUC19 backbone vector. The ~5 kbp expression cassette containing band was separated from the pUC19 backbone DNA by gel electrophoresis, excised from the gel, and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. 30 μ L elution buffer was used for the elution step. An equimolar ratio of pMhCt095 and pMhCt096 linear transformation DNA totaling 10 μ L were used to transform strain yMhCt010 (*supra*). Transformants were selected on ura selection plates and placed at 37°C for growth. Approximately twelve transformants were picked the following day and restreaked for single colonies to ura selection plates and grown at 37°C overnight, and then a single colony was picked from each of the streaks generated by each initial transformant and restreaked to ura selection plates. After another night of growth at 37°C, a final single colony was picked from each streak and restreaked to a ura selection plate and grown overnight at 37°C. After this second round of single colony purification and outgrowth, genomic DNA was prepared for use in PCR to verify the desired targeted integration occurred as described herein. Correct targeting of the pMhCt095 and pMhCt096 fragments to the adh1202 locus was verified using primers 0611718 and 0611632 (*supra*). Primer 0611718 binds in the PDC terminator region present in pMhCt096, while primer 0611632 binds in adh1202 locus DNA 3' of the region targeted and amplifies in the anti-sense direction. Generation of an approximately 727 bp band from PCRs with these primers indicated the occurrence of the desired integration event at the adh1202 locus.

[00391] A PCR reaction (25 μ L) contained 0.5 μ L genomic DNA for the strain to be screened, 1X Crimson Taq™ Reaction Buffer (New England Biolabs), 25 pmol of the sense primer, 25 pmol of the anti-sense primer, 200 μ M each of dATP, dCTP, dGTP, and dTTP, and 0.625 units of Crimson Taq™ DNA polymerase (New England Biolabs). The PCR was

performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 95°C for 30 seconds followed by 30 cycles each at 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 2.5 minutes, with a final extension at 68°C for 10 minutes. Following thermocycling, the PCR reaction products were separated by 1% agarose gel electrophoresis in TAE buffer and the sizes of the bands visualized and interpreted as described above. Two independently isolated transformants giving the desired 727 bp band were designated yMhCt019 or 95/96 2 (see genotype in Table 21).

Table 21: Transformant genotype

Strain	Parent strain	Genotype
yMhCt019	yMhCt010	adh1202Δ::(<i>PDC_{promo}</i> -Opt.SaPanD r10, <i>ENO1_{promo}</i> -Opt.SaPanD r1, URA3, <i>TDH3_{promo}</i> -Opt.SaPanD r5, <i>PGK1_{promo}</i> -Opt.SaPanD r2)/ADH1202 <i>pdca</i> ::(<i>PDC_{promo}</i> -Opt.SaPanD <i>ENO1_{promo}</i> -Opt.ScUGA1, URA3-Scar, <i>TDH3_{promo}</i> -Opt.ScYMR226C)/ <i>pdca</i> ::(<i>PDC_{promo}</i> -Opt.SaPanD, <i>ENO1_{promo}</i> -Opt.ScUGA1, URA3-Scar, <i>TDH3_{promo}</i> -Opt.ScYMR226C) <i>ura3</i> -/ura3-
95/96 2		

[00392] A *ura*⁻ derivative of yMhCt019 then was isolated as described herein. Genomic DNAs from several FOA resistant colonies of yMhCt019 were screened by PCR for the desired loop-out event with the primers 0611815 and 0612795. Primer 0611815 anneals in the RKL terminator of the left-hand construct and amplifies toward the URA3 promoter, while primer 0612795 anneals within 3' *adh1202* homology (of pMhCt096 or endogenous *adh1202* locus) back toward 5' region. PCR reactions were carried out as described for the isolation of yMhCt019 above except that the length of the extension phase was changed to 3.5 minutes. Generation of a 3.7 kbp band with these primers indicates that the desired loop-out event has occurred and only the URA3 promoter scar remains at the modified *adh1202* locus, while an approximately 5.1 kbp band would indicate the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. A strain that gave the desired 3.7 kbp band was kept and designated yMhCt021.

[00393] In order to isolate a derivative of yMhCt021 homozygous for the multiple panD expression cassette at *adh1202*, yMhCt021 was transformed with linearized pMhCt095 and pMhCt096 as described above. After two rounds of single colony purification and outgrowth, genomic DNA was prepared for use in PCR to verify the desired targeted integration occurred. Correct targeting of the pMhCt095 and pMhCt096 fragments to the remaining wild-type *adh1202* locus of yMhCt021 was verified with the primers 0612891 and 0612893. Primer 0612891 anneals in the 3' region of SaPanD r1 plus half of the *PacI* site after r1 stop of pMhCt095. Primer 0612893 anneals in the extreme 5' region of SaPanD r5, includes the *NheI* site and leader of pMhCt096, and amplifies in reverse complementary direction.

[00394] Generation of a 3.2 kbp band with these primers indicates the presence of an intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette as expected from the second integration event via pMhCt095 and pMhCt096 on the remaining adh1202 wild-type locus of yMhCt021, while an approximately 1.7 kbp band would indicate the presence of the URA3 scar site at the other adh1202 locus (from the initial integration event and subsequent URA3 marker loop-out). PCR reactions were carried out as described for the isolation of yMhCt019 above except that the length of the extension phase was changed to 3.5 minutes. A strain that gave both of these band sizes was designated yMhCt022 (see genotype in Table 22).

Table 22: Transformant genotype

Strain	Parent strain	Genotype
yMhCt022	yMhCt021	adh1202Δ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD r10, $ENO1_{\text{promo}}$ -Opt.SaPanD r1, URA3, $TDH3_{\text{promo}}$ -Opt.SaPanD r5, $PGK1_{\text{promo}}$ -Opt.SaPanD r2)/adh1202Δ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD r10, $ENO1_{\text{promo}}$ -Opt.SaPanD r1, URA3-Scar, $TDH3_{\text{promo}}$ -Opt.SaPanD r5, $PGK1_{\text{promo}}$ -Opt.SaPanD r2) pdcΔ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.ScUGA1, URA3-Scar, $TDH3_{\text{promo}}$ -Opt.ScYMR226C)/pdcΔ::($P_{DC_{\text{promo}}}$ -Opt.SaPanD, $ENO1_{\text{promo}}$ -Opt.ScUGA1, URA3-Scar, $TDH3_{\text{promo}}$ -Opt.ScYMR226C) ura3-/ura3-

[00395] Strains were grown in shake flasks and CFE were prepared and assayed for aspartate decarboxylase (ADC) activity as herein. The experimental results are shown in Table 23A.

Table 23A: Transformant enzyme activity data

Strain	Gene Overexpressed	ADC activity
MBin500 (control)	N/A	0.00
yMhCt019	ADC (SEQ ID NOs: 130, 145, 146, and 147), gabT (SEQ ID NO: 141),	2.18
95/96 2	3-HPDH (SEQ ID NO: 144)	2.52

[00396] The aspartate 1-decarboxylase (ADC), beta-alanine aminotransferase (BAAT) and 3HP dehydrogenase (3-HPDH) activities in CFE prepared from strains MBin500 (control), yMhCt019, 95/96-2, yMhCt008 and yMhCt022 were compared. The results of this experiment are shown in Table 23B.

Table 23B: Transformant enzyme activity data

Strain	Genes Overexpressed	Gene Sources	ADC Activity	BAAT Activity	3HP DH Activity
MBin500 (control)	N/A	N/A	0.002	0.61	0.4

yMhCt019	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.789	14.53	72.0
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			
95/96-2	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.891	20.42	73.2
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			
yMhCt008	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	0.272	13.14	61.1
	ADC (SEQ ID NO: 130)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			
yMhCt022	YMR226c (SEQ ID NO: 144)	<i>S. cerevisiae</i>	1.233	15.44	66.7
	ADC (SEQ ID NOs: 130, 145, 146 and 147)	<i>S. avermitilis</i>			
	UGA1 (SEQ ID NO: 141)	<i>S. cerevisiae</i>			

[00397] Strains yMhCt019 and 95/96 2 were also analyzed by SDS-PAGE as described herein. Both strains showed a protein band at 53 kD, 29 kD, ~14 kD, and at ~3 kD. The sizes of the 53 kD and 29 kD protein bands are consistent with the sizes of the proteins encoded by the UGA1 and YMR226c genes, respectively. The combined sizes of the 14 and 3 kD protein bands are consistent with the post-translationally cleaved protein encoded by the panD gene. The 53 kD, 29 kD, 14 kD and 3 kD proteins were not observed in the SDS-PAGE analysis of the control strain MBin500.

[00398] Strains MBin500 and yMhCt019 were evaluated in bioreactors for 3-HP production, using the method described herein. Control strain MBin500 produced no detectable 3-HP (average of two independent fermentations). Strain yMhCt019 produced 5.23 g/L 3-HP (average of three independent fermentations).

Example 3A-10: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus

[00399] Additional constructs were designed to incorporate four copies of nucleotides encoding an alternate ADC from *B. licheniformis* (SEQ ID NO: 139) at the adh1202 locus. In a similar approach to that described above, a left-hand and a right-hand construct were designed to allow homologous recombination at the *I. orientalis* adh1202 locus.

Construction of a left-hand fragment

[00400] The plasmid pMhCt095 (*supra*) was digested with *Xba*I and *Pac*I and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 7.3 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00401] A *Bacillus licheniformis* aspartate decarboxylase (ADC) panD gene was codon-optimized for expression in *I. orientalis* (version 1; SEQ ID NO: 149) and synthetically

constructed into plasmid 1110206 (GeneArt®). Plasmid 1110206 was digested with *Xba*I and *Pac*I and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 380 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00402] The ~380 bp purified fragment was ligated into the 7.3 kbp pMhCt095 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 60.5 ng of the digested pMhCt095, 6.3 ng of the 380 bp fragment from 1110206, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 1.5 hr at room temperature and a 3 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *Xba*I and *Pac*I. A clone yielding the correct digested band size was designated pMeJi309.

[00403] The plasmid pMeJi309 was digested with *Nhe*I and *Asc*I and purified by 1% agarose gel electrophoresis in TBE buffer. A band of approximately 7.3 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00404] The *Bacillus licheniformis* aspartate decarboxylase panD gene was again codon-optimized for expression in *I. orientalis* (version 2; SEQ ID NO: 148) and synthetically constructed into plasmid 1110205 (GeneArt®). A PCR was performed on a mixture containing 3 µL 1110205, 25 pM each of primers 0612695 and 0612724, 1X *pf*x amplification buffer (Invitrogen), 2mM MgSO₄, 1.25 Units Platinum® *pf*x DNA polymerase (Invitrogen) in a final volume of 50 µL. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 2 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 3 minutes.

[00405] The PCR product from the amplification reactions was purified by 1% agarose gel electrophoresis in TBE buffer. The approximately 400 bp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to manufacturer's instructions. The purified PCR product was inserted into the digested pMeJi309 vector above using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a reaction containing 93 ng pMeJi309 vector fragment, 52 ng PCR product above, 2 µL 1X IN-FUSION™ reaction buffer (Clontech), and 1 µL of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. A 2.5 µL sample of the

reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *Xba*I and *Pac*I. A clone yielding the correct digested band size was confirmed to be correct by DNA sequencing and designated pMeJi310-2 (Figure 14).

Construction of a right-hand fragment

[00406] The plasmid pMhCt096 (*supra*) was digested with *Xba*I and *Pac*I and purified by 1% agarose gel electrophoresis in TBE buffer. A band at approximately 4.8 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00407] The *Bacillus licheniformis* aspartate decarboxylase panD gene was again codon-optimized for expression in *I. orientalis* (version 3; SEQ ID NO: 151) and synthetically constructed into plasmid 1110208 (GeneArt®). Plasmid 1110208 was digested with *Xba*I and *Pac*I and purified by 1% agarose gel electrophoresis in TBE buffer, and an approximately 380 bp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00408] The 380 bp fragment above was ligated into the 7.3 kbp pMhCt096 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 72.2 ng digested pMhCt096, 6.9 ng 380 bp fragment from 1110208, 1 µL 10X ligation buffer with 10mM ATP, and 1 µL T4 ligase. The reaction was incubated for 1 and a half hours at room temperature and a 3 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *Nhe*I and *Asc*I. A clone yielding correct digested band size was designated pMeJi311.

[00409] The plasmid pMeJi311 was digested with enzymes *Nhe*I and *Asc*I and purified by 1% agarose gel electrophoresis in TBE buffer, and an approximately 7.3 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00410] The *Bacillus licheniformis* aspartate decarboxylase panD gene was again codon-optimized for expression in *I. orientalis* (version 4; SEQ ID NO: 150) and synthetically constructed into plasmid 1110207 (GeneArt®). A PCR was performed on a mixture containing 3 µL 1110207, 25 pM each of 0612698 and 0612725, 1X *pfx* amplification buffer (Invitrogen), 2 mM MgSO₄, 1.25 units Platinum® *pfx* DNA polymerase (Invitrogen) in a final

volume of 50 μ L. The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for 1 cycle at 95°C for 2 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 3 minutes.

[00411] The PCR product from the amplification reaction was purified by 1% agarose gel electrophoresis in TBE buffer. A band at approximately 400 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to manufacturer's instructions. The purified PCR product was inserted into the digested pMeJi311 vector above using an IN-FUSION™ Advantage PCR Cloning Kit (Clontech) in a reaction containing 65.1 ng of the pMeJi311 *NheI* to *Ascl* digested vector fragment, 85 ng of the PCR product above, 2 μ L 1X IN-FUSION™ reaction buffer (Clontech), and 1 μ L of IN-FUSION™ enzyme (Clontech). The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, and then placed on ice. A 2.5 μ L sample of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated for two days at room temperature. Several of the resulting transformants were screened for proper insertion by restriction digest using *NheI* and *Ascl*. A clone yielding the correct digested band size was confirmed to be correct by DNA sequencing and designated pMeJi312-2 (Figure 15).

Integration of left-hand and right-hand fragments

[00412] Plasmid pMeJi310-2 was digested with *HpaI* and *Sac II* and plasmid pMeJi312-2 was digested with *EcoRI* and *SacII* as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the two approximately 5 kbp bands were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00413] *I. orientalis* CNB1 was transformed with the digested pMeJi310-2 and pMeJi312-2 DNA and correct locus targeting and transformation was verified by Crimson Taq (New England Biolabs) PCR as described in herein. Primers 0612794 and 0611245 yielded an approximately 3.17kbp band; primers 612479 and 0611632 yielded an approximately 1.48 kbp band; and primers 611248 and 612795 yielded an approximately 2.3 kbp band. A strain which gave the expected bands for proper integration of the expression cassette was designated MeJi409-2. A *ura-* derivative of strain MeJi409-2 was then obtained as described above.

[00414] Strains MBin500 and MeJi409-2 were evaluated in fermentation bioreactors for 3-HP production, using the method described herein. Control strain MBin500 produced no detectable 3-HP (average of two independent fermentations). Strain MeJi409-2 (one fermentation) produced 4.62 g/L. In order to account for differences in the amount of cell

mass in these fermentations compared to other (e.g., future) fermentations, the 3-HP concentration per unit of cell mass (expressed as [g/L 3-HP]/[g/L dry cell weight]) was calculated to be 0.20 for MeJi409-2.

Example 3A-11: Yeast strains expressing aspartate 1-decarboxylase (ADC) and aspartate aminotransferase (AAT) at the adh9091 locus

[00415] The nucleotide sequence (SEQ ID NO: 13) that encodes the *I. orientalis* aspartate aminotransferase (AAT) of SEQ ID NO: 14 was PCR amplified from *I. orientalis* genomic DNA using the primers 0611268 and 0611269. The PCR reaction (50 μ L) contained 50 ng of strain *I. orientalis* genomic DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611268 and 0611269, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 1278 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 1278 bp, with an *Nru*I restriction site at the 5' end of the fragment and a *Pac*I restriction site at its 3' end.

[00416] The resulting 1278 bp fragment above comprising the AAT gene CDS (SEQ ID NO: 13) was then cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *Bam* HI digestion. A clone yielding the desired band sizes was confirmed by sequencing and designated pGMEr111.

[00417] Plasmids pGMEr121 and pGMEr111 were double-digested with restriction enzymes *Pac*I and *Nru*I. The resulting 7695 bp vector fragment, from plasmid pGMEr121, and the resulting 1272 bp insert fragment comprising the AAT coding sequence, from plasmid pGMEr111, were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel, and purified using the QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00418] A ligation reaction was then set up with 3 μ L of vector fragment, 4 μ L of insert fragment, 2 μ L of sterile dd water, 10 μ L of 2X Quick Ligase Buffer and 1 μ L of Quick T4 Ligase (Quick Ligation Kit, New England Biolabs) and performed according to the manufacturer's instructions. A 5 μ L aliquot of the ligation reaction above was transformed into XL10-Gold® Ultracompetent *E. coli* cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *SmaI/PpuMI* double digestion. A clone yielding the desired band sizes was confirmed by sequencing and designated pGMEr126 (Figure 16).

[00419] Plasmids pGMEr126 comprises the *I. orientalis* AAT expression cassette, in which the gene transcription is controlled by the *I. orientalis* TDH3 promoter and the TKL terminator, flanked by the truncated 3' region of the URA3 coding sequence and the URA3 promoter, upstream; and by the 3' homology region with the *I. orientalis* adh9091 locus, downstream.

[00420] The *S. avermitilis* panD gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130) was PCR amplified from the pMA-T vector received from GeneArt® using the primers 061166 and 0611662. The PCR reaction (50 μ L) contained 50 ng of strain plasmid DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611661 and 0611662, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1.5 μ L of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 453 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The total length of the resulting PCR fragment was approximately 453 bp with an *NruI* restriction site at the 5' end of the fragment and an *Apal* restriction site at the 3' end.

[00421] The resulting 453 bp fragment, comprising the codon-optimized version of *S. avermitilis* panD gene, was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *EcoRI* digestion. A plasmid yielding the desired band sizes was confirmed by sequencing and designated pGMEr127.

[00422] Plasmids pGMEr127 and pGMEr125(a) were digested with restriction enzymes *NruI* and *Apa I*. Before stopping the digestion reactions 1 μ L of Calf Intestinal Alkaline Phosphatase (New England Biolabs) was added to the pGMEr125(a) digestion in order to de-phosphorylate the ends and prevent self-ligation. The resulting 8188 bp vector fragment, from plasmid pGMEr125(a) (*supra*), and the 440 bp insert fragment, comprising the codon-optimized version of the *S. avermitilis* panD gene (SEQ ID NO: 130) from plasmid pGMEr127 (*supra*), were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00423] A ligation reaction was then set up with 4 μ L of vector fragment, 4 μ L of insert fragment, 9 μ L of 2X Quick Ligase Buffer and 1 μ L of Quick T4 Ligase (New England Biolabs) and performed according to the manufacturer's instructions. A 5 μ L aliquot of the ligation reaction above was transformed into XL10-Gold® Ultracompetent *E. coli* cells (Stratagene) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired insert by *Bam*HI digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr130 (Figure 17).

[00424] Plasmid pGMEr130 comprises a construct made of the following fragments: the 5' flank of the *I. orientalis* adh9091 locus, an empty expression cassette with the *I. orientalis* PDC promoter/ TAL terminator, the panD expression cassette (containing SEQ ID NO: 130) under control of the *I. orientalis* ENO1 promoter and the RKI terminator, and the truncated 5' fragment of the URA3 marker gene under control of the *I. orientalis* URA3 promoter.

[00425] To determine whether yeast strain *I. orientalis* CNB1 was able to express the *I. orientalis* codon-optimized version of *S. avermitilis* panD gene (SEQ ID NO: 130) and the *I. orientalis* AAT gene (SEQ ID NO: 13), the expression plasmids pGMEr130 and pGMEr126 were constructed. Plasmid pGMEr130 comprises (from 5'-3') the 5' flanking region for genomic integration of the construct at the *I. orientalis* adh9091 locus, the panD expression cassette under control by the ENO1 promoter and the RKI terminator, and the truncated 5' portion of the URA3 selection marker driven by the URA3 promoter. Plasmid pGMEr126 comprises (from 5'-3') the 3' portion of the URA3 selection marker, the AAT gene expression cassette under control by the TDH3 promoter and the TKL terminator, and the 3' flank for genomic integration of the construct at the *I. orientalis* adh9091 locus. All promoters and terminators were derived from *I. orientalis*.

[00426] Plasmid pGMEr126 was digested with restriction enzyme *EcoRI*, which excised a 4758 bp fragment of interest, while plasmid pGMEr130 was digested with restriction enzyme *Hind III* creating a 5034 bp fragment needed for transformation. The 4758 bp and the 5034

bp fragments were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel, and purified using the QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00427] *I. orientalis* CNB1 was cultured and co-transformed as described herein with approximately 500 ng of both the 4758 bp and 5034 bp linear fragments. Eight transformant strains were obtained and then cultured in shake flasks. The resulting broths were used to run an SDS-PAGE, Tris-HCl (Bio-Rad Laboratories) gel to detect the expression of the *S. avermitilis* panD gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130) and of the *I. orientalis* AAT gene (SEQ ID NO: 13). A positive strain was designated yGMEr008 and its broth was also used to determine the ADC and the AAT activity levels as described above.

Example 3A-12: Yeast strains expressing pyruvate carboxylase (PYC), aspartate 1-decarboxylase (ADC) and aspartate aminotransferase (AAT) at the adh9091 locus

[00428] The nucleotide sequence that encodes the *I. orientalis* pyruvate carboxylase (PYC) of SEQ ID NO: 2 was PCR amplified from *I. orientalis* genomic DNA using the primers 0611266 and 0611267. The PCR reaction (50 µL) contained 50 ng of *I. orientalis* genomic DNA, 1X Phusion HF buffer (New England Biolabs), 50 pmol each of primers 0611266 and 0611267, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 µL of 100% DMSO (New England Biolabs) and 1 unit of Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 98°C for 2 minutes followed by 35 cycles each at 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. Following thermocycling, the PCR reaction products were separated by 0.8% agarose gel electrophoresis in TBE buffer where an approximately 3557 bp PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The resulting PCR fragment had an *Xba*I restriction site at the 5' end of the fragment and a *Pac*I restriction site at its 3' end.

[00429] The resulting 3557 bp fragment, comprising the *I. orientalis* PYC gene CDS (SEQ ID NO: 1), was cloned into pCR2.1-TOPO vector and transformed into One-Shot TOP10 *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *Eco*RI digestion. Six clones yielding the desired band sizes were confirmed and designated pGMEr132.7, pGMEr132.14, pGMEr132.16, pGMEr132.25, pGMEr132.27 and pGMEr132.30. Sequencing analysis revealed that plasmid pGMEr132.14 has the proper

PYC CDS but was missing the *Xba*I restriction site at the CDS 5' end. Since this restriction site is needed to insert the PYC CDS in expression plasmid pGMEr125, the 315 bp *Hind*III fragment of plasmid pGMEr132.14 (comprising the 5' end on the PYC CDS with the altered *Xba*I site) was replaced with the 315 bp *Hind*III fragment from plasmid pGMEr132.7, which has a unaltered 5' end of the PYC CDS including the correct *Xba*I site. The resulting 7173 bp *Hind*III vector fragment, from plasmid pGMEr132.14, and the 315 bp *Hind*III insert fragment, from plasmid pGMEr132.7, were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel, and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00430] A ligation reaction was then set up with 4 µL of vector fragment, 5 µL of insert fragment, 10 µL of 2X Quick Ligase Buffer and 1 µL of Quick T4 Ligase (New England Biolabs) and performed according to the manufacturer's instructions. A 5 µL aliquot of the ligation reaction above was transformed into One-Shot TOP10 *E.coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion and orientation of the desired insert by *Bam*HI and *Xba*I double digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr133.

[00431] In order to insert the *I. orientalis* PYC CDS downstream of the PDC promoter in plasmid pGMEr125(b), plasmids pGMEr125(b) and pGMEr133 (*supra*) were digested with *Pac*I and *Xba*I. The resulting 8188 bp vector fragment, from plasmid pGMEr125(b), and the 3553 bp insert fragment, comprising the *I. orientalis* PYC CDS (SEQ ID NO: 1) from plasmid pGMEr133, were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer, excised from the gel, and purified using the QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00432] A ligation reaction was then set up with 3 µL of vector fragment, 6 µL of insert fragment, 10 µL of 2X Quick Ligase Buffer and 1 µL of Quick T4 Ligase (New England Biolabs) and performed according to the manufacturer's instructions. A 5 µL aliquot of the ligation reaction above was transformed into One-Shot TOP10 *E.coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion of the desired fragment by *Bam*HI digestion. A clone yielding the desired band sizes was confirmed and designated pGMEr136.

[00433] Plasmid pGMEr136 comprises the 5' flank of the *I. orientalis* adh9091 locus, the *I. orientalis* PYC gene expression cassette (SEQ ID NO: 1) under control of the *I. orientalis* PDC promoter and TAL terminator, an empty expression cassette with an *I. orientalis* ENO1

promoter / RKI terminator, and the truncated 5' fragment of the *I. orientalis* URA3 marker gene under control of the URA3 promoter.

[00434] About 5 µg of plasmid pGMEr136 (*supra*) and 4 µg of plasmid pGMEr127 were digested with restriction enzymes *Apal* and *Nru* I. The resulting 11729 bp vector fragment, from plasmid pGMEr136, and the resulting insert fragment comprising the *S. avermitilis* panD gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130) (436 bp) from plasmid pGMEr127, were purified by 0.8% agarose gel electrophoresis in 1X TBE buffer using a NucleoSpin® Extract II (Macherey-Nagel) according to the manufacturer's instructions.

[00435] A ligation reaction was then set up comprising 5 µL of vector fragment, 4 µL of insert fragment, 9 µL of 2X Quick Ligase Buffer and 1 µL of Quick T4 Ligase (New England Biolabs). The reaction was incubated at room temperature for 1 hour. A 5 µL aliquot of the ligation reaction above was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for the desired insert by *Bam*HI digestion. A clone yielding the desired band sizes was chosen and designated pGMEr137 (Figure 18).

[00436] Plasmid pGMEr137 comprises the *I. orientalis* PYC gene (SEQ ID NO: 1) under transcriptional control of the *I. orientalis* PDC promoter and TAL terminator, the *S. avermitilis* panD gene codon-optimized for expression in *I. orientalis* (SEQ ID NO: 130) under transcriptional control of the *I. orientalis* ENO1 promoter and RKI terminator, the URA3 promoter followed by the 5' end of the URA3 marker and the 5' flanking region of the *I. orientalis* adh9091 locus.

[00437] Plasmid pGMEr126 (*supra*) was digested with restriction enzyme *Eco*RI, which excised a 4758 bp fragment of interest; while plasmid pGMEr137 (*supra*) was digested with restriction enzymes *Hpa*I and *Nhe*I creating a 8400 bp fragment. Both the 4758 bp and the 8400 bp fragments were separated by 0.8% agarose gel electrophoresis in 1X TBE buffer; the bands were excised from the gel and purified using the NucleoSpin® Extract II (Macherey-Nagel) according to the manufacturer's instructions. *I. orientalis* CNB1 was cultured and co-transformed with approximately 500 ng of both the 4758 bp and 8400 bp linear fragments as described herein, resulting in transformant yGMEr009.

Example 3A-13: Yeast strains expressing aspartate 1-decarboxylase (ADC), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the *pdc* locus; and expressing pyruvate carboxylase (PYC), aspartate 1-decarboxylase (ADC), and aspartate aminotransferase (AAT) at the *adh9091* locus

[00438] To increase pyruvate carboxylase (PYC) and aspartate aminotransferase (AAT) activity in a strain already over-expressing an aspartate aminotransferase (AAT), a β -alanine aminotransferase (BAAT), and a 3-HP dehydrogenase (3-HPDH), strain yMhCt010 (*supra*) was transformed with linear fragments of pGMEr137 (*supra*) and pGMEr126 (*supra*) as described above. After two rounds of single colony purification and outgrowth, genomic DNA was prepared for use in PCR to verify the desired targeted integration occurred as described above. Correct targeting of the pGMEr137 and pGMEr126 fragments to the *adh9091* locus was confirmed using the primers 0611814 and 0612055. Primer 0611814 anneals in the 3' end of the TDH3 promoter of pGMEr126 and amplifies in the 3' direction. Primer 0612055 anneals 3' of the *adh9091* 3' flanking homology present in pGMEr126, so amplification of a PCR product with this primer pair will only occur if the integration DNA targeted to the correct locus via homologous recombination. The presence of an approximately 3066 bp band from a PCR containing primers 0611814 and 0612055 indicates the desired integration of pGMEr126 and pGMEr137 fragments occurred at the *adh9091* locus.

[00439] After two rounds of single colony purification and outgrowth of several independent transformants of yMhCt010 with linear fragments of pGMEr137 and pGMEr126, genomic DNA was prepared for use in PCR to verify the desired targeted integration occurred as described above. Three independently isolated strains that gave an approximately 3066 bp band from a PCR containing primers 0611814 and 0612055 were designated yMhCt020, GMErin010#2, and GMErin010#3. These strains contain a polynucleotide (SEQ ID NO: 130) encoding the corresponding ADC (SEQ ID NO: 17) at both of the *pdc* loci and one of the *adh9091* loci; a polynucleotide (SEQ ID NO: 141) encoding the corresponding *gabT* (SEQ ID NO: 24) at both of the *pdc* loci; a polynucleotide (SEQ ID NO: 144) encoding the corresponding 3-HPDH (SEQ ID NO: 129) at both of the *pdc* loci; a polynucleotide (SEQ ID NO: 1) encoding the corresponding PYC (SEQ ID NO: 2) at one of the *adh9091* loci; and a polynucleotide (SEQ ID NO: 13) encoding the corresponding AAT (SEQ ID NO: 14) at one of the *adh9091* loci (see Table 24).

Table 24: Transformant genotypes

Strain	Parent strain	Genotype
yMhCt020	yMhCt010	$adh9091\Delta::(PDC_{\text{promo}}\text{-}pycCNB1, ENO1_{\text{promo}}\text{-}SaPanD(\text{reverse}), URA3, TDH3_{\text{promo}}\text{-}aat)/ADH9091$ $pdc\Delta::(PDC_{\text{promo}}\text{-}Opt.SaPanD, ENO1_{\text{promo}}\text{-}Opt.ScUGA1, URA3\text{-}Scar, TDH3_{\text{promo}}\text{-}Opt.ScYMR226C)/pdc\Delta::(PDC_{\text{promo}}\text{-}Opt.SaPanD,$
GMErin010 #2		
GMErin010 #3		

		ENO1 _{promo} -Opt.ScUGA1, URA3-Scar, TDH3 _{promo} - Opt.ScYMR226C) ura3-/ura3-
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[00440] Strains yMhCt020, GMErin010#2, and GMErin010#3 were grown in shake flasks and CFEs were prepared and assayed for PYC, AAT and 3-HPDH activities as described in herein. The experimental results shown in Table 25.

Table 25: Transformant enzyme activity data

Strain	Gene Overexpressed	PYC activity	AAT activity	ADC activity	3-HPDH activity
MBin500 (control)	N/A	0.14	0.03	0.00	0.14
yMhCt008	ADC (SEQ ID NO: 130), gabT (SEQ ID NO: 141), 3-HPDH (SEQ ID NO: 144)	0.22	2.05	0.64	2.95
GMEr009-2	ADC (SEQ ID NO: 130), PYC (SEQ ID NO: 1), AAT (SEQ ID NO: 13)	1.15	24.62	0.05	0.07
yMhCt020	ADC (SEQ ID NO: 130), gabT (SEQ ID NO: 141), 3-HPDH (SEQ ID NO: 144),	2.74	35.02	0.56	1.68
GMErin010 #2	PYC (SEQ ID NO: 1), AAT (SEQ ID NO: 13),	1.97	40.32	0.59	1.87
GMErin010 #3	PYC (SEQ ID NO: 1), AAT (SEQ ID NO: 13),	1.50	23.79	0.42	3.30

[00441] The strains in Table 25 were also analyzed by SDS-PAGE as described herein. MBin500 and GMEr009-2 showed a protein band at ~64 kD that was absent in the four other samples. The mass of these proteins is consistent with their identity as the native pyruvate decarboxylase in *I. orientalis* CNB1. Strains yMhCt008, yMhCt020, GMErin010 #2, and GMErin010 #3 showed bands at 53 kD and 29 kD. The mass of these proteins is consistent with mass of the proteins encoded by the UGA1 and YMR226c genes, respectively. Strains GMEr009-2, yMhCt020, GMErin010 #2, and GMErin010 #3 all showed bands at 46.3 kD. The mass of these proteins is consistent with mass of the protein encoded by the AAT gene.

Example 3A-14: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at both adh1202 loci.

[00442] A ura3- derivative of MeJi409-2 containing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 (*supra*) was isolated using the FOA counter-selection loop-out protocol described above. Genomic DNA of several FOA resistant colonies of parent strain MeJi409-2 was screened by PCR for the desired loop-out event with primers 0611815 and 0611817. Primer 0611815 anneals in the RKL terminator of the left-hand construct and amplifies toward the ura3 promoter. Primer 0611817 anneals in TDH3 promoter and amplifies back toward the ura3 cassette. The presence of an 828 bp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind

after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.2 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions with Crimson Taq™ DNA polymerase (New England Biolabs) were carried out as described above. One FOA resistant colony from parent strain MeJi409-2, designated MeJi411, gave the desired 828 bp band.

Integration of the left-hand and right-hand fragments

[00443] Plasmid pMeJi310-2 was digested with *HpaI* and *SacII* and plasmid pMeJi312-2 was digested with *EcoRI* and *SacII* as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the two approximately 5 kbp bands were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00444] MeJi411 was transformed with the digested pMeJi310-2 and pMeJi312-2 DNA and correct loci targeting and transformation was verified by Crimson Taq (New England Biolabs) PCR as described herein. Primers 0611225 and 0611632 yielded an approximately 5 kbp band; primers 0611815 and 0611632 yielded an approximately 6 kbp band with the ura marker, and a 4.5 kbp band without. Primers 0611631 and 0612579 yield an approximately 936 bp band when the wildtype *adh1202* locus is still intact (strains that did not show this band were selected). A strain which gave the expected bands for proper integrating of the expression cassette was designated MeJi412.

Example 3A-15: Yeast strains expressing four copies of nucleotides encoding an aspartate 1-decarboxylase (ADC) at the *adh1202* locus, with two copies of the nucleotides encoding the aspartate 1-decarboxylase (ADC) under the control of the a PDC promoter and two copies under the control of a TDH3 promoter.

[00445] This example describes constructs designed to incorporate four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the *adh1202* locus with two copies of under control of the *I. orientalis* PDC promoter and two copies under the control of the *I. orientalis* TDH3 promoter. In a similar approach to that described above, a left-hand and a right-hand constructs were designed to allow homologous recombination at the *I. orientalis* CNB1 *adh1202* locus.

Construction of a left-hand fragment

[00446] The plasmid pMeJi310-2 (*supra*; see Figure 14) was digested with *XbaI* and *StuI* followed by treatment with CIP and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 6.7 kbp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00447] The PDC promoter was excised from pMeJi310-2 by digestion with *NotI* followed by a fill-in reaction with Klenow and subsequent digestion with *NheI*. A band at approximately 708 bp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00448] The 708 bp of purified fragment was ligated into the 6.7 kbp pMeJi310-2 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 6.7 kbp fragment from pMeJi310-2, 1 or 5 µL of the 708 bp fragment from pMeJi310-2, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated overnight at 16°C and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *ApaI*. A clone yielding correct digested band size was designated pMiBa137.

Construction of a right-hand fragment

[00449] The plasmid pMeJi312-2 (*supra*) was digested with *XbaI* and *StuI* followed by treatment with CIP and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 6.8 kbp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00450] The TDH3 promoter was excised from pMeJi312-2 by digestion with *PmeI* and *NheI*. A band at approximately 966 bp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00451] The 966 bp of purified fragment was ligated into the 6.8 kbp pMeJi312-2 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 6.8 kbp fragment from pMeJi312-2, 1 or 5 µL of the 966 bp fragment from pMeJi312-2, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for approximately 6 hours at 16°C and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *SaII*. A clone yielding correct digested band size was designated pMiBa136.

Integration of the left-hand and right-hand fragments

[00452] Plasmid pMiBa137 was digested with *HpaI* and *SacII* and plasmid pMiBa136 was digested with *EcoRI* and *SacII* as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the two approximately 5 kbp bands were excised from the

gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00453] *I. orientalis* CNB1 was transformed with the digested pMIBa137 and pMIBa136 DNA and correct loci targeting and transformation was verified by Crimson Taq (New England Biolabs) PCR as described in herein. Primers 0611717 and 0611631 yielded bands of approximately 2.5 kbp and 955 bp; primers 0611718 and 0611632 yielded an approximately 733 bp band; primers 0612794 and 0611245 yielded an approximately 2.7 kbp band; primers 0611225 and 0612795 yielded an approximately 4.2 kbp band. A strain which gave the expected bands for proper integration of the expression cassette was designated MIBa351.

Removal of the ura marker from MIBa351

[00454] A ura⁻ derivative of MIBa351 was isolated as described above. Genomic DNAs from several FOA resistant colonies of MIBa351 were screened by PCR for the desired loop-out event with primers 0611815 and 0611817. Primer 0611815 anneals in the RKI terminator of the left-hand construct and amplifies toward the ura3 promoter. Primer 0611817 anneals in TDH3 promoter and amplifies back toward the ura3 cassette. The presence of an 828 bp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.2 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions with Crimson Taq™ DNA polymerase (New England Biolabs) were carried out as described above. FOA resistant colonies that yielded the 828 bp fragment with the above primers were further tested with primers 0612794 and 0611245, which yield a 2.7 kbp product, and primers 0611815 and 0612795, which yield a 4 kbp product, to confirm that the four copies of the nucleotide sequence SEQ ID NO: 138 encoding the *B. licheniformis* ADC of SEQ ID NO: 139 remained intact. One FOA resistant colony from parent strain MIBa351, designated MIBa353, gave the desired PCR products with all 3 primer sets.

Construction of a reverse expression cassette right-hand fragment

[00455] Plasmid pMIBa136 contains two expression cassettes going in the forward orientation. To ease screening of homozygous strains, a new plasmid was constructed where the panDbl expression cassettes of pMIBa136 were placed in the reverse orientation. The plasmid pMIBa136 (*supra*) was digested with *NotI* and *PmeI* followed by a fill-in reaction with Klenow and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. Bands at approximately 3.4 and 4.4 kbp were excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The

4.4 kbp fragment from pMIBa136 was treated with CIP and purified using the QIAQUICK® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

[00456] The 3.4 kbp purified fragment from pMIBa136 was ligated into the 4.4 kbp pMIBa136 vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 4.4 kbp fragment from pMIBa136, 1 or 5 µL of the 3.4 kbp fragment from pMIBa136, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated overnight at 16°C and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *PacI* and *EcoRI*. A clone yielding correct digested band size was designated pMIBa138.

Integration of left-hand and right-hand fragments

[00457] Plasmid pMIBa137 was digested with *HpaI* and *SacII* and plasmid pMIBa138 was digested with *EcoRI* and *SacII* as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the two approximately 5 kbp bands were excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00458] MIBa353 was transformed with the digested pMIBa137 and pMIBa138 DNA and correct loci targeting and transformation was verified by PCR using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. Primers 0611718 and 0611632 yielded an approximately 733 bp band (to confirm the first integration is still present); primers 0612367 and 0611632 yielded an approximately 960 bp band (to confirm that the second copy integrated); primers 0611631 and 0612579 yielded an approximately 936 bp band if the wildtype *adh1202* locus is still present (lack of this band confirms loss of wt *adh1202* locus). Two strains which gave the expected bands for proper integrating of the expression cassette were saved and designated MIBa355 and MIBa356.

Aspartate 1-decarboxylase activity in MeJi409-2, MeJi412, MIBa351 and MIBa355

[00459] Strains MeJi409-2, MeJi412, MIBa351 and MIBa355 were grown in shake flasks and CFE were prepared and assayed for aspartate 1-decarboxylase (ADC) activity as described herein. The results are shown in Table 26. The activity for each strain is an average of two independent shake flask cultures. Strains MeJi409-2, MeJi412, MIBa351 and MIBa355 were also tested in bioreactors for 3-HP production, using the methods described herein. The results from these bioreactor experiments are also shown in Table 26. In order to account for differences in cell mass in these fermentations, the 3-HP production performance shown in Table 26 is expressed as 3-HP concentration per unit of cell mass

(expressed as g/L 3-HP/g/L dry cell weight). The results show that as the level of ADC activity in the cells increased, the 3-HP production performance increased.

Table 26: Transformant ADC activity and 3-HP production data

Strain	Gene overexpressed	ADC activity	3-HP/DCW
MBin500	N/A	0	0
MeJi409-2	ADC (SED ID NO: 138)	0.629	0.19
MeJi412		1.151	0.43
MIBa351		0.659	0.32
MIBa355		1.173	0.52

Example 3A-16: Plasmid construction for expressing pyruvate carboxylase (PYC) at the PDC locus

[00460] Plasmid pANN28 containing the nucleotide sequence of SEQ ID NO: 1 (encoding the *I. orientalis* PYC of SEQ ID NO: 2) for integration at the PDC locus was constructed as described below.

[00461] The upstream and downstream flanking regions of *I. orientalis* PDC were amplified by PCR using genomic DNA as a template (Pfu polymerase, Stratagene) according to the manufacturer's instructions. The primers oANN7 and oANN8 allowed the incorporation of unique restriction sites flanking the upstream region and the primers oANN9 and oANN10 allowed the incorporation of unique restriction sites flanking the downstream region. The PCR products were purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 800 bp for each PCR product was excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were cloned into TOPO vectors (Invitrogen) and transformed into electro-competent *E. coli* DH10B cells (Invitrogen) according to manufacturer's instructions. Several of the resulting transformants were screened for proper insertion by colony PCR with the same primers used to create the PCR products. Positive clones were further confirmed by sequencing. A clone yielding the correct PDC downstream flank was designated pANN04. A clone yielding the correct PDC upstream flank was designated pANN07.

[00462] Plasmid pANN04 was digested with *Apal* and *SacI* (for use as vector/backbone); plasmid pANN04 was digested with *NotI* and *SacI*; plasmid pANN07 was digested with *NotI* and *Apal*. Each fragment was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 3.5 kbp for the vector, and approximately 1 kbp for each insert were excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified products were ligated using T4

ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 49 ng of the vector, 120 ng of the downstream insert, 41 ng of the upstream insert, 1 μ L 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* OneShot TOP10 cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oANN7 and oANN10 (yielding a band of approximately 1.7 kbp). A clone yielding the correct insertion was designated pANN12.

[00463] The *I. orientalis* PYC coding sequence (SEQ ID NO: 1) from pGMEr137 (*supra*) was modified by site directed mutagenesis to eliminate three EcoRI restriction sites which do not alter the amino acid sequence of the encoded enzyme. Plasmid pGMEr137 was used as a template with primers oANN13, oANN14 and oANN15 used to elimination of the above mentioned restriction sites using a Multi change kit (Stratagene) according to the manufacturer's instructions. Several of the resulting transformants were screened by restriction digest using EcoRI. Positive clones were further confirmed by sequencing. A clone yielding the correct pyc coding sequence was designated pANN14.

[00464] Plasmid pJY39 (Figure 29) was digested with XhoI and PaeI; plasmid pACN5 (*supra*; see Figure 19) was digested with XhoI and XbaI; plasmid pANN14 was digested with XbaI and PaeI. Each fragment was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 8 kbp for the vector, approximately 700 bp for the first insert, and approximately 3.6 kbp for the second insert encoding the PYC were excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified products were ligated using T4 ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 51 ng of the vector, 49 ng of the first insert, 210 ng of the second insert, 1 μ L 10X ligation buffer with 10 mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* OneShot TOP10 cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oJLJ57 and oJLJ43 (yielding a band of approximately 1 kbp), primers oJLJ45 and oANN16 (yielding a band of approximately 730 bp), and primers oANN20 and oJY45 (yielding a band of approximately 1.2 kbp). A clone yielding the correct insertion was designated pANN15.

[00465] Plasmids pANN12 and pANN15 were digested with NotI. Plasmid pANN15 was additionally digested with NcoI for further fractionation of the backbone and improved separation of desired fragment. The digested pANN12 was purified using a Qiagen kit according to the manufacturer's instructions. The NotI fragments were purified by agarose gel electrophoresis in TBE buffer as described herein. The bands of approximately 5 kbp (from pANN12) and approximately 6.3 kbp (from pANN15) were gel purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

[00466] The purified product from pANN15 was ligated into the pANN12 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 50 ng of the vector, 115 ng of the insert, 1 μ L 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 1.5 hr at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* OneShot TOP10 cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oANN20 and oJY45 (yielding a band of approximately 1.2 kbp). Clones yielding correct insertion were further screened by restriction enzyme digestion with SacI/EcoRI and with SacI/EcoRV in order to differentiate the insert orientation. A clone yielding the ura3 marker near the upstream PDC flank was designated pANN27. A clone yielding the ura3 marker near the downstream PDC flank was designated pANN28.

Example 3A-17: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus and pyruvate carboxylase (PYC) at the pdc locus

[00467] This example describes the construction of yeast strains expressing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the adh1202 locus and a nucleotide encoding the *I. orientalis* PYC of SEQ ID NO: 2 at the pdc locus.

Removal of the ura marker from MeJi412

[00468] A ura⁻ derivative of MeJi412 was isolated as described above. Several FOA resistant colonies of MeJi412 were screened by colony PCR for the desired loop-out event with primers 0611815 and 0611817. Primer 0611815 anneals in the RKI terminator of the left-hand construct and amplifies toward the ura3 promoter. Primer 0611817 anneals in TDH3 promoter and amplifies back toward the ura3 cassette. The presence of an 869 bp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.6 kbp indicates the presence of the intact URA3

promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions with Phire® Plant Direct PCR Kit (Finnzymes) were carried out as described above. FOA resistant colonies that yielded the 869 bp fragment with the above primers were further tested with primers 0612794 and 0611817, which yield a 3.8 kbp product, and primers 611815 and 612795, which yield a 3.7 kbp product, to confirm that the four copies of the nucleotide sequence SEQ ID NO: 138 encoding the *B. licheniformis* ADC of SEQ ID NO: 139 remained intact. One FOA resistant colony from parent strain MeJi412, designated MeJi413, gave the desired PCR products with all 3 primer sets.

Integration of fragment

[00469] Plasmid pANN28 (*supra*) was digested with Ascl and SacI and purified by agarose gel electrophoresis in TBE buffer. The band at approximately 7.1 kbp was excised from the gel and purified using a gel purification kit (Qiagen) according to the manufacturer's instructions.

[00470] Strain MeJi413 was transformed with the digested and purified fragment from pANN28 and correct loci targeting and transformation was verified by colony PCR (Failsafe, mix E, Epicenter) according to the manufacturer's instructions. Primers oANN12 and oJLJ44 yielded an approximately 1 kbp band; primers oANN11 and oANN16 yielded an approximately 1.3 kbp band. A strain which gave the expected bands for proper integration of the expression cassette was designated yANN35.

[00471] A ura⁻ derivative of yANN35 then was isolated as described above. Several FOA resistant colonies were screened by colony PCR for the desired loop-out event with primers oANN12 and oJY44. Primer oANN12 anneals outside of the downstream flanking region. Primer oJY44 anneals to the TAL terminator. The presence of a 1.5 kbp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.8 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions with Failsafe DNA polymerase (Epicenter) were carried out as described above. Isolates positive for this event were further confirmed by colony PCR with primers oANN16 and oANN11. One FOA resistant colony was designated yANN37.

[00472] Strain yANN37 was transformed with the digested and purified fragments from pANN28 and correct loci targeting and transformation was verified by colony PCR (Failsafe, mix E, Epicenter) according to the manufacturer's instructions. The preliminary screen was done with primers oHJJ116 and oHJJ117 which are specific for the PDC gene. A band of approximately 500 bp indicates the presence of the gene and thus a negative result for the

desired integration. Isolates that were positive for deletion of PDC were further confirmed with additional PCR reactions. Primers oANN11 and oANN16 yielded an approximately 1.3 kbp band. Primers oANN12 and oJLJ44 yielded an approximately 1 kbp band; primers oANN12 and oJY44 yielded an approximately 1.5 kbp band and an approximately 2.9 kbp band (corresponding to the first and second integration events respectively).

[00473] Additionally, the previous integration events at the *adh1202* locus were confirmed by colony PCR as described above. Primers 0611631 and 0611245 yielded an approximately 3.8 kbp band. Primers 0611245 and oNovo3 yielded an approximately 3 kbp band. Primers 0611815 and 0612795 yielded an approximately 3.6 kbp band. A strain which gave the expected bands for proper integration of the expression cassette was designated yANN41.

Example 3A-18: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the *adh1202* locus and pyruvate carboxylase (PYC) at the *pdh* locus

[00474] This example describes the construction of yeast strains expressing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the *adh1202* locus (with two copies under the control of the a PDC promoter and two copies under the control of a TDH3 promoter) and a nucleotide encoding the *I. orientalis* PYC of SEQ ID NO: 2 at the *pdh* locus.

Removal of ura marker from MIBa355

[00475] A *ura*⁻ derivative of MIBa355 was isolated as described above. Genomic DNA from several FOA resistant colonies of MIBa355 were screened by PCR for the desired loop-out event with primers 0611815 and 0611718. The presence of an approximately 500 bp band indicates the presence of only the *ura3* scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 1.9 kbp indicated the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR was performed using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. FOA resistant colonies that yielded the approximately 500 bp fragment with the above primers were further tested with primers 0611631 and 0611245, which yield a 3.5 kbp product, and primers 0611815 and 0611632, which yield a 4.5 kbp product, to confirm that the four copies of the nucleotide sequence SEQ ID NO: 138 encoding the *B. licheniformis* ADC of SEQ ID NO: 139 remained intact. They were also tested with PCR using primers 0611815 and 0611817 to confirm that the first modification at *adh1202* was present. These PCR primers yielded a 828 bp

fragment. One FOA resistant colony from parent strain MIBa355, designated MIBa357, gave the desired PCR products with all four primer sets.

[00476] The plasmid pANN28 (*supra*) was digested with *Ascl* and *Sacl* and purified by 1% agarose gel electrophoresis in TBE buffer. Approximately 7.1 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00477] MIBa357 was transformed with the digested pANN28 DNA and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit (Finnzymes). Primers 0611622 and 0611552 yielded an approximately 850 bp band; primers 0611245 and 0612794 yielded an approximately 2.8 kbp band; primers 0611815 and 0612795 yielded an approximately 3.9 kbp band. A strain which gave the expected bands for proper integrating of the expression cassette was designated McTs241.

[00478] A *ura*⁻ derivative of McTs241 then was isolated as described previously. Several FOA resistant colonies of McTs241 were screened by PCR for the desired loop-out event with primers 0614233 and 0611554 and lack of growth on *ura* minus selection plates. The presence of an 4.6 kbp band indicated the presence of only the *ura3* scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 5.9 kbp indicated the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR reactions using Phire Plant Direct PCR Kit (Finnzymes) were carried out as described above. One FOA resistant colony from parent strain McTs241 that had the desired loop-out event was designated McTs247.

[00479] To create the homozygous integration McTs247 was transformed with the digested pANN28 DNA and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit (Finnzymes). As a first screen transformants were screen by PCR with primers 0611552 and 0611553 which should yield an approximately 850 bp band only if the *pdv* locus is intact and thus the homozygous integration of *PYC* at the *PDC* locus did not occur. Of those that were negative for a band from this PCR were then screened by additional PCR with primers 0611555 and 0611554. With these primers a product should only amplify 1.4 kbp band if *PDC* is intact and thus not a homozygous integration of *PYC* at *PDC* locus. Further screening of transformants was done by PCR using primers 0611622 and 0611552 yielding an approximately 850 bp band; primers 0611245 and 0612794 yielded an approximately 2.8 kbp band; primers 0611815 and 0612795 yielded an approximately 3.9 kbp band. A strain which gave the expected bands for proper integrating of the expression cassette was designated McTs253.

Example 3A-19: PYC activity, ADC activity and 3-HP production performance of strains MeJi412, yANN35, yANN41, MIBa355, McTs241 and McTs253.

[00480] Strains MeJi412, yANN35, yANN41, MIBa355, McTs241 and McTs253 were grown in shake flasks and CFE were prepared and assayed for pyruvate carboxylase (PYC) activity and aspartate 1-decarboxylase (ADC) activity as described herein. The results are shown in Table 27. Strains MeJi412, yANN35, yANN41, MIBa355, McTs241 and McTs253 were also tested in bioreactors for 3-HP production, using the methods described herein. The results from these bioreactor experiments are also shown in Table 27. In order to account for differences in cell mass in these fermentations, the 3-HP production performance shown is expressed as 3-HP concentration per unit of cell mass (expressed as g/L 3-HP/g/L dry cell weight). The results show that as the level of PYC activity in the cells increased, the 3-HP production performance increased.

Table 27: Transformant PYC and ADC activity and 3-HP production performance

Strain	Gene Overexpressed	PYC activity	ADC activity	3-HP/DCW
MeJi412	ADC (SED ID NO: 138)	6.8	1.151	0.43
yANN35	ADC (SED ID NO: 138) PYC (SEQ ID NO: 1)	47.2	1.090	0.64
yANN41	ADC (SED ID NO: 138) PYC (SEQ ID NO: 1)	49.0	1.263	1.30
MIBa355	ADC (SED ID NO: 138)	6.9	1.173	0.52
McTs241	ADC (SED ID NO: 138) PYC (SEQ ID NO: 1)	24.8	1.119	0.76
McTs253	ADC (SED ID NO: 138) PYC (SEQ ID NO: 1)	55.5	1.347	1.30

Example 3A-20: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus and deletion of beta-alanine aminotransferase (BAAT)

[00481] This example describes the construction and performance of yeast strains expressing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the adh1202 locus and deletion of the native *I. orientalis* gene encoding the BAAT (PYD4) of SEQ ID NO: 20.

Construction of I. orientalis BAAT (PYD4) deletion plasmid

[00482] The plasmid pMIBa123 (*supra*) was digested with NotI, KpnI, Apal and the purified by 1% agarose gel electrophoresis in TBE buffer as described herein. Two bands at approximately 3.6 kbp and 3.8 kbp were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's

instructions. These two pieces comprised the plasmid backbone and the ura selection cassette with *S. cerevisiae* 3-HPDH gene (YMR226c) of SEQ ID NO: 144.

[00483] A PCR product for the upstream *I. orientalis* PYD4 homology was generated by PCR amplification using *I. orientalis* MBin500 genomic DNA prepared as described previously using primers 0613178 and 0613180. The downstream *I. orientalis* PYD4 homology piece was prepared by PCR amplification from MBin500 genomic DNA using primers 0613179 and 0613181. Fifty pmoles of each primer was used in a PCR reaction containing 0.5 μ L of MBin500 genomic DNA as template, 0.2mM each dATP, dGTP, dCTP, dTTP, 1X Expand High Fidelity Buffer (Roche), 3.5 U Expand High Fidelity Enzyme Mix (Roche) in a final volume of 50 μ L. The amplification reaction was performed in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf) programmed for one cycle at 95°C for 3 minutes; and 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After the cycles, the reaction was incubated at 72°C for 5 minutes and then cooled at 10°C until further processed. The approximately 800 bp band from PCR using primers 0613178 and 0613180 and the approximately 900 bp band from PCR using primers 0613179 and 0613181 were purified by 1% agarose gel electrophoresis in TBE buffer. The bands were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00484] The PYD4 upstream PCR product, PYD4 downstream PCR product, and pMIBa123 NotI/KpnI/ApaI digested plasmid were assembled in a reaction with IN-FUSION HD™ (Clontech Laboratories, Inc.) according to manufacturer's instructions. From the In-FUSION reaction 2 μ L was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. After a recovery period, two 100 μ L aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 μ g of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and sequencing. A plasmid with the correct sequence were verified by sequencing and named pMcTs61.

[00485] The plasmid pMcTs61 still contains the PDC promoter, YMR226c gene from *S. cerevisiae*, and the PDC terminator. To remove these undesired segments, pMcTs61 was digested with EcoRI and XhoI followed by addition of Klenow fragment to create blunt ends. The 7.1 kbp fragment was purified by 1% agarose gel electrophoresis in TBE buffer. The band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The digested blunt plasmid was ligated together using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The

reaction mixture contained 1X T4 DNA ligase buffer, 1 μ L T4 DNA ligase, 5 μ L pMcTs61 digested and blunted purified DNA in total volume of 20 μ L. The reaction was incubated at room temperature for 2 hours. A 10 μ L sample of the ligation reaction was used to transform ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. After a recovery period, two 100 μ L aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 μ g of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates. Clones were analyzed by colony PCR. Template DNA from each colony was prepared by dissolving 1 colony in 50 μ L sterile water, heated at 95°C for 10 minutes, then cooled on ice until use. Primers 0612911 and 0612909 were used to screen the transformants. The PCR reaction with these primers would amplify a 1 kbp band if the plasmid was correct. Ten pmoles of each primer was used in a PCR reaction containing 2 μ L colony DNA template, 0.1mM each dATP, dGTP, dCTP, dTTP, 1X Crimson Taq Reaction Buffer (New England Biolabs), 1 U Crimson Taq DNA Polymerase (New England Biolabs) in a final volume of 20 μ L. The amplification reaction was performed in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf) programmed for one cycle at 95°C for 3 minutes; and 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes. After the cycles, the reaction was incubated at 72°C for 5 minutes and then cooled at 10°C until further processed. From 5 μ L of the PCR reaction a 1 kbp PCR fragment was visualized on a 1% TAE-agarose gel with ethidium bromide in TAE buffer. One transformant with the correct size PCR product was selected and named pMcTs64 (Figure 30). Plasmid DNA of pMcTs64 was prepared using a BIOROBOT® 9600 (Qiagen).

Deletion of native I. orientalis BAAT (PYD4) from MeJi413 using pMcTs64 construct

[00486] Plasmid pMcTs64 (*supra*; see Figure 30) was digested with Apal, NcoI, KpnI and purified by 1% agarose gel electrophoresis in TBE buffer. Approximately 3.3 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00487] Strain MeJi413 (*supra*) was transformed with the digested pMcTs64 DNA and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit (Finnzymes). Primers 0612908 and 0613242 yield an approximately 1.7 kbp band; primers 0613241 and 0612909 yield an approximately 1.5 kbp band to confirm the integration of the deletion cassette. Primers 0611815 and 0611632 yield an approximately 4.2 kbp band; primers 0611817 and 0611631 yield an approximately 4.8 kbp band to confirm the ADC cassette at the ADH1202 locus was still intact. A strain which gave the expected bands for proper integrating of the deletion cassette and ADC cassette was designated McTs225.

[00488] A ura⁻ derivative of McTs225 then was isolated as described previously. Several FOA resistant colonies of McTs225 were screened by PCR for the desired loop-out event with primers 0612911 and 0612910. The presence of an 1.1 kbp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.5 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. Primers 0611815 and 0611632 yield an approximately 4.2 kbp band; primers 0611817 and 0611631 yield an approximately 4.8 kbp band to confirm the ADC cassette at the adh1202 locus was still intact. PCR reactions using Phire Plant Direct PCR Kit (Finnzymes) were carried out as described above. One FOA resistant colony from parent strain McTs225 that had the desired loop-out event was designated McTs228.

[00489] To create a homozygous deletion of the native gene encoding the *I. orientalis* BAAT (PYD4) of SEQ ID NO: 20, McTs228 was transformed with digested pMcTs64 and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit (Finnzymes). Two primer sets were used to screen by PCR for PYD4 locus deletion. Primers 0613550 and 0612910 yield an approximately 700 bp band only if the PYD4 locus is intact which would indicate that homozygous deletion of PYD4 did not occur. Additionally transformants were screen with primers 0612911 and 0613551 which yield an approximately 600 bp band if PYD4 was not deleted. Transformants that were negative for the *I. orientalis* PYD4 locus were further screened with primers 0613242 and 0613243 yielding an approximately 3.5 kbp and 2.1 kbp band; primers 0612908 and 0613243 yielded an approximately 1.7 kbp band; primers 0612909 and 0612911 yielded an approximately 950 bp band. The ADC cassette at adh1202 locus was confirmed to still be intact with primers 0611817 and 0611631 yielding an approximate 4.8 kbp and primers 611815 and 612712 yielding an approximate 4.2 kbp band. A strain which gave the expected bands for proper integrating of the expression cassette was designated McTs236.

[00490] A ura⁻ derivative of McTs236 then was isolated as described previously. Several FOA resistant colonies of McTs236 were screened by PCR for the desired loop-out event with primers 0613242 and 0613243 yielding an approximately 2.1 kbp band. The ADC cassette at ADH1202 locus was confirmed to still be intact with primers 0611245 and 0612794 yielding an approximate 3 kbp and primers 0611815 and 0612795 yielding an approximate 3.6 kbp band. A strain which gave the expected bands for proper integrating of the expression cassette was designated McTs245.

[00491] Strains MIBa372 and McTs245 were tested in bioreactors for 3-HP production, using the methods described herein. In order to account for differences in cell mass in these

fermentations, the 3-HP production performance is expressed as 3-HP concentration per unit of cell mass (expressed as g/L 3-HP/g/L dry cell weight). The g/L 3-HP/g/L dry cell weight for strains MIBa372 and McTs245 were 1.66 and 0.16, respectively. These results suggest that the native PYD4 gene in *I. orientalis* is responsible for the conversion of beta-alanine to malonate semialdehyde, since deletion of this gene led to a 10-fold decrease in 3-HP production performance.

Example 3A-21: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus and deletion of 3-HP dehydrogenase (3-HPDH)

[00492] This example describes the construction and performance of yeast strains expressing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the adh1202 locus and deletion of the native *I. orientalis* gene encoding the 3-HPDH of SEQ ID NO: 26.

Construction of I. orientalis 3-HPDH deletion plasmid

[00493] The plasmid pMIBa123 (*supra*) was digested with NotI, KpnI, Apal and the purified by 1% agarose gel electrophoresis in TBE buffer as described herein. Two bands at approximately 3.6 kbp and 3.8 kbp were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. These two pieces comprised the plasmid backbone and the ura selection cassette with *S. cerevisiae* 3-HPDH gene of SEQ ID NO: 144.

[00494] A PCR product for the upstream *I. orientalis* 3-HPDH homology was amplified from *I. orientalis* MBin500 genomic DNA prepared as described previously using primers 0613183 and 0613184. A downstream *I. orientalis* 3-HPDH homology piece was amplified from *I. orientalis* MBin500 genomic DNA using primers 0613185 and 0613186. Fifty pmol of each primer was used in a PCR reaction containing 0.5µl of MBin500 genomic DNA as template, 0.2 mM each dATP, dGTP, dCTP, dTTP, 2% DMSO, 1X Phusion HF Buffer (Finnzymes), 2U Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) in a final volume of 50µl. The amplification reaction was performed in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf Scientific, Inc., Westbury, NY, USA) programmed for one cycle at 95°C for 3 minutes; and 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After the cycles, the reaction was incubated at 72°C for 5 minutes and then cooled at 4°C until further processed. The approximately 640 bp band from PCR of primers 0613183 and 0613184 and the approximately 670 bp band of the PCR from primers 0613185 and 0613186 was purified by 1% agarose gel electrophoresis in TBE buffer. The bands were

excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00495] The *I. orientalis* 3-HPDH upstream PCR product, *I. orientalis* 3-HPDH downstream PCR product, and pMIBa123 NotI/KpnI/ApaI digested plasmid were assembled in a reaction with IN-FUSION HD™ (Clontech Laboratories, Inc.) according to manufacturer's instructions. From the In-FUSION reaction 2 µL was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. After a recovery period, two 100 µl aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and sequencing. A plasmid with the correct sequence was verified by sequencing and named pMcTs60.

[00496] The plasmid pMcTs60 still contains the PDC promoter, YMR226c gene from *S. cerevisiae*, and the PDC terminator. To remove these undesired segments, pMcTs60 was digested with NotI and XbaI and the approximately 5 kbp band containing the 3-HPDH homology regions and the plasmid backbone were purified by 1% agarose gel electrophoresis in TBE buffer. The bands were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The *ura3* selection cassette was amplified with 2 PCR reactions one with primers 0613416 and 0613417 and the other with primers 0613418 and 0613419. Fifty pmol of each primer was used in a PCR reaction containing 0.5 µL of pMcTs60 plasmid DNA as template, 0.2 mM each dATP, dGTP, dCTP, dTTP, 1X Expand High Fidelity Buffer (Roche), 3.5 U Expand High Fidelity Enzyme Mix (Roche) in a final volume of 50 µL. The amplification reaction was performed in an EPPENDORF® MASTERCYCLER® 5333 (Eppendorf Scientific, Inc.) programmed for one cycle at 95°C for 3 minutes; and 30 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After the cycles, the reaction was incubated at 72°C for 5 minutes and then cooled at 4°C until further processed. The approximately 700 bp band from PCR of primers 0613416 and 0613417 and the approximately 1 kbp band of the PCR from primers 0613418 and 0613419 was purified by 1% agarose gel electrophoresis in TBE buffer. The bands were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The plasmid backbone containing the *I. orientalis* 3-HPDH homology regions and the *ura3* cassette PCR products were assembled in a reaction with IN-FUSION HD™ (Clontech Laboratories, Inc.) according to manufacturer's instructions. From the In-FUSION reaction 2 µL was transformed into Solo Pack Gold Super Competent Cells (Stratagene) according to manufacturer's instructions. After a recovery period, two 100

μL aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 μg of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and a plasmid with the correct restriction digest pattern was named pMcTs65 (Figure 31).

Deletion of native I. orientalis 3-HPDH from MeJi413 using pMcTs65 construct

[00497] Plasmid pMcTs65 (*supra*; see Figure 31) was digested with Apal, Sph, KpnI and purified by 1% agarose gel electrophoresis in TBE buffer. Approximately 2.9 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00498] Strain MeJi413 (*supra*) was transformed with the digested pMcTs65 DNA and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit (Finnzymes). Primers 0613034 and 0613035 yielded an approximately 2.7 kbp band to confirm the integration of the deletion cassette. Primers 0611815 and 0611632 yielded an approximately 4.2 kbp band; primers 0611817 and 0611631 yielded an approximately 4.8 kbp band to confirm the ADC cassette at the adh1202 locus was still intact. A strain which gave the expected bands for proper integrating of the deletion cassette and ADC cassette was designated McTs229.

[00499] A ura⁻ derivative of McTs229 then was isolated as described previously. Several FOA resistant colonies of McTs229 were screened by PCR for the desired loop-out event with primers 0613034 and 0613241. The presence of an 1.4 kbp band indicates the presence of only the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 2.8 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. The presence of a 1.9 kbp band indicates the wild-type locus which is present in these transformants since these are heterozygous for the deletion. Primers 0611815 and 0611632 yielded an approximately 4.2 kbp band; primers 0611631 and 0612366 yielded an approximately 4.5 kbp band to confirm the ADC cassette at the ADH1202 locus was still intact. PCR reactions using Phire Plant Direct PCR Kit (Finnzymes) were carried out as described above. One FOA resistant colony from parent strain McTs225 that had the desired loop-out event was designated McTs238.

[00500] To create a homozygous deletion of the native gene encoding the *I. orientalis* 3-HPDH of SEQ ID NO: 26, McTs238 was transformed with digested pMcTs65 and correct loci targeting and transformation was verified by PCR using Phire Plant Direct PCR Kit

(Finnzymes). Two primer sets were used to screen by PCR for YMR226c locus deletion. Primers 0613034 and 0613747 would yield an approximately 500 bp band if the 3-HPDH locus is intact to indicate that homozygous deletion of 3-HPDH did not occur. Additionally transformants were screened with primers 0613746 and 0613241 which would yield an approximately 660 bp band if 3-HPDH was not deleted. Transformants that were wild-type negative for the *I. orientalis* 3-HPDH locus were further screened with primers 0613034 and 0613241 yielding an approximately 2.8 kbp and 1.4 kbp band; primers 0612908 and 0613241 yielded an approximately 1.5 kbp band; primers 0613034 and 0612909 yielded an approximately 1 kbp band. The ADC cassette at ADH1202 locus was confirmed to still be intact with primers 0611245 and 0612794 yielding an approximate 3 kbp and primers 0611815 and 0612795 yielding an approximate 3.6 kbp band. A strain which had the expected bands for proper integrating of the expression cassette was designated McTs244.

[00501] A ura⁻ derivative of McTs244 when as isolated as described previously. Several FOA resistant colonies of McTs244 were screened by PCR for the desired loop-out event with primers 0613034 and 0613241 yielding an approximately 1.4 kbp band. The ADC cassette at ADH1202 locus was confirmed to still be intact with primers 0611245 and 0612794 yielding an approximately 3 kbp band and primers 0611815 and 0612795 yielding an approximately 3.6 kbp band. One FOA resistant colony from parent strain McTs244 that had the desired loop-out event was designated McTs259.

[00502] Strains MIBa372 and McTs244 were tested in bioreactors for 3-HP production, using the methods described herein. In order to account for differences in cell mass in these fermentations, the 3-HP production performance is expressed as 3-HP concentration per unit of cell mass (expressed as g/L 3-HP/g/L dry cell weight). The g/L 3-HP/g/L dry cell weight for strains MIBa372 and McTs259 were 1.66 and < 0.1, respectively. These results indicate that the native 3-HPDH gene in *I. orientalis* is responsible for the conversion of malonate semialdehyde to 3-HP, since deletion of this gene abolished 3-HP production.

Example 3A-22: Yeast strains expressing pyruvate carboxylase (PYC), aspartate aminotransferase (AAT), β -alanine aminotransferase (BAAT), and 3-hydroxypropionic acid dehydrogenase (3-HPDH) at the *pdh* locus; and aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the *adh1202* locus

[00503] Additional constructs were designed to incorporate the nucleotide sequence SEQ ID NO: 1 encoding the *I. orientalis* PYC of SEQ ID NO: 2, the nucleotide sequence SEQ ID NO: 13 encoding the *I. orientalis* AAT of SEQ ID NO: 14, the nucleotide sequence SEQ ID NO: 142 encoding the *S. kluyveri* BAAT of SEQ ID NO: 21, and the nucleotide sequence SEQ ID NO: 144 encoding the *S. cerevisiae* 3-HPDH of SEQ ID NO: 129 at the *I. orientalis*

pdg locus in strains that also contain four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the adh1202 locus. In a similar approach to that described above, a left-hand and a right-hand construct were designed to allow homologous recombination at the *I. orientalis* CNB1 pdg locus. These constructs were prepared and transformed into MIBa357 as described below.

Construction of a left-hand fragment

[00504] The nucleotide sequence SEQ ID NO: 13 encoding the *I. orientalis* AAT of SEQ ID NO: 14 was amplified by PCR using plasmid pGMEr126 (Figure 16) as a template according to the manufacturer's instructions (Pfu polymerase, Stratagene). The primers oANN1 and oANN2 allowed the incorporation of unique restriction sites flanking the gene coding sequence. The PCR product was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 1.3 kbp was excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified PCR product was digested with Apal and NruI and gel purified as described herein.

[00505] The plasmid pGMEr135 (identical to pGMEr136 above, except that the ENO1 promoter/RKI terminator insert is in opposite orientation) was digested with Apal and NruI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 11.7 kbp was excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

[00506] The purified 1.3 kbp PCR product was ligated into the 11.7 kbp pGMEr135 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 49.8 ng of the vector, 354 ng of the 1.3 kbp insert, 1 μ L 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* DH10B cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oHJ2 and oANN1 (yielding a band of approximately 2.3 kbp) and primers oANN5 and oANN6 (yielding a band of approximately 877 bp). The sequence of the aat fragment amplified by PCR was also confirmed. A clone yielding correct insertion and sequence was digested with Apal and NruI and gel purified as described herein. A band of approximately 1.3 kbp was excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

[00507] The plasmid pGMEr137 (*supra*; see Figure 18), containing the desired nucleotide sequence SEQ ID NO: 1 encoding the *I. orientalis* PYC of SEQ ID NO: 2, was digested with Apal and NruI and purified by agarose gel electrophoresis in TBE buffer as described herein.

A band of approximately 11.7 kbp was excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

[00508] The purified 1.3 kbp PCR product was ligated into the 11.7 kbp pGMEr137 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 49.4 ng of the vector, 54 ng of the 1.3 kbp insert, 1 μ L 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* DH10B cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oHJ2 and oANN1 (yielding a band of approximately 2.3 kbp) and primers oANN5 and oANN6 (yielding a band of approximately 877 bp). A clone yielding correct insertion and sequence was designated pANN02.

Construction of a left-hand fragment with the AAT encoding sequence in the opposite orientation

[00509] The plasmid pANN02 was digested with PmeI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 10.3 kbp and a band of approximately 2.7 kbp were excised from the gel and purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions. The 10.3 kbp vector fragment from pANN02 was dephosphorylated with CIP (New England Biolabs) and purified with a purification kit (Qiagen) according to the manufacturer's instructions. The 2.7 kbp fragment from pANN02 was ligated into the dephosphorylated 10.3 kbp linearized vector from pANN02 using T4 ligase (New England Biolabs) in a total reaction volume of 10 μ L composed of 36 ng of the vector, 28 ng of the insert, 1 μ L 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 μ L T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 2 μ L aliquot of the reaction was transformed into electro-competent *E. coli* TOP10 cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on LB + Kanamycin plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by colony PCR with primers oJY44 and oHJ1 (yielding a band of approximately 1.3 kbp). A clone yielding correct insertion was designated pANN5.

Construction of a right-hand fragment

[00510] A right-hand construct containing two *B. licheniformis* ADC coding regions and the *I. orientalis* PDC locus 3' targeting flanking DNA was constructed as follows. The pMhCt071 plasmid (a plasmid identical to pMhCt077 above except that the *S. cerevisiae* 3-HPDH ORF is not codon optimized for *I. orientalis*) was digested with PmeI and PacI, treated with 10

units calf intestinal phosphatase (New England Biolabs), and purified by 0.9% agarose gel electrophoresis in TAE buffer, and an approximately 4.7 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit according to the manufacturer's instructions.

[00511] The plasmid pMeJi312-2 (*supra*; see Figure 15) was digested with PmeI and PacI to extract two *B. licheniformis* ADC expression cassettes and purified by 0.9% agarose gel electrophoresis in TAE buffer. An approximately 2.8 kbp band was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit according to the manufacturer's instructions.

[00512] The fragment containing dual *B. licheniformis* ADC coding regions from pMeJi312-2 was then ligated into the linearized pMhCt071 vector fragment in a ligation reaction (20 µL) containing 1X Quick ligation buffer (New England Biolabs), 1 µL 4.7 kbp fragment of pMhCt071 vector, 3 µL 2.8 kbp insert from pMeJi312-2, and 1 µL Quick T4 DNA ligase (New England Biolabs). The ligation reaction was incubated for 5 min at room temperature, and then the tube was placed on ice. 5 µL of this reaction was used to transform SoloPack Gold SuperCompetent Cells (Agilent Technologies) according to the manufacturer's instructions. Transformants were plated onto 2X YT+ amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper ligation of the desired fragments by ApaLI digestion. A clone yielding the desired band sizes was kept and designated pMhCt110.

[00513] The plasmid pMhCt110 was digested with XbaI and PacI followed by treatment with CIP and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 7.1 kbp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00514] The codon-optimized *S. cerevisiae* 3-HPDH coding sequence of SEQ ID NO: 144 was excised from pMIBa123 (*supra*) by digestion with XbaI and PacI and purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 814 bp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The 814 bp purified fragment was ligated into the 7.1 kbp fragment from pMhCt110 using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the digested pMhCt110, 1 or 7 µL of the 814 bp fragment from pMIBa123, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 30 minutes at room temperature and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated over the weekend at room temperature. Several of the resulting transformants were screened for

proper insertion by restriction digest using 2 combinations of enzymes XbaI and PaeI and AseI and PaeI. A clone yielding correct digested band sizes from each digest was designated pMIBa142.

[00515] Plasmid pMIBa142 was digested with AseI followed by a fill-in reaction with Klenow and subsequent digestion with NheI and CIP treatment. The digestion was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 7.5 kbp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00516] The nucleotide sequence SEQ ID NO: 142 encoding the *S. kluyveri* BAAT of SEQ ID NO: 21, was excised from pMIBa124 (*supra*) by digestion with PaeI followed by a fill-in reaction with Klenow and subsequent digestion with XbaI. The digestion was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A band at approximately 1.4 kbp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00517] The 1.4 kbp purified fragment from pMIBa124 was ligated into the 7.5 kbp fragment from pMIBa142 using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the digested pMIBa142, 7 µL of the 1.4 fragment from pMIBa124, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 2.5 hours at 16°C and the entire ligation was transformed into Sure cells (Agilent) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated overnight at 37°C. Several of the resulting transformants were screened for proper insertion by restriction digest with StuI and PmeI. A clone yielding correct digested band size was designated pMIBa144.

Integration of a left-hand and right-hand fragments into MIBa357

[00518] Plasmid pANN5 was digested with NotI and NheI and plasmid pMIBa144 was digested with NotI as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the 8.2 kbp fragment from pANN5 and the 6 kbp fragment from pMIBa144 were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to manufacturer's instructions.

[00519] MIBa357 was transformed with the digested pANN5 and pMIBa144 DNA and correct loci targeting and transformation was verified by PCR using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. Primers 0611552 and 0613695 yielded an approximately 4.1 kbp band (to confirm the left half integration at the *pdv* locus); primers 0612358 and 0611554 yielded an approximately 2.5 kbp band (to confirm the right half integration at the *pdv* locus); 0611245 and 0611631 yielded an approximately 3.5 kbp band (to confirm the left half 4X ADC integration remained at the

adh1202 locus); and primers 0611815 and 0611632 yielded an approximately 4.6 kbp band (to confirm the right half 4X ADC integration remained at the adh1202 locus). One isolate which gave the expected bands for proper integrating of the expression cassette at the pdc locus and retained the expression cassette at the adh1202 locus was saved and designated MIBa360.

Removal of ura marker from MIBa360

[00520] A ura⁻ derivative of MIBa360 was isolated as described above. Genomic DNAs from several FOA resistant colonies of MIBa360 were screened by PCR for the desired loop-out event with primers 0611815 and 0613689. The presence of an approximately 1.9 kbp band indicates the removal of the ura marker with the ura3 scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 3.3 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. PCR was performed using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. Two FOA resistant colonies that yielded the approximately 1.9 kbp fragment with the above primers were saved and designated MIBa363 and MIBa364.

Construction of a reverse expression cassette right-hand fragment

[00521] Plasmid pMIBa144 contains the desired ADC and 3-HPDH expression cassettes going in the forward orientation. To ease screening of homozygous strains, a new plasmid was constructed where the ADC expression cassette of pMIBa144 was placed in the reverse orientation. The plasmid pMIBa144 (*supra*) was digested with *Stu*I and *Pme*I purified by 1% agarose gel electrophoresis in TBE buffer as described herein. Bands at approximately 6.1 kbp and 2.8 kbp were excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The 6.1 kbp fragment from pMIBa144 was treated with *CIP* and purified using the QIAQUICK® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

[00522] The 2.8 kbp purified fragment from pMIBa144 was ligated into the 6.1 kbp pMIBa144 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 6.1 kbp fragment from pMIBa144, 7 µL of the 2.8 kbp fragment from pMIBa144, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 4 hours at 16°C and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting

transformants were screened for proper insertion by restriction digest using SphI and XbaI. A clone yielding correct digested band size was designated pMiBa146.

Integration of a left-hand and right-hand fragments into MiBa363

[00523] Plasmid pANN5 was digested with NotI and NheI and plasmid pMiBa146 was digested with NotI as described herein. These were purified by 1% agarose gel electrophoresis in TBE buffer, and the 8.2 kbp fragment from pANN5 and the 6.2 kbp fragment from pMiBa146 were excised from the gel and purified the QIAQUICK® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

[00524] MiBa363 was transformed with the digested pANN5 and pMiBa144 DNA and correct loci targeting and transformation was verified by PCR using the Phire® Plant Direct PCR kit (Finnzymes) or Kapa Robust DNA polymerase according to the manufacturer's instructions. To confirm integrations at *pdv* locus, the following primer pairs were used. Primers 0613689 and 0611815 yielded an approximately 1.9 kbp band; primers 0612366 and 0611554 yielded an approximately 2.5 kbp band; 0613688 and 0611815 yielded an approximately 3.2 kbp band; 0611622 and 0611552 yielded an approximately 945 bp band. To check the integrations at *adh1202* the following primer pairs were used. Primers 0611245 and 0612794 yielded an approximately 2.8 kbp band and primers 0611815 and 0612795 yielded an approximately 3.9 kbp band. Two isolates which gave the expected bands for proper integrating of the expression cassette at the *pdv* locus and retained the expression cassette at the *adh1202* locus were saved and designated MiBa372 and MiBa373.

Removal of ura marker from MiBa372

[00525] A *ura*⁻ derivative of MiBa372 was isolated as described above. Genomic DNAs from several FOA resistant colonies of MiBa372 were screened by PCR for the desired loop-out event with primers 0611815 and 0613688. The presence of an approximately 2.1 kbp band indicates the removal of the *ura* marker with the *ura3* scar site (a single URA3 promoter left behind after homologous recombination between the two URA3 promoters in the parent strain) as desired, while a band of approximately 3.3 kbp indicates the presence of the intact URA3 promoter-URA3 ORF-URA3 terminator-URA3 promoter cassette, indicating the desired recombination event did not occur. FOA resistant colonies of MiBa372 were also screened by PCR with primers 0611815 and 0613689 (amplifies 1.9 kbp fragment) to confirm modification of the first chromosome and 0611552 and 0611553 (amplifies 850 bp fragment if the *pdv* locus is present) to confirm loss of the *pdv* locus. PCR was performed using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. An FOA resistant colony that yielded the approximately 1.9 kbp fragment with 0611815 and 0613689, but did not amplify fragments with 0613688 and 0611815, or with

0611552 and 0611553 was saved and designated MIBa375. The genotype of MIBa375 is shown in Table 28.

Table 28: Transformant genotype

Strain	Genotype
MIBa375	adh1202Δ::(PDC _{promo} -Opt.Blpand PDC _{promo} -Opt.Blpand Ura3-Scar TDH3 _{promo} Opt.Blpand TDH3 _{promo} Opt.Blpand)/adh1202Δ::(PDC _{promo} -Opt.Blpand PDC _{promo} -Opt.Blpand Ura3-Scar [TDH3 _{promo} Opt.Blpand TDH3 _{promo} Opt.Blpand]reverse) pdcΔ::(PDC _{promo} -CNB1pyc ENO1 _{promo} -CNB1aat Ura3-Scar TDH3 _{promo} -OptSkPYD4 PGK1 _{promo} -OptScYMR226c/ pdcΔ::(PDC _{promo} -CNB1pyc ENO1 _{promo} -CNB1aat Ura3-Scar TDH3 _{promo} -OptSkPYD4 PGK1 _{promo} -OptScYMR226c ura3-/ura3-

Example 3A-23: Yeast strains deleted for the glycerol 3-phosphate dehydrogenase (GPD) gene.

Deletion of the GPD gene in strain MIBa375

[00526] Additional constructs were designed to delete both copies of glycerol 3-phosphate dehydrogenase gene (SEQ ID NO: 117, which encodes GPD of SEQ ID NO: 118) from the host *I. orientalis* genome. These constructs contained approximately 1003 bp of nucleotide sequence homologous to the sequence upstream of the *GPD* gene and approximately 852 bp of sequence homologous to the sequence downstream of the *GPD* gene, with a T_{PDC}-URA3 marker cassette (PDC terminator-URA3 promoter-URA3 gene-URA3 terminator-URA3 promoter) cloned in between.

[00527] The regions upstream and downstream of *GPD* were amplified from *I. orientalis* CNB1 genomic DNA using *Pfu* DNA polymerase as per manufacturer's specifications. The upstream region contained an *Apal* site; this was eliminated by PCR using overlapping primers designed with a mismatch to one of the nucleotides in the *Apal* recognition sequence. Primer pairs oACN48/oACN51 and oACN49/oACN50 were used to amplify these two overlapping fragments for the upstream region. These PCR products were separated on and excised from a 1% agarose gel and purified using a Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, and were used as template for a second round of PCR with primers oACN48/oACN49 (having forward *Apal*/reverse *NotI* sites). The downstream region was amplified with primers oACN52/oACN53 (having forward *NotI*/reverse *SacI* sites) from *I. orientalis* genomic DNA using *Pfu* DNA polymerase as per manufacturer's specifications. Both PCR products were gel purified and cloned separately into the vector pCR®-BluntII-TOPO® (Invitrogen). Isolates were confirmed to have the desired insert by restriction digest of plasmid DNA, and were verified by sequencing. Vector pACN58 contained the cloned upstream fragment and

pACN59 contained the downstream fragment. Plasmid pACN58 was digested with *Apal/NotI* to release the upstream flank, plasmid pACN59 was digested with *SacI/NotI* to release the downstream flank, and pACN59 was digested with *Apal/SacI* to provide the vector backbone. The three desired fragments were separated on a 1% agarose gel, excised and purified, and ligated in a 3-piece ligation reaction using T4 ligase (New England Biolabs). The ligation reaction was transformed into *E.coli* TOP10 electrocompetent cells and transformants were confirmed by restriction digest of plasmid DNA. During this procedure, an additional *SacI* site in the downstream region was detected, which resulted in a downstream region of 853 bp (as opposed to 1 kbp). Two isolates with the desired inserts were named pACN62 and pACN63.

[00528] The T_{PDC1} -*URA3* cassette was isolated from vector pJLJ8 (Figure 32) using a *NotI* digest and gel purification. This fragment was then ligated into pACN62 (*supra*) that had been digested with *NotI* and dephosphorylated, and the ligation was transformed into *E. coli* DH10B electrocompetent cells (Invitrogen). Colonies with the *URA3* insert were confirmed by PCR using primers oACN48/oJLJ44 and oACN48/oJLJ46. Primer oJLJ44 anneals at the end of the downstream *URA3* promoter and amplifies outward from the T_{PDC1} -*URA3* cassette. Primer oJLJ46 anneals at the 5' end of the PDC terminator and amplifies outward from the T_{PDC1} -*URA3* cassette. Vector pHJJ56 contains the *URA3* facing the downstream *GPD* region and pHJJ57 contains the *URA3* facing the upstream *GPD* region.

[00529] Plasmids pHJJ56 and pHJJ57 were linearized by digestion with *KpnI* and *Apal* and the fragments containing the deletion cassette were purified by gel extraction. Linearized pHJJ56 was transformed into the *ura*- strain MIBa375. Single colonies were restreaked for purification and tested by PCR for the desired *GPD* deletion using primers oJLJ44, oJLJ46, oACN54 and oACN55. Cells were lysed in 40 μ L Y-Lysis buffer and 2 μ L Zymolyase (ZymoResearch) at 37°C for 30 minutes and 1 μ L of the lysis reaction used in a 25 μ L PCR reaction. PCR reactions used Failsafe DNA polymerase and Buffer E according to manufacturer's specifications, with an annealing temperature of 55°C and the following cycling profile: 1 cycle at 94°C for 2 minutes; 29 cycles each at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 3 minutes. Strains with one copy of the *GPD* knockout produced bands of approximately 0.9 and 1.2 kbp and were named yHJN1 and yHJN2.

[00530] Strains yHJN1 and yHJN2 were grown overnight in YPD media and plated onto ScD-2X FOA media to select to loss of the *URA3* marker. Single colonies were purified on YPD and patched to ScD-*ura* and YPD media to confirm the *ura*- phenotype. *Ura*- colonies were confirmed to have retained the knockout using the same PCR reaction used to confirm

the first integration. A ura⁻ derivative of yHJN1 was named yHJN3 and a ura⁻ derivative of yHJN2 was named yHJN4.

[00531] Linearized pHJJ57 was transformed into yHJN3 and yHJN4 and single colonies were purified on ScD-ura media. The presence of two copies of the *GPD* knockout was confirmed by PCR using primers oJLJ44, oACN54, and oACN55 in one reaction, and primers oJLJ46, oACN54, and oACN55 in a second reaction. Primer oACN54 anneals to a region approximately 37 bp upstream of the upstream flanking sequence for *GPD*, while oACN55 anneals to a region approximately 24 bp downstream of the downstream flank. The former reaction produces bands of approximately 900 and 1050 bp if both copies of the *GPD* are deleted, and the latter reaction produces bands of approximately 1025 and 1200 bp. Colonies with two copies of the *GPD* knockout grew more slowly on ScD-ura plates than those with a single copy of the deletion. Strains having both copies of the *GPD* gene deleted were named yHJN7 (derived from yHJN3) and yHJN8 (derived from yHJN4).

[00532] Strains MIBa372, yHJN7 and yHJN8 were tested in bioreactors for glycerol production, using the methods described herein. Strain MIBa372 produced 29.5 g/L glycerol in 48 hours. No detectable glycerol was produced by strains yHJN7 or yHJN8 during the fermentation. The absence of glycerol in the final fermentation broth may provide advantages in the recovery and purification of 3-HP from the fermentation broth.

3A-24: Yeast strains expressing aspartate 1-decarboxylase (ADC) from four nucleotide sequences at the adh1202 locus, 3-HP dehydrogenase (3-HPDH) at the adh9091 locus and deletion of native 3-HP dehydrogenase (3-HPDH)

Plasmid construction for integration of I. orientalis 3-HPDH at the adh9091 locus

[00533] The nucleotide sequences of SEQ ID NO: 25 encoding the *I. orientalis* 3-HPDH of SEQ ID NO: 26 and SEQ ID NO: 19 encoding the *I. orientalis* BAAT (PYD4) of SEQ ID NO: 20 were amplified from MBin500 *I. orientalis* genomic DNA prepared as described previously using primers 0611954 and 0611957 (for 3-HPDH) or 0611997 and 0611998 (PYD4). Primer 0611954 adds a kozak sequence (TAAA) and *NheI* site to the 5' end, and primer 0611957 adds a *PacI* site to the 3' end of 3-HPDH during amplification. Primer 0611997 adds a kozak sequence (TAAA) and *PacI* site to the 5' end, and primer 0611998 adds a *PacI* site to the 3' end of PYD4 during amplification. Fifty pmoles of each primer was used in a PCR reaction containing 50 ng of MBin500 genomic DNA as template, 0.2 mM each dATP, dGTP, dCTP, dTTP, 1X Expand High Fidelity Buffer (Roche), and 2.6 units of Expand High Fidelity DNA Polymerase (Roche) in a final volume of 50 µL. The PCR was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific) programmed for one cycle at 95°C for 3 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and

72°C for 1 minute (3-HPDH PCR) or 2 minutes (PYD4 PCR), with a final extension at 72°C for 5 minutes. Following thermocycling, the PCR reaction products were separated by 1.0% agarose gel electrophoresis in TAE buffer where an approximately 831 bp 3-HPDH or 1.4 kbp PYD4 PCR product was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Five µl of the purified 3-HPDH or PYD4 was cloned into pCR2.1 (Invitrogen) using a TOPO-TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The transformations were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by digestion with *EcoRI*. Clones yielding the desired insert size were confirmed to be correct by DNA sequencing. One clone containing 3-HPDH was designated pMBin190, and another containing PYD4 was designated pMBin193.

[00534] Plasmid pMBin193 was digested with *XbaI* and *PacI* and run on a 1.0% agarose gel in TAE buffer where the 1.4 kbp PYD4 band was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The digested PYD4 fragment was ligated into the *XbaI* and *PacI* restricted linear pMBa107 plasmid (*supra*) using T4 DNA ligase. The ligation product was transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by digestion with *SnaBI* or *EcoRI*. One clone yielding the desired band sizes was designated pMBin203. Plasmid pMBin203 contains *NotI* sites that flank the following expression cassette: PDC promoter and terminator up and downstream of the PYD4 CDS, the URA3 promoter, the URA3 ORF, and the URA3 terminator followed by the URA3 promoter.

[00535] Plasmid pMBin203 was digested with *NotI* and separated on a 1.0% agarose gel in TAE buffer where the approximately 4.1 kbp fragment (containing the PDC promoter, PYD4 CDS, the PDC terminator, and the URA3 selection marker) was excised from the gel and purified using a QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Plasmid pHJJ27 (containing 5' and 3' homology regions to the *adh9091* locus; see Figure 21) was digested with *NotI*, treated with CIP and separated on a 1.0% agarose gel in TAE buffer where the approximately 5.7 kbp linear plasmid was purified as described above. The fragment from pMBin203 was then ligated into the *NotI* restricted pHJJ27 using T4 DNA ligase as described above. The ligation product was transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated onto 2X YT + amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened by digestion with *PstI*. One clone yielding the desired band sizes was designated pMBin204. Plasmid pMBin204 allows targeting of PYD4 to the *adh9091* locus.

[00536] The nucleotide sequence of SEQ ID NO: 25 encoding the *I. orientalis* 3-HPDH of SEQ ID NO: 26 was removed from plasmid pMBin190 (*supra*) by digestion with NheI and PaeI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 827 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. The plasmid pMBin204 (*supra*) was digested with XbaI and PaeI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 8.4 kbp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00537] The purified approximately 827 bp *I. orientalis* 3-HPDH gene product above was ligated into the 8.4 kbp pMBin204 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 20 µL composed of 1 µL of the 8.4 kbp vector, 10 µL of the 827 bp insert, 2 µL 10X ligation buffer with 10 mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 18 hours at 16°C and a 10 µL aliquot of the reaction was transformed into One Shot TOP10 cells (Invitrogen) according to manufacturer's instructions. After a recovery period, two 100 µL aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per ml. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and a plasmid with the correct restriction digest pattern was designated pMcTs90.

Plasmid construction for integration of S. cerevisiae 3-HPDH at the adh9091 locus

[00538] The 826 bp wild-type nucleotide sequence encoding the *S. cerevisiae* 3-HPDH of SEQ ID NO: 129 was PCR amplified from JGI69 genomic DNA and amended with an XbaI site on the 5' end of the gene and a PaeI site on the 3' end of the gene. The amplification reaction was performed using Platinum® Pfx DNA polymerase (Invitrogen) according to manufacturer's instructions. A Master PCR reaction containing 1.125 µL of *S. cerevisiae* genomic DNA, 112.5 pM each of primers 611191 and 611199, 1X Pfx amplification buffer (Invitrogen), 2 mM MgSO₄, 0.2 mM dNTP mix, 5 Units Platinum® Pfx DNA polymerase (Invitrogen) in a final volume of 200 µL. The mix was aliquoted into eight tubes and gradient PCR performed. The amplification reactions were incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific Inc.) programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, Gradient 40-55°C for 30 seconds, and 72°C for 2 minutes; and 1 cycle at 72°C for 3 minutes.

[00539] The 826 bp wild-type YMR226c PCR gene product was purified by 1% agarose gel electrophoresis in TBE buffer as described herein. A fragment of approximately 826 bp was

excised from the gel and extracted from the agarose using a QIAQUICK® Gel Extraction Kit (Qiagen). The PCR product was digested overnight at 37°C with *Xba*I and *Pac*I then purified using the QIAQUICK® PCR purification Kit (Qiagen).

[00540] The plasmid pMIBa100 (*supra*) was digested with *Xba*I and *Pac*I followed by treatment with CIP resulting in an approximately 6.8 kbp linear fragment. The digestion was purified using the QIAQUICK® PCR purification Kit (Qiagen) according to the manufacturer's instructions.

[00541] The 826 bp YMR226c purified and digested PCR fragment was ligated into the 6.8 kbp pMIBa100 linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 6.7 kbp fragment from pMIBa100, 1 µL or 7 µL of the 826 bp YMR226c PCR product, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for approximately 4 hours at 16°C and the entire reaction was transformed into Sure chemically competent cells (Aglient) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *Xba*I and *Pac*I. Correct clones by digest were confirmed by DNA sequencing. A clone yielding correct digested band size and DNA sequence was designated pMIBa101.

[00542] Plasmid pHJJ76-no ura (*supra*) was digested with *Not*I followed by treatment with CIP. The linear 5.2 kbp fragment was purified using a QIAQUICK® PCR Purification Kit (Qiagen).

[00543] The YMR226c expression cassette was excised from pMIBa101 by digestion with *Not*I. A band at approximately 3546 bp was excised from the gel and purified using QIAQUICK® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

[00544] The 3546 bp purified fragment from pMIBa101 was ligated into the 5.2 kbp pHJJ76-no ura linearized vector using T4 ligase (New England Biolabs) in a total reaction volume of 10 µL composed of 1 µL of the 5.2 kbp fragment from pHJJ76-no ura, 1 µL or 5 µL of the 3546 bp fragment from pMIBa101, 1 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated overnight at 16°C and a 4 µL aliquot of the reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. Transformants were plated on 2X YT+amp plates and incubated at 37°C overnight. Several of the resulting transformants were screened for proper insertion by restriction digest using *Xba*I and *Kpn*I. A clone yielding correct digested band size was designated pMIBa109.

[00545] The wild-type nucleotide sequence encoding the *S. cerevisiae* 3-HPDH of SEQ ID NO: 129 was removed from plasmid pMIBa109 by digestion with *Xba*I and *Pac*I and purified

by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 818 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00546] The purified approximately 818 bp *S. cerevisiae* 3-HPDH gene product was ligated into the 8.4 kbp pMBin204 linearized vector above using T4 ligase (New England Biolabs) in a total reaction volume of 20 µL composed of 1 µL of the 8.4 kbp vector, 10 µL of the 818 bp insert, 2 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 18 hours at 16°C and a 10 µL aliquot of the reaction was transformed into One Shot TOP10 cells (Invitrogen) according to manufacturer's instructions. After a recovery period, two 100 µL aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and a plasmid with the correct restriction digest pattern was designated pMcTs91.

Plasmid construction for integration of M. sedula 3-HPDH at the adh9091 locus

[00547] An *I. orientalis* codon-optimized nucleotide sequence of SEQ ID NO: 343 encoding the *M. sedula* 3-HPDH of SEQ ID NO: 29 was synthesized by GeneArt® resulting in the plasmid 11AAE2AP. The synthetic gene was digested from the plasmid with XbaI and PacI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 959 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00548] The purified approximately 959 bp *M. sedula* 3-HPDH gene product was ligated into the 8.4 kbp pMBin204 linearized vector above using T4 ligase (New England Biolabs) in a total reaction volume of 20 µL composed of 1 µL of the 8.4 kbp vector, 16 µL of the 959 bp insert, 2 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 1 hour at room temperature and a 10 µL aliquot of the reaction was transformed into Solo Pack Gold Super Competent cells (Agilent) according to manufacturer's instructions. After a recovery period, two 100 µL aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per mL. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and a plasmid with the correct restriction digest pattern was designated pMcTs76.

Plasmid construction for integration of E. coli 3-HPDH at adh9091 locus

[00549] An *I. orientalis* codon-optimized nucleotide sequence of SEQ ID NO: 143 encoding the *E. coli* 3-HPDH of SEQ ID NO: 27 was synthesized by GeneArt® resulting in the plasmid 1045168. The synthetic gene was digested from the plasmid with XbaI and PacI and purified by agarose gel electrophoresis in TBE buffer as described herein. A band of approximately 761 bp was excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions.

[00550] The purified approximately 761 bp *E. coli* 3-HPDH gene product was ligated into the 8.4 kbp pMBin204 linearized vector above using T4 ligase (New England Biolabs) in a total reaction volume of 20 µL composed of 1 µL of the 8.4kbp vector, 16 µL of the 761 bp insert, 2 µL 10X ligation buffer with 10mM ATP (New England Biolabs), and 1 µL T4 ligase (New England Biolabs). The reaction was incubated for 1 hr at room temperature and a 10 µL aliquot of the reaction was transformed into Solo Pack Gold Super Competent cells (Agilent) according to manufacturer's instructions. After a recovery period, two 100 µL aliquots from the transformation reaction were plated onto 150 mm 2X YT plates supplemented with 100 µg of ampicillin per ml. The plates were incubated overnight at 37°C. Putative recombinant clones were selected from the selection plates and plasmid DNA was prepared from each one using a BIOROBOT® 9600 (Qiagen). Clones were analyzed by restriction digest and a plasmid with the correct restriction digest pattern was designated pMcTs77.

Integration of 3-HPDH fragments at adh9091 in McTs259

[00551] Plasmids pMcTs76, pMcTs77, pMcTs91 were digested with KpnI and ApaI, and plasmid pMcTs90 was digested with SacI and ApaI as described herein. The resulting digestion products were purified by 1% agarose gel electrophoresis in TBE buffer, and the 5.5 kbp fragment from pMcTs76, the 5.3 kbp fragment from pMcTs77, the 5.4 kbp fragment from pMcTs90, and 5.4 kbp fragment from pMcTs91 were excised from the gel and purified using a NUCLEOSPIN® Extract II Kit (Macherey-Nagel) the according to the manufacturer's instructions.

[00552] Strain McTs259 which expressing four copies of nucleotides encoding the *B. licheniformis* ADC of SEQ ID NO: 139 at the adh1202 locus and deletion of the native *I. orientalis* gene encoding the 3-HPDH of SEQ ID NO: 26 (*supra*), was transformed with the digested pMcTs76, pMcTs77, pMcTs90, or pMcTs91 DNA. The correct loci targeting and transformation was verified by PCR using the Phire® Plant Direct PCR kit (Finnzymes) according to the manufacturer's instructions. To confirm integrations at adh9091 locus, the following primer pairs were used. Primers 0614627 and 0612909 yielded an approximately 3.47 kbp band for fragment from pMcTs76 integrated, approximately 3.27 kbp band for fragment from pMcTs77 integrated, approximately 3.34 kbp band for fragment from pMcTs90 integrated, approximately 3.33 kbp band for fragment from pMcTs91 integrated; primers

0612908 and 0614626 yielded an approximately 1.97 kbp band. To check the integrations at adh1202 the following primer pairs were used. Primers 0611245 and 0612794 yielded an approximately 3.0 kbp band and primers 0611815 and 0612795 yielded an approximately 3.6 kbp band. To check the deletion of the native *I. orientalis* 3-HPDH gene the following primers were used. Primers 0613034 and 0613241 yielded an approximately 1.4 kbp band. Isolates which gave the expected bands for proper integrating of the expression cassette at the adh9091 locus, retained the expression cassette at the adh1202 and retained the deletion *I. orientalis* 3-HPDH locus were saved and designated McTs261 (pMcTs76 fragment), McTs263 (pMcTs77 fragment), McTs267 (pMcTs90 fragment), and McTs269 (pMcTs91 fragment) as shown in Table 29.

Table 29: Transformant constructs

Construction Plasmid	Gene	Gene Source	Gene Product SEQ ID NO	Integration construct	Transformant
pMBin190	3-HPDH (YMR226c)	<i>I. orientalis</i>	26	pMcTs90	McTs267
11AAE2AP	3-HPDH (Msed_1993)	<i>M. sedula</i>	29	pMcTs76	McTs261
pMIBa109	3-HPDH (YMR226c)	<i>S. cerevisiae</i>	129	pMcTs91	McTs269
1045168	3-HPDH (ydfG)	<i>E. coli</i>	27	pMcTs77	McTs263

[00553] The transformant strains were tested for 3-HP production using the shake flask method described above. The heterozygous transformants McTs267, McTs269, and McTs263 produced 0.149 (+/- 0.024), 0.168 (+/- 0.052), and 0.162 (+/- 0.018) g/L 3-HP per g/L dry cell weight, respectively. Native strain MeJi412 produced 0.263 (+/- 0.026) g/L 3-HP per g/L dry cell weight, and the 3-HPDH deletion strain produced no detectable 3-HP. The heterozygous transformant McTs261 did not produce detectable 3-HP with this experiment. These results suggest that even heterozygous 3-HPDH transformants can restore some 3-HPDH activity of 3-HPDH deletion strain using either exogenous or endogenous 3-HPDH gene sequences.

Example 3B: Modified yeast strains expressing malate pathway genes

[00554] Yeast cells that produce 3-HP via a pathway that utilizes PEP, OAA, and malate intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of a PPC, malate dehydrogenase, and malate decarboxylase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3C: Modified yeast strains expressing malonate semialdehyde pathway genes

[00555] Yeast cells that produce 3-HP via a pathway that utilizes PEP, OAA and malonate semialdehyde intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of a PPC, 2-keto acid decarboxylase, KGD, BCKA, indolepyruvate decarboxylase, 3-HPDH (including malonate semialdehyde reductase), HIBADH, and 4-hydroxybutyrate dehydrogenase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3D: Modified yeast strains expressing malonyl-CoA pathway genes

[00556] Yeast cells that produce 3-HP via a pathway that utilizes PEP, OAA, malonyl-CoA, and, optionally, malonate semialdehyde intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of a PPC, OAA formatylase, malonyl-CoA reductase, CoA acylating malonate semialdehyde dehydrogenase, 3-HPDH (including malonate semialdehyde reductase), HIBADH, and 4-hydroxybutyrate dehydrogenase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3E: Modified yeast strains expressing malonyl-CoA pathway genes

[00557] Yeast cells that produce 3-HP via a pathway that utilizes pyruvate, acetyl-CoA, malonyl-CoA, and, optionally, malonate semialdehyde intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of a PDH, acetyl-CoA carboxylase, malonyl-CoA reductase, CoA acylating malonate semialdehyde dehydrogenase, 3-HPDH (including malonate semialdehyde reductase), HIBADH, and 4-hydroxybutyrate dehydrogenase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3F: Modified yeast strains expressing alanine pathway genes

[00558] Yeast cells that produce 3-HP via a pathway that utilizes pyruvate, alanine, β -alanine, and, optionally, malonate semialdehyde, β -alanyl-CoA, acrylyl-CoA, and 3-HP-CoA intermediates can be engineered by expressing one or more enzymes involved in the pathway. The expressed genes may include one or more of an alanine dehydrogenase, pyruvate/alanine aminotransferase, alanine 2,3 aminomutase, CoA transferase, CoA

synthetase, β -alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, 3-hydroxyisobutyryl-CoA hydrolase, BAAT, 3-HPDH (including malonate semialdehyde reductase), HIBADH, and 4-hydroxybutyrate dehydrogenase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3G: Modified yeast strains expressing lactate pathway genes

[00559] Yeast cells that produce 3-HP via a pathway that utilizes pyruvate, lactate, lactyl-CoA, acrylyl-CoA, and 3-HP-CoA intermediates can be engineered by expressing one or more enzymes involved in this pathway. The expressed genes may include one or more of an LDH, CoA transferase, CoA synthetase, lactyl-CoA dehydratase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, and 3-hydroxyisobutyryl-CoA hydrolase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3H: Modified yeast strains expressing glycerol pathway genes

[00560] Yeast cells that produce 3-HP via a pathway that utilizes glycerol and 3-HPA intermediates can be engineered by expressing one or more enzymes involved in this pathway. The expressed genes may include one or more of a glycerol dehydratase and aldehyde dehydrogenase gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

Example 3I: Modified yeast strains expressing β -alanyl CoA pathway genes

[00561] Yeast cells that produce 3-HP via a pathway that utilizes PEP or pyruvate, β -alanine, β -alanyl-CoA, acrylyl-CoA, 3-HP-CoA, and, optionally OAA, aspartate, and alanine intermediates can be engineered by expressing one or more enzymes involved in this pathway. The expressed genes may include one or more of a PPC, PYC, AAT, ADC, CoA transferase, CoA synthetase, β -alanyl-CoA ammonia lyase, 3-HP-CoA dehydratase, 3-HP-CoA hydrolase, 3-hydroxyisobutyryl-CoA hydrolase, alanine dehydrogenase, pyruvate/alanine aminotransferase, and AAM gene. The expressed genes may be derived from a gene that is native to the host cell, or they may be derived from a source gene that is non-native to the host cell.

[00562] In some aspects, the yeast cells or methods of use thereof may be described by the following numbered paragraphs:

[B1] A genetically modified yeast cell comprising an active 3-HP fermentation pathway, wherein the cell comprises one or more exogenous 3-HP pathway genes selected from:

- an exogenous PPC gene;
- an exogenous PYC gene;
- an exogenous AAT gene;
- an exogenous ADC gene;
- an exogenous BAAT or gabT gene; and
- an exogenous 3-HPDH gene.

[B2] The genetically modified yeast cell of paragraph B1, comprising an exogenous AAT gene.

[B3] The genetically modified yeast cell of paragraph B1 or B2, comprising an exogenous PYC gene.

[B4] The genetically modified yeast cell of any of paragraphs B1-B3, comprising an exogenous ADC gene.

[B5] The genetically modified yeast cell of any of paragraphs B1-B4, comprising an exogenous BAAT or gabT gene.

[B6] The genetically modified yeast cell of any of paragraphs B1-B5, comprising an exogenous 3-HPDH gene.

[B7] The genetically modified yeast cell of paragraph B1, comprising:

- an exogenous PYC gene;
- an exogenous AAT gene;
- an exogenous ADC gene;
- an exogenous BAAT or gabT gene; and
- an exogenous 3-HPDH gene.

[B8] The genetically modified yeast cell of any of paragraphs B1-B7, comprising an exogenous PPC gene.

[B9] The genetically modified yeast cell of any of paragraphs B1-B7, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 2, 3, 4, 5, 6, 7, and 8.

[B10] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 2.

[B11] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3.

[B12] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 4.

[B13] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 5.

[B14] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 6.

[B15] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 7.

[B16] The genetically modified yeast cell of paragraph B9, wherein the exogenous PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 8.

[B17] The genetically modified yeast cell of any of paragraphs B1-B16, wherein the exogenous PYC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 1.

[B18] The genetically modified yeast cell of any of paragraphs B1-B17, wherein the AAT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 14, 15, and 16.

[B19] The genetically modified yeast cell of paragraph B18, wherein the AAT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 14.

[B20] The genetically modified yeast cell of paragraph B18, wherein the AAT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 15.

[B21] The genetically modified yeast cell of paragraph B18, wherein the AAT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 16.

[B22] The genetically modified yeast cell of any of paragraphs B1-B21, wherein the AAT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 13.

[B23] The genetically modified yeast cell of any of paragraphs B1-B22, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 17, 18, 133, 135, 137, and 139.

[B24] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 17.

[B25] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 18.

[B26] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 133.

[B27] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 135.

[B28] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 137.

[B29] The genetically modified yeast cell of paragraph B23, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%,

95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 139.

[B30] The genetically modified yeast cell of any of paragraphs B1-B29, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 130, 131, 132, 134, 136, and 138.

[B31] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 130.

[B32] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 131.

[B33] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 132.

[B34] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 134.

[B35] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 136.

[B36] The genetically modified yeast cell of paragraph B30, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 138.

[B37] The genetically modified yeast cell of any of paragraphs B1-B36, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 20, 21, 22, 23, and 24.

[B38] The genetically modified yeast cell of paragraph B37, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%,

85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 20.

[B39] The genetically modified yeast cell of paragraph B37, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 21.

[B40] The genetically modified yeast cell of paragraph B37, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 22.

[B41] The genetically modified yeast cell of paragraph B37, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 23.

[B42] The genetically modified yeast cell of paragraph B37, wherein the exogenous BAAT or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 24.

[B43] The genetically modified yeast cell any of paragraphs B1-B42, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 19, 140, 141, and 142.

[B44] The genetically modified yeast cell any of paragraph B43, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 19.

[B45] The genetically modified yeast cell any of paragraph B43, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 140.

[B46] The genetically modified yeast cell any of paragraph B43, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 141.

[B47] The genetically modified yeast cell any of paragraph B43, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%,

75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 142.

[B48] The genetically modified yeast cell of any of paragraphs B1-B47, wherein said exogenous BAAT gene or exogenous gabT is a BAAT gene that is also a gabT gene.

[B49] The genetically modified yeast cell of any of paragraphs B1-B48, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs: 26, 27, 28, 29, 30, 31, 32, 33, 34, and 129.

[B50] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 26.

[B51] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 27.

[B52] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 28.

[B53] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 29.

[B54] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 30.

[B55] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 31.

[B56] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 32.

[B57] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 33.

[B58] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 34.

[B59] The genetically modified yeast cell of paragraph B49, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 129.

[B60] The genetically modified yeast cell of any of paragraphs B1-B59, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 25, 143, 144, and 343.

[B61] The genetically modified yeast cell of paragraph B60, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 25.

[B62] The genetically modified yeast cell of paragraph B60, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 143.

[B63] The genetically modified yeast cell of paragraph B60, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 144.

[B64] The genetically modified yeast cell of paragraph B60, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 343.

[B65] The genetically modified yeast cell of any of paragraphs B1-B64, wherein the 3-HPDH gene is also a HIBADH gene.

[B66] The genetically modified yeast cell of any of paragraphs B1-B65, wherein the 3-HPDH gene is also a 4-hydroxybutyrate dehydrogenase gene.

[B67] The genetically modified yeast cell of any of paragraphs B1-B66, wherein the PPC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 10, 11, and 12.

[B68] The genetically modified yeast cell of paragraph B67, wherein the PPC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 10.

[B69] The genetically modified yeast cell of paragraph B67, wherein the PPC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 11.

[B70] The genetically modified yeast cell of paragraph B67, wherein the PPC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 12.

[B71] The genetically modified yeast cell of any of paragraphs B1-B70, wherein the PPC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence of SEQ ID NO: 9.

[B72] The genetically modified yeast cell of any of paragraphs B1-B71, wherein said yeast cell is Crabtree-negative.

[B73] The genetically modified yeast cell of any of paragraphs B1-B72, wherein the yeast cell belongs to a genus selected from *Issatchenkia*, *Candida*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Saccharomyces*.

[B74] The genetically modified yeast cell of paragraph B73, wherein the yeast cell belongs to a clade selected from the *I. orientalis*/*P. fermentans* clade and the *Saccharomyces* clade.

[B75] The genetically modified yeast cell of paragraph B73, wherein the yeast cell is selected from *I. orientalis*, *C. lambica*, and *S. bulderi*.

[B76] The genetically modified yeast cell of any of paragraphs B1-B75, wherein said cell further comprises one or more deletions or disruptions of a native gene selected from PDC, ADH, GAL6, CYB2A, CYB2B, GPD, GPP, ALD, and PCK genes.

[B77] The genetically modified yeast cell of paragraph B76, wherein one or more of the deletions or disruptions results from insertion of one or more of the exogenous 3-HP pathway genes.

[B78] The genetically modified yeast cell of any of paragraphs B1-B77, wherein one or more of the exogenous 3-HP pathway genes are operatively linked to one or more exogenous regulatory elements.

[B79] The genetically modified yeast cell of paragraph B78, wherein the one or more regulatory elements are foreign to the one or more 3-HP pathway genes.

[B80] The genetically modified yeast cell of any of paragraphs B1-B79, wherein the exogenous PYC gene is operatively linked to an exogenous promoter that is foreign to the PYC gene.

[B81] The genetically modified yeast cell of any of paragraphs B1-B80, wherein the exogenous AAT gene is operatively linked to an exogenous promoter that is foreign to the AAT gene.

[B82] The genetically modified yeast cell of any of paragraphs B1-B81, wherein the exogenous ADC gene is operatively linked to an exogenous promoter that is foreign to the ADC gene.

[B83] The genetically modified yeast cell of any of paragraphs B1-B82, wherein the exogenous BAAT or gabT gene is operatively linked to an exogenous promoter that is foreign to the BAAT or gabT gene.

[B84] The genetically modified yeast cell of any of paragraphs B1-B83, wherein the exogenous 3-HPDH gene is operatively linked to an exogenous promoter that is foreign to the 3-HPDH gene.

[B85] The genetically modified yeast cell of any of paragraphs B1-B84, wherein the exogenous PPC gene is operatively linked to an exogenous promoter that is foreign to the PPC gene.

[B86] The genetically modified yeast cell of any of paragraphs B1-B85, wherein the cell is capable of growing at a pH of less than 4 in media containing 75 g/L or greater 3-HP.

[B87] The genetically modified yeast cell of any of paragraphs B1-B86, wherein the cell is a 3-HP-resistant yeast cell.

[B88] The genetically modified yeast cell of any of paragraphs B1-B87, wherein the cell has undergone mutation and/or selection, such that the mutated and/or selected cell possess a higher degree of resistance to 3-HP than a wild-type cell of the same species.

[B89] The genetically modified yeast cell of paragraph B88, wherein the cell has undergone mutation and/or selection before being genetically modified with the one or more exogenous 3-HP pathway genes.

[B90] The genetically modified yeast cell of paragraph B88 or B89, wherein the cell has undergone selection in the presence of lactic acid or 3-HP.

[B91] The genetically modified yeast cell of paragraph B91, wherein the selection is chemostat selection.

[B92] A method of producing 3-HP comprising:

- (i) culturing the genetically modified yeast cell of any of paragraphs B1-B91 in the presence of medium comprising at least one carbon source; and
- (ii) isolating 3-HP from the culture.

[B93] The method of paragraph B92, wherein said carbon source is selected from glucose, xylose, arabinose, sucrose, fructose, cellulose, glucose oligomers, and glycerol.

[B94] The method of paragraph B92 or B93, wherein the medium is at a pH of less than 5, e.g., in the range of about 1.5 to about 4.5, about 2.0 to about 4.0, or about 2.0 to about 3.5.

What is claimed is:

1. A genetically modified yeast cell comprising an active 3-HP fermentation pathway, wherein the cell comprises one or more exogenous 3-HP pathway genes selected from:

- an exogenous PPC gene;
- an exogenous PYC gene;
- an exogenous AAT gene;
- an exogenous ADC gene;
- an exogenous BAAT or gabT gene; and
- an exogenous 3-HPDH gene.

2. The genetically modified yeast cell of claim 1, comprising an exogenous PYC gene.

3. The genetically modified yeast cell of claim 2, wherein the PYC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 2, 3, 4, 5, 6, 7, and 8.

4. The genetically modified yeast cell of claim 2 or 3, wherein the PYC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 1.

5. The genetically modified yeast cell of any one of claims 1-4, comprising an exogenous AAT gene.

6. The genetically modified yeast cell of any one of claim 5, wherein the AAT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 14, 15, and 16.

7. The genetically modified yeast cell of claim 5 or 6, wherein the AAT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to the nucleotide sequence of SEQ ID NO: 13.

8. The genetically modified yeast cell of any one of claims 1-7, comprising an exogenous ADC gene.

9. The genetically modified yeast cell of claim 9, wherein the ADC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 17, 18, 133, 135, 137, and 139.

10. The genetically modified yeast cell of claim 8 or 9, wherein the ADC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 130, 131, 132, 134, 136, and 138.

11. The genetically modified yeast cell of any one of claims 1-10, comprising an exogenous BAAT gene or an exogenous gabT gene.

12. The genetically modified yeast cell of claim 11, wherein the BAAT gene or gabT gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 20, 21, 22, 23, and 24.

13. The genetically modified yeast cell of claim 11 or 12, wherein the BAAT gene or gabT gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 19, 140, 141, and 142.

14. The genetically modified yeast cell of any one of claims 11-13, wherein said BAAT gene or gabT is a BAAT gene that is also a gabT gene.

15. The genetically modified yeast cell of any one of claims 1-14, comprising an exogenous 3-HPDH gene.

16. The genetically modified yeast cell of claim 15, wherein the 3-HPDH gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 26, 27, 28, 29, 30, 31, 32, 33, 34, and 129.

17. The genetically modified yeast cell of claim 15 or 16, wherein the 3-HPDH gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence selected from SEQ ID NOs: 25, 143, 144, and 343.

18. The genetically modified yeast cell of any one of claims 15-17, wherein the 3-HPDH gene is also a HIBADH gene.

19. The genetically modified yeast cell of any one of claims 15-18, wherein the 3-HPDH gene is also a 4-hydroxybutyrate dehydrogenase gene.

20. The genetically modified yeast cell of any one of claims 1-19, comprising an exogenous PPC gene.

21. The genetically modified yeast cell of claim 20, wherein the PPC gene encodes a polypeptide with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to an amino acid sequence selected from SEQ ID NOs: 10, 11, and 12.

22. The genetically modified yeast cell of claim 20 or 21, wherein the PPC gene comprises a nucleotide sequence with at least 60%, e.g., at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity to a nucleotide sequence of SEQ ID NO: 9.

23. The genetically modified yeast cell of any of claims 1-22, wherein said yeast cell is Crabtree-negative.

24. The genetically modified yeast cell of any of claims 1-22, wherein the yeast cell belongs to a genus selected from *Issatchenkia*, *Candida*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Torulaspora*, *Zygosaccharomyces*, and *Saccharomyces*.

25. The genetically modified yeast cell of claim 24, wherein the yeast cell belongs to a clade selected from the *I. orientalis*/*P. fermentans* clade and the *Saccharomyces* clade.

26. The genetically modified yeast cell of claim 24, wherein the yeast cell is selected from *I. orientalis*, *C. lambica*, and *S. bulderi*.

27. The genetically modified yeast cell of any of claims 1-26, wherein said cell further comprises one or more deletions or disruptions of a native gene selected from PDC, ADH, GAL6, CYB2A, CYB2B, GPD, GPP, ALD, and PCK genes.

28. The genetically modified yeast cell of claim 27, wherein one or more of the deletions or disruptions results from insertion of one or more of the exogenous 3-HP pathway genes.

29. The genetically modified yeast cell of any of claims 1-28, wherein one or more of the exogenous 3-HP pathway genes are operatively linked to one or more exogenous regulatory elements.

30. The genetically modified yeast cell of any of claims 1-29, wherein the cell is capable of growing at a pH of less than 4 in media containing 75 g/L or greater 3-HP.

31. The genetically modified yeast cell of any of claims 1-30, wherein the cell is a 3-HP-resistant yeast cell.

32. The genetically modified yeast cell of any of claims 1-31, wherein the cell has undergone mutation and/or selection, such that the mutated and/or selected cell possess a higher degree of resistance to 3-HP than a wild-type cell of the same species.

33. The genetically modified yeast cell of claim 32, wherein the cell has undergone mutation and/or selection before being genetically modified with the one or more exogenous 3-HP pathway genes.

34. The genetically modified yeast cell of claim 32 or 33, wherein the cell has undergone selection in the presence of lactic acid or 3-HP.

35. The genetically modified yeast cell of claim 34, wherein the selection is chemostat selection.

36. A method of producing 3-HP comprising:

(i) culturing the genetically modified yeast cell of any of claims 1-35 in the presence of medium comprising at least one carbon source; and

(ii) isolating 3-HP from the culture.

37. The method of claim 36, wherein said carbon source is selected from glucose, xylose, arabinose, sucrose, fructose, cellulose, glucose oligomers, and glycerol.

38. The method of claim 36 or 37, wherein the medium is at a pH of less than 5, e.g., in the range of about 1.5 to about 4.5, about 2.0 to about 4.0, or about 2.0 to about 3.5.

1/32

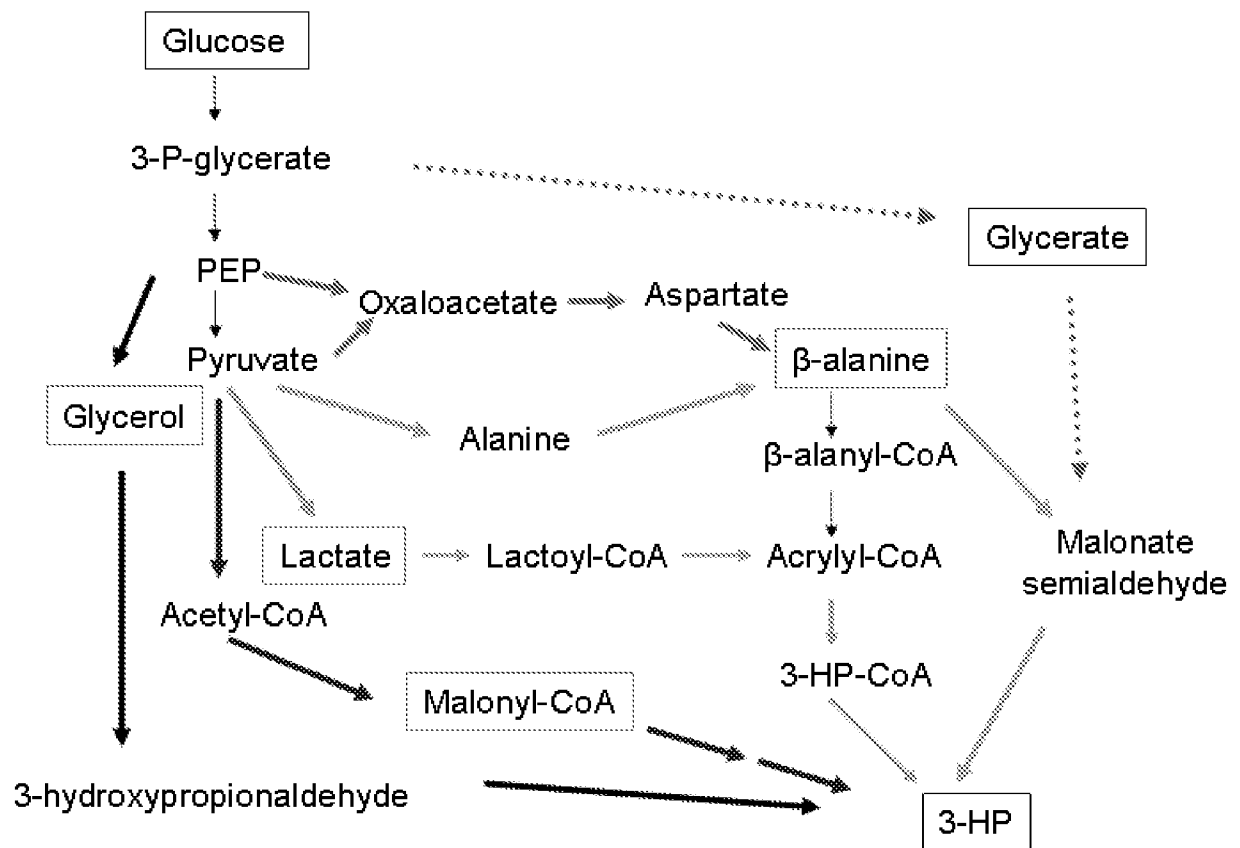


Fig. 1

2/32

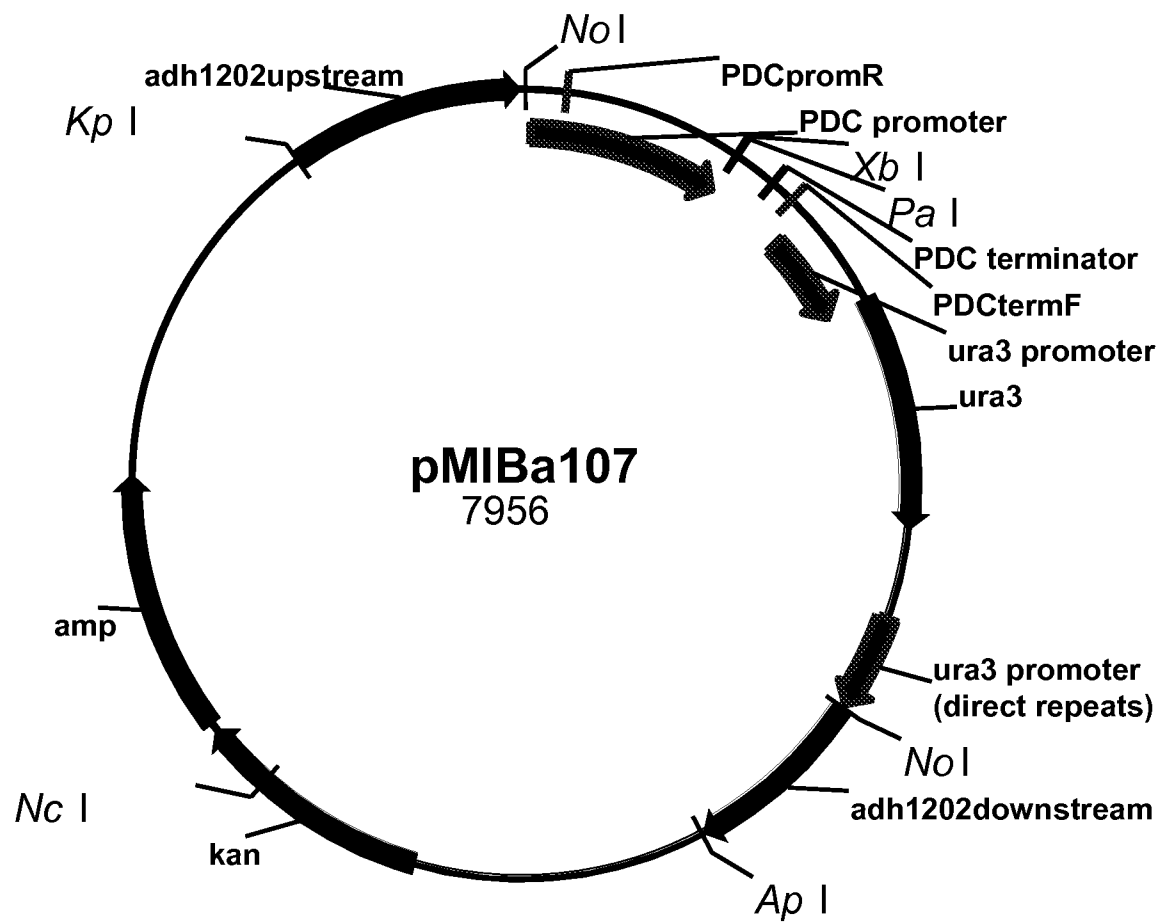


Fig. 2

3/32

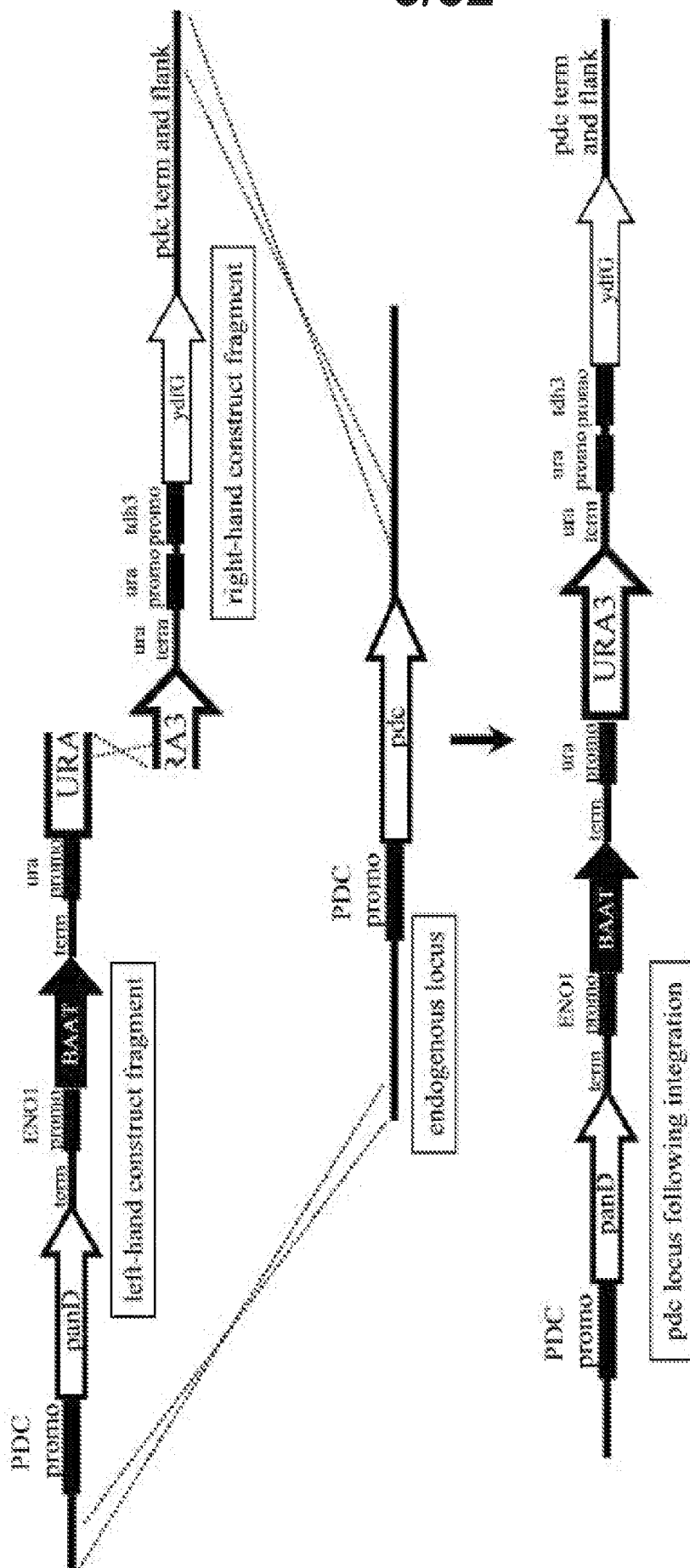


Fig. 3

4/32

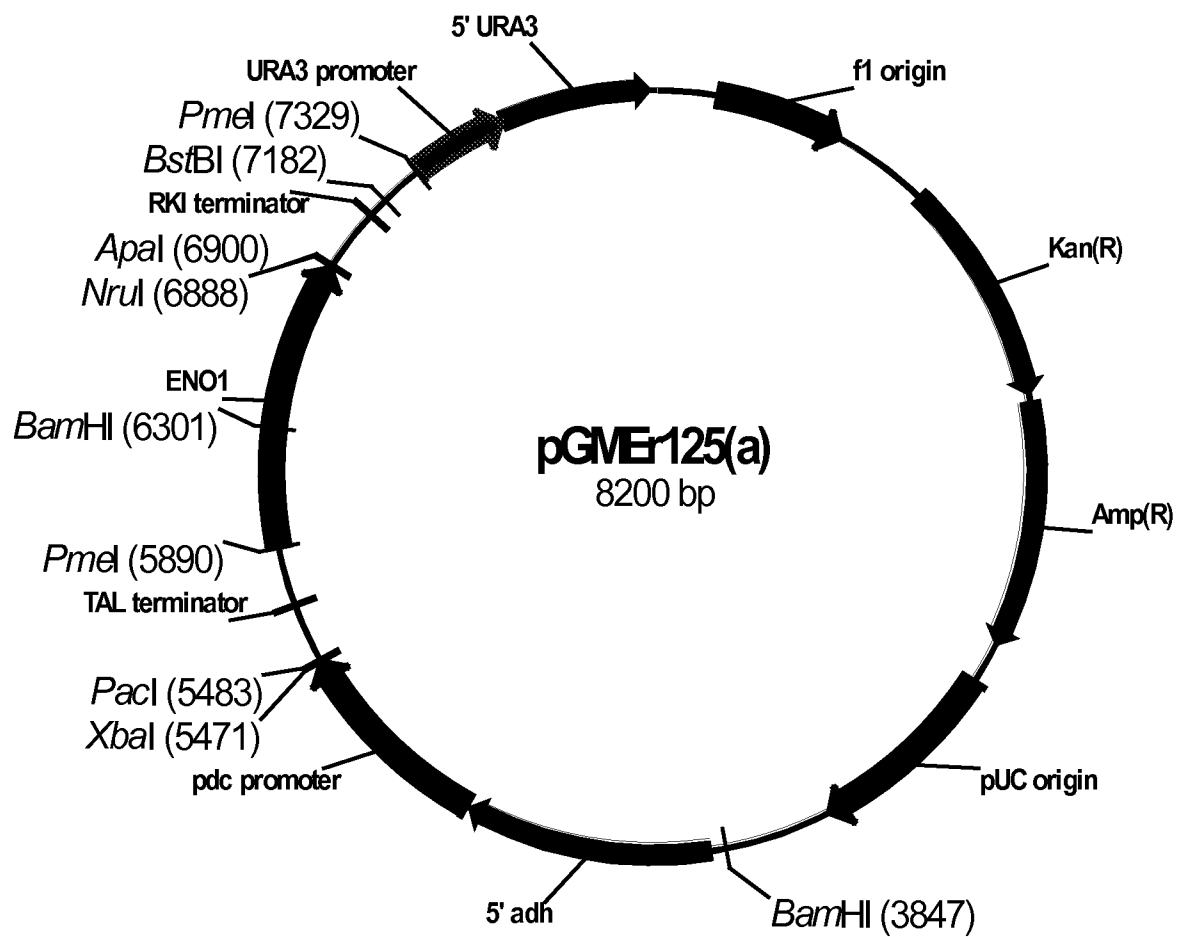


Fig. 4

5/32

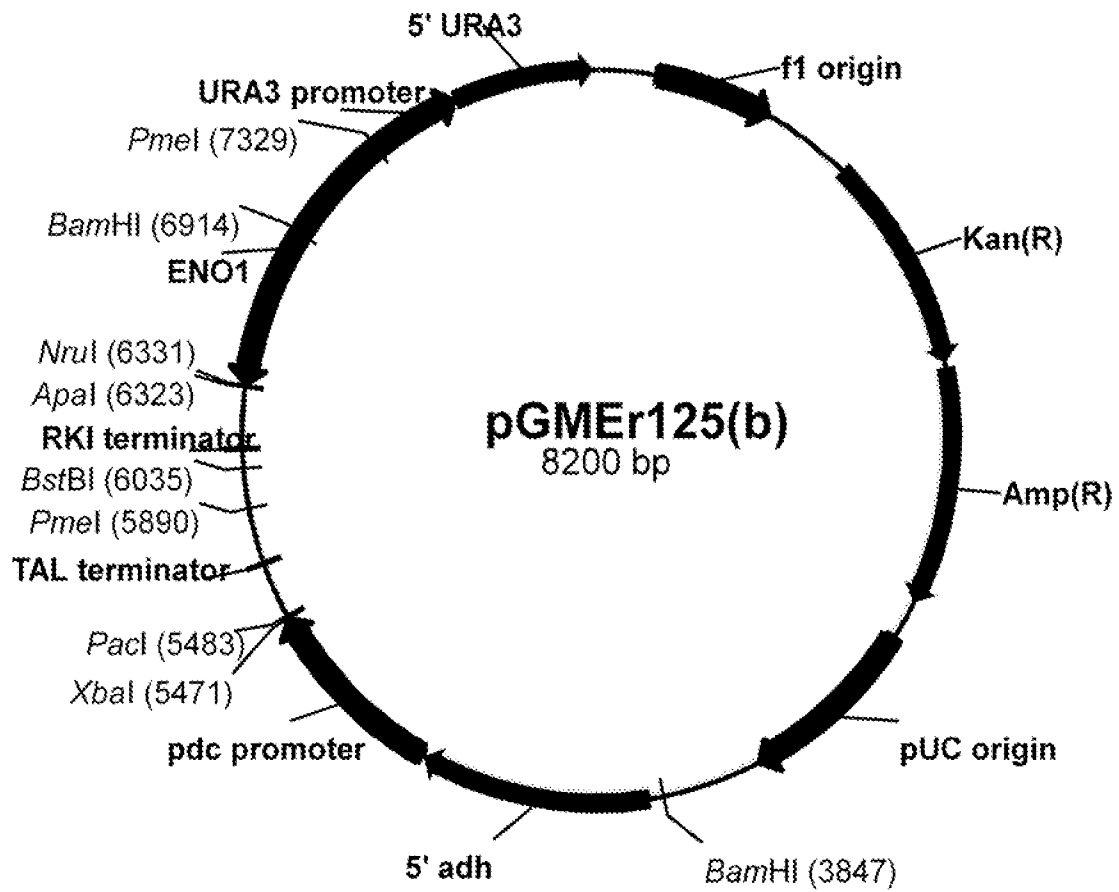


Fig. 5

6/32

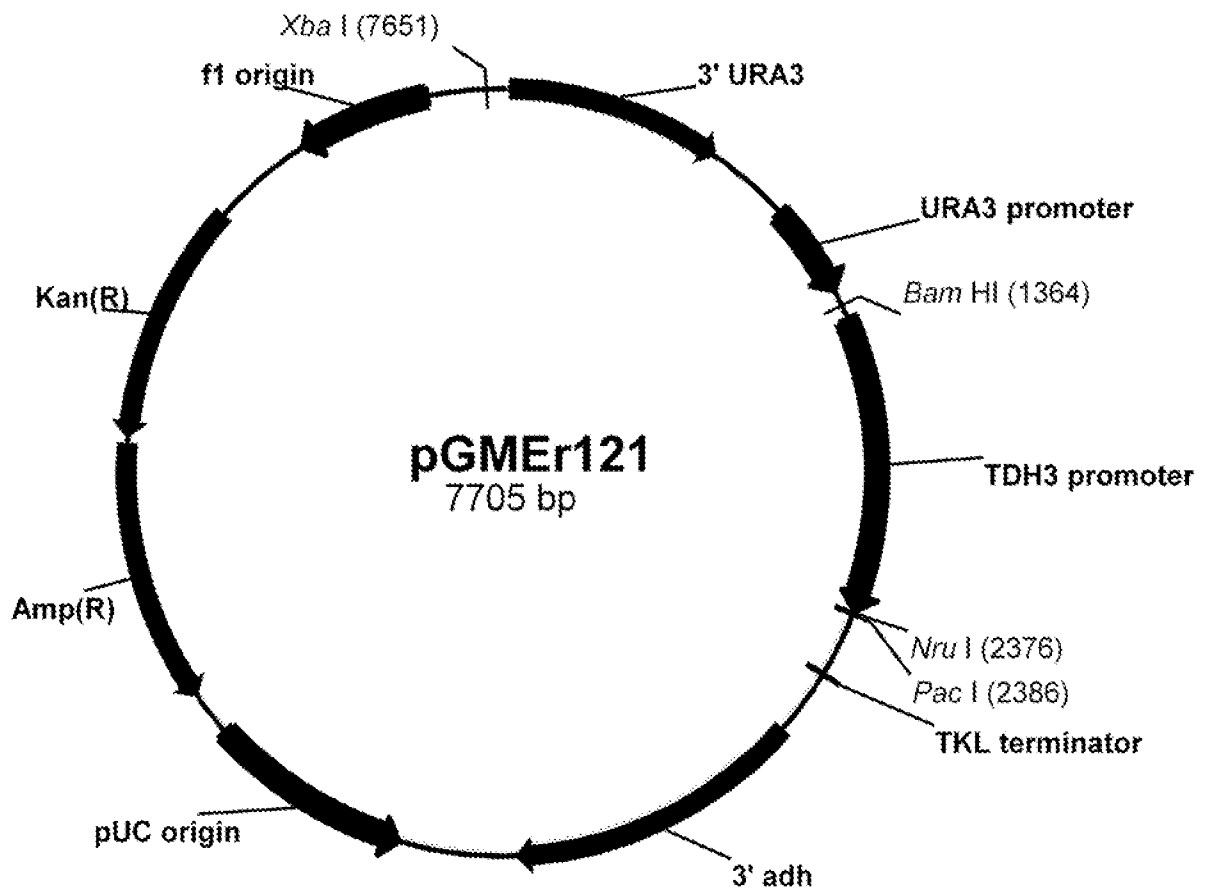


Fig. 6

7/32

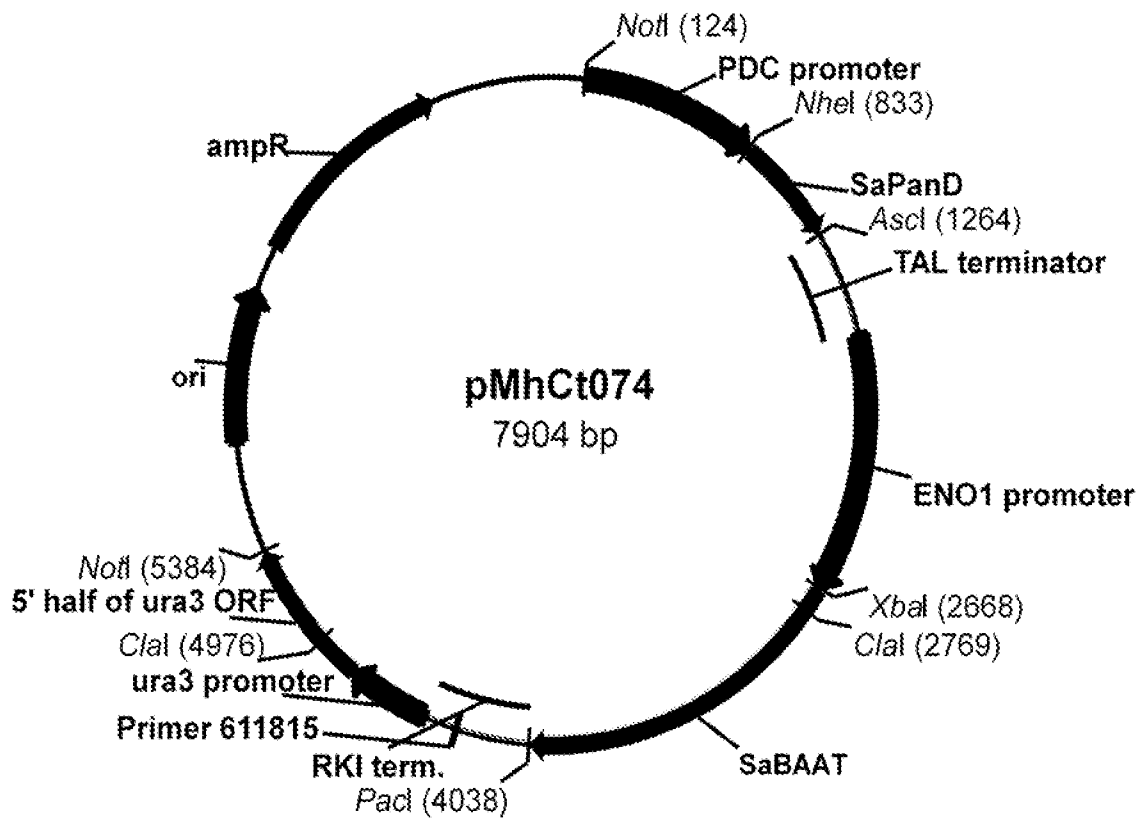


Fig. 7

8/32

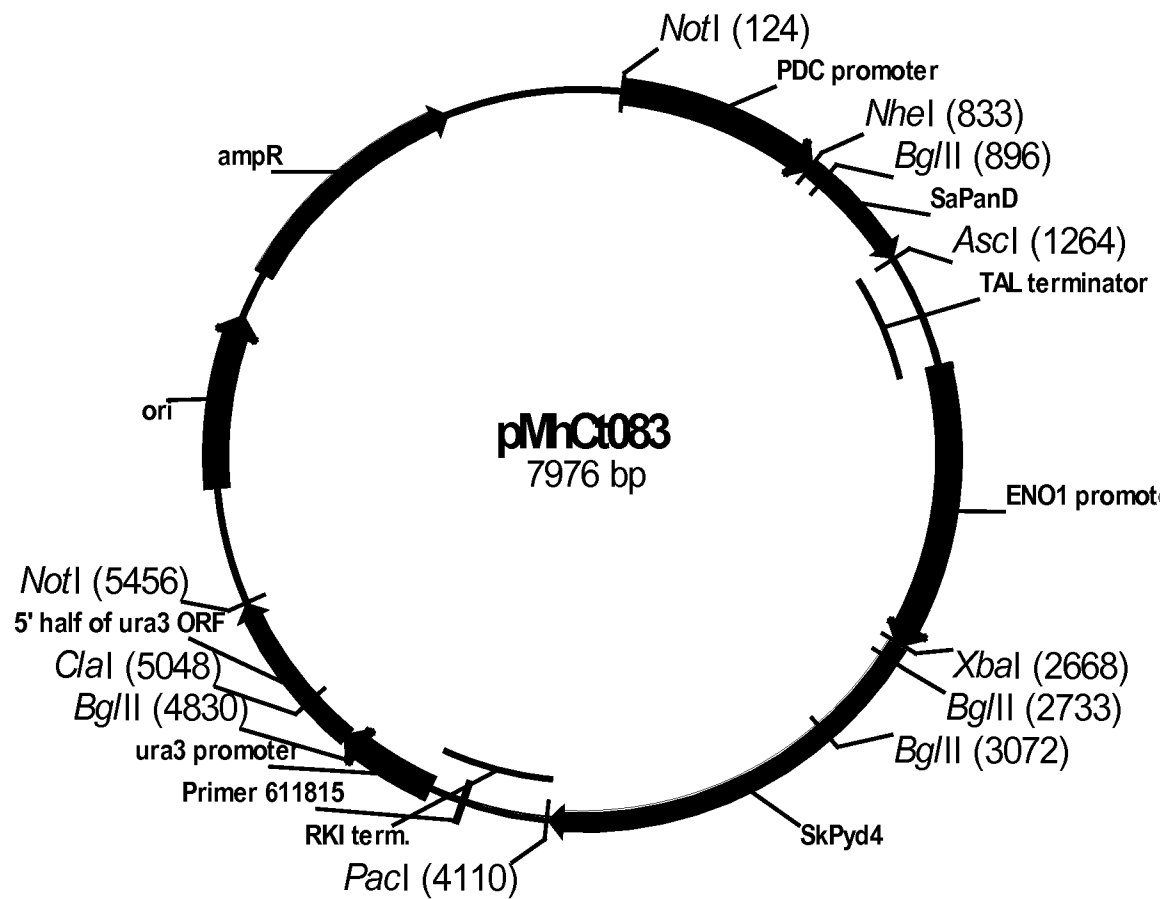


Fig. 8

9/32

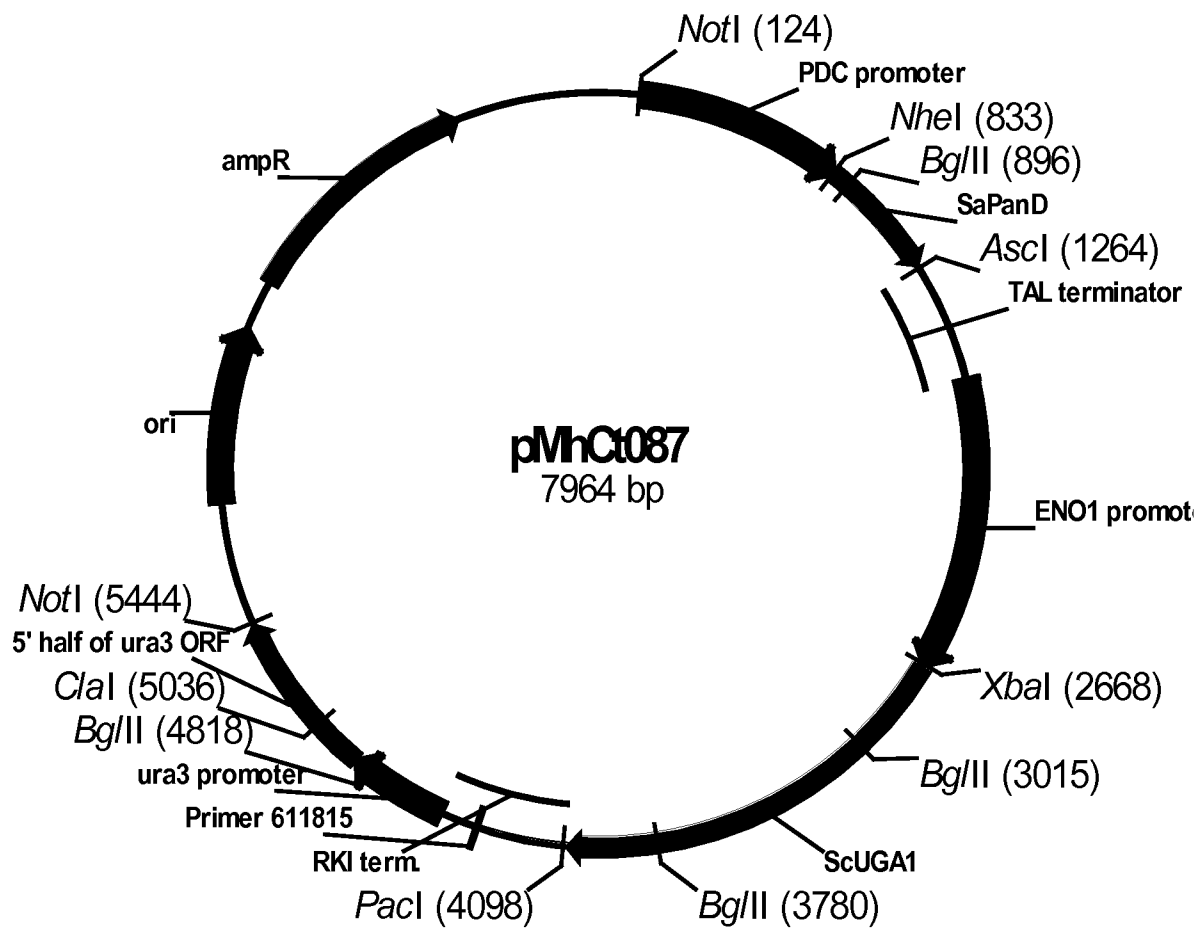


Fig. 9

10/32

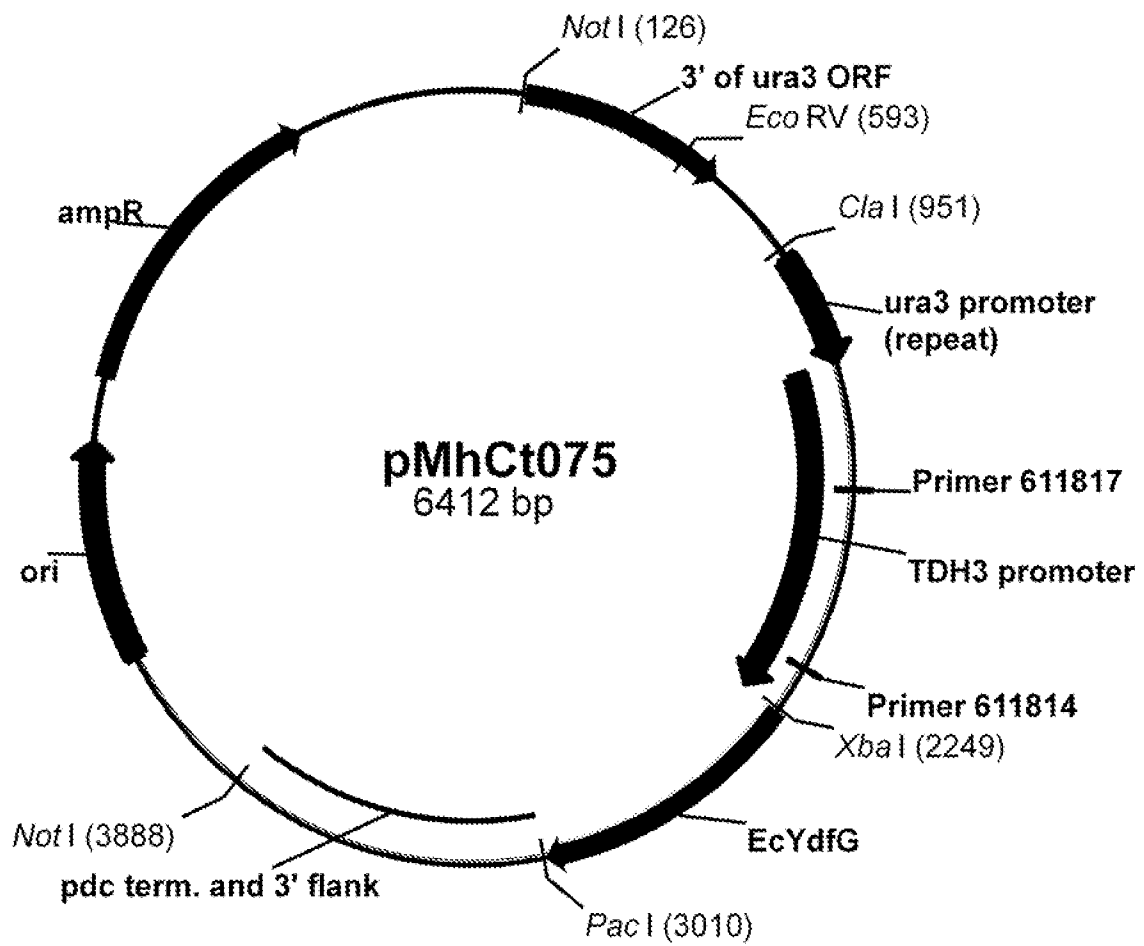


Fig. 10

11/32

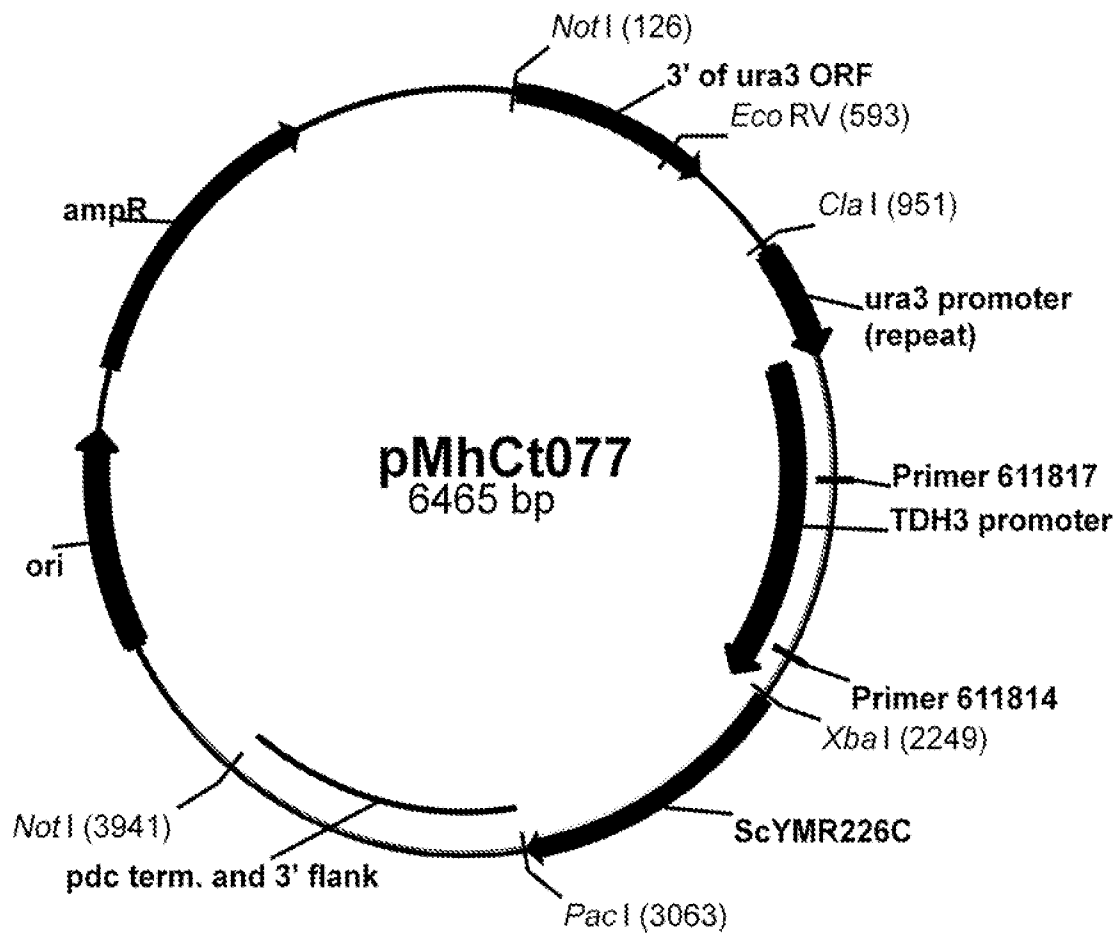


Fig. 11

12/32

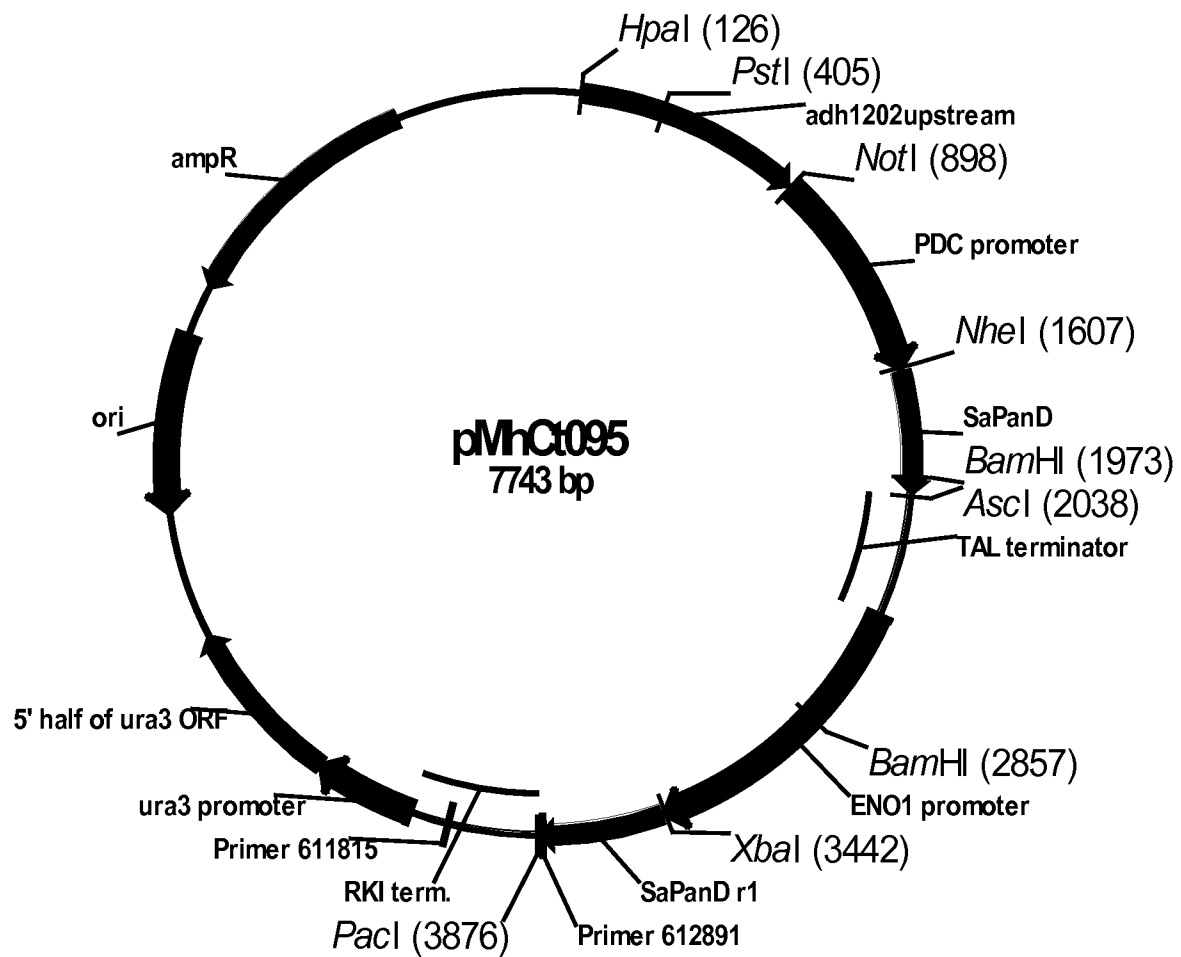


Fig. 12

13/32

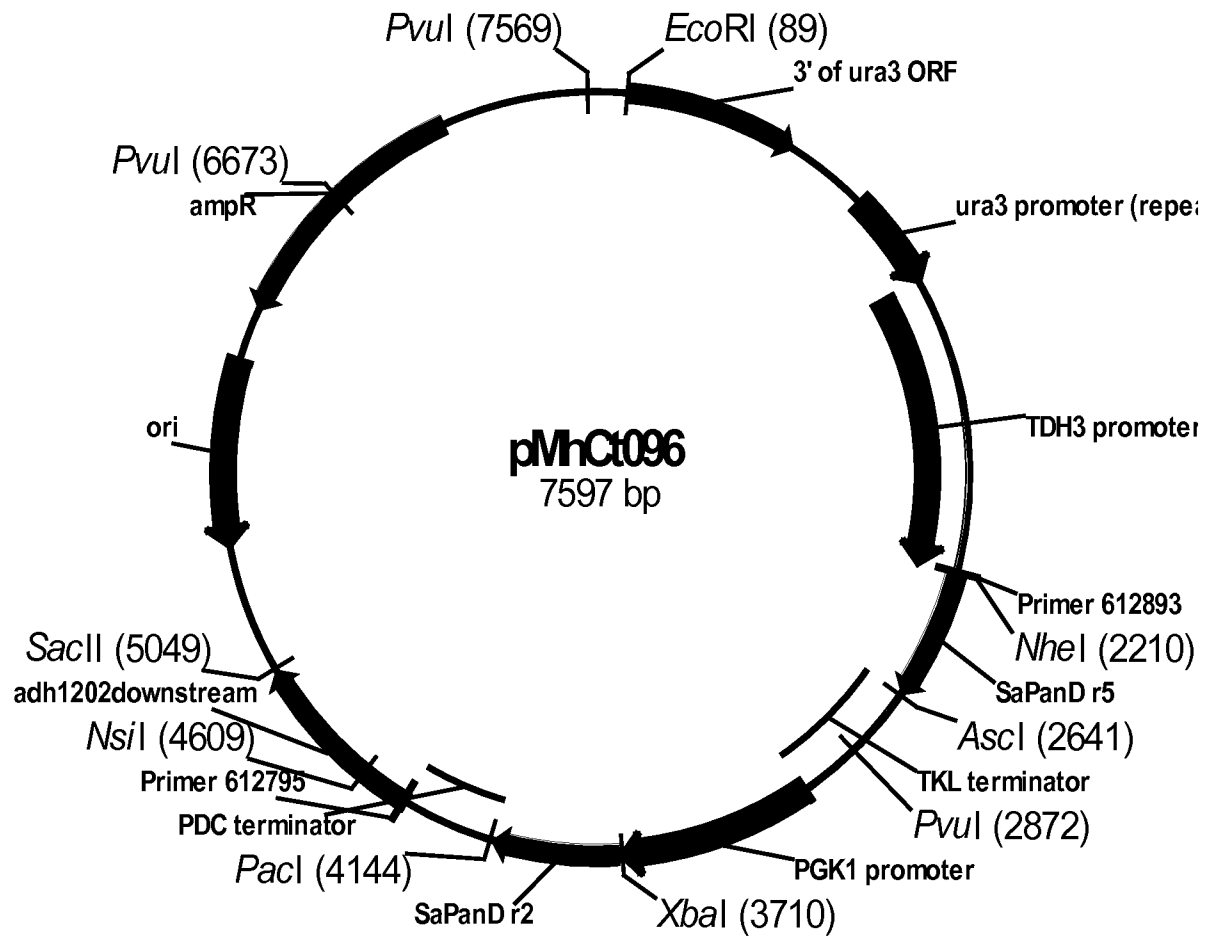


Fig. 13

14/32

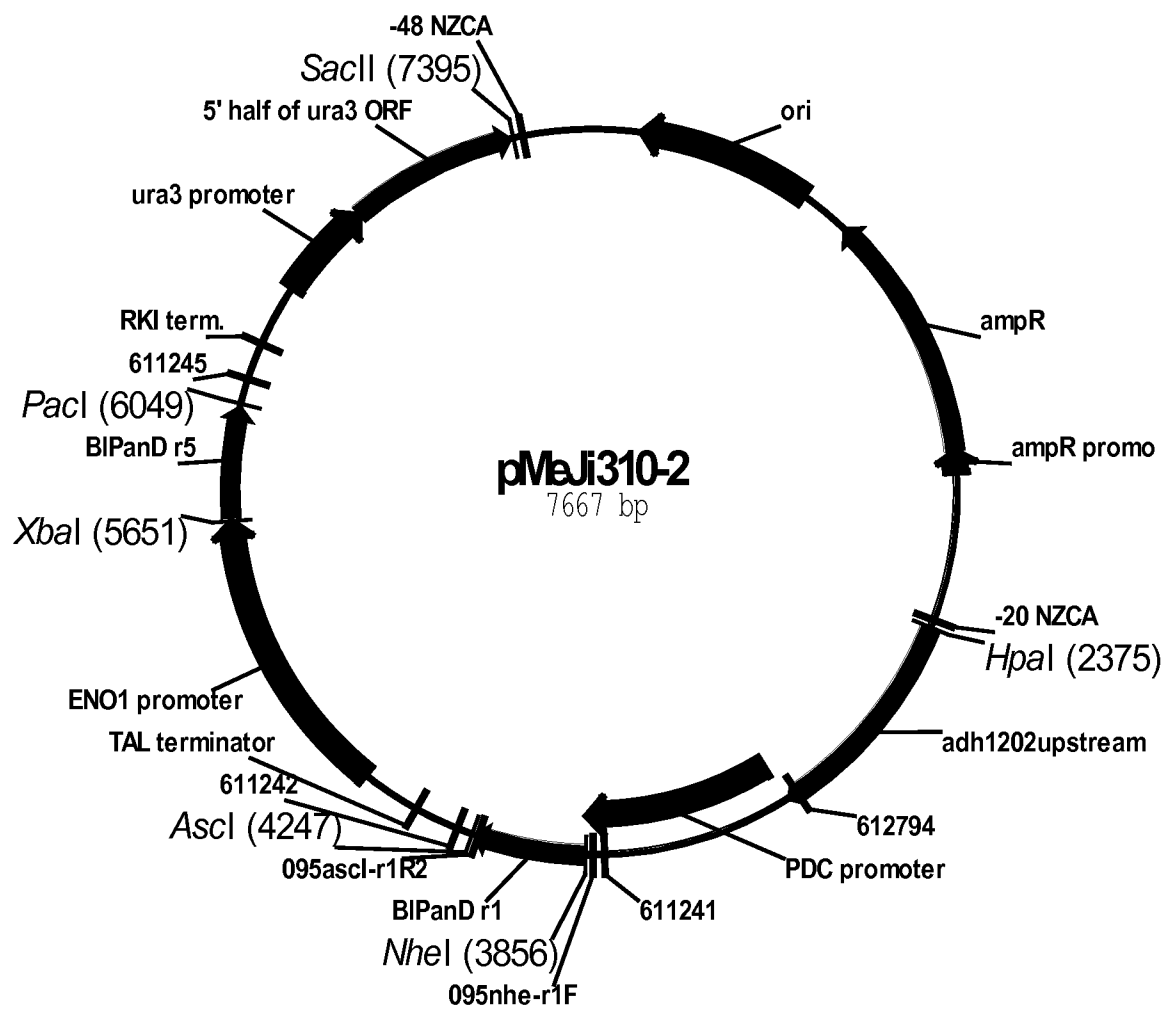


Fig. 14

15/32

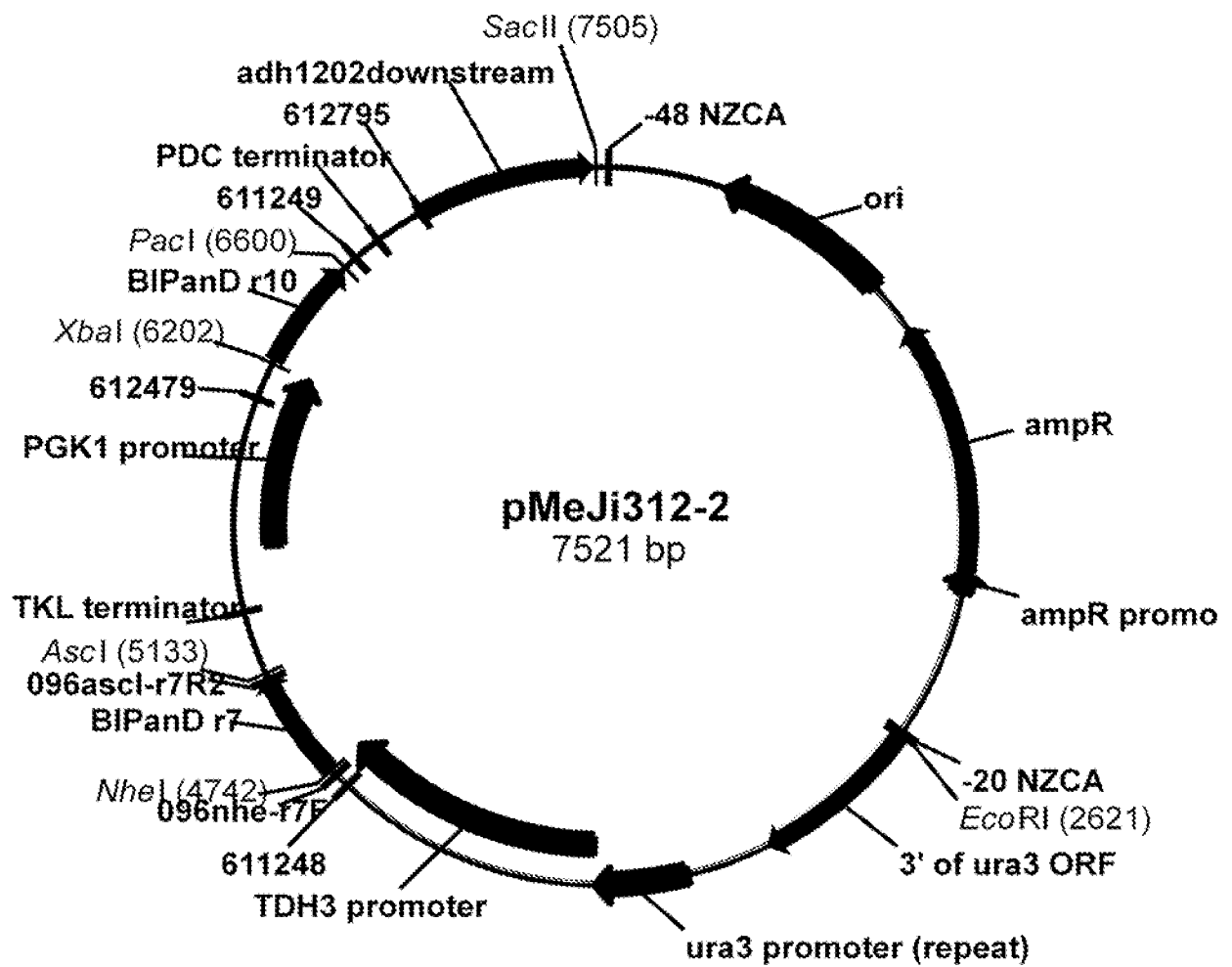


Fig. 15

16/32

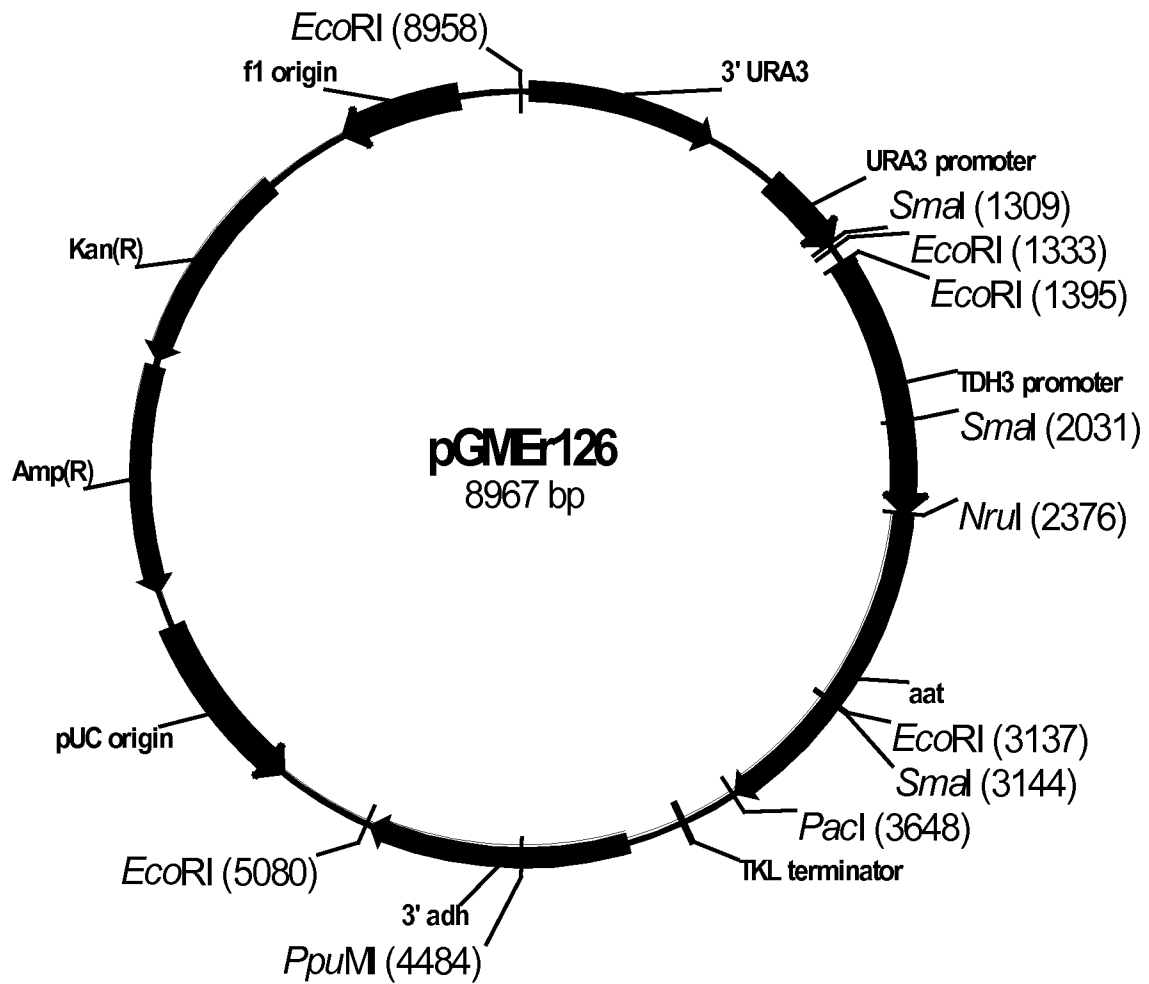


Fig. 16

17/32

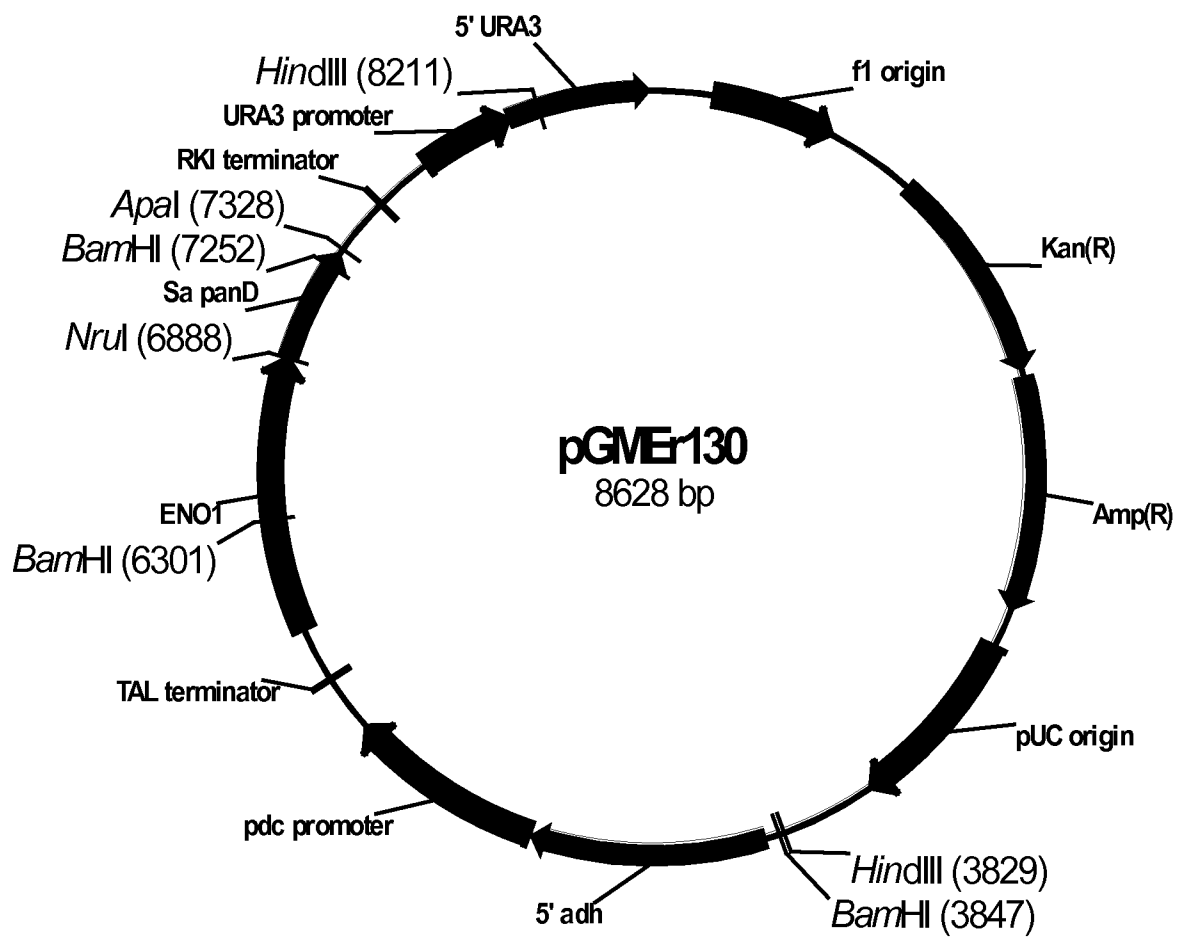


Fig. 17

18/32

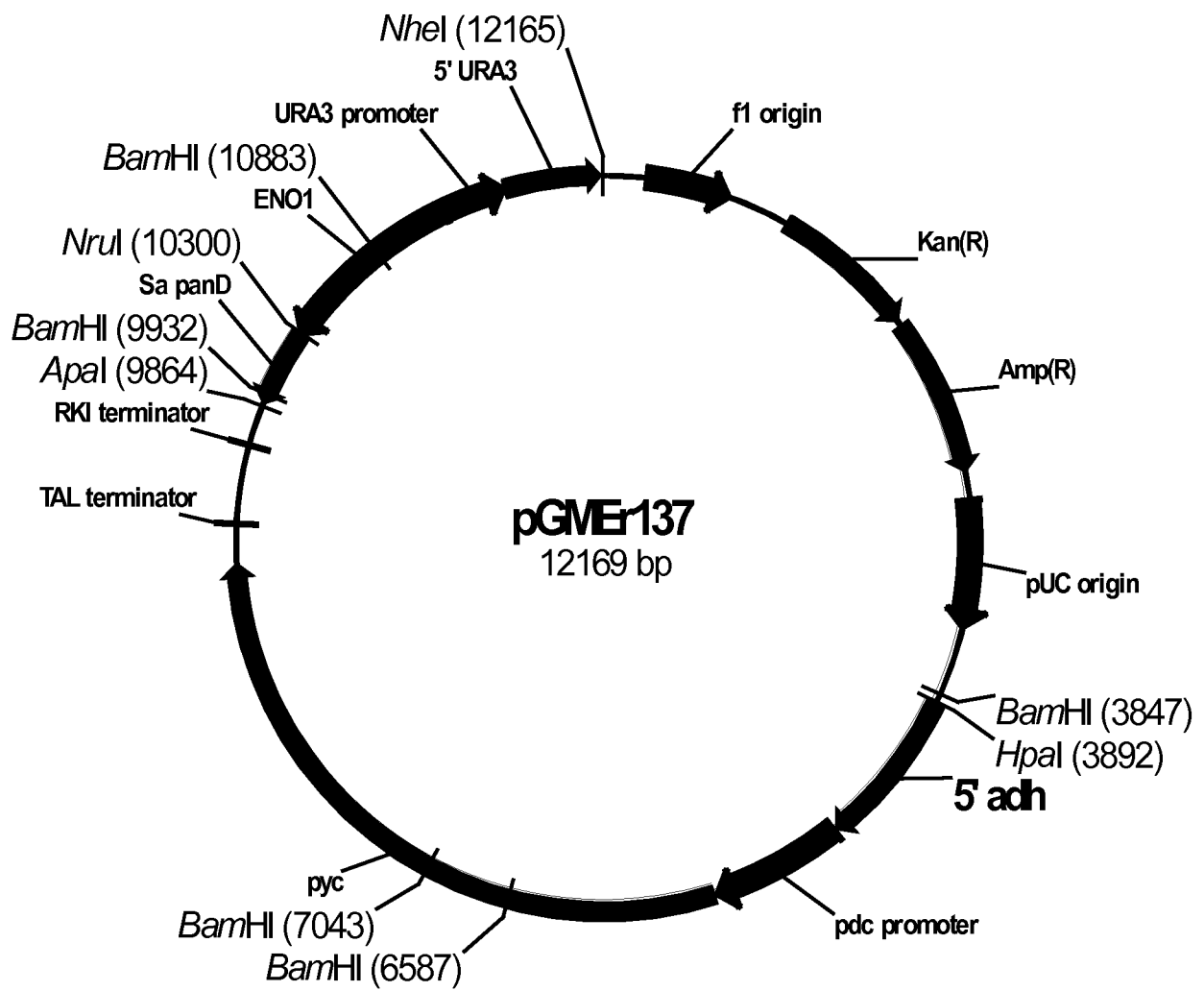


Fig. 18

19/32

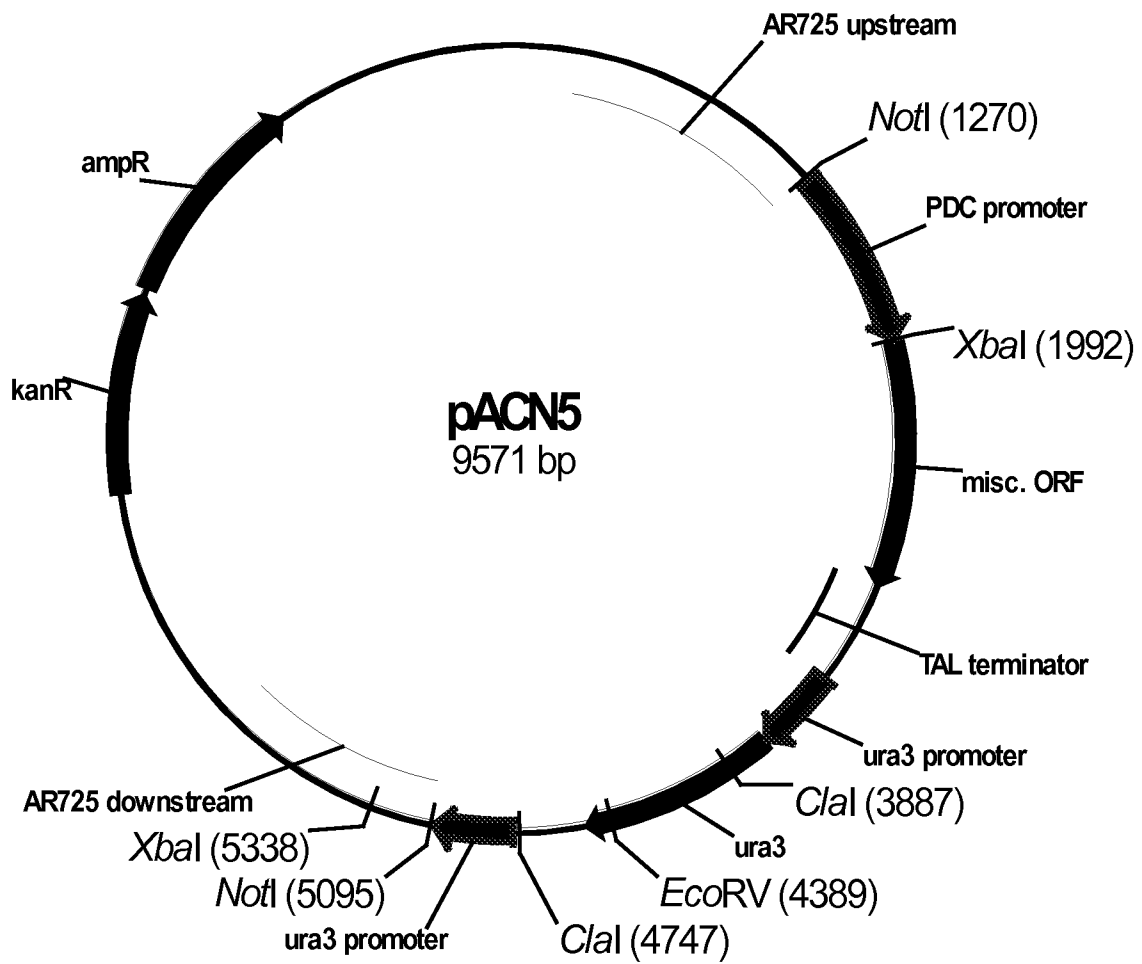


Fig. 19

20/32

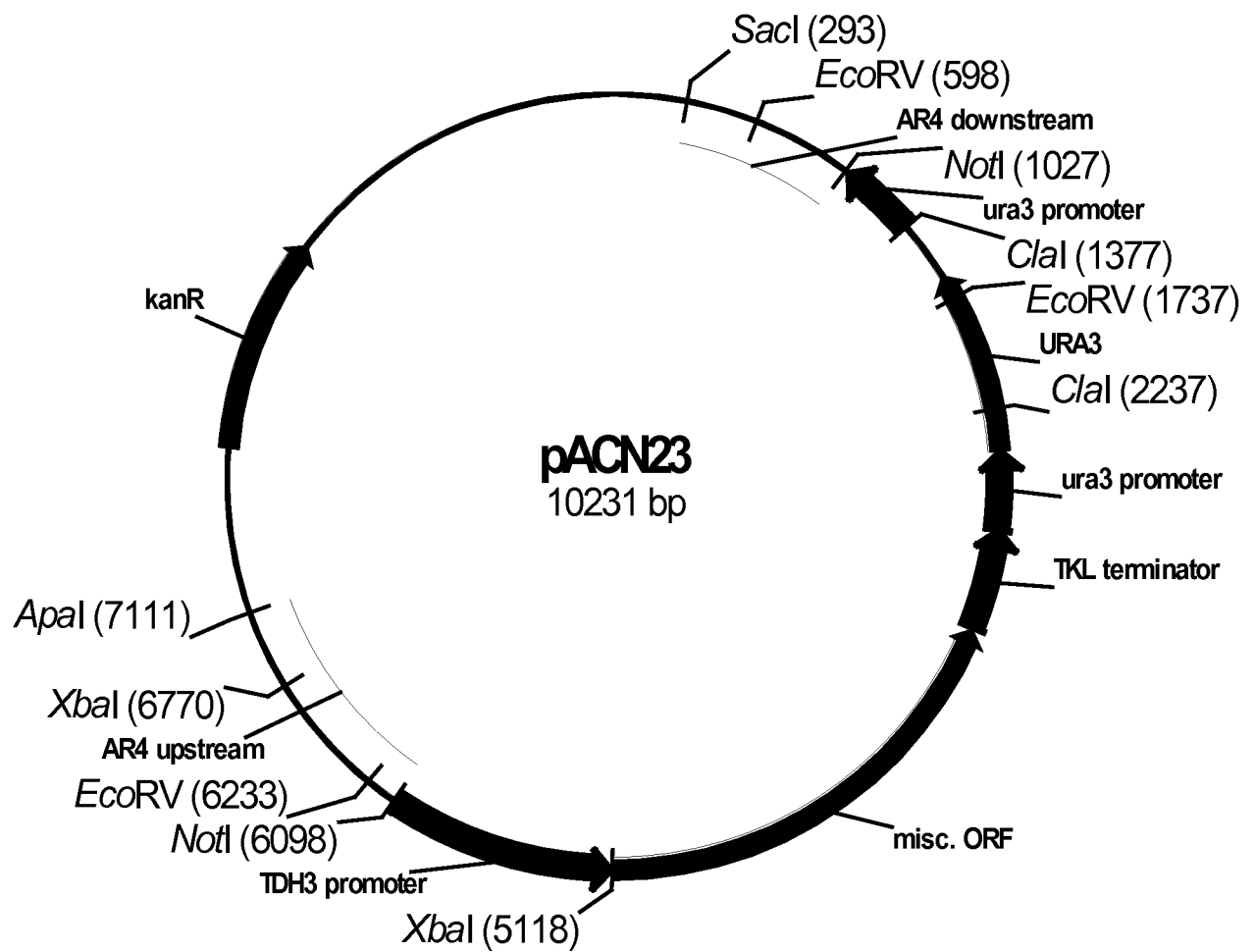


Fig. 20

21/32

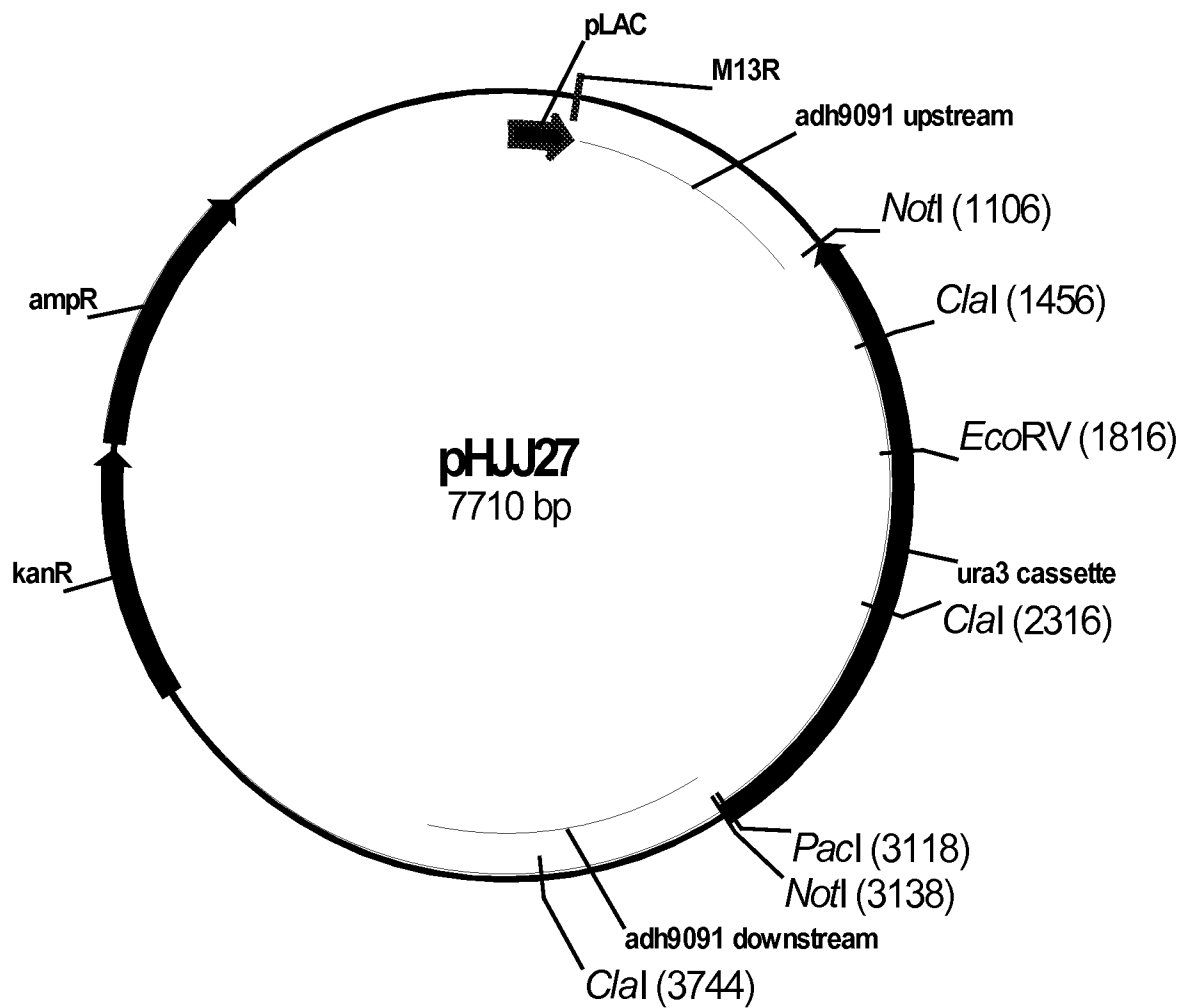


Fig. 21

22/32

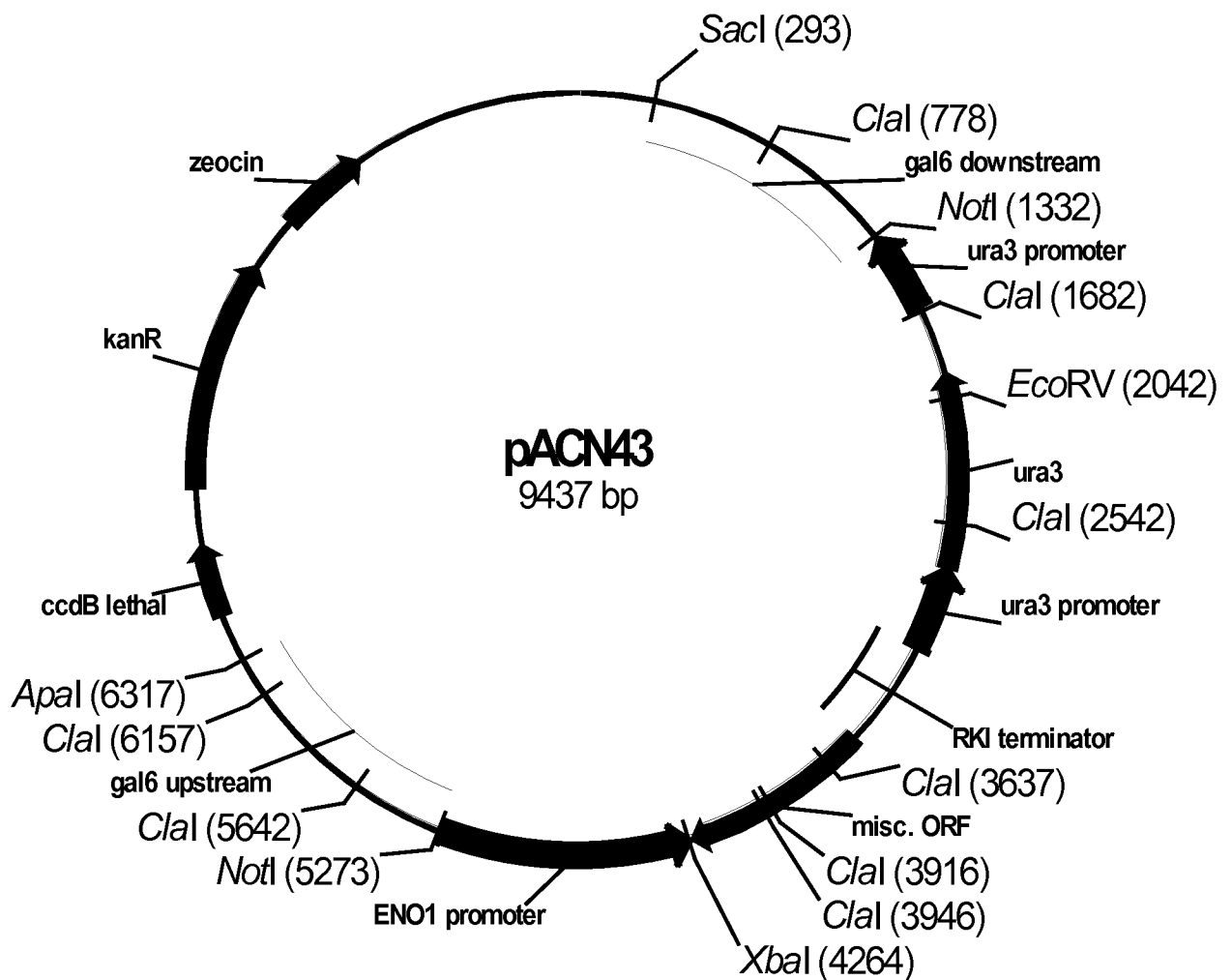


Fig. 22

23/32

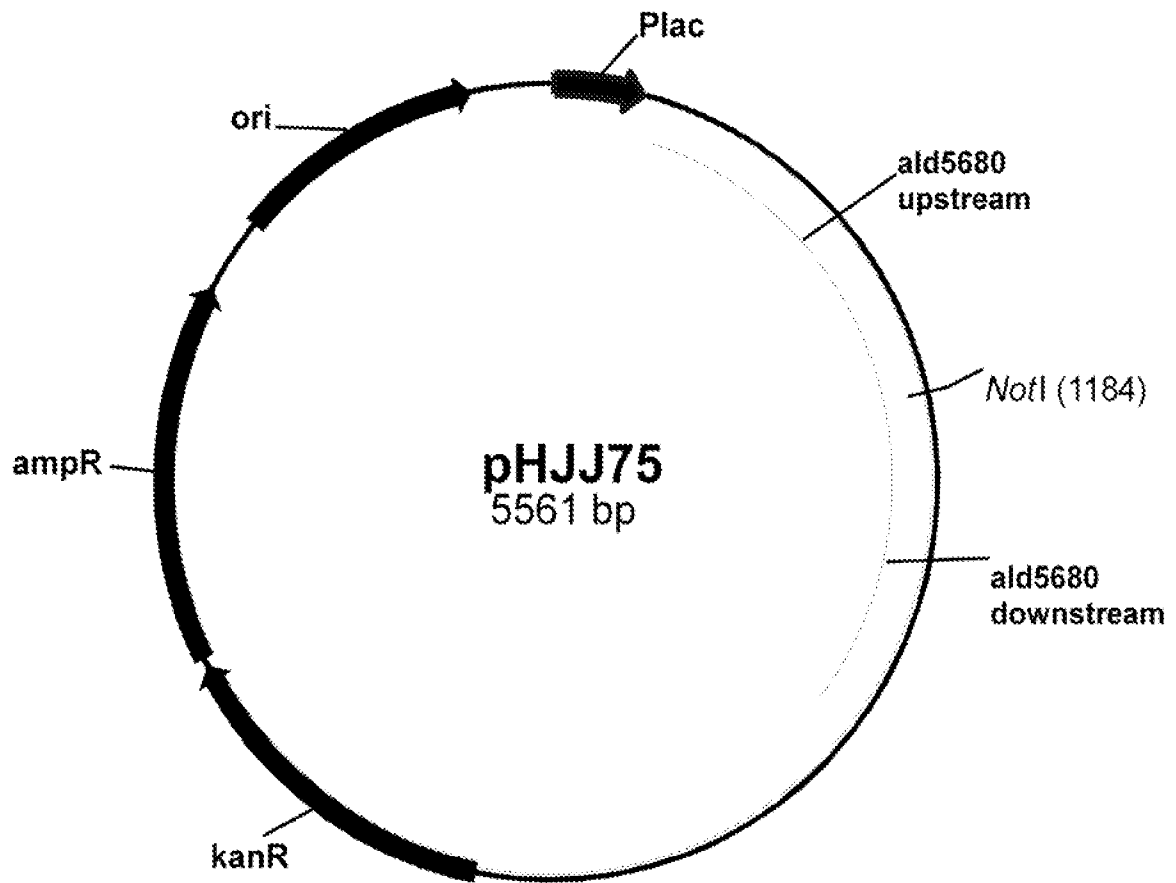


Fig. 23

24/32

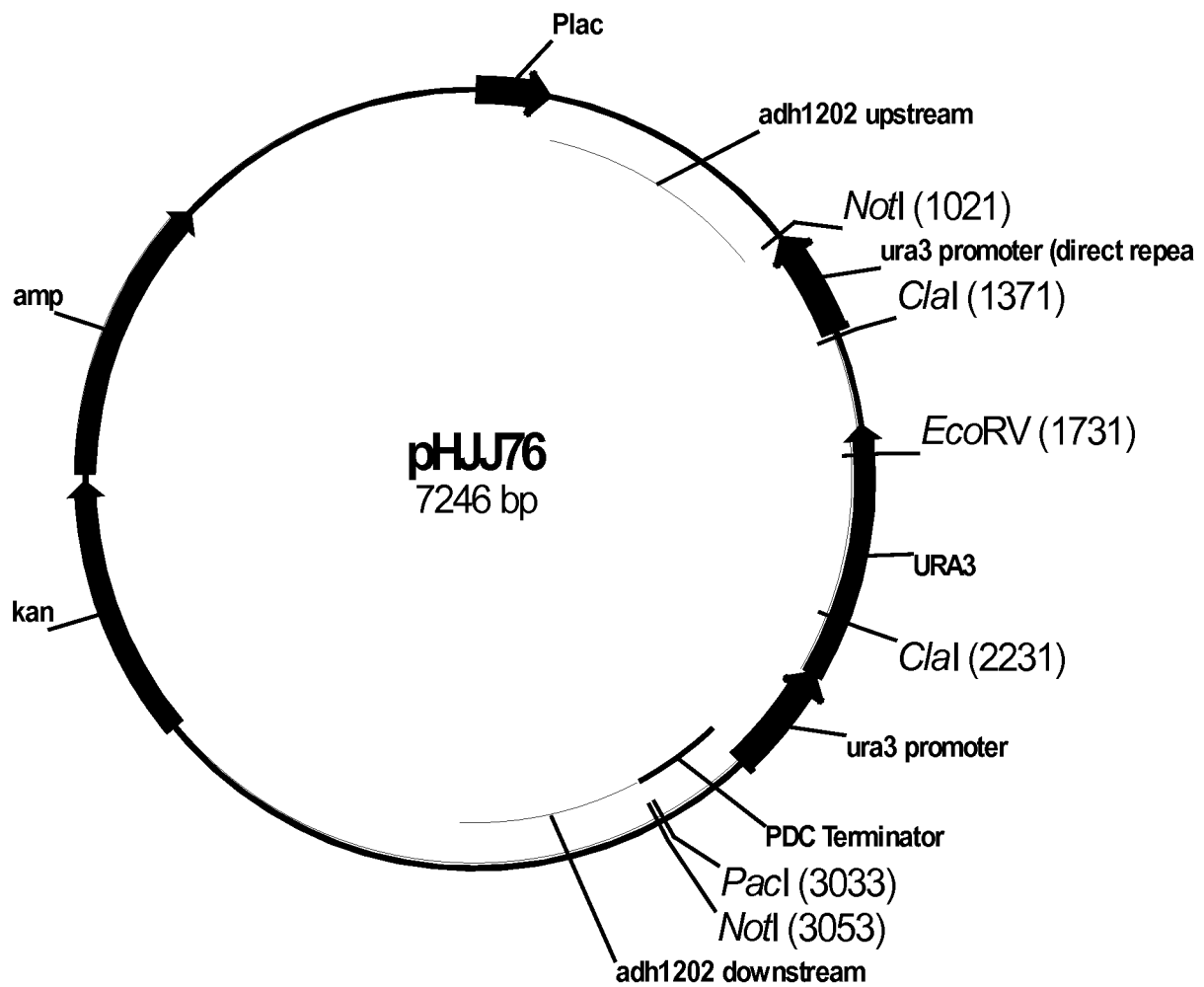


Fig. 24

25/32

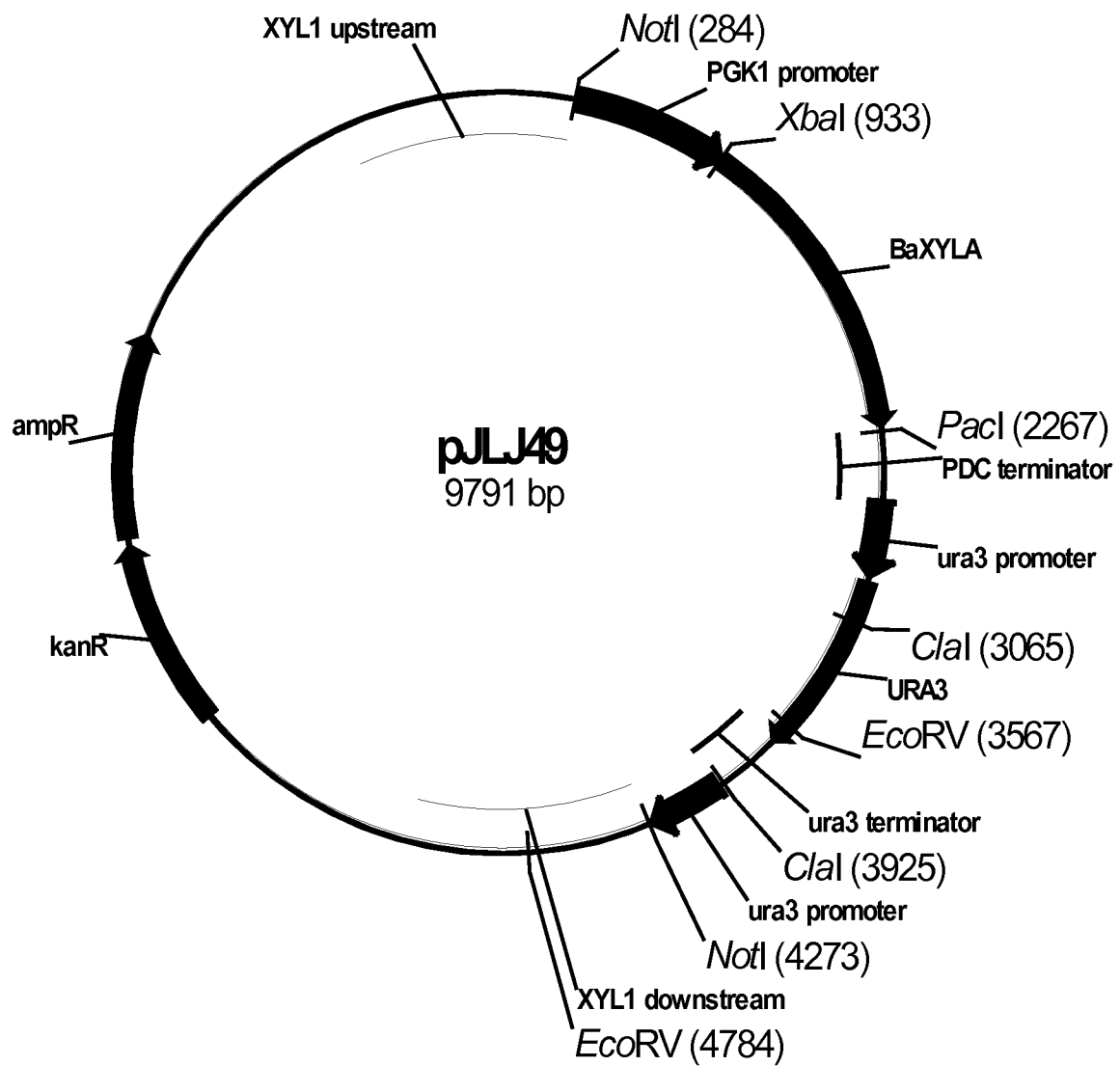


Fig. 25

26/32

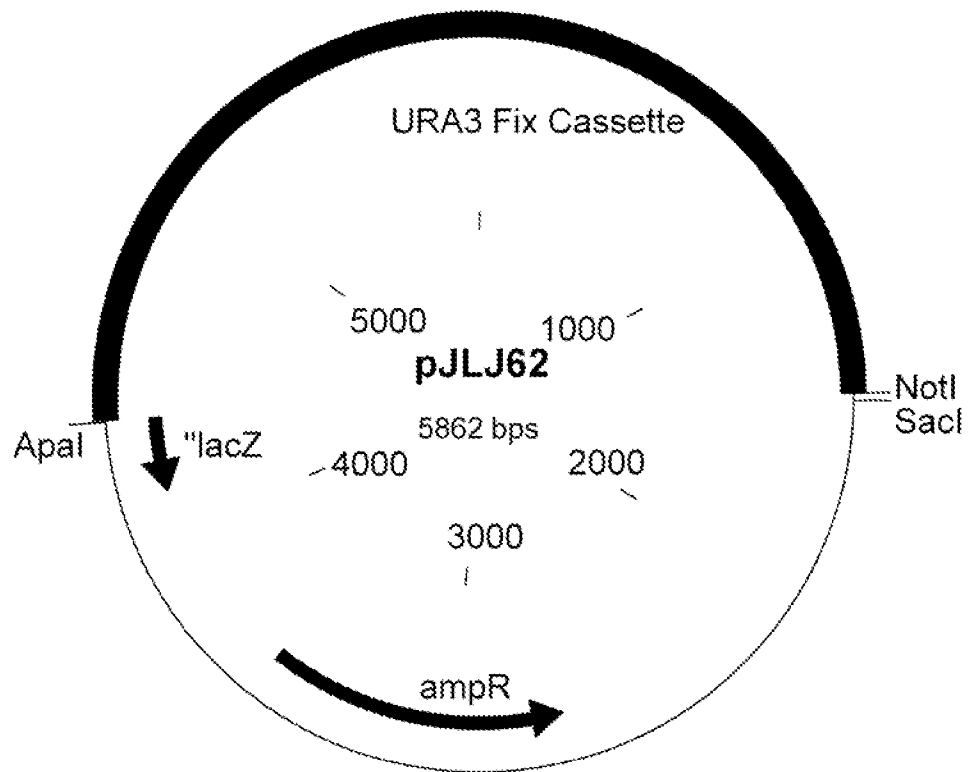


Fig. 26

27/32

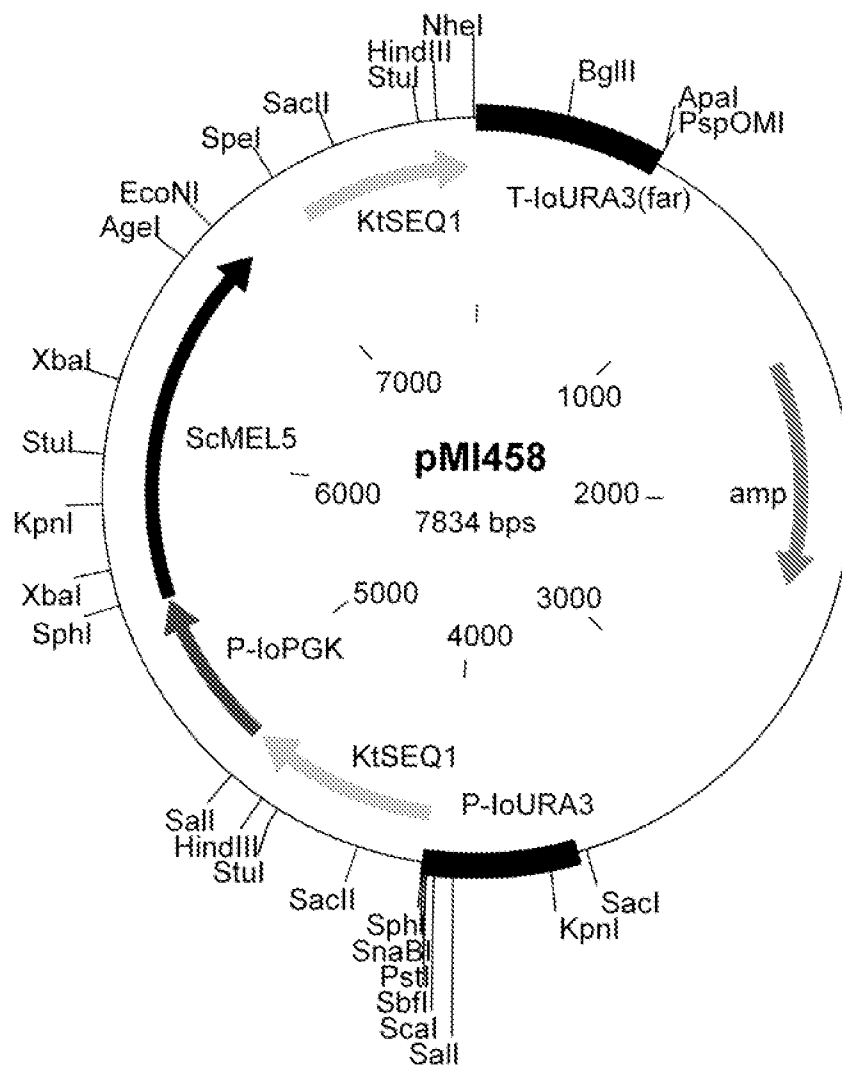


Fig. 27

28/32

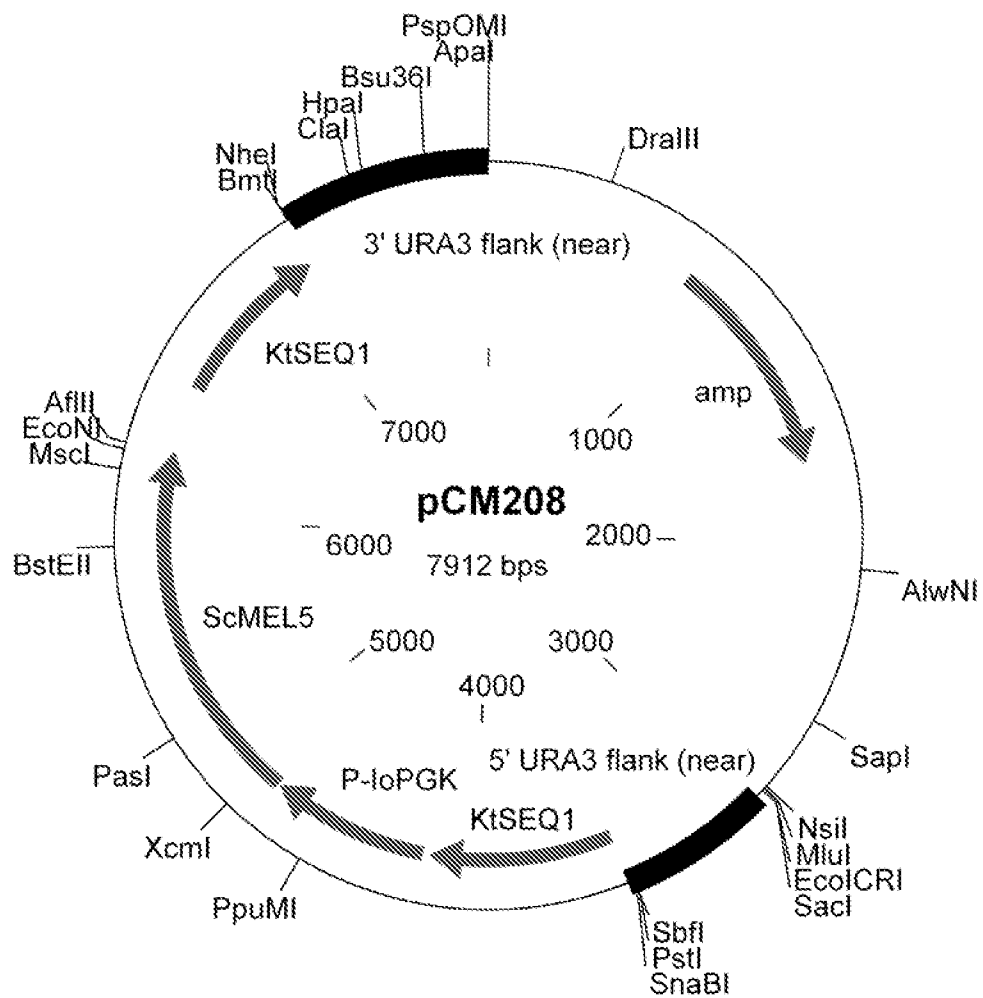


Fig. 28

29/32

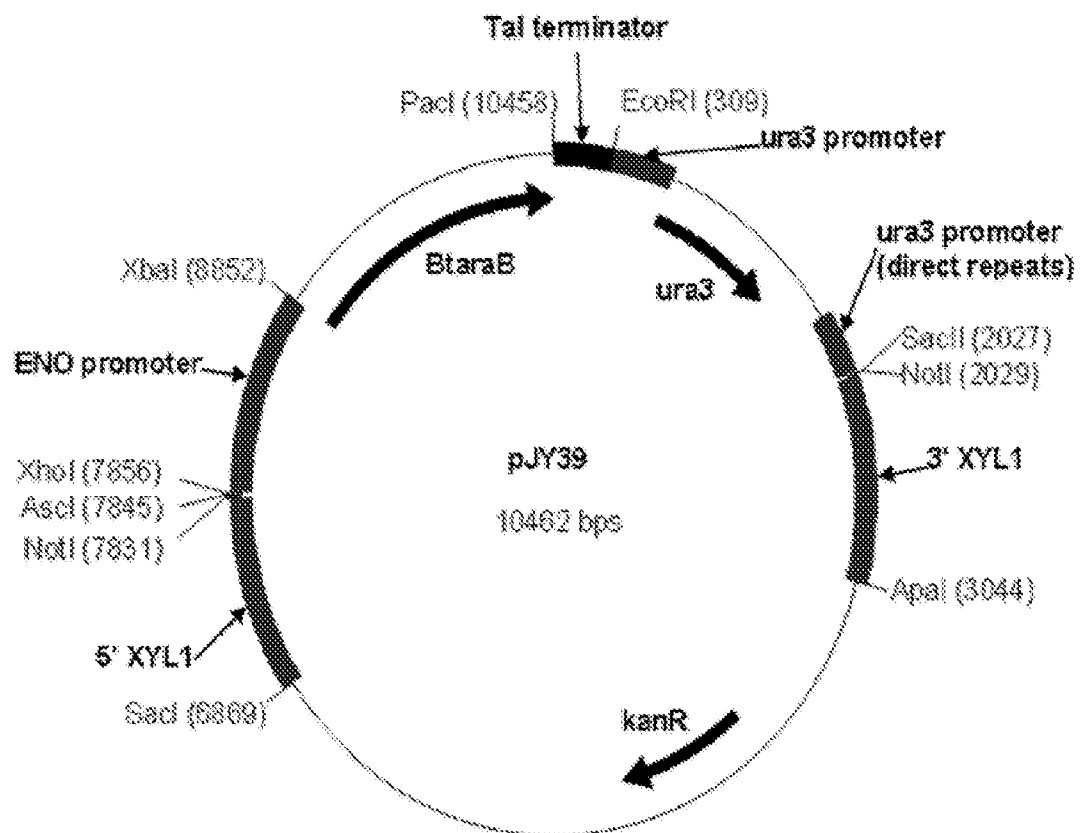


Fig. 29

30/32

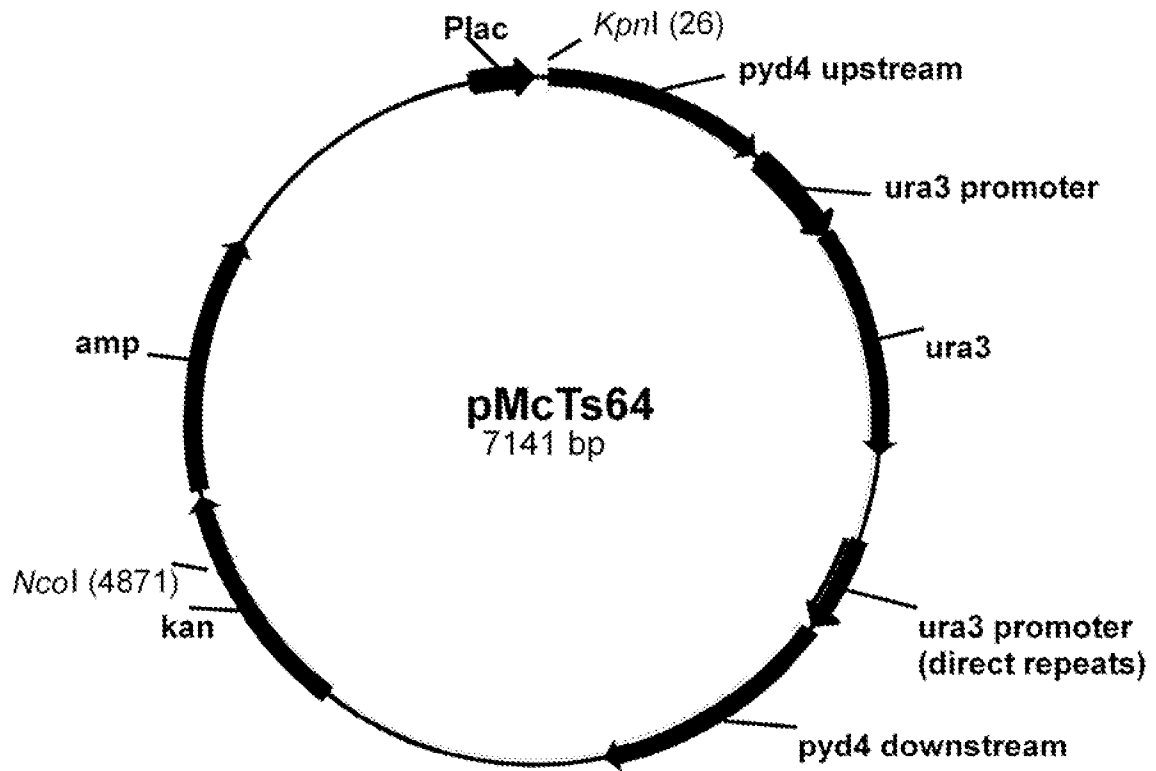


Fig. 30

31/32

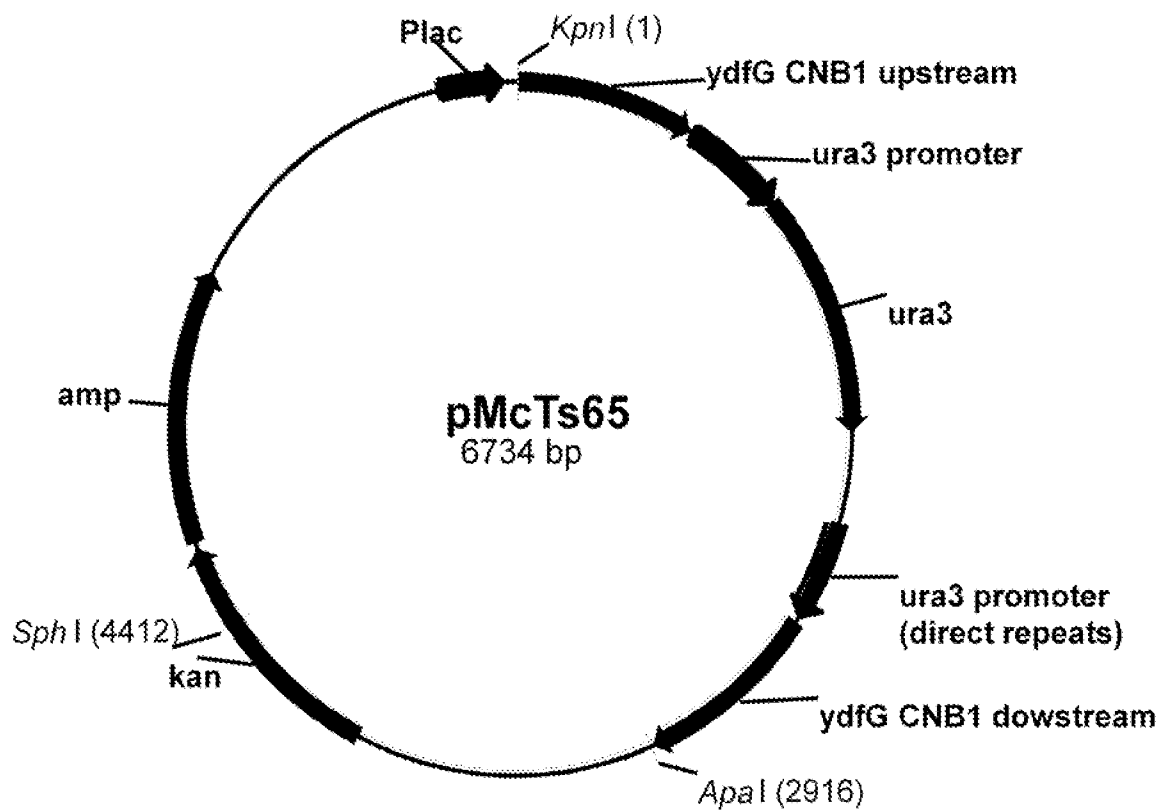
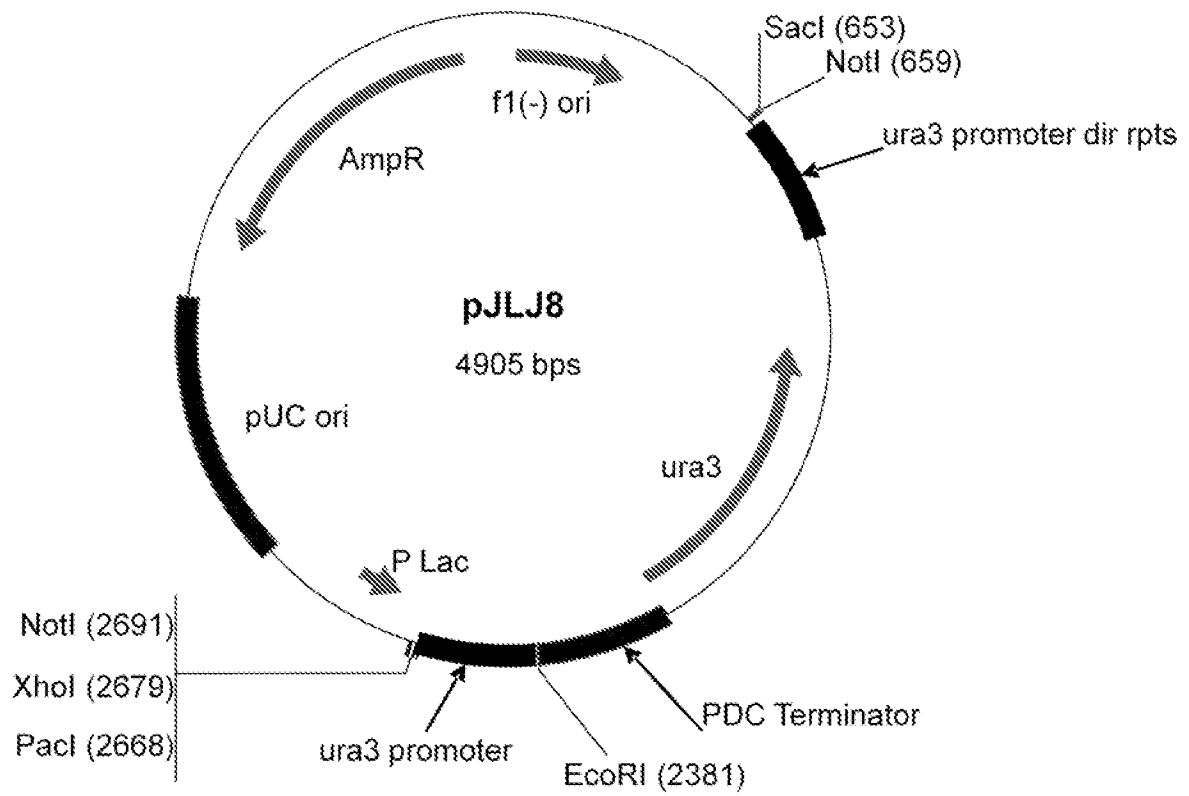


Fig. 31

32/32**Fig. 32**