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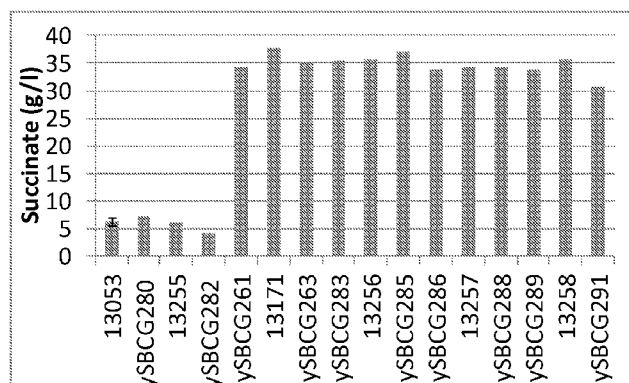


Figure 65

(57) Abstract: The present application provides genetically modified yeast cell comprising an active succinate fermentation path-
way, as well as methods of using these cells to produce succinate.

COMPOSITIONS AND METHODS FOR SUCCINATE PRODUCTION

RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Patent Application No. 61/436,185, filed January 25, 2011, the disclosure of which is incorporated by reference herein in its entirety.

BACKGROUND

[0002] Succinate (butanedioic acid) is a four carbon dicarboxylic acid that plays a key role in the citric acid cycle. Succinate was recently listed by the US Department of Energy at the top of its list of value added chemicals from biomass. Succinate is a precursor for a number of compounds, including tetrahydrofuran, 1,4-butanediol, and γ -butyrolactone. Succinate has a wide variety of potential applications including use in liquid antigels, heat transfer fluids, the solvents gamma butyrolactone (GBL) and dimethyl isosorbide, pigments, the polyesters poly-butylene succinate (PBS) and PEIT, synthesis intermediates and plasticizers.

[0003] Succinate has traditionally been derived from maleic anhydride, which is produced by oxidation of butane. In recent years, there have been several attempts to move away from these traditional production methods to biological production methods. Biological production provides several advantages over derivation from petrochemical sources, including increased efficiency and cost effectiveness and decreased environmental impact.

[0004] Previously developed biological succinate production methods have primarily utilized bacterial fermentation hosts. Although several bacterial species have been used successfully to produce succinate, bacteria present certain drawbacks for large-scale organic acid production. As organic acids are produced, the fermentation medium becomes increasingly acidic. These lower pH conditions result in lower costs for organic acid production, because the resultant product is partially or wholly in the acid form. However, most bacteria do not perform well in strongly acidic environments, and therefore either die or begin producing so slowly that they become economically unviable. 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.

[0005] There has been increasing interest in recent years around the use of yeast to ferment sugars to organic acids. Yeast 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. Yeast are also not susceptible to

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

[0006] Although yeast 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 in yeast 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 yeast possess the combination of sufficiently high volumetric and specific sugar utilization rates with the ability to grow robustly under low pH conditions. The Department of Energy has estimated that production rates of approximately 2.5 g/L/hour are necessary, using a minimal media, for economic fermentations of organic acid (<http://www1.eere.energy.gov/biomass/pdfs/35523.pdf>).

[0007] The yeast strains that have been developed thus far for succinate production have not exhibited high enough yields for economic production on an industrial scale. Therefore, there is a need for improved yeast strains that generate succinate on a large scale in a more cost-effective manner.

SUMMARY

[0008] Provided herein in certain embodiments are genetically modified yeast cells comprising an active succinate fermentation pathway from phosphoenolpyruvate or pyruvate to succinate. In certain embodiments, the yeast cells provided herein are succinate resistant. In certain embodiments, the active succinate fermentation pathway includes at least the following reactions 1) conversion of pyruvate and/or phosphoenolpyruvate to oxaloacetate, 2) conversion of oxaloacetate to malate, 3) conversion of malate to fumarate, and 4) conversion of fumarate. In certain embodiments, the pathway also includes export of succinate from inside the cell to the extracellular environment. Each of the reactions in the active succinate fermentation pathway is catalyzed by one or more enzymes, which in turn are encoded by one or more exogenous or endogenous succinate fermentation pathway genes. In certain embodiments, all of the enzymes catalyzing reactions in the active succinate fermentation pathway are encoded by endogenous genes. In other embodiments, all of the enzymes catalyzing reactions in the active succinate fermentation pathway are encoded by exogenous genes. In still other embodiments, the enzymes catalyzing reactions

in the active succinate fermentation pathway are encoded by a mix of endogenous and exogenous genes.

[0009] In certain embodiments, the genetically modified yeast cells provided herein comprise one or more endogenous genes encoding enzymes that catalyze various reactions in the active succinate fermentation pathway, and in certain of these embodiments the cells comprise one or more copies of endogenous pyruvate carboxylase, phosphoenolpyruvate carboxylase, malate dehydrogenase, fumarase, fumarate reductase, and/or succinate exporter genes. In certain of these embodiments, the endogenous genes are operatively linked to endogenous regulatory elements only. In other embodiments, one or more of the endogenous genes are operatively linked to one or more exogenous regulatory elements.

[0010] In certain embodiments, the genetically modified yeast cells provided herein comprise one or more exogenous genes encoding enzymes that catalyze various reactions in the active succinate fermentation pathway, and in certain of these embodiments the cells comprise one or more copies of exogenous pyruvate carboxylase, phosphoenolpyruvate carboxylase, malate dehydrogenase, fumarase, fumarate reductase, and/or succinate exporter genes. In certain of these embodiments, the exogenous genes are operatively linked to exogenous regulatory elements only. In other embodiments, one or more of the exogenous genes are operatively linked to one or more endogenous regulatory elements.

[0011] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous pyruvate carboxylase gene. In certain of those embodiments where the cells comprise an exogenous pyruvate carboxylase gene, the exogenous pyruvate carboxylase gene is derived from a yeast source gene such as an *Issatchenkia orientalis*, *Saccharomyces cerevisiae*, or *Kluyveromyces marxianus* source gene, and in certain of these embodiments the exogenous pyruvate carboxylase gene 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 the yeast source gene. In certain embodiments, the exogenous pyruvate carboxylase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:8, 10, or 12, 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 NOs:8, 10, or 12. In certain embodiments, the exogenous pyruvate carboxylase gene comprises the nucleotide sequence of SEQ ID NOs:7, 9, or 11, 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 NOs:7, 9, or 11. In certain other embodiments where the cells comprise an exogenous pyruvate carboxylase

gene, the exogenous pyruvate carboxylase gene is derived from a fungal source gene other than a *Rhizopus oryzae* source gene.

[0012] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous phosphoenolpyruvate carboxylase gene. In certain of these embodiments where the cells comprise an exogenous phosphoenolpyruvate carboxylase gene, the exogenous phosphoenolpyruvate carboxylase gene is derived from a bacterial source gene such as an *Escherichia coli* or *Mannheimia succiniciproducens* source gene, and in certain of these embodiments the exogenous phosphoenolpyruvate carboxylase gene 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 the bacterial source gene. In certain embodiments, the exogenous phosphoenolpyruvate carboxylase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:4 or 6, 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 NOs:4 or 6. In certain embodiments, the exogenous phosphoenolpyruvate carboxylase gene comprises the nucleotide sequence of SEQ ID NOs:3 or 5, 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 NOs:3 or 5.

[0013] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous malate dehydrogenase gene. In certain of these embodiments where the cells comprise an exogenous malate dehydrogenase gene, the exogenous malate dehydrogenase gene is derived from a yeast source gene such as an *I. orientalis*, *Zygosaccharomyces rouxii*, or *K. marxianus* source gene, and in certain of these embodiments the exogenous malate dehydrogenase gene 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 the yeast source gene. In certain embodiments, the exogenous malate dehydrogenase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:14, 16, 18, 168, 20, 22, or 24, 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 NOs:14, 16, 18, 168, 20, 22, or 24. In certain embodiments, the exogenous malate dehydrogenase gene comprises the nucleotide sequence of SEQ ID NOs:13, 15, 17, 167, 19, 21, or 23, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence of SEQ ID

NOs:13, 15, 17, 167, 19, 21, or 23. In certain other embodiments where the cells comprise an exogenous malate dehydrogenase gene, the exogenous malate dehydrogenase gene is derived from a bacterial source gene such as an *E. coli* source gene, and in certain of these embodiments the exogenous malate dehydrogenase gene 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 the bacterial source gene. In certain embodiments, the exogenous malate dehydrogenase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO:170, 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:170. In certain embodiments, the exogenous malate dehydrogenase gene comprises the nucleotide sequence of SEQ ID NO:169, 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:169. In certain other embodiments where the cells comprise an exogenous malate dehydrogenase gene, the exogenous malate dehydrogenase gene is derived from a fungal source gene such as a *R. oryzae* source gene, and in certain of these embodiments the exogenous malate dehydrogenase gene 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 the fungal source gene. In certain embodiments, the exogenous malate dehydrogenase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO:172, 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:172. In certain embodiments, the exogenous malate dehydrogenase gene comprises the nucleotide sequence of SEQ ID NO:171, 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:171.

[0014] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous fumarase gene. In certain of these embodiments where the cells comprise an exogenous fumarase gene, the exogenous fumarase gene is derived from a yeast source gene such as an *I. orientalis* source gene, and in certain of these embodiments the exogenous fumarase gene 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 the yeast source gene. In certain embodiments, the exogenous fumarase gene encodes a

polypeptide that comprises the amino acid sequence of SEQ ID NO:2, 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:2. In certain embodiments, the exogenous fumarase gene comprises 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.

[0015] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous fumarate reductase gene. In certain of these embodiments where the cells comprise an exogenous fumarate reductase gene, the exogenous fumarase gene is derived from a yeast source gene such as an *S. cerevisiae*, *Saccharomyces mikatae*, *Kluyveromyces polyspora*, or *K. marxianus* source gene, and in certain of these embodiments the exogenous fumarate reductase gene 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 the yeast source gene. In certain embodiments, the exogenous fumarate reductase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:26, 28, 30, or 32, 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 NOs:26, 28, 30, or 32. In certain embodiments, the exogenous fumarate reductase gene comprises the nucleotide sequence of SEQ ID NOs:25, 27, 29, or 31, 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 NOs:25, 27, 29, or 31. In certain other embodiments where the cells comprise an exogenous fumarate reductase gene, the exogenous fumarate reductase gene is derived from a protozoan source gene such as a *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania braziliensis*, or *Leishmania mexicana* source gene, and in certain of these embodiments the exogenous fumarate reductase gene 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 the protozoan source gene. In certain embodiments, the exogenous fumarate reductase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:174, 176, 178, or 180, 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 NOs:174, 176, 178, or 180. In certain embodiments, the exogenous fumarate reductase gene comprises the nucleotide sequence of SEQ ID NOs:173, 175, 177, or 179, 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 NOs:173, 175, 177, or 179.

[0016] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous succinate exporter gene. In certain of these embodiments where the cells comprise an exogenous succinate exporter gene, the exogenous succinate exporter gene is derived from a fungal source gene such as an *Schizosaccharomyces pombe* or *Aspergillus oryzae* source gene, and in certain of these embodiments the exogenous succinate exporter gene 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 the fungal source gene. In certain embodiments, the exogenous succinate exporter gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NOs:182 or 184, 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 NOs:182 or 184. In certain embodiments, the exogenous succinate exporter gene comprises the nucleotide sequence of SEQ ID NOs:181 or 183, 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 NOs:181 or 183.

[0017] In certain embodiments, the genetically modified yeast cells provided herein comprise, in addition to an active succinate fermentation pathway, an active reduction pathway from glucose-6-phosphate to ribulose-5-phosphate. In certain embodiments, this pathway includes at least the following reactions 1) conversion of glucose 6-phosphate to 6-phosphogluconolactone, 2) conversion of 6-phosphogluconolactone to 6-phosphogluconate, and 3) conversion of 6-phosphogluconate to ribulose 5-phosphate. Each of the reactions in the active reduction pathway is catalyzed by one or more enzymes, which in turn are encoded by one or more exogenous or endogenous reduction pathway genes. In certain embodiments, all of the enzymes catalyzing reactions in the active reduction pathway are encoded by endogenous genes. In other embodiments, all of the enzymes catalyzing reactions in the active reduction pathway are encoded by exogenous genes. In still other embodiments, the enzymes catalyzing reactions in the active reduction pathway are encoded by a mix of endogenous and exogenous genes.

[0018] In certain embodiments, the genetically modified yeast cells provided herein comprise one or more endogenous genes encoding enzymes that catalyze various reactions in the active reduction pathway, and in certain of these embodiments the cells comprise one

or more copies of endogenous glucose 6-phosphate dehydrogenase, gluconolactonase, and/or 6-phosphogluconate dehydrogenase genes. In certain of these embodiments, the endogenous genes are operatively linked to endogenous regulatory elements only. In other embodiments, one or more of the endogenous genes are operatively linked to one or more exogenous regulatory elements.

[0019] In certain embodiments, the genetically modified yeast cells provided herein comprise one or more exogenous genes encoding enzymes that catalyze various reactions in the active reduction pathway, and in certain of these embodiments the cells comprise one or more copies of exogenous glucose 6-phosphate dehydrogenase, gluconolactonase, and/or 6-phosphogluconate dehydrogenase genes. In certain of these embodiments, the exogenous genes are operatively linked to exogenous regulatory elements only. In other embodiments, one or more of the exogenous genes are operatively linked to one or more endogenous regulatory elements.

[0020] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous glucose 6-phosphate dehydrogenase gene. In certain of these embodiments where the cells comprise an exogenous glucose 6-phosphate dehydrogenase gene, the exogenous glucose 6-phosphate dehydrogenase gene is derived from a yeast source gene such as an *I. orientalis* source gene, and in certain of these embodiments the exogenous glucose 6-phosphate dehydrogenase gene 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 the yeast source gene. In certain embodiments, the exogenous glucose 6-phosphate dehydrogenase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO:34, 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:34. In certain embodiments, the exogenous glucose 6-phosphate dehydrogenase gene comprises the nucleotide sequence of SEQ ID NO:33, 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:33.

[0021] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous gluconolactonase gene. In certain of these embodiments where the cells comprise an exogenous gluconolactonase gene, the exogenous gluconolactonase gene is derived from a yeast source gene such as an *I. orientalis* source gene, and in certain of these embodiments the exogenous gluconolactonase gene 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 the yeast source gene. In certain embodiments, the exogenous gluconolactonase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO:36, 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:36. In certain embodiments, the exogenous gluconolactonase gene comprises the nucleotide sequence of SEQ ID NO:35, or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence of SEQ ID NO:35.

[0022] In certain embodiments, the genetically modified yeast cells provided herein comprise an endogenous and/or exogenous 6-phosphogluconate dehydrogenase gene. In certain of these embodiments where the cells comprise an exogenous 6-phosphogluconate dehydrogenase gene, the exogenous 6-phosphogluconate dehydrogenase gene is derived from a yeast source gene such as an *I. orientalis* source gene, and in certain of these embodiments the exogenous 6-phosphogluconate dehydrogenase gene 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 the yeast source gene. In certain embodiments, the exogenous 6-phosphogluconate dehydrogenase gene encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO:38, 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:38. In certain embodiments, the exogenous 6-phosphogluconate dehydrogenase gene comprises the nucleotide sequence of SEQ ID NO:37, 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:37.

[0023] In certain embodiments, the genetically modified yeast cells provided herein comprise a deletion or disruption of one or more endogenous genes. In certain of these embodiments, the cells comprise a deletion or disruption of an endogenous pyruvate carboxykinase, malic enzyme, pyruvate decarboxylase, and/or succinate importer gene.

[0024] In certain embodiments, the genetically modified yeast cells are derived from host yeast cells that exhibit a relatively high degree of succinate resistance. In certain embodiments, the cells provided herein belong to the genus *Issatchenkia*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Kluyveromyces*, *Saccharomyces*, *Debaryomyces*, or *Saccharomycopsis*, and in certain of these embodiments the cells belong to the *Pichia*

fermentans/*I. orientalis* clade. In certain embodiments, the cells belong to the species *I. orientalis*, *Candida lambica*, *Candida sorboxylosa*, *Candida zemplinina*, *Candida geochares*, *Pichia membranifaciens*, *Zygosaccharomyces kombuchaensis*, *Candida sorbosivorans*, *K. marxianus*, *Candida vanderwaltii*, *Candida sorbophila*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces lentus*, *Saccharomyces bayanus*, *Saccharomyces bulderi*, *Debaryomyces castellii*, *Candida boidinii*, *Candida etchellsii*, *Kluyveromyces lactis*, *Pichia jadinii*, *Pichia anomala*, or *Saccharomycopsis crataegensis*.

[0025] Provided herein in certain embodiments are methods of producing succinate by culturing the genetically modified yeast cells provided herein in the presence of at least one carbon source, then isolating the succinate from the culture. In certain embodiments, the carbon source is one or more of glucose, xylose, arabinose, sucrose, fructose, cellulose, glucose oligomers, and glycerol. Also provided herein are processes for converting succinate produced by the methods provided herein to a product such as poly-butylene succinate (PBS), other polymers, cosmetics, foodstuff, feed, or pharmaceuticals.

BRIEF DESCRIPTION OF DRAWING]

- [0026]** Figure 1: pMI449, CYB2A deletion construct.
- [0027]** Figure 2: pMI454, CYB2A deletion construct.
- [0028]** Figure 3: pBH165, GPD1 deletion construct.
- [0029]** Figure 4: pCM188, GPD1 deletion construct.
- [0030]** Figure 5: pCA89, CYB2B deletion construct.
- [0031]** Figure 6: pMI457, PGK:MEL5 construct.
- [0032]** Figure 7: pCA90, CYB2B deletion construct.
- [0033]** Figure 8: pHJJ17.
- [0034]** Figure 9: pVB10, PDC:cre construct
- [0035]** Figure 10: pVB32.
- [0036]** Figure 11: pKF031, PGK:MEL5 construct.
- [0037]** Figure 12: pKF044, CYB2A construct.
- [0038]** Figure 13: pKWB18, ATO2 deletion construct
- [0039]** Figure 14: pKWB21, PDC1 deletion construct.
- [0040]** Figure 15: pKF043, *I. orientalis* PYC1 expression construct.
- [0041]** Figure 16: pKF045, *I. orientalis* PYC1 expression construct.
- [0042]** Figure 17: pKWB14, *S. cerevisiae* PYC1 expression construct.
- [0043]** Figure 18: pKWB15, *S. cerevisiae* PYC1 expression construct.
- [0044]** Figure 19: pKWB16, *K. marxianus* PYC1 expression construct.
- [0045]** Figure 20: pKWB17, *K. marxianus* PYC1 expression construct
- [0046]** Figure 21: pHJJ23

- [0047]** Figure 22: pGPB11, ADHa deletion construct.
- [0048]** Figure 23: pGPB28, ADHa deletion construct.
- [0049]** Figure 24: pGPB14, ADHa deletion construct.
- [0050]** Figure 25: pGPB34, , ADHa deletion construct.
- [0051]** Figure 26: pGPB20, 22, 25, and 26, FRD1 expression constructs.
- [0052]** Figure 27: pGPB36, 37, 39, and 40, FRD1 expression constructs.
- [0053]** Figure 28: pGPB30, FUM1 expression constructs.
- [0054]** Figure 29: pGPB42, FUM1 expression constructs.
- [0055]** Figure 30: pGPB44, FUM1 expression constructs.
- [0056]** Figure 31: pGPB47, FUM1 expression constructs.
- [0057]** Figure 32: pKF031, ZWF1 expression construct.
- [0058]** Figure 33: pKF044, ZWF1 expression construct.
- [0059]** Figure 34: pKF033, ZWF1 expression construct.
- [0060]** Figure 35: pGPB056, ZWF1 expression construct.
- [0061]** Figure 36: pKWB19, MAE1 deletion construct.
- [0062]** Figure 37: pKWB24, MAE1 deletion construct.
- [0063]** Figure 38: pKWB29, MAE1 deletion construct.
- [0064]** Figure 39: pKWB20, PCK1 deletion construct.
- [0065]** Figure 40: pKWB25, PCK1 deletion construct.
- [0066]** Figure 41: pKWB30, PCK1 deletion construct.
- [0067]** Figure 42: pKWB23.
- [0068]** Figure 43: pKWB28.
- [0069]** Figure 44: Malate production by GC-FID (g/L) in strain 12657 (control) and strains 12601, 12620, and 12625 expressing exogenous malate dehydrogenase.
- [0070]** Figure 45: Succinate production by GC-FID (g/L) in strains 12791/12791 (control) and strains 17824/12826 expressing exogenous fumarate.
- [0071]** Figure 46: pVMB54
- [0072]** Figure 47: pKWB86.
- [0073]** Figure 48: pKWB87.
- [0074]** Figure 49: Growth of RIOR43690 deletion strains on YNB-succinate plates.
- [0075]** Figure 50: pKWB95.
- [0076]** Figure 51: pKWB96.
- [0077]** Figure 52: pKWB97.
- [0078]** Figure 53: pVMB108.
- [0079]** Figure 54: pVMB109.

[0080] Figure 55: pVMB116.

[0081] Figure 56: pVMB117.

[0082] Figure 57: Succinate production (g/L) in strain 12868 (control) and strains 13125 and 13051 expressing exogenous succinate transporter.

[0083] Figure 58: Malate production (g/L) in strain 13053 (control) and 13221 (malic enzyme deletion).

[0084] Figure 59: pHJJ77.

[0085] Figure 60: pGPB119.

[0086] Figure 61: pGPB126, 159, 160, and 161.

[0087] Figure 62: pGPB127.

[0088] Figure 63: pGPB168.

[0089] Figure 64: pGPB148.

[0090] Figure 65: Succinate production (g/L) in yeast strains expressing various fumarate reductases.

[0091] Figure 66: Succinate production (g/L) at 90 hour fermentation time at pH 3.0, 10% CO₂, OUR (mmol/h) variable.

[0092] Figure 67: pGPB43.

DETAILED DESCRIPTION

[0093] 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. All references cited herein are incorporated by reference in their entirety.

Abbreviations

[0094] α -KGDH, α -ketoglutarate dehydrogenase; CYB2, L-(+)-lactate:ferricytochrome c oxidoreductase; CYC, iso-2-cytochrome c; ENO1, enolase; FRD, fumarate reductase; FUM, fumarase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPD, glycerol 3-phosphate dehydrogenase; G6PD, glucose 6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; MAE, malic enzyme; MAE, malic anion exporter; MDH, malate dehydrogenase; OAA, oxaloacetate; OUR, oxygen uptake rate; PCK, phosphoenolpyruvate carboxykinase; PDC, pyruvate decarboxylase; PEP, phosphoenolpyruvate; 6PGDH, 6-phosphogluconate dehydrogenase; PGK, phosphoglycerate kinase; PFL, pyruvate formate lyase; PPC, phosphoenolpyruvate carboxylase; PYC, pyruvate carboxylase; RKI, ribose 5-phosphate ketol-isomerase; TAL, transaldolase; TCA, tricarboxylic acid; TEF, translation

elongate factor; TKL, transketolase; URA3, orotidine 5'-phosphate decarboxylase; XDH, xylitol dehydrogenase; XR, xylose reductase; 6PGDH, 6-phosphogluconate dehydrogenase.

Description

[0095] Provided herein are genetically modified yeast cells for the production of succinate, methods of making these yeast cells, and methods of using these cells to produce succinate. "Succinate" as used herein includes salt and acid forms of succinate.

[0096] There are three primary fermentation pathways for producing succinate from a microorganism: reductive TCA, oxidative TCA, and glyoxylate shunt.

[0097] The reductive TCA pathway begins with carboxylation of the three carbon glycolytic intermediate phosphoenolpyruvate (PEP) or pyruvate to oxaloacetate (OAA) (by PEP carboxylase (PPC) and pyruvate carboxylase (PYC), respectively). OAA is converted to malate by malate dehydrogenase (MDH), malate is converted to fumarate by fumarase (FUM, also known as fumarate hydratase), and fumarate is converted to succinate by fumarate reductase (FRD). When written from the perspective of redox state, the net stoichiometry for this succinate production pathway is: $1 \text{ glucose (C}_6\text{H}_{12}\text{O}_6) + 2 \text{ CO}_2 + 2 (\text{NADH} + \text{H}^+) \rightarrow 2 \text{ succinic acid (C}_4\text{H}_6\text{O}_4) + 2 \text{ H}_2\text{O} + 2 \text{ NAD}^+$. The reductive TCA pathway provides the highest succinate yield of the three succinate fermentation pathways, but it results in a net deficit in reducing power (NADH). This means that in isolation the pathway results in a redox imbalance. In order to provide redox balance, the reductive TCA pathway can be combined with one or both of the oxidative TCA or glyoxylate shunt pathways, or with one or more unrelated pathways that produce NADH or NADPH.

[0098] The oxidative TCA pathway begins with the conversion of OAA and acetyl-CoA to citrate by citrate synthase. OAA can be generated from carboxylation of PEP or pyruvate, while acetyl-CoA is generated from the decarboxylation of pyruvate by PDH or pyruvate formate lyase (PFL). Citrate is converted to isocitrate by aconitase, isocitrate is converted to α -ketoglutarate by isocitrate dehydrogenase (IDH), α -ketoglutarate is converted to succinyl-CoA by α -ketoglutarate dehydrogenase (α -KGDH), and succinyl-CoA is converted to succinate by succinyl coenzyme A synthetase (succinate thiokinase). The net stoichiometry for this succinate production pathway is: $1 \text{ glucose (C}_6\text{H}_{12}\text{O}_6) + 2 \text{ H}_2\text{O} + 5 \text{ NAD}^+ \rightarrow 1 \text{ succinic acid (C}_4\text{H}_6\text{O}_4) + 2 \text{ CO}_2 + 5 (\text{NADH} + \text{H}^+)$. This pathway has a lowest carbon yield of the three succinate fermentation pathways, but the highest yield of reducing power.

[0099] Like the oxidative TCA pathway, the glyoxylate shunt pathway begins with the generation of citrate from OAA and acetyl-CoA and the conversion of citrate to isocitrate. Isocitrate is converted to glyoxylate and succinate by isocitrate lyase. Glyoxylate is condensed with acetyl-CoA to form malate by malate synthase, and the resultant malate is converted to succinate via a fumarate intermediate. The net stoichiometry for this succinate

production pathway is: 1 glucose($\text{C}_6\text{H}_{12}\text{O}_6$) + $\frac{2}{3}$ H_2O + $2\frac{2}{3}$ NAD^+ $\rightarrow$ $1\frac{1}{3}$ succinic acid ($\text{C}_4\text{H}_6\text{O}_4$) + $\frac{2}{3}$ CO_2 + $2\frac{2}{3}$ ($\text{NADH} + \text{H}^+$).

[00100] Previous attempts to produce succinate from microorganisms at commercially viable levels have utilized bacterial fermentation hosts. These bacterial hosts are either native succinate producers or non-native succinate producers that have been genetically engineered to produce succinate. Examples of native succinate producers are *Actinobacillus succinogenes* (see, e.g., US Patent 5,504,004) and *M. succiniciproducens*, each of which primarily utilizes a reductive TCA pathway. *A. succinogenes* and *M. succiniciproducens* both produce relatively high titers of succinate, but they also produce various organic acid by-products. The presence of these by-products decreases yield and complicates succinate recovery. An example of a non-native succinate producer is *E. coli*. Although *E. coli* is capable of producing trace levels of succinate naturally, genetic modification is required to obtain useful titers. Significant efforts have been made previously to increase succinate yield in genetically modified *E. coli* by decreasing the formation of other organic acids and combining different succinate fermentation pathways. Although *E. coli* strains have been developed that produce fewer organic acid by-products, they still produce lower succinate titers than the native producers. In addition, *E. coli* requires aerobic conditions to grow, but produces succinic acid at high yields only under anaerobic conditions. This means that succinate production in *E. coli* requires a two-phase fermentation.

[00101] One drawback common to all of the bacterial hosts developed to date for succinate production is relatively poor performance in strongly acidic environments. However, allowing the low pH conditions to develop as organic acids are produced is preferred for commercial succinate production. The ideal host for commercial succinate production should produce high levels of succinate and relatively low levels of other organic acids, and should possess a high degree of pH resistance and the ability to both grow and ferment under anaerobic or substantially anaerobic conditions.

[00102] As disclosed herein, a set of yeast cells from various species were tested for succinate resistance. Cells exhibiting succinate resistance were further evaluated based on their growth rates and glucose consumption rates in media containing varying concentrations of succinate. Based on these experiments, a set of ideal host cells for succinate production were identified. These host cells were then genetically modified to contain an active succinate fermentation pathway, resulting in a set of genetically modified yeast cells that produce succinate under low pH conditions.

[00103] Provided herein in certain embodiments are genetically modified succinate-resistant yeast cells having at least one active succinate fermentation pathway from PEP or pyruvate

to succinate. A yeast cell having an "active succinate fermentation pathway" as used herein produces active enzymes necessary to catalyze each reaction in a succinate fermentation pathway, and therefore is capable of producing succinate in measurable yields when cultured under fermentation conditions in the presence of at least one fermentable sugar. A yeast cell having an active succinate fermentation pathway comprises one or more succinate fermentation pathway genes. A "succinate fermentation pathway gene" as used herein refers to the coding region of a nucleotide sequence that encodes an enzyme involved in an active succinate fermentation pathway.

[00104] In certain embodiments, the yeast cells provided herein have a reductive TCA active succinate fermentation pathway that proceeds through PEP or pyruvate, OAA, malate, and fumarate intermediates. In these embodiments, the yeast cells comprise a set of succinate fermentation pathway genes comprising MDH, FUM, FRD, one or both of PPC and PYC genes, and, optionally, a succinate exporter gene.

[00105] In those embodiments where the yeast cells provided herein have a reductive TCA active succinate fermentation pathway, the cells may further have an active reduction pathway. An "active reduction pathway" as used herein produces NADH or NADPH from NAD or NADP, respectively, thereby helping to balance out redox imbalances generated by a reductive TCA pathway. A yeast cell having an active reduction pathway comprises one or more reduction pathway genes. A "reduction pathway gene" as used herein refers to the coding region of a nucleotide sequence that encodes an enzyme involved in an active reduction pathway.

[00106] In certain embodiments, the yeast cells provided herein have a pentose phosphate active reduction pathway that proceeds through glucose 6-phosphate, 6-phosphogluconolactone, 6-phosphogluconate, and ribulose 5-phosphate intermediates. In these embodiments, the yeast cells comprise a set of reduction pathway genes comprising glucose 6-phosphate dehydrogenase (G6PD), gluconolactonase, and 6-phosphogluconate dehydrogenase (6PGDH) genes.

[00107] In certain embodiments, the yeast cells provided herein may have one or more active succinate fermentation pathways, or portions of such pathways, that are not reductive TCA active succinate fermentation pathways. In these embodiments, the other pathways or portions thereof may be present in addition to or in lieu of the reductive TCA pathway. For example, the cells may comprise a reductive TCA active succinate fermentation pathway and all or a part of an oxidative TCA or glyoxylate shunt active succinate fermentation pathway.

[00108] The succinate fermentation pathway and reduction 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.

[00109] 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 MDH gene that is identical to an endogenous MDH gene may be inserted into a yeast cell, resulting in a modified cell with a non-native (increased) number of MDH gene copies. Similarly, an exogenous PDC promoter that is identical to an endogenous PDC promoter can be inserted into a yeast cell such that it is operatively linked to an endogenous gene such as an MDH gene, resulting in altered expression of the endogenous gene. 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 MDH gene from a particular species may be inserted into a yeast cell of another species. Similarly, an exogenous PDC promoter from a particular species may be inserted into a yeast cell of another species.

[00110] 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.

[00111] In certain embodiments, the genetically modified yeast cells provided herein comprise one or more exogenous succinate fermentation and/or reduction pathway genes. In certain embodiments, the yeast cells comprise a single exogenous gene. In other embodiments, the 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 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.

[00112] In certain embodiments, the yeast cells provided herein comprise one or more endogenous succinate fermentation and/or reduction 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 succinate fermentation pathway genes.

[00113] In certain embodiments, the yeast cells provided herein comprise one or more endogenous succinate fermentation and/or reduction pathway genes and one or more exogenous succinate fermentation and/or reduction pathway genes. In these embodiments, the yeast cells may have an active succinate fermentation pathway that comprises one or more endogenous succinate fermentation pathway genes and one or more exogenous succinate fermentation pathway genes. For example, a yeast cell may comprise endogenous copies of PYC, MDH, and/or FUM genes and exogenous copies of FRD and/or PPC genes. In certain embodiments, the yeast cells may comprise both endogenous and exogenous copies of a single succinate fermentation pathway gene. For example, a yeast cell may comprise both endogenous and exogenous copies of an MDH gene.

[00114] Succinate fermentation and/or reduction 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).

[00115] Regulatory elements linked to succinate fermentation and/or reduction pathway genes in the yeast cells provided herein may be endogenous or exogenous. For example, an endogenous succinate fermentation pathway gene may be operatively linked to only endogenous regulatory elements, or it may be linked to one or more exogenous regulatory elements. Endogenous genes operatively linked to one or more exogenous regulatory elements may exhibit higher expression levels than the same genes linked to only endogenous regulatory elements. Similarly, an exogenous succinate fermentation pathway gene may be inserted into a yeast cell such that it is operatively linked to endogenous regulatory elements only, or it may be linked to one or more exogenous regulatory elements. For example, an exogenous gene may be introduced into the cell as part of an exogenous 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 sequence 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. 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.

[00116] Examples of promoters that may be linked to one or more succinate fermentation and/or reduction pathway genes in the yeast cells provided herein include, but are not limited to, promoters for pyruvate decarboxylase (PDC1), phosphoglycerate kinase (PGK), xylose reductase (XR), xylitol dehydrogenase (XDH), L-(+)-lactate-cytochrome c oxidoreductase (CYB2), translation elongation factor-1 or -2 (TEF1, TEF2), enolase (ENO1),

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and orotidine 5'-phosphate decarboxylase (URA3) genes. In these examples, the succinate fermentation and/or reduction 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.

[00117] Examples of terminators that may be linked to one or more succinate fermentation and/or reduction 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 succinate fermentation and/or reduction 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, succinate fermentation and/or reduction pathway 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.

[00118] 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 succinate fermentation 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.

[00119] 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 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 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.

[00120] An integration or deletion construct may comprise one or more selection marker cassettes cloned into the construct between the two target DNA 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 (melibiose) 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 a 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 above.

[00121] 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, a homologous recombination event 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.

[00122] 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.

[00123] 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 direction with respect to one another. The repeat sequences are advantageously about 25 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.

[00124] An exogenous succinate fermentation or reduction pathway gene in the modified yeast cells provided herein may be derived from a source gene from any suitable source organism. 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 source gene encodes a polypeptide that 1) is identical to a polypeptide encoded by the source 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 source gene, and/or 3) has the same function in a succinate

fermentation or reduction pathway as the polypeptide encoded by the source gene. For example, a FUM gene that is derived from an *I. orientalis* FUM 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 malate to fumarate. A gene derived from a source 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 source gene. In certain embodiments, a gene derived from a source gene may comprise a nucleotide sequence that is identical to the coding region of the source gene. For example, a FUM gene that is derived from an *I. orientalis* FUM 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.

[00125] In certain embodiments of the modified yeast cells provided herein, an exogenous succinate fermentation or reduction 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 a native *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 gene may comprise a nucleotide sequence that differs from the coding region of a native gene, but nonetheless encodes a polypeptide that is identical to the polypeptide encoded by the native gene. In still other embodiments, the exogenous 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 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 the coding region of one or more native genes. In still other embodiments, the exogenous gene may encode a polypeptide that has less than 50% sequence identity to a polypeptide encoded by a native gene, but which nonetheless has the same function as the native polypeptide in a succinate fermentation or reduction pathway (i.e., the ability to catalyze the same reaction between reaction intermediates).

[00126] In other embodiments, an exogenous succinate fermentation or reduction 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 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 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 gene is derived from a non-yeast source, the exogenous gene sequence may be codon optimized for expression in a yeast host cell.

[00127] In those embodiments where the exogenous succinate fermentation or reduction 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 gene from the source organism. In certain of these embodiments, the exogenous gene may be identical to a native 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 the coding region of a native gene from the source organism. In other embodiments, the exogenous 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 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 the coding region of one or more native genes from the source organism. In still other embodiments, the exogenous gene may encode a polypeptide that has less than 50% sequence identity to a polypeptide encoded by a native gene from the source organism, but which nonetheless has the same function as the native polypeptide from the source organism in an active succinate fermentation or active reduction pathway. An exogenous 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.

[00128] In certain embodiments, the genetically modified yeast cells provided herein have a reductive TCA active succinate fermentation pathway that proceeds via PEP or pyruvate, OAA, malate, and fumarate intermediates. In these embodiments, the cells comprise one or more succinate fermentation pathway genes encoding enzymes selected from the group consisting PPC, PYC, MDH, FUM, FRD, and/or succinate exporter genes. In certain

embodiments, the cells also have one or more active reduction pathways. In these embodiments, the cells comprise one or more reduction pathway genes encoding enzymes selected from the group consisting of G6PD, gluconolactonase, and 6PGDH. In certain embodiments, the cells may comprise all or part of an active oxidative TCA or glyoxylate shunt succinate fermentation pathway. In these embodiments, the cells comprise one or more genes encoding enzymes selected from the group consisting of citrate synthase, PDH, PFL, aconitase, IDH, α -KGDH, succinate thiokinase, isocitrate lyase, and malate synthase. In certain embodiments, the cells have reduced activity of endogenous succinate dehydrogenase (SDH), which catalyzes the back-reaction of succinate to fumarate.

[00129] 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 to OAA. In certain embodiments, a PPC gene may be derived from a bacterial source. For example, a PPC gene may be derived from an *E. coli* PPC gene encoding the amino acid sequence set forth in SEQ ID NO:4 or a *M. succiniciproducens* PPC gene encoding the amino acid sequence set forth in SEQ ID NO:6. 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:4 or 6. In certain embodiments, a bacterial-derived PPC gene may comprise the nucleotide sequence set forth in SEQ ID NOs:3 or 5, 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 NOs:3 or 5. In other embodiments, a PPC gene may be derived from a plant source.

[00130] 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 to OAA. 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:8, an *S. cerevisiae* PYC1 gene encoding the amino acid sequence set forth in SEQ ID NO:10, or a *K. marxianus* PYC1 gene encoding the amino acid sequence set forth in SEQ ID NO:12. 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:8, 10, or 12. In certain embodiments, a yeast-derived PYC gene may comprise the nucleotide sequence set forth in SEQ ID NOs:7, 9, or 11, 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 NOs:7, 9, or 11. In other embodiments, a PYC gene may be derived from a fungal source other than *R. oryzae*.

[00131] A "malate dehydrogenase gene" or "MDH 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, an MDH gene may be derived from a yeast source. For example, the MDH gene may be derived from an *I. orientalis* MDH1, MDH2, or MDH3 gene encoding the amino acid sequence set forth in SEQ ID NOs:14, 16, or 18, respectively, a *Z. rouxii* MDH gene encoding the amino acid sequence set forth in SEQ ID NO:168, or a *K. marxianus* MDH1, MDH2, or MDH3 gene encoding the amino acid sequence set forth in SEQ ID NOs:20, 22, or 24, respectively. 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, 16, 18, 168, 20, 22, or 24. In certain embodiments, a yeast-derived MDH gene may comprise the nucleotide sequence set forth in SEQ ID NOs:13, 15, 17, 167, 19, 21, or 23 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NOs:13, 15, 17, 167, 19, 21, or 23. In certain embodiments, an MDH gene may be derived from a bacterial source. For example, the MDH gene may be derived from an *E. coli* MDH gene encoding the amino acid sequence set forth in SEQ ID NO:170. 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:170. In certain embodiments, a bacterial-derived MDH gene may comprise the nucleotide sequence set forth in SEQ ID NO:169 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:169. In certain embodiments, an MDH gene may be derived from a fungal source. For example, the MDH gene may be derived from an *R. oryzae* MDH gene encoding the amino acid sequence set forth in SEQ ID NO:172. 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:172. In certain embodiments, a fungal-derived MDH gene may comprise the nucleotide sequence set forth in SEQ ID NO:171 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:171.

[00132] A "fumarase gene" or "FUM gene" as used herein refers to any gene that encodes a polypeptide with fumarase activity, meaning the ability to catalyze the conversion of malate to fumarate. In certain embodiments, a FUM gene may be derived from a yeast source. For example, the FUM gene may be derived from an *I. orientalis* FUM 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, a yeast-derived FUM 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, a FUM gene may be derived from a bacterial source outside of the *Mannheimia* genus.

[00133] A "fumarate reductase gene" or "FRD gene" as used herein refers to any gene that encodes a polypeptide with fumarate reductase activity, meaning the ability to catalyze the conversion of fumarate to succinate. In certain embodiments, an FRD gene may be derived from a yeast source. For example, the FRD gene may be derived from an *S. cerevisiae* FRD1 gene encoding the amino acid sequence set forth in SEQ ID NO:26, a *S. mikatae* FRD1 gene encoding the amino acid sequence set forth in SEQ ID NO:28, a *K. polyspora* FRD1 gene encoding the amino acid sequence set forth in SEQ ID NO:30, or a *K. marxianus* FRD1 gene encoding the amino acid sequence set forth in SEQ ID NO:32. 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:26, 28, 30, or 32. In certain embodiments, a yeast-derived FRD gene may comprise the nucleotide sequence set forth in SEQ ID NOs:25, 27, 29, or 31, 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 NOs:25, 27, 29, or 31. In certain embodiments, an FRD gene may be derived from protozoan source. For example, the FRD gene may be derived from a *T. brucei* FRD gene encoding the amino acid sequence set forth in SEQ ID NO:174, a *T. cruzi* FRD gene encoding the amino acid sequence set forth in SEQ ID NO:176, a *L. braziliensis* FRD gene encoding the amino acid sequence set forth in SEQ ID NO:178, or a *L. mexicana* FRD gene encoding the amino acid sequence set forth in SEQ ID NO:180. 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:174, 176, 178, or 180. In certain embodiments, a

protozoan-derived FRD gene may comprise the nucleotide sequence set forth in SEQ ID NOs:173, 175, 177, or 179, 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 NOs:173, 175, 177, or 179.

[00134] A "succinate exporter gene" as used herein refers to any gene that encodes a polypeptide with succinate export activity, meaning the ability to transport succinate out of a cell and into the extracellular environment. In certain embodiments, a succinate exporter gene may be derived from a fungal source. For example, the succinate exporter gene may be derived from a *S. pombe* malic anion exporter (MAE) gene encoding the amino acid sequence set forth in SEQ ID NO:182 or an *A. oryzae* malic anion transporter encoding the amino acid sequence set forth in SEQ ID NO:184. 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:182 or 184. In certain embodiments, a fungal-derived succinate exporter gene may comprise the nucleotide sequence set forth in SEQ ID NOs:181 or 183, 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 NOs:181 or 183.

[00135] A "glucose 6-phosphate dehydrogenase" or "G6PD gene" as used herein refers to any gene that encodes a polypeptide with glucose 6-phosphate dehydrogenase activity, meaning the ability to catalyze the conversion of glucose 6-phosphate to 6-phosphogluconolactone. In certain embodiments, a G6PD gene may be derived from a yeast source. For example, the G6PD gene may be derived from an *I. orientalis* G6PD gene (ZWF1) 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 of SEQ ID NO:34. In certain embodiments, a yeast-derived G6PD gene may comprise the nucleotide sequence set forth in SEQ ID NO:33 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:33.

[00136] A "gluconolactonase gene" as used herein refers to any gene that encodes a polypeptide with gluconolactonase activity, meaning the ability to catalyze the conversion of 6-phosphogluconolactone to 6-phosphogluconate. In certain embodiments, a gluconolactonase gene may be derived from a yeast source. For example, the

gluconolactonase gene may be derived from an *I. orientalis* gluconolactonase gene encoding the amino acid sequence set forth in SEQ ID NO:36. 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:36. In certain embodiments, a yeast-derived gluconolactonase gene may comprise the nucleotide sequence set forth in SEQ ID NO:35 or a nucleotide sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the nucleotide sequence set forth in SEQ ID NO:35.

[00137] A "6-phosphogluconate dehydrogenase gene" or "6PGDH gene" as used herein refers to any gene that encodes a polypeptide with 6-phosphogluconate dehydrogenase activity, meaning the ability to catalyze the conversion of 6-phosphogluconate to ribulose-5-phosphate. In certain embodiments, a 6PGDH gene may be derived from a yeast source. For example, the 6PGDH gene may be derived from an *I. orientalis* 6PGDH gene encoding the amino acid sequence set forth in SEQ ID NO:38. 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:38. In certain embodiments, a yeast-derived 6PGDH gene may comprise the nucleotide sequence set forth in SEQ ID NO:37 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:37.

[00138] 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.

[00139] 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 succinate production.

[00140] 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.

[00141] In certain embodiments, the modified yeast cells provided herein comprise a deletion or disruption of one or more native genes encoding an enzyme involved in ethanol fermentation or consumption, including for example pyruvate decarboxylase (PDC, catalyzes the conversion of pyruvate to acetaldehyde) and/or alcohol dehydrogenase 1 (ADH1, catalyzes the conversion of acetaldehyde to ethanol) or 2 (ADH2, catalyzes the conversion of ethanol to acetaldehyde). Such modifications decrease the ability of the yeast cell to produce ethanol, thereby maximizing succinate production. In certain embodiments wherein the modified yeast cell is *I. orientalis*, the cells comprise a deletion or disruption of a PDC gene encoding the amino acid sequence of SEQ ID NO:40, an ADH_a gene encoding the amino acid sequence of SEQ ID NO:42, and/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:40 or 42. In certain of these embodiments, the deleted or disrupted gene may comprise the nucleotide sequence of SEQ ID NOs:39 or 41, 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 NOs:39 or 41.

[00142] In certain embodiments, the 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 by-products such as acetate or diols, including for example glycerol 3-phosphate dehydrogenase (GPD, catalyzes the conversion of dihydroxyacetone phosphate to glycerol 3-phosphate). In certain embodiments wherein the modified yeast cell is *I. orientalis*, the cells comprise a deletion or disruption of a GPD gene encoding the amino acid sequence of SEQ ID NO:44 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 NO:44. In certain of these embodiments, the deleted or disrupted GPD gene may comprise the nucleotide sequence of SEQ ID NO:43 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:43.

[00143] In certain embodiments, the 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 succinate fermentation pathway. For example, in certain embodiments the modified yeast cells provided herein comprise a deletion or disruption of a native PEP carboxykinase (PCK) gene, which encodes an enzyme that converts OAA to PEP. In certain embodiments wherein the modified yeast cell is *I. orientalis*, the cells comprise a deletion or disruption of a PCK gene encoding the amino acid sequence of SEQ ID NO:46 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 NO:46. In certain of these embodiments, the deleted or disrupted PCK gene may comprise the nucleotide sequence of SEQ ID NO:45 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:45. In another example, the modified yeast cells provided herein comprise a deletion or disruption of a native malic enzyme (MAE) gene, which encodes an enzyme that converts malate to pyruvate. In certain embodiments wherein the modified yeast cell is *I. orientalis*, the cells comprise a deletion or disruption of an MAE gene encoding the amino acid sequence of SEQ ID NO:48 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 NO:48. In certain of these embodiments, the deleted or disrupted MAE gene may comprise the nucleotide sequence of SEQ ID NO:47 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:47. In another example, the modified yeast cells provided herein comprise a deletion or disruption of a native succinate importer gene, which as used herein refers to any gene that encodes a polypeptide that allows for growth on and consumption of succinate. In certain embodiments wherein the modified yeast cell is *I. orientalis*, the cells comprise a deletion or disruption of a succinate importer gene RIOR43690 encoding the amino acid sequence of SEQ ID NO:186 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 NO:186. In certain of these embodiments, the deleted or disrupted RIOR43690 gene may comprise the nucleotide sequence of SEQ ID NO:185 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:185.

[00144] 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 succinate fermentation pathway product or intermediate.

[00145] 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 succinate fermentation pathway, including for example native genes encoding an enzyme selected from the group consisting of ammonia transport outward (ATO), L-lactate cytochrome-c oxidoreductase (CYB2A or CYB2B, catalyzes the conversion of lactate to pyruvate), and alcohol dehydrogenase (ADHa or ADHb, catalyzes the conversion between acetaldehyde and ethanol). Deletion or disruption of neutral genes allows for insertion of one or more exogenous genes without affecting succinate fermentation pathways. In certain embodiments wherein the modified yeast cell is *S. orientalis*, the cells comprise a deletion or disruption of a CYB2A gene encoding the amino acid sequence of SEQ ID NO:50, a CYB2B gene encoding the amino acid sequence of SEQ ID NO:52, an ATO2 gene encoding the amino acid sequence of SEQ ID NO:54 an ADHb gene encoding the amino acid sequence of SEQ ID NO:217, and/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:50, 52, 54, or 217. In certain of these embodiments, the deleted or disrupted gene may comprise the nucleotide sequence of SEQ ID NOs:49, 51, 53, or 216 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 NOs:49, 51, 53, or 216.

[00146] In certain embodiments, the genetically modified yeast cells provided herein are succinate resistant yeast cells. A "succinate-resistant yeast cell" as used herein refers to a yeast cell that exhibits a growth rate in media containing 75 g/L or greater succinate at pH 2.8 that is at least 50% as high as its growth rate in the same media containing 0 g/L succinate (see, e.g., the cells disclosed in Example 1 below). In certain of these embodiments, the yeast cells may exhibit succinate resistance in their native form. In other embodiments, the cells may have undergone mutation and/or selection before, during, or after introduction of genetic modifications related to an active succinate fermentation

pathway, such that the mutated and/or selected cells possess a higher degree of resistance to succinate than wild-type cells of the same species. In certain embodiments, mutation and/or selection may be carried out on cells that exhibit succinate 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 succinate in order to determine their potential as industrial hosts for succinate production. In addition to succinate resistance, the yeast cells provided herein may have undergone mutation and/or selection for resistance to one or more additional organic acids or to other fermentation products, by-products, or media components.

[00147] Selection for resistance to succinate or other compounds may be accomplished using methods well known in the art. For example, selection may be carried out using a chemostat. A chemostat is a device 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)).

[00148] As disclosed herein, yeast strains exhibiting succinate resistance were identified based on their growth rate and glucose consumption rates in succinate containing media. One such succinate resistant strain was *I. orientalis* strain CD1822. Strain CD1822 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 glucose 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 DM 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, with no pH adjustment in DM defined medium. Strain CD1822 was a single isolate selected based on the measured fermentation rates and growth rates.

[00149] Yeast strains exhibiting the best combinations of growth and glucose consumption in succinate media as disclosed in the examples below are preferred host cells for various genetic modifications relating to succinate fermentation pathways. Yeast genera that possess the potential for a high degree of succinate resistance, as indicated by growth in the presence of 150 g/L succinate at a pH of 2.8, include for example *Issatchenkia* and *Candida*. Other yeast genera with the potential for a relatively high degree of succinate resistance, as indicated by growth in the presence of 100 g/L succinate, include for example *Pichia*, *Zygosaccharomyces*, *Kluyveromyces*, *Saccharomyces*, *Debaryomyces*, and *Saccharomycopsis*. Species exhibiting a high degree of succinate resistance included *I. orientalis* (also known as *Candida krusei*), *C. lambica* (also known as *Pichia fermentans*), *C. sorboxylosa*, *C. zemlinina*, *C. geochares*, *P. membranifaciens*, *Z. kombuchaensis*, *C. sorbosivorans*, *K. marxianus*, *C. vanderwaltii*, *C. sorbophila*, *Z. bisporus*, *Z. lentus*, *S. bayanus*, *S. bulderi*, *D. castellii*, *C. boidinii*, *C. etchellsii*, *K. lactis*, *P. jadinii*, *P. anomala*, and *S. crataegensis*. *I. orientalis* and *C. lambica* belong to the *I. orientalis*/*P. fermentans* clade. Specific strains exhibiting succinate resistance included *I. orientalis* strains PTA-6658, 60585, and 24210, *C. lambica* strain 38617, and *C. sorboxylosa* strain 24120.

[00150] 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 succinate 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.

[00151] 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* or *Candida*, and in certain of these embodiments the host cell belongs to the *I. orientalis*/*P. fermentans* clade. In certain of embodiments, the host cell is *I. orientalis* or *C. lambica*.

[00152] The *I. orientalis*/*P. fermentans* clade is the most terminal clade that contains at least the species *I. orientalis*, *Pichia galeiformis*, *Pichia* sp. YB-4149 (NRRL designation), *Candida ethanolica*, *Pichia 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.

[00153] 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 *C. 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)).

[00154] The ideal yeast cell for succinate production is ideally capable of conducting fermentation 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 conducting fermentation at low pH levels.

[00155] A suitable host cell may possess one or more favorable characteristics in addition to succinate resistance and/or low pH growth capability. For example, potential host cells exhibiting succinate 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 succinate fermentation or reduction pathway, or they may be evaluated after one or more such modifications have taken place.

[00156] The level of gene expression and/or the number of exogenous genes to be utilized in a given cell will vary depending upon the identity of the host cell. For fully genome-sequenced yeasts, whole-genome stoichiometric models may be used to determine which enzymes should be expressed to develop a desired pathway succinate fermentation pathway. Whole-genome stoichiometric models are described in, for example, Hjersted *Biotechnol Bioeng* 97:1190 (2007) and Famili *Proc Natl Acad Sci USA* 100:13134 (2003).

[00157] 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 known in the art. 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 succinate fermentation or reduction pathway. Experiments may be conducted in which 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 succinate 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.

[00158] In certain embodiments, methods are provided for producing succinate from a genetically modified yeast cell as provided herein. In certain embodiments, these methods comprise providing a modified yeast cell as provided herein with at least one carbon source and culturing the yeast cell such that succinate is produced. The carbon source may be any carbon source that can be fermented by the yeast cell. Examples include, but are not limited to, twelve carbon sugars such as sucrose, hexose sugars such as glucose or fructose, glycan, starch, or other polymer of glucose, glucose oligomers such as maltose, maltotriose and isomaltotriose, panose, and fructose oligomers, and pentose sugars such as xylose, xylan, other oligomers of xylose, or arabinose. 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.

[00159] In certain embodiments, culturing of the cells provided herein to produce succinate may be divided up into phases. For example, the cell culture process may be divided into a cultivation phase, a production phase, and a recovery phase. The following represent examples of specific conditions that may be used for each of these phases. One of ordinary skill in the art will recognize that these conditions may be varied based on factors such as the species of yeast being used, the desired yield, or other factors.

[00160] 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 is a DM medium containing 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 is adjusted may be allowed to

range freely during cultivation, or may be buffered if necessary to prevent the pH from falling below or rising above predetermined levels. For example, the medium may be buffered to prevent the pH of the solution from falling below around 2.0 or rising above around 8.0 during cultivation. In certain of these embodiments, the medium may be buffered to prevent the pH of the solution from falling below around 3.0 or rising above around 7.0, and in certain of these embodiments the medium may be buffered to prevent the pH of the solution from falling below around 4.0 or rising above around 6.0. In certain embodiments, the fermentation medium is inoculated with sufficient yeast cells that are the subject of the evaluation to produce an OD₆₀₀ of 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 25-50 °C, and the cultivation time may be up to around 120 hours. During cultivation, aeration and agitation conditions are selected to produce a desired oxygen uptake rate. In one example, conditions are selected to produce an oxygen uptake rate of around 2-25 mmol/L/hr, preferably from around 5-20 mmol/L/hr, and more preferably from around 8-15 mmol/L/hr. "Oxygen uptake rate" or "OUR" as used herein refers to the volumetric rate at which oxygen is consumed during the fermentation. Inlet and outlet oxygen concentrations can be measured with exhaust gas analysis, for example by mass spectrometers. OUR can be calculated by one of ordinary skill in the art using the Direct Method described in *Bioreaction Engineering Principles* 2nd Edition, 2003, Kluwer Academic/Plenum Publishers, p. 449, equation 1.

[00161] In one example, the concentration of cells in the fermentation medium is typically in the range of about 0.1 to 20, preferably from about 0.1 to 5, even more preferably from about 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 can be varied throughout fermentation as a process control (see, e.g., WO03/102200). 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 about 2 to 25 mmol/L/hr, preferably from about 5 to 20 mmol/L/hr, and more preferably from about 8 to 15 mmol/L/hr. The medium may be buffered during the production phase such that the pH is maintained in a range of about 2.0 to about 8.0, about 3.0 to about 7.0, or about 3.5 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.

[00162] 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.

[00163] In other embodiments, the pH of the fermentation medium may be permitted to drop during cultivation from a starting pH that is at or above the lower pKa (4.207) of succinate, typically 8 or higher, to at or below the lower pKa of the acid fermentation product, such as in the range of about 2.0 to about 4.2, in the range of from about 3.0 to about 4.2, or in the range from about 3.8 to about 4.2.

[00164] In still other embodiments, fermentation may be carried out to produce a product acid by adjusting the pH of the fermentation broth to at or below the lower pKa of the product acid prior to or at the start of the fermentation process. The pH may thereafter be maintained at or below the lower pKa of the product acid throughout the cultivation. In certain embodiments, the pH may be maintained at a range of about 2.0 to about 4.2, in the range of from about 3.0 to about 4.2, or in the range from about 3.8 to about 4.2.

[00165] 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, succinate and ethanol may be co-produced. In these embodiments, ethanol may be produced at a yield of greater than 10%, greater than 25%, or greater than 50%.

[00166] In certain embodiments of the methods provided herein, the final yield of succinate 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. In certain embodiments, the cells provided herein are capable of converting at least 80% or at least 90% by weight of a carbon source to succinate. The concentration, or titer, of succinate 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 succinate may be increased by increasing or decreasing the temperature of the fermentation medium, particularly during the production phase.

[00167] 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

Example 1A: Selection of host yeast cells based on succinate tolerance:

[00168] A set of wild-type yeast strains were tested for their ability to grow in the presence of succinate.

[00169] The range of succinate concentrations to utilize in primary screening procedures was determined by evaluating the ability of seven wild-type yeast strains (*Candida sonorensis*, *Candida zemplinina*, *I. orientalis* strain PTA-6658, *K. lactis*, *K. marxianus*, *S. cerevisiae* strain CENPK 113-7D, and *I. orientalis* strain CD1822) to grow on media containing varying levels of succinate. 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 succinate to an OD₆₀₀ of 0.05. Plates were covered with a gas permeable membrane and incubated in a 30°C/300 rpm shaker overnight. The optical densities of 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 succinate concentration that one or more of the strains grew in (150 g/L) was chosen as the upper range for primary screening procedure.

[00170] For the primary screening procedure, 91 yeast strains were screened for growth on microtiter plates at 0 g/L, 100 g/L, or 150 g/L succinate and pH 2.8 using the same protocol used for range finding. Solubility issues presented challenges when trying to test succinate concentrations greater than 150 g/L, so low pH rather than higher succinate concentration was used to test more stringent conditions. For these samples, strains were screened for growth at 150 g/L succinate and pH 2.5.

[00171] 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 of each well were measured at 600 nm in a GENios model plate reader, and plates were observed visually for growth.

[00172] A similar protocol was run to evaluate growth at lactic acid concentration of 0 g/L, 30 g/L, 45 g/L, and 60 g/L. Table 1 summarizes the highest concentrations of succinate or lactic acid at which growth was observed.

Table 1: Primary screen for growth on succinate or lactic acid:

ATCC#	Genus/species	Lactic acid (g/L)	Succinate (g/L)
PTA-6658	<i>Issatchenkia orientalis</i>	60	150 (pH 2.5)
CD1822 (Cargill collection)	<i>Issatchenkia orientalis</i>	60	150 (pH 2.5)
PYCC 04-501	<i>Candida zemplinina</i>	60	100
Cargill	<i>Candida geochares</i>	60	100
NCYC 2696	<i>Pichia membranifaciens</i>	60	100
NCYC2897	<i>Zygosaccharomyces kombuchaensis</i>	60	100
mya-402	<i>Saccharomyces bulderi</i>	60	0
38619	<i>Candida sorbosivorans</i>	60	100
NCYC 535	<i>Schizosaccharomyces pombe</i>	60	0
52486	<i>Kluyveromyces marxianus</i>	45	100
113-7D	<i>Saccharomyces cerevisiae</i> - CENPK	45	0
MUCL 300000	<i>Candida vanderwaltii</i>	45	100
Cargill	<i>Candida sorbophila</i>	45	100
NCYC 3134	<i>Zygosaccharomyces bisporus</i>	45	100
NCYC 2928	<i>Zygosaccharomyces lentus</i>	45	100
NCYC 734	<i>Saccharomyces ludwigii</i>	45	0
60585	<i>Issatchenkia orientalis</i>	45	150 (pH 2.8)
46330	<i>Yarrowia lipolytica</i>	45	0
36946	<i>Zygosaccharomyces bailii</i>	45	0
60592	<i>Candida milleri</i>	45	0
38617	<i>Candida lambica</i>	45	150 (pH 2.8)
20306	<i>Candida rugosa</i>	45	0
28525	<i>Candida valida</i>	45	0
20347	<i>Candida zeylanoides</i>	45	0
24210	<i>Issatchenkia orientalis</i>	45	150 (pH 2.5)
20282	<i>Kodamaea ohmeri</i>	45	0
90739	<i>Saccharomyces bayanus</i>	45	100
MYA-404	<i>Saccharomyces bulderi</i>	45	0/100
MUCL 31237	<i>Saccharomycopsis javensis</i>	45	0

32109	<i>Candida sonorensis</i>	30	0
PYCC 70-1022	<i>Debaryomyces castellii</i>	30	100
PYCC 70-104	<i>Candida boidinii</i>	30	100
PYCC 60-8	<i>Candida etchellsii</i>	30	100
44691	<i>Candida kefyr</i>	30	0
34890	<i>Zygosaccharomyces rouxii</i>	30	0
60591	<i>Candida milleri</i>	30	0
24120	<i>Candida sorboxylosa</i>	30	150 (pH 2.5)
28526	<i>Pichia fermentans</i>	30	0
96784	<i>Saccharomyces cerevisiae</i>	0/30	0
52709	<i>Kluyveromyces thermotolerans</i>	0/30	0
NCYC 614	<i>Pachysolen tannophilus</i>	0/30	0
CBS 8452	<i>Wickerhamiella occidentalis</i>	0/30	0
18735	<i>Candida blankii</i>	0/30	0
8585	<i>Kluyveromyces lactis</i>	0	100
9950	<i>Pichia jadinii</i>	0	100
38623	<i>Candida fluvialis</i>	0	0
20033	<i>Saccharomyces capsularis</i>	0	NG
20284	<i>Candida famata</i>	0	NG
20118	<i>Candida guilliermondii</i>	0	0
20178	<i>Candida intermedia</i>	0	0
20179	<i>Candida parapsilosis</i>	0	0
96309	<i>Candida pseudolambica</i>	0	0
20280	<i>Debaryomyces polymorphus</i>	0	0
20277	<i>Dekkera anomala</i>	0	0
10563	<i>Dekkera lambica</i>	0	0
20030	<i>Hyphopichia burtonii</i>	0	0
9889	<i>Metschnikowia pulcherrima</i>	0	0
2102	<i>Pichia anomala</i>	0	0/100
24116	<i>Pichia nakasei</i>	0	0
16768	<i>Pichia silvicola</i>	0	0
34024	<i>Pichia strasburgensis</i>	0	0
2261	<i>Pichia tannicola</i>	0	0
76514	<i>Saccharomyces uvarum</i>	0	0
52714	<i>Torulaspora delbrueckii</i>	0	0
90197	<i>Yamadazyma guilliermondii</i>	0	0
20321	<i>Yamadazyma halophila</i>	0	0
MUCL 44417	<i>Saccharomycopsis crataegensis</i>	0	100
NRRL Y-7290	<i>Saccharomycopsis vini</i>	0	0
9950	<i>Pichia jadinii</i>	0	100
CBS 6054	<i>Pichia stipitis</i>	0	0
NCYC 2389	<i>Candida shehatae</i>	0	0

201225	<i>Yamadazyma stipitis</i>	0	0
10660	<i>Schizosaccharomyces japonicus</i>	0	NG
12659	<i>Lipomyces starkeyi</i>	0	0
42479	<i>Torulaspora pretoriensis</i>	0	0
90624	<i>Debaryomyces hansenii</i>	NG	NG
20117	<i>Candida catenulata</i>	NG	NG
96927	<i>Candida lactiscondensi</i>	NG	NG
36592	<i>Candida pignaliae</i>	NG	NG
34087	<i>Citeromyces matritensis</i>	NG	NG
36591	<i>Kluyveromyces yarrowii</i>	NG	NG
20292	<i>Nematospora coryli</i>	NG	NG
28778	<i>Pichia fluxuum</i>	NG	NG
58362	<i>Pichia toletana</i>	NG	NG
96272	<i>Bulleromyces albus</i>	NG	NG
MUCL 47216 or MUCL 31253B	<i>Candida tenuis</i>	NG	NG
20361	<i>Candida methanosorbosa</i>	NG	NG
NCYC 813	<i>Brettanomyces naardenensis</i>	NG	NG/0
76214	<i>Myxozyma kluyveri</i>	NG	NG
56306	<i>Lipomyces tetrasporus</i>	NG	NG
56465	<i>Candida naeodendra</i>	NG	NG

[00173] All six strains that exhibited growth at 150 g/L succinate were selected for secondary screening. For the first secondary screen, growth rates were measured in YPD media containing 0 g/L succinate at pH 3.0 or 75 g/L succinate at pH 2.85. 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. Results are summarized in Table 2.

Table 2: Growth rate in succinate:

Strain	0 g/L succinate (pH 3.0) (h ⁻¹)	75 g/L succinate (pH 2.85) (h ⁻¹)
<i>Issatchenkia orientalis</i> ATCC PTA-6658	0.71	0.50
<i>Issatchenkia orientalis</i> CD1822	0.69	0.47
<i>Issatchenkia orientalis</i> ATCC 60585	0.73	0.46
<i>Candida lambica</i> ATCC 38617	0.81	0.48
<i>Candida sorboxylosa</i> ATCC 24120	0.66	0.36

<i>Issatchenkia orientalis</i> ATCC 24210	0.74	0.45
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[00174] For the second secondary screen, glucose consumption was measured in YPD media containing 0 g/L succinate at pH 3.0 or 75 g/L succinate at pH 2.85. 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 (Yellow Springs Instruments, YSI). The resulting data was plotted and glucose consumption rates were established. Results are summarized in Table 3.

Table 3: Glucose consumption rate in succinate:

Strain	0 g/L succinate (pH 3.0) (g L ⁻¹ h ⁻¹)	75 g/L succinate (pH 2.85) (g L ⁻¹ h ⁻¹)
<i>Issatchenkia orientalis</i> ATCC PTA-6658	>4.2 g/L/h	>2.3 g/L/h
<i>Issatchenkia orientalis</i> CD1822	>4.2 g/L/h	>2.3 g/L/h
<i>Issatchenkia orientalis</i> ATCC 60585	>4.2 g/L/h	2.3-2.8 g/L/h
<i>Candida lambica</i> ATCC 38617	>4.2 g/L/h	>2.3 g/L/h
<i>Candida sorboxylosa</i> ATCC 24120	3.1 g/L/h	0.7 g/L/h
<i>Issatchenkia orientalis</i> ATCC 24210	>4.2 g/L/h	>2.3 g/L/h

[00175] To identify the most attractive candidates for succinate production, 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. 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 of 0 to 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. In accordance with the description above, the final score for each strain was calculated as follows:

$$\text{Final score for strain X} = \frac{(\text{actual growth rate in highest acid concentration} - \text{slowest growth rate}) * 0.25}{(\text{fastest growth rate} - \text{slowest growth rate})}$$

$$\begin{aligned}
 &+ \\
 &(\text{slope of growth rates of strain X} - \text{lowest growth rate slope}) * 0.25 \\
 &(\text{largest growth rate slope} - \text{lowest growth rate slope}) \\
 &+ \\
 &(\text{actual glycolytic rate in highest acid concentration} - \text{slowest glycolytic rate}) * 0.50 \\
 &(\text{fastest glycolytic rate} - \text{slowest glycolytic rate})
 \end{aligned}$$

[00176] Normalized values for each category and the final weighted score for each strain are summarized in Table 4.

Table 4: Normalized strain grades in succinate:

Strain	Growth rate @ 75g/L succinate	Growth rate slope	Glycolic rate	Weighted score
<i>Issatchenkia orientalis</i> ATCC PTA-6658	1.00	1.00	0.76	0.88
<i>Issatchenkia orientalis</i> CD1822	0.79	0.94	0.76	0.81
<i>Issatchenkia orientalis</i> ATCC 60585	0.71	0.50	1.00	0.80
<i>Candida lambica</i> ATCC 38617	0.86	0.00	0.76	0.60
<i>Candida sorboxylosa</i> ATCC 24120	0.00	0.25	0.00	0.06
<i>Issatchenkia orientalis</i> ATCC 24210	0.64	0.31	0.79	0.63

[00177] The same procedures were utilized to screen, rate, and score the original 91 yeast strains from the primary screen with media containing 0, 30, 45, and 60 g/L lactic acid at pH 3.0 (~80% free acid). Due to difficulties in properly scoring some very weak growth that occasionally occurred at 60 g/L, 21 strains were re-tested in the primary screen. Of these 21 strains, eight were eliminated due to very slow growth relative to the rest of the test group. The remaining 13 strains were advanced into secondary screening, and normalized values and weighted and summed scores were derived for each strain. These results are summarized in Table 5.

Table 5: Normalized strain grades in lactic acid:

Strain	Growth rate 50 g/L lactic acid	Growth rate slope	Glycolic rate	Weighted score
<i>Candida lambica</i> ATCC 38617	0.92	1	1	0.98
<i>Issatchenkia orientalis</i> ATCC PTA-6658	0.94	0.95	1	0.97
<i>Issatchenkia orientalis</i> CD1822	1.00	0.86	1	0.97
<i>Issatchenkia orientalis</i> ATCC 24210	0.89	0.73	1	0.91
<i>Candida zemplinina</i>	0.22	0.95	1	0.79

<i>Saccharomyces bulderi</i> ATCC MYA-404	0.47	0.45	1	0.73
<i>Saccharomyces bayanus</i>	0.08	0.91	0.96	0.73
<i>Saccharomyces bulderi</i> ATCC MYA-402	0.5	0.23	1	0.68
<i>Candida milleri</i> ATCC 60592	0	0.64	0.92	0.62
<i>Candida sorbosivorans</i>	0.28	0.95	0.59	0.60
<i>Kodamaea ohmeri</i>	0.42	0	0.76	0.49
<i>Candida geochares</i>	0.17	0.27	0.69	0.46
<i>Saccharomyces javensis</i>	0.11	0.68	0	0.20

[00178] Of the strains tested in lactic acid, only *S. javensis* did not achieve a 2.5 g/L/hr glucose utilization rate at pH 2.85 in media with 50 g/L lactic acid. While *I. orientalis* and *C. lambica* showed tolerance for both succinic and lactic acids, there were a number of species and strains that were tolerant for only one of the acids. Additionally, the rank order of the strains is different for each acid. This is even more clearly illustrated in the primary screen results (Table 1), where more strains were included. The most succinate tolerant strains are scattered among the top three tiers for lactic acid tolerance. Further, one of the strains that grew at the highest lactic acid concentration in the primary screen and scored highly in the secondary screen (*S. bulderi*) did not grow even at the lowest non-zero concentration of succinic acid tested. Thus, tolerance to lactic acid was shown to be a very poor predictor of tolerance to succinic acid, meaning that ideal strains for succinate production cannot be identified based on tolerance to lactic acid. This is further highlighted by comparing the strains that showed succinate 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 (*C. sonorensis*, *C. methanosorbosa*, *C. parapsilosis*, *C. naeodendra*, *C. krusei*, and *C. blankii*) 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 150 g/L succinate at either pH 2.5 or pH 2.8.

Example 1B: Mutagenesis and selection of mutant strains having succinate resistance:

[00179] Yeast cells selected in Example 1A are subjected to mutagenesis and exposed to selection pressure in order to identify mutants with high succinate tolerance.

[00180] 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, cell can be mutagenized using UV irradiation.

[00181] To select for succinate resistant mutant strains, aliquots of the EMS-treated cell suspension (approximately 2×10^8 of mutagenized cells) are plated onto a potato glucose agar (PDA) or another media containing succinate 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 succinate 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 succinate production requirements so that it has the necessary attributes for commercial succinate production.

Example 2: Deletion of both alleles of CYB2A, GPD1, and CYB2B from *I. orientalis* strain CD1822:

[00182] Both alleles of CYB2A, GPD1, and CYB2B are deleted from *I. orientalis* strain CD1822. As discussed above, CD1822 is an evolved lactic acid resistant strain isolated from a chemostat that also exhibited a high degree of succinate tolerance.

Example 2A: Deletion of both CYB2A alleles from *I. orientalis* strain CD1822:

[00183] Plasmids pMI449 (Figure 1) and pMI454 (Figure 2) are used to delete both copies of the L-lactate:ferricytochrome c oxidoreductase (CYB2A) gene (SEQ ID NO:49) in *I. orientalis* strain CD1822, a lactic acid resistant strain of *I. orientalis* isolated from the environment. pMI449 and pMI454 were both described in WO07/106524. Each plasmid contains 5' and 3' flanking regions from *I. orientalis* CYB2A separated by a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to a PGK promoter. This selection marker cassette is flanked on either end by a sequence ("KtSEQ") from *K. thermotolerans*. The 5' and 3' CYB2A flanking regions in pMI449 correspond to nucleotides from 913 to 487 bp upstream of the start of the predicted ORF and nucleotides from 90 to 676 bp downstream of the stop codon of the predicted ORF, respectively. The 5' and 3' CYB2A flanking regions in pMI454 correspond to nucleotides from 466 to 7 bp upstream of

the predicted ORF and nucleotides from 402 bp upstream to 77 bp downstream of the predicted stop codon, respectively.

[00184] The first CYB2A allele is deleted by transforming strain CD1822 with pMI449 digested with *SacI* using lithium acetate transformation (Gietz *Met Enzymol* 350:87 (2002)). Transformants are selected on yeast nitrogen base (YNB) + 2% melibiose plate containing x- α -gal (5-bromo-4-chloro-3-indolyl- α -D-galactoside). Blue-colored transformants are visible after around 4 days of growth at 30°C. Transformants are picked and plated for single colonies on Yeast Extract/Peptone/20 g/L glucose plates (YPD) containing x- α -gal. A single blue colony for each transformant is picked and re-streaked to YPD plates. Genomic DNA is isolated from the purified transformants, and replacement of the CYB2A gene is confirmed by PCR. To obtain strains where the MEL5 marker has undergone spontaneous recombination to excise it from the chromosome, the transformant is grown for several rounds in liquid YPD (100 g/L glucose) at 250 rpm and 30°C. A dilution series is plated onto YPD plates overlaid with x- α -gal, and grown overnight at 30°C. A white colony (indicative of the loop-out of the MEL5 marker cassette) is selected and re-streaked to YPD + x- α -gal plates. A white colony is selected and genomic DNA is prepared. Disruption of one allele of the native CYB2A gene is verified by PCR using primers oMM173 (SEQ ID NO:58) and oTM123 (SEQ ID NO:62).

[00185] The second CYB2A allele is deleted from this transformant by transforming with pMI454 digested with *SacI*. Transformants are obtained and purified as described above and analyzed by PCR for the absence of a 1000 bp CYB2A-specific PCR product using primers oMM175 (SEQ ID NO:60) and oMM176 (SEQ ID NO:61). The MEL5 marker derived from plasmid pMI454 is looped out of a transformant having a deletion of both CYB2A alleles via recombination as before, and confirmed by PCR using primers oMM172 (SEQ ID NO:57) and oMM173. This transformant is designated strain 2610.

Example 2B: Deletion of both GPD1 alleles from *I. orientalis* strain 2610:

[00186] Plasmid pBH165 (Figure 3) is used to delete one allele of the GPD1 gene (SEQ ID NO:43) from *I. orientalis* strain 2610. pBH165, which was described in WO07/106524, contains upstream and downstream fragments of the *I. orientalis* GPD1 gene separated by a selection marker cassette comprising *S. cerevisiae* MEL5 operatively linked to a PGK promoter and surrounded by KtSEQ flanking sequences. The upstream and downstream fragments of the GPD1 gene correspond to nucleotides from 1 to 302 bp and from 322 to 608 bp downstream of the start codon, respectively.

[00187] Strain 2610 is transformed with pBH165 digested with *NdeI* and *EcoRI* using lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plate overlaid with x- α -gal. Blue-colored transformants are visible after around 4 to 7 days of

growth at 30°C. Transformants are picked and plated for single colonies on YPD plates containing x- α -gal, and a single transformant is picked and re-streaked to YPD plates. Genomic DNA is isolated from the transformants, and disruption of one allele of the GPD1 gene is confirmed by one or more PCR reactions. The resulting transformant is designated strain 2639.

[00188] To obtain strains where the MEL5 marker has undergone spontaneous recombination to excise it from the chromosome, strain 2639 is grown for several rounds in liquid YPD (100g/L glucose) at 250 rpm and 30°C. A dilution series is plated onto YPD plates overlaid with x- α -gal, and grown overnight at 30°C. A white colony (indicative of the loop-out of the MEL5 marker cassette) is selected and re-streaked to YPD + x- α -gal plates. A white colony is selected and streaked onto a YPD plate. Disruption of one allele of the native GPD1 gene and loss of the MEL5 marker is verified by PCR. The resultant transformant is designated strain 2643.

[00189] Plasmid pCM188 (Figure 4) is used to delete the second GPD1 allele from *I. orientalis* strain 2643. pCM188 was generated by amplifying a 3' flanking region (corresponding to nucleotides from 1169 to 1770 bp downstream of the GPD1 gene start codon) of the GPD1 gene using primers CMO588 (SEQ ID NO:65) and CMO589 (SEQ ID NO:66). The primers included nucleotides for incorporating an XmaI site at the 5' end and an EcoRI site at the 3' end of the amplified DNA. The resultant product is digested with XmaI and EcoRI and ligated to similarly digested pBH165 to generate pCM188, which contains upstream and downstream fragments of the *I. orientalis* GPD1 gene separated by a selection marker cassette comprising *S. cerevisiae* MEL5 operatively linked to a PGK promoter and surrounded by KtSEQ flanking sequences.

[00190] Strain 2643 is transformed with pCM188 digested with NdeI and EcoRI using lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plate overlaid with x- α -gal. Blue-colored transformants are visible after around 4 to 7 days of growth at 30°C. Transformants are picked and plated for single colonies on YPD plates containing x- α -gal, and a single transformant is picked and re-streaked to YPD plates. Genomic DNA is isolated from the transformants, and disruption of the second GPD1 allele is confirmed by one or more PCR reactions. The resulting transformant is designated strain 2644.

[00191] To obtain strains where the MEL5 marker has undergone spontaneous recombination to excise it from the chromosome, strain 2644 is grown for several rounds in liquid YPD (100g/L glucose) at 250 rpm and 30°C. A dilution series is plated onto YPD plates overlaid with x- α -gal, and grown overnight at 30°C. A white colony (indicative of the loop-out of the MEL5 marker cassette) is selected and re-streaked to YPD + x- α -gal plates.

A white colony is selected and streaked onto a YPD plate. Disruption of the second allele of the native GPD1 gene and loss of the MEL5 marker is verified by PCR. The resultant transformant is designated strain 2652.

Example 2C: Deletion of both CYB2B alleles from *I. orientalis* strain 2652:

[00192] Plasmids pCA89 and pCA90 are used to delete one allele of the CYB2B gene (SEQ ID NO:51) from *I. orientalis* strain 2652. pCA89 (Figure 5) is generated by amplifying a 5' flanking region of CYB2B using PCR primers oCA385 (SEQ ID NO:67) and oCA386 (SEQ ID NO:68), which incorporates SacI and SbfI restriction sites into the PCR product, then digesting the product and ligating to pMI457 cut with the same enzymes. pMI457 (Figure 6) contains a MEL5 gene operatively linked to a PGK promoter and surrounded by KtSEQ flanking sequences. pCA90 (Figure 7) is generated by amplifying a 3' flanking region of CYB2B using PCR primers oCA387 (SEQ ID NO:69) and oCA388 (SEQ ID NO:70), which incorporates NheI and PspOMI restriction sites into the PCR product, then digesting the product and ligating to pMI457 cut with the same enzymes.

[00193] Strain 2652 is transformed with pCA89 digested with SacI and AgeI and pCA90 digested with XcmI and ApaI using lithium acetate transformation. The two plasmid fragments are able to recombine during transformation to form a functional MEL5 gene. Transformants are selected on YNB + 2% melibiose plate overlaid with x- α -gal. Blue-colored transformants are visible after around 4 to 7 days of growth at 30°C. Transformants are picked and plated for single colonies on YPD plates containing x- α -gal, and a single transformant is picked and re-streaked to YPD plates. Genomic DNA is isolated from the transformants, and replacement of a first CYB2B allele is confirmed by one or more PCR reactions. The resulting transformant is designated strain 2719.

[00194] To obtain strains where the MEL5 marker has undergone spontaneous recombination to excise it from the chromosome, strain 2719 is grown for several rounds in liquid YPD (100g/L glucose) at 250 rpm and 30°C. A dilution series is plated onto YPD plates overlaid with x- α -gal, and grown overnight at 30°C. A white colony (indicative of the loop-out of the MEL5 marker cassette) is selected and re-streaked to YPD + x- α -gal plates. A white colony is selected and streaked onto a YPD plate. Disruption of a first allele of the native CYB2B gene and loss of the MEL5 marker is verified by PCR. The resultant transformant is designated strain 2721.

[00195] The second CYB2B allele is deleted from strain 2721 by transforming with the same pCA89 and pCA90 fragments used to transform strain 2652. Transformants are obtained and purified as described above, and strains from which the MEL5 marker has been excised by recombination are generated using the methods described above. Disruption of both

CYB2B alleles and loss of the MEL5 gene is verified by PCR. The resultant transformant is designated strain 2732.

[00196] The various CYB2A, GPD1, and CYB2B deletion strains generated in Example 2 are summarized in Table 6.

Table 6: *I. orientalis* CYB2A, GPD1, and CYB2B deletion strains:

Strain name	Description	Parent strain
CD1822	Lactic acid-resistant parent strain	--
2610	CYB2A deletion (2)	
2639/2643	CYB2A deletion (2) GPD1 deletion (1)	2610
2644/2652	CYB2A deletion (2) GPD1 deletion (2)	2643
2719/2721	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (1)	2652
2732	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2)	2721

Example 3: Construction of cre expression plasmids pVB10 and pVB32:

[00197] The cre recombinase gene is synthesized using the native cre protein sequence as a reference. This gene is PCR amplified from template DNA (Blue Heron Biotechnologies) representing a codon-optimized version of the bacteriophage P1 CRE gene (SEQ ID NO:55, encoding polypeptide of SEQ ID NO:56) using PCR primers oVB5 (SEQ ID NO:72) and oVB6 (SEQ ID NO:73) and cloned into pCR2.1-TOPO (Invitrogen) to produce pVB15a. pVB15a is digested with BamHI and PaeI to generate a 1 kb cre fragment, and this fragment is ligated into similarly digested pHJJ17 (Figure 8). The resultant vector, pVB10 (Figure 9), contains the cre gene operatively linked to a PDC promoter and terminator. The vector also contains a URA3 selection marker gene from *I. orientalis*.

[00198] To replace the URA3 selection marker in pVB10 with the SUC2 selection marker, a SUC2 expression cassette was amplified from pTMC82. This SUC2 expression cassette contains the *S. cerevisiae* SUC2 gene operatively linked to the *I. orientalis* PGK1 promoter and the *S. cerevisiae* CYC1 terminator. Amplification was performed using primers oTM298 (SEQ ID NO:63) and oTM299 (SEQ ID NO:64), which add NsiI and Sall restriction sites to the product. The PCR product was digested with NsiI and Sall and ligated to pVB10 digested with Sall and PstI (NsiI and PstI have compatible cohesive ends) to produce pVB32 (Figure 10).

Example 4: Insertion of PYC1 at the PDC1 locus in *I. orientalis* strain 2732:

[00199] A PYC1 expression cassette is inserted at one or both PDC1 alleles in *I. orientalis* strain 2732 (Example 2).

Example 4A: Construction of PDC1 deletion construct pKWB21:

[00200] Upstream and downstream regions of the PDC1 gene (SEQ ID NO:39) were amplified in order to generate a PDC1 deletion construct. The upstream and downstream regions correspond to nucleotides from 496 bp upstream to the start codon of PDC1 and from the stop codon to 539 bp downstream, respectively. Amplification of the upstream region is performed using primers oKW70 (forward, SEQ ID NO:92) and oKW71 (reverse, SEQ ID NO:93), which adds a PmeI restriction site and NdeI, NotI, and SacI restriction sites, respectively, to the product. Amplification of the downstream region is performed using primers oKW72 (forward, SEQ ID NO:94) and oKW73 (reverse, SEQ ID NO:95), which adds NdeI, NotI, and SacI restriction sites and a PmeI restriction site, respectively, to the product. The two fragments are amplified independently, then assembled into a full-length product with a 2-stage PCR protocol. The first stage uses 10 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with no primers, and the second stage uses 20 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with upstream forward and downstream reverse primers. The full-length product is gel purified, cloned into pCR-BluntII (Invitrogen), and sequenced. The plasmid confirmed to have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final PDC1 deletion construct is designated pKWB21 (Figure 14).

Example 4B: Construction of *I. orientalis* PYC1 expression constructs pKF043 and pKF045:

[00201] The PYC1 gene from *I. orientalis* (SEQ ID NO:7) is amplified from genomic DNA using Phusion polymerase and primers oKF245 (SEQ ID NO:134) and oKF246 (SEQ ID NO:135), which contain an MluI site and an SbfI site, respectively. After amplification, the product is gel purified, digested with MluI and SbfI, and ligated to similarly digested pKF031 and pKF044. pKF031 (Figure 11) and pKF044 (Figure 12) are constructed from pUC19 backbones, and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKF031 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKF044 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites.

[00202] The plasmids are transformed into *E. coli*, and transformants are selected on LB plates containing 100 µg/ml carbenicillin and screened using primers flanking the NotI site of pKF031 and pKF044 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)).

Quickchange PCR is performed using primers oKW96 (SEQ ID NO:110) and oKW97 (SEQ ID NO:111) to eliminate an internal NdeI site (T2847C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKF043 (MEL5 marker) (Figure 15) and pKF045 (CYB2A marker) (Figure 16).

Example 4C: Construction of *S. cerevisiae* PYC1 expression constructs pKWB14 and pKWB15:

[00203] The PYC1 gene from *S. cerevisiae* (SEQ ID NO:9) is amplified from genomic DNA using Phusion polymerase and primers oKW29 (SEQ ID NO:80) and oKW30 (SEQ ID NO:81), both of which contain at their 5' end 23 bp flanking the NotI site in pKF031 and pKF044 to enable directional ligation-less cloning. After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044.

Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed using primers oKW81 (SEQ ID NO:102) and oKW82 (SEQ ID NO:103) to eliminate an internal NdeI site (T2838C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB14 (MEL5 marker) (Figure 17) and pKWB15 (CYB2A marker) (Figure 18).

Example 4D: Construction of *K. marxianus* PYC1 expression constructs pKWB16 and pKWB17:

[00204] *K. marxianus* is streaked on YPD plates, and after around 3 days the PYC1 gene (SEQ ID NO:11) is amplified from genomic DNA by colony PCR using primers oKW85 (SEQ ID NO:106) and oKW86 (SEQ ID NO:107). After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044. Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed using primers oKW83 (SEQ ID NO:104) and oKW84 (SEQ ID NO:105) to eliminate an internal SacI site (T1446A). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB16 (contains MEL5 marker) (Figure 19) and pKWB17 (contains CYB2A marker) (Figure 20).

Example 4E: Insertion of *I. orientalis* PYC1 at the first and second PDC1 loci in *I. orientalis* strain 2732:

[00205] pKF043 and pKF045 are both amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end. pKF043 is amplified using primers oKF243 (SEQ ID NO:132) and oKF244 (SEQ ID NO:133), and pKF045 is amplified using primers oKF255 (SEQ ID NO:137) and oKF244 (SEQ ID NO:133). Each of these primers contains on their 5' end 65 bp of sequence specific to the 65 bp immediately upstream and downstream of the

PDC1 locus in *I. orientalis*. This recombination sequence enables double recombination and integration at the PDC1 locus.

[00206] The PCR product amplified from pKF043 is used to transform *I. orientalis* strain 2732. Transformants are selected on YNB + melibiose+ x- α -gal and, and integration of PYC1 at a first PDC1 allele is confirmed by PCR using primers oCM566 (SEQ ID NO:138), oKF151 (SEQ ID NO:129), oKF252 (SEQ ID NO:136), and oCM587 (SEQ ID NO:139). The correct heterozygous strain is designated SSK10.

[00207] Strain SSK10 is transformed with the PCR product from pKF045 amplification to generate a homozygous strain with PYC1 inserted at both PDC1 alleles. Integration is confirmed by PCR using the primers oCM566 (SEQ ID NO:138), oMM174 (SEQ ID NO:59), oCM587 (SEQ ID NO:139), and oCA397 (SEQ ID NO:71). The correct homozygous strain is designated 12339.

[00208] For marker recycling, *I. orientalis* 12339 was grown to around OD₆₀₀ of 1.0 in YP +100g/L glucose (50ml media in a 250ml flask; 30 °C/250rpm). Cells were transformed with pVB32 using lithium acetate transformation, and transformants were selected on YNB + 2% sucrose plates overlaid with x- α -gal. After 4 to 5 days, white colonies were streaked to YP + 20g/L glucose plates overlaid with x- α -gal and grown at 37 °C for 2 days. Genomic DNA from white colonies was screened for retention of the expression cassette at the *I. orientalis* PDC1 locus and for loss of the selectable markers using PCR primers oGPB9 (SEQ ID NO:140), oGPB10 (SEQ ID NO:141), oGPB11 (SEQ ID NO:142), and oGPB12 (SEQ ID NO:143). Positive transformants were confirmed to have lost the marker by a phenotypic screen showing no growth on YNB + 2% lactic, 2% melabiose, or 2% sucrose. The homozygous strain with both markers removed was designated 12429.

Example 4F: Insertion of *S. cerevisiae* PYC1 at first and second PDC1 loci in *I. orientalis* strain 12429:

[00209] pKWB14 and pKWB15 are both digested with NdeI/SacI to liberate the fragment containing the marker cassette, ENO promoter, PYC1 gene, and terminator. These fragments are cloned into pKWB21 digested with NdeI and SacI, and the resultant plasmid is transformed into *E. coli*. Transformants are selected on LB + kanamycin, and colonies are screened with M13F and M13R primers. Clones having the desired insert are designated pKWB45 (MEL5 marker) and pKWB46 (CYB2B marker).

[00210] pKWB45 is digested with PmeI, gel purified, and transformed into *I. orientalis* strain 12429. Transformants are selected on YNB + lactate or YNB + melibiose + x-a-gal and screened by PCR using flanking primers oKW70 (SEQ ID NO:92) and oKW73 (SEQ ID NO:95) and nested primers oGPB53 (SEQ ID NO:149), oGPB55 (SEQ ID NO:151), oKW121 (SEQ ID NO:121), oKW122 (SEQ ID NO:122) to verify correct insertion at the PDC1 locus.

A heterozygous strain with the *S. cerevisiae* PYC1 gene inserted at a first PDC1 locus is designated ySBCK9.

[00211] Strain ySBCK9 is transformed with pKWB46 digested with PmeI, and transformants are screened for integration as above. Strains homozygous for *S. cerevisiae* PYC1 at the PDC1 loci are designated ySBCK10.

[00212] Marker recycling is carried out, and the homozygous strain with both markers removed is designated ySBCK11.

Example 4G: Insertion of *K. marxianus* PYC1 at first and second PDC1 loci in *I. orientalis* strain 12429:

[00213] pKWB16 and pKWB17 are both digested with NdeI/SacI to liberate the fragment containing the marker cassette, ENO promoter, PYC gene, and terminator. These fragments are cloned into pKWB21 digested with NdeI and SacI, and the resultant plasmid is transformed into *E. coli*. Transformants are selected on LB + kanamycin, and colonies are screened with M13F and M13R primers. Clones having the desired insert are designated pKWB47 (MEL5 marker) and pKWB48 (CYB2A marker).

[00214] pKWB47 is digested with PmeI, gel purified, and transformed into *I. orientalis* 12429. Transformants are selected on YNB + lactate or YNB + melibiose and screened by PCR using flanking primers oKW70 (SEQ ID NO:92) and oKW73 (SEQ ID NO:95) and nested primers oGPB53 (SEQ ID NO:149), oGPB55 (SEQ ID NO:151), oKW83 (SEQ ID NO:104), oKW84 (SEQ ID NO:105) to verify correct insertion at the PDC1 locus. A heterozygous strain *K. marxianus* PYC1 gene inserted at a first PDC1 locus is designated ySBCK12.

[00215] Strain ySBCK12 is transformed with pKWB48 digested with PmeI, and transformants are screened for integration as above. Strains homozygous for *K. marxianus* PYC1 are designated ySBCK13. Marker recycling is carried out, and the homozygous strain with both markers removed is designated ySBCK14.

[00216] The various PYC1 insertion/PDC1 deletion strains generated in Example 4 are summarized in Table 7.

Table 7: *I. orientalis* PYC1 insertion strains:

Strain name	Description	Parent strain
SSK10	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (1)	2732
12339/12429	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2)	SSK10
ySBCK9	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>S. cerevisiae</i> PYC1 insertion at PDC1 (1)	12429
ySBCK10/ySBCK11	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>S. cerevisiae</i> PYC1 insertion at PDC1 (2)	ySBCK9
ySBCK12	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>K. marxianus</i> PYC1 insertion at PDC1 (1)	12429
ySBCK13/ ySBCK14	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>K. marxianus</i> PYC1 insertion at PDC1 (2)	ySBCK12

Example 5: Insertion of MDH at the ATO2 locus in *I. orientalis* strain 12429:

[00217] An MDH expression cassette is inserted at one or both ATO2 alleles of *I. orientalis* strain 12429 (Example 4).

Example 5A: Construction of ATO2 deletion construct pKWB18:

[00218] Upstream and downstream regions of *I. orientalis* ATO2 (SEQ ID NO:53) were amplified in order to generate an ATO2 deletion construct. The upstream and downstream regions correspond to nucleotides from 419 bp upstream to the start codon of ATO2 and from the stop codon to 625 bp downstream, respectively. Amplification of the upstream region is performed using primers oKW66 (forward, SEQ ID NO:88) and oKW67 (reverse, SEQ ID NO:89), which adds a PmeI restriction site and NdeI, NotI, and SacI restriction sites, respectively, to the product. Amplification of the downstream region is performed using primers oKW68 (forward, SEQ ID NO:90) and oKW69 (reverse, SEQ ID NO:91), which adds NdeI, NotI, and SacI restriction sites and a PmeI restriction site, respectively, to the product. The two fragments are amplified independently, then assembled into a full-length product with a two stage PCR protocol. The first stage uses 10 cycles (98°C 10s, 55°C 20s, 72°C 60s) with no primers, and the second stage uses 20 cycles (98°C 10s, 55°C 20s, 72°C 60s) with upstream forward and downstream reverse primers. The full-length product is gel purified, cloned into pCR-BluntII (Invitrogen), and sequenced. The plasmid confirmed to

have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid-borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final ATO2 deletion construct is designated pKWB18 (Figure 13).

Example 5B: Construction of *I. orientalis* MDH expression constructs pKWB2-pKWB7:

[00219] The MDH1, MDH2, and MDH3 genes from *I. orientalis* (SEQ ID NOs:13, 15, and 17, respectively) are amplified from genomic DNA using primers designed for ligation-less cloning into the NotI site of pKF031 and pKF044. MDH1 is amplified using primers oKW13 (SEQ ID NO:74) and oKW14 (SEQ ID NO:75), MDH2 is amplified using primers oKW15 (SEQ ID NO:76) and oKW16 (SEQ ID NO:77), and MDH3 is amplified using primers oKW114 (SEQ ID NO:118) and oKW18 (SEQ ID NO:79). After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044. Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 and oKW95. Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB2 (MDH1, MEL5 marker), pKWB3 (MDH2, MEL5 marker), pKWB4 (MDH3, MEL5 marker), pKWB5 (MDH1, CYB2A marker), pKWB6 (MDH2, CYB2A marker), and pKWB7 (MDH3, CYB2A marker).

Example 5C: Construction of *K. marxianus* MDH expression constructs pKWB8-pKWB13:

[00220] The MDH1, MDH2, and MDH3 genes from *K. marxianus* (SEQ ID NOs:19, 21, and 23, respectively) are amplified from genomic DNA using primers designed for ligation-less cloning into the NotI site of pKF031 and pKF044. MDH1 is amplified using primers oKW100 (SEQ ID NO:112) and oKW101 (SEQ ID NO:113), MDH2 is amplified using primers oKW102 (SEQ ID NO:114) and oKW103 (SEQ ID NO:115), and MDH3 is amplified using primers oKW104 (SEQ ID NO:116) and oKW105 (SEQ ID NO:117). After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044. Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed on MDH2 using primers oKW132 (SEQ ID NO:123), oKW133 (SEQ ID NO:124), oKW134 (SEQ ID NO:125), and oKW135 (SEQ ID NO:126) to eliminate internal SacI sites (G609A and G819A). Quickchange PCR is performed on MDH3 using primers oKW136 and oKW137 to eliminate an internal NdeI site (T18C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB8 (MDH1, MEL5 marker), pKWB9 (MDH2, MEL5 marker), pKWB10 (MDH3, MEL5 marker), pKWB11 (MDH1, CYB2a marker), pKWB12 (MDH2, CYB2a marker), and pKWB13 (MDH3, CYB2a marker).

Example 5D: Insertion of *I. orientalis* MDH1, MDH2, and MDH3 at first and second ATO2 loci in *I. orientalis* strain 12429:

[00221] pKWB2, pKWB3, pKWB4, pKWB5, pKWB6, and pKWB7 are digested with NdeI and SacI to liberate the fragment containing selectable marker, ENO promoter, *I. orientalis* MDH1, 2, or 3, and terminator. These fragments are cloned into NdeI/SacI digested pKWB18 (ATO2 deletion construct), followed by selection on LB + kanamycin. Colonies are screened with M13F (SEQ ID NO:152) and M13R (SEQ ID NO:153) primers to confirm correct clones, which are designated pKWB33 (MDH1, MEL5), pKWB34 (MDH2, MEL5), pKWB35 (MDH3, MEL5), pKWB36 (MDH1, CYB2A), pKWB37 (MDH2, CYB2A), and pKWB38 (MDH3, CYB2A).

[00222] pKWB33, pKWB34, and pKWB35 are digested with PmeI and the appropriate fragments transformed into *I. orientalis* strain 12429 by lithium acetate transformation. Transformants are selected by growth on YNB + lactate or YNB + melibiose and screened by PCR with primers flanking the ATO2 locus (oKW66 (SEQ ID NO:88) and oKW69 (SEQ ID NO:91)) along with nested primers specific to each MDH (oKW13 (SEQ ID NO:74), oKW14 (SEQ ID NO:75), oKW15 (SEQ ID NO:76), oKW16 (SEQ ID NO:77), oKW114 (SEQ ID NO:118), oKW18 (SEQ ID NO:79)). Colonies with the correct insertion of MDH at a first ATO2 locus are designated ySBCK15 (MDH1), ySBCK18 (MDH2), and ySBCK21 (MDH3).

[00223] ySBCK15, ySBCK18, and ySBCK21 are transformed with pKWB36, pKWB37, and pKWB38, respectively, and transformants are screened for MDH integration as above. Strains homozygous for *I. orientalis* MDH at the ATO2 loci are designated ySBCK16 (MDH1), ySBCK19 (MDH2), and ySBCK22 (MDH3).

[00224] Marker recycling is carried out using pVB32, and homozygous strains with both markers removed are designated ySBCK17 (MDH1), ySBCK20 (MDH2), and ySBCK23 (MDH3).

Example 5E: Insertion of *K. marxianus* MDH1, MDH2, and MDH3 at first and second ATO2 loci in *I. orientalis* strain 12429:

[00225] pKWB8, pKWB9, pKWB10, pKWB11, pKWB12, and pKWB13 are digested with NdeI and SacI to liberate the fragment containing selectable marker, ENO promoter, *K. marxianus* MDH1, 2, or 3, and terminator. These fragments are cloned into NdeI/SacI digested pKWB18 (ATO2 deletion construct), transformed into *E. coli*, and selected on LB + kanamycin. Colonies are screened with M13F (SEQ ID NO:152) and M13R (SEQ ID NO:153) primers to confirm correct clones, which are designated pKWB39 (MDH1, MEL5), pKWB40 (MDH2, MEL5), pKWB41 (MDH3, MEL5), pKWB42 (MDH1, CYB2A), pKWB43 (MDH2, CYB2A), pKWB44 (MDH3, CYB2A).

[00226] pKWB39, pKWB40, and pKWB41 are digested with PmeI and the appropriate fragments are used to transform *I. orientalis* strain 12429 by lithium acetate transformation. Transformants are selected by growth on YNB + lactate or YNB + melibiose and screened by PCR with primers flanking the ATO2 locus (oKW66 (SEQ ID NO:88) and oKW69 (SEQ ID NO:91)) along with nested primers specific to each MDH (oKW100 (SEQ ID NO:112), oKW101 (SEQ ID NO:113), oKW102 (SEQ ID NO:114), oKW103 (SEQ ID NO:115), oKW104 (SEQ ID NO:116), oKW105 (SEQ ID NO:117)). Colonies with the correct insertion of MDH at a first ATO2 locus are designated ySBCK24 (MDH1), ySBCK27 (MDH2), and ySBCK30 (MDH3).

[00227] pKWB42, pKWB43, and pKWB44 are digested with PmeI and transformed into ySBCK24, ySBCK27, and ySBCK30, respectively, and transformants are screened for MDH integration as above. Strains homozygous for *K. marxianus* MDH at the ATO2 loci are designated ySBCK25 (MDH1), ySBCK28 (MDH2), and ySBCK31 (MDH3).

[00228] Marker recycling is carried out using pVB32, and homozygous strains with both markers removed are designated ySBCK26 (MDH1), ySBCK29 (MDH2), and ySBCK32 (MDH3).

[00229] The various MDH insertion/ATO2 deletion strains generated in Example 5 are summarized in Table 8.

Table 8: *I. orientalis* MDH insertion strains:

Strain name	Description	Parent strain
ySBCK15	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (1)	12429
ySBCK16/ySBCK17	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2)	ySBCK15
ySBCK18	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (1)	12429
ySBCK19/ySBCK20	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2)	ySBCK18
ySBCK21	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (1)	12429

ySBCK22/ySBCK23	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2)	ySBCK21
ySBCK24	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (1)	12429
ySBCK25/ySBCK26	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2)	ySBCK24
ySBCK27	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (1)	12429
ySBCK28/ySBCK29	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2)	ySBCK27
ySBCK30	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (1)	12429
ySBCK31/ySBCK32	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2)	ySBCK30

Example 6: Insertion of FRD1 at the ADHa locus in *I. orientalis* strains 12429, ySBCK17, ySBCK20, ySBCK23, ySBCK26, ySBCK29, and ySBCK32:

[00230] An FRD1 expression cassette is inserted at one or both ADHa alleles of *I. orientalis* strains 12429 (Example 4) and strains ySBCK17, ySBCK20, ySBCK23, ySBCK26, ySBCK29, and ySBCK32 (Example 5).

Example 6A: Construction of ADHa deletion constructs pGPB11, pGPB14, pGPB28, and pGPB34:

[00231] pKF044 was used as a template for quickchange mutagenesis using oligonucleotides oKW64 (SEQ ID NO:86) and oKW65 (SEQ ID NO:87) to delete an EcoRI site at nucleotide 932 of the *I. orientalis* CYB2A gene. The resulting plasmid was designated pKW49. pKW49 was digested with EcoRI and BglII and the resultant fragment ligated to EcoRI and BamHI digested pHJJ23 (Figure 21). The resulting ADHa deletion construct, designated pGPB11 (Figure 22), contains the *I. orientalis* PDC1 promoter (amplified using

primers oJLJ3 (SEQ ID NO:156) and oJLJ19 (SEQ ID NO:157)) and terminator (amplified using primers oJLJ1 (SEQ ID NO:154) and oJLJ2 (SEQ ID NO:155)) and a CYB2A marker element between an 858 bp fragment corresponding to the region immediately 5' of the *I. orientalis* AHD2a open reading frame (amplified using primers oHJJ71 (SEQ ID NO:159) and oHJJ72 (SEQ ID NO:160)) and a 996 bp fragment corresponding to the region immediately 3' of the *I. orientalis* ADHa open reading frame (amplified using primer oHJJ73 (SEQ ID NO:161) and oHJJ74 (SEQ ID NO:162)).

[00232] pKF046, which contains an *S. cerevisiae* MEL5 marker gene operatively linked to a *I. orientalis* PGK promoter and an *S. cerevisiae* MEL5 terminator and flanked by LoxP sites, was used as a template for quickchange mutagenesis using oligonucleotides oKW74 (SEQ ID NO:96) and oKW75 (SEQ ID NO:97) to delete an EcoRI site at nucleotide 2392 of the plasmid. The resulting plasmid was designated pKW50. pKW50 was digested with EcoRI and BglII and the resultant fragment ligated to EcoRI and BamHI digested pHJJ23. The resulting plasmid, designated pGPB14 (Figure 24), contains the same elements as pGPB11, but with the CYB2A selectable marker element replaced by the *S. cerevisiae* MEL5 selectable marker element.

[00233] pGPB11 and pGPB14 were each digested with EcoRI and BamHI to remove those portions of the plasmids corresponding to the PDC promoter and terminator, and each plasmid backbone was blunted with Klenow fragment and ligated to recircularize the plasmid. The plasmids were then transformed into *E. coli*. Plasmid isolated from positive colonies was designated pGPB28 (Figure 23, derived from pGPB11) and pGPB34 (Figure 25, derived from pGPB14).

Example 6B: Construction of FRD1 expression constructs pGPB20, pGPB22, pGPB25, pGPB26, pGPB36, pGPB37, pGPB39, and pGPB40:

[00234] Expression cassettes for the FRD1 gene from various sources were inserted into the ADHa deletion construct pGPB11. Sources for the FRD1 gene were *S. cerevisiae* (SEQ ID NO:25), *S. mikatae* (SEQ ID NO:27), *K. polyspora* (SEQ ID NO:29), and *K. marxianus* (SEQ ID NO:31). The latter three genes were all codon optimized to *I. orientalis*.

[00235] Plasmids containing *S. cerevisiae*, *S. mikatae*, *K. polyspora*, or *K. marxianus* FRD1 genes were digested with XbaI and PacI, and the FRD1 fragments were ligated to similarly digested pGPB11. The resulting plasmids, which contained the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and also contained the *I. orientalis* CYB2A selectable marker, were designated pGPB20 (*S. mikatae* FRD1), pGP22 (*K. marxianus* FRD1), pGPB25 (*K. polyspora* FRD1), and pGPB26 (*S. cerevisiae* FRD1) (Figure 26).

[00236] pGPB20, pGP22, pGPB25, and pGPB26 were digested with BamHI and NdeI and ligated to similarly digested pGPB14. The resulting plasmids, which contained the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and also contained the *S. cerevisiae* MEL5 selectable marker were designated pGPB36 (*S. mikatae* FRD1), pGP37 (*K. marxianus* FRD1), pGPB39 (*K. polyspora* FRD1), and pGPB40 (*S. cerevisiae* FRD1) (Figure 27).

Example 6C: Insertion of *K. marxianus* FRD1 at first ADHa loci of *I. orientalis* strain 12429:

[00237] pGPB22 is digested with SacI and ApaI and transformed into *I. orientalis* strain 12429 by lithium acetate transformation. Transformants are selected on YNB + 2% lactic plates overlaid with x- α -gal. After around six days, white transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. White colonies are picked, and genomic DNA is isolated and screened by PCR to confirm integration of the FRD1 expression cassette at the ADHa locus using primers oGPB47 (SEQ ID NO:147), oJLJ43 (SEQ ID NO:158), oKW64 (SEQ ID NO:86), and oGPB46 (SEQ ID NO:146). Strains with the correct integration of the FRD1 gene are designated ySBCG4, ySBCG5, and ySBCG6.

Example 6D: Insertion of FRD1 at first and second ADHa loci of *I. orientalis* strains

ySBCK17, ySBCK20, ySBCK23, ySBCK26, ySBCK29, and ySBCK32:

[00238] pGPB20 (*S. mikatae* FRD1), pGPB22 (*K. marxianus* FRD1), pGPB25 (*K. polyspora* FRD1), and pGPB26 (*S. cerevisiae* FRD1) are digested with SacI and ApaI and transformed into strains ySBCK17, ySBCK20, ySBCK23, ySBCK26, ySBCK29, and ySBCK32 by lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FRD1 expression cassette at a first ADHa locus using primers oGPB47 (SEQ ID NO:147), oJLJ43 (SEQ ID NO:158), oKW64 (SEQ ID NO:86), and oGPB46 (SEQ ID NO:146). The resulting strains are designated ySBCGH1-24.

[00239] pGPB36 (*S. mikatae* FRD1), pGPB37 (*K. marxianus* FRD1), pGPB39 (*K. polyspora* FRD1), and pGPB40 (*S. cerevisiae* FRD1) are digested with SacI and ApaI and transformed into strains ySBCGH1-24 by lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FRD1 expression cassette at the second ADHa locus using primers oGPB47 (SEQ ID NO:147), oJLJ43 (SEQ ID NO:158), oGPB54 (SEQ ID NO:150), and oGPB46 (SEQ ID NO:146). The resulting strains are designated ySBCGH25-48.

[00240] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH49-72.

[00241] The various FRD1 insertion/ADHa deletion strains generated in Example 6 are summarized in Table 9.

Table 9: *I. orientalis* FRD1 insertion strains:

Strain name	Description	Parent strain
ySBCG4/ySBCG5/ySBCG6	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	12429
ySBCGH1	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK17
ySBCGH25/ySBCGH49	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH1
ySBCGH2	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK17
ySBCGH26/ySBCGH50	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH2
ySBCGH3	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK17
ySBCGH27/ySBCGH51	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH3
ySBCGH4	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK17

ySBCGH28/ySBCGH52	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCGH4
ySBCGH5	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK20
ySBCGH29/ySBCGH53	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH5
ySBCGH6	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK20
ySBCGH30/ySBCGH54	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH6
ySBCGH7	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK20
ySBCGH31/ySBCGH55	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH7
ySBCGH8	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK20
ySBCGH32/ySBCGH56	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCGH8

ySBCGH9	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK23
ySBCGH33/ySBCGH57	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH9
ySBCGH10	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK23
ySBCGH34/ySBCGH58	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH10
ySBCGH11	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK23
ySBCGH35/ySBCGH59	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH11
ySBCGH12	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK23
ySBCGH36/ySBCGH60	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCGH12
ySBCGH13	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK26

ySBCGH37/ySBCGH61	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH13
ySBCGH14	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK26
ySBCGH38/ySBCGH62	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH14
ySBCGH15	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK26
ySBCGH39/ySBCGH63	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH15
ySBCGH16	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK26
ySBCGH40/ySBCGH64	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCGH16
ySBCGH17	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK29
ySBCGH41/ySBCGH65	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH17

ySBCGH18	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK29
ySBCGH42/ySBCGH66	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH18
ySBCGH19	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK29
ySBCGH43/ySBCGH67	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH19
ySBCGH20	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK29
ySBCGH44/ySBCGH68	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2)	ySBCGH20
ySBCGH21	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	ySBCK32
ySBCGH45/ySBCGH69	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCGH21
ySBCGH22	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	ySBCK32

ySBCGH46/ySBCGH70	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCGH22
ySBCGH23	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	ySBCK32
ySBCGH47/ySBCGH71	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCGH23
ySBCGH24	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	ySBCK32
ySBCGH48/ySBCGH72	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCGH24

Example 7: Insertion of single copies of *I. orientalis* MDH genes at the ATO2 locus in *I. orientalis* strain ySBCG5:

[00242] *I. orientalis* MDH1, -MDH2, or MDH3 expression cassettes are inserted at one or both ATO2 alleles of *I. orientalis* strain ySBCG5 (Example 6).

Example 7A: Insertion of *I. orientalis* MDH1 at a first ATO2 locus of *I. orientalis* strain ySBCG5:

[00243] A PCR product amplified with the primers oKF254 and oKF202 using pKWB2 as the template is transformed into strain ySBCG5 by lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plates overlaid with x- α -gal. Blue-colored transformants are visible after around 6 days of growth at 30°C. Transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. Blue colonies are picked, and genomic DNA is isolated and screened for correct integration of the MDH1 expression cassette at the ATO2 locus by PCR using primers oGPB55 (SEQ ID NO:151), oKW66 (SEQ ID NO:88), oKW69 (SEQ ID NO:91), and oGPB54 (SEQ ID NO:150). Sister strains with the correct integration of the MDH1 gene are designated ySBCG22 and ySBCG23.

Example 7B: Insertion of *I. orientalis* MDH2 at a first ATO2 loci of *I. orientalis* strain ySBCG5:

[00244] A PCR product amplified with the primers oKF254 and oKF202 using pKWB3 as the template is transformed into strain ySBCG5 by lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plates overlaid with x- α -gal. Blue-colored transformants are visible after around 6 days of growth at 30°C. Transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. Blue colonies are picked, and genomic DNA is isolated and screened for correct integration of the MDH2 expression cassette at the ATO2 locus by PCR using primers oGPB55 (SEQ ID NO:151), oKW66 (SEQ ID NO:88), oKW69 (SEQ ID NO:91), and oGPB54 (SEQ ID NO:150). Strains with the correct integration of the MDH2 gene are designated ySBCG25 and ySBCG26.

[00245] The various MDH insertion/ATO2 deletion strains generated in Example 7 are summarized in Table 10.

Table 10: *I. orientalis* MDH insertion strains:

Strain name	Description	Parent strain
ySBCG22/ySBCG23	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1) <i>I. orientalis</i> MDH1 insertion at ATO2 (1)	ySBCG5
ySBCG25/ySBCG26	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1) <i>I. orientalis</i> MDH2 insertion at ATO2 (1)	ySBCG5

Example 8: Insertion of *I. orientalis* FUM1 genes at the CYB2A locus in *I. orientalis* strains ySBCGH49-72:

[00246] *I. orientalis* FUM1 expression cassettes are inserted at both alleles of CYB2A of *I. orientalis* strains ySBCGH49-72 (Example 6).

Example 8A: Construction of *I. orientalis* FUM1 expression constructs pGPB30, pGPB42, pGPB44, and pGPB47:

[00247] An expression cassette for the *I. orientalis* FUM1 gene (SEQ ID NO:1) is inserted into the ADHa deletion construct pGPB11. PCR primers oGPB38 (SEQ ID NO:144) and oGPB40 (SEQ ID NO:145) are used to amplify FUM1 using *I. orientalis* genomic DNA as the template. The 5' primer adds an XbaI site at the start site of the coding sequence and the 3' primer adds a PaeI site 3' of the stop codon. The resulting PCR product is digested with XbaI and PaeI and ligated to similarly digested pGPB11. The resulting plasmid, which

contains the FUM1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and the CYB2A selectable marker, is designated pGPB30 (Figure 28).

[00248] pGPB30 is digested with BamHI and NdeI and ligated into similarly digested pGPB14. The resulting plasmid is designated pGPB44 (Figure 30).

[00249] The expression cassette from pGPB30 is excised using NotI and ligated to the NotI cut pKW22. The resulting plasmid is designated pGPB42 (Figure 29).

[00250] The expression cassette from pGPB44 is excised using NotI and ligated to the NotI cut pKW22. The resulting plasmid is designated pGPB47 (Figure 31).

Example 8B: Insertion of *I. orientalis* FUM1 at one or both *I. orientalis* CYB2B loci:

[00251] Integration of the first copy of the FUM1 expression cassette at the CYB2B locus is performed using plasmids containing the CYB2A selectable marker. pGPB42 is digested with SacI and ApaI and transformed into *I. orientalis* strains ySBCGH49-72 using lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FUM1 expression cassette at the first CYB2B locus using primers oKW117 (SEQ ID NO:119), oJLJ43 (SEQ ID NO:158), oKW120 (SEQ ID NO:120), and oGPB46 (SEQ ID NO:146). The resulting strains are designated ySBCGH73-96.

[00252] Integration of the second copy of the FUM1 expression cassette at the CYB2B locus is performed using plasmids containing the MEL5 selectable marker. pGPB47 is digested with SacI and ApaI and transformed into ySBCGH73-96 using lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FUM1 expression cassette at the second CYB2B locus using primers oKW117 (SEQ ID NO:119), oJLJ43 (SEQ ID NO:158), oKW120 (SEQ ID NO:120), and oGPB46 (SEQ ID NO:146). The resulting strains are designated ySBCGH97-120.

[00253] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH121-144.

[00254] The various FUM1 insertion/CYB2B deletion strains generated in Example 8 are summarized in Table 11.

Table 11: *I. orientalis* FUM1 insertion strains:

Strain name	Description	Parent strain
ySBCGH73	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH49
ySBCGH97/ySBCGH121	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH73
ySBCGH74	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH50
ySBCGH98/ySBCGH122	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH74
ySBCGH75	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH51
ySBCGH99/ySBCGH123	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH75
ySBCGH76	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH52

ySBCGH100/ySBCGH124	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH76
ySBCGH77	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH53
ySBCGH101/ySBCGH125	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH77
ySBCGH78	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH54
ySBCGH102/ySBCGH126	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH78
ySBCGH79	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH55
ySBCGH103/ySBCGH127	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH79

ySBCGH80	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH56
ySBCGH104/ySBCGH128	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH80
ySBCGH81	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH57
ySBCGH105/ySBCGH129	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH81
ySBCGH82	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH58
ySBCGH106/ySBCGH130	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH82
ySBCGH83	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH59

ySBCGH107/ySBCGH131	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH83
ySBCGH84	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH60
ySBCGH108/ySBCGH132	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH84
ySBCGH85	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH61
ySBCGH109/ySBCGH133	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH85
ySBCGH86	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH62
ySBCGH110/ySBCGH134	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH86

ySBCGH87	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH63
ySBCGH111/ySBCGH135	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH87
ySBCGH88	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH64
ySBCGH112/ySBCGH136	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH88
ySBCGH89	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH65
ySBCGH113/ySBCGH137	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH89
ySBCGH90	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH66

ySBCGH114/ySBCGH138	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH90
ySBCGH91	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH67
ySBCGH115/ySBCGH139	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH91
ySBCGH92	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH68
ySBCGH116/ySBCGH140	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH92
ySBCGH93	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH69
ySBCGH117/ySBCGH141	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH93

ySBCGH94	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH70
ySBCGH118/ySBCGH142	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH94
ySBCGH95	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH71
ySBCGH119/ySBCGH143	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH95
ySBCGH96	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	ySBCGH72
ySBCGH120/ySBCGH144	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH96

Example 9: Insertion of ZWF1 at the GPD1 locus in *I. orientalis* strains ySBCGH121-144:

[00255] A ZWF1 expression cassette is inserted at one or both GPD1 alleles in *I. orientalis* strains ySBCGH121-144 (Example 8).

Example 9A: Construction of *I. orientalis* ZWF1 expression constructs pKF033 and pGPB56:

[00256] The ZWF1 gene from *I. orientalis* (SEQ ID NO:33) is amplified from genomic DNA using Phusion polymerase and primers oKF168 (SEQ ID NO:131) and oKF163 (SEQ ID NO:130), which contain an MluI site and an SbfI site, respectively. After amplification, the product is gel purified, digested with MluI and SbfI, and ligated to similarly digested pKF031

(SEQ ID NO:127) and pKF044 (SEQ ID NO:128). pKF031 (Figure 32) and pKF044 (Figure 33) are constructed from pUC19 backbones, and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKF031 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKF044 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites.

[00257] The plasmids are transformed into *E. coli*, and transformants are selected on LB plates containing 100 µg/ml carbenicillin and screened using primers flanking the NotI site of pKF031 and pKF044 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKF033 (MEL5 marker) (Figure 34) and pGPB056 (CYB2A marker) (Figure 35).

Example 9B: Insertion of *I. orientalis* ZWF1 at the first and second GPD1 loci in *I. orientalis* strains ySBCGH121-144:

[00258] pKF033 and pGPB56 are both amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end using primers oGPBH1 (SEQ ID NO:163) and oGPBH2 (SEQ ID NO:164). Each of these primers contains on their 5' end 65 bp of sequence specific to the 65 bp immediately upstream and downstream of the GPD1 locus in *I. orientalis*. This recombination sequence enables double recombination and integration at the GPD1 locus.

[00259] The PCR product amplified from pKF033 is used to transform *I. orientalis* strains ySBCGH121-144. Transformants are selected on YNB + melibiose+ x-α-gal, and integration of ZWF1 at a first GPD1 allele is confirmed by PCR using primers oGPBH3 (SEQ ID NO:165), oGPBH4 (SEQ ID NO:166), oGPB55 (SEQ ID NO:151), and oGPB11 (SEQ ID NO:142). The correct heterozygous strains are designated ySBCGH145-168.

[00260] To generate homozygous strains with ZWF1 inserted at both GPD1 alleles, strains ySBCGH145-168 are transformed with the PCR product amplified from pGPB56. Transformants are selected on YNB + 2% lactic acid + x-α-gal, and integration of ZWF1 is confirmed by PCR using the primers oGPBH3 (SEQ ID NO:165), oGPBH4 (SEQ ID NO:166), oGPB53 (SEQ ID NO:149), and oGPB52 (SEQ ID NO:148). The correct homozygous strains are designated ySBCGH169-192.

[00261] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH193-216.

[00262] The various ZWF1 insertion/GPD1 deletion strains generated in Example 9 are summarized in Table 12.

Table 12: *I. orientalis* ZWF1 insertion strains:

Strain name	Description	Parent strain
ySBCGH145	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH121
ySBCGH169/ySBCGH193	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH145
ySBCGH146	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH122
ySBCGH170/ySBCGH194	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH146
ySBCGH147	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH123
ySBCGH171/ySBCGH195	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH147

ySBCGH148	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH124
ySBCGH172/ySBCGH196	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH148
ySBCGH149	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH125
ySBCGH173/ySBCGH197	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH149
ySBCGH150	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH126
ySBCGH174/ySBCGH198	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH150

ySBCGH151	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH127
ySBCGH175/ySBCGH199	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH151
ySBCGH152	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH128
ySBCGH176/ySBCGH200	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH152
ySBCGH153	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH129
ySBCGH177/ySBCGH201	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH153

ySBCGH154	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH130
ySBCGH178/ySBCGH202	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH154
ySBCGH155	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH131
ySBCGH179/ySBCGH203	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH155
ySBCGH156	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH132
ySBCGH180/ySBCGH204	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH156

ySBCGH157	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH133
ySBCGH181/ySBCGH205	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH157
ySBCGH158	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH134
ySBCGH182/ySBCGH206	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH158
ySBCGH159	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH135
ySBCGH183/ySBCGH207	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH159

ySBCGH160	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH136
ySBCGH184/ySBCGH208	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH160
ySBCGH161	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH137
ySBCGH185/ySBCGH209	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH161
ySBCGH162	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH138
ySBCGH186/ySBCGH210	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH162

ySBCGH163	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH139
ySBCGH187/ySBCGH211	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH163
ySBCGH164	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH140
ySBCGH188/ySBCGH212	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH164
ySBCGH165	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH141
ySBCGH189/ySBCGH213	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH165

ySBCGH166	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH142
ySBCGH190/ySBCGH214	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH166
ySBCGH167	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH143
ySBCGH191/ySBCGH215	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH167
ySBCGH168	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	ySBCGH144
ySBCGH192/ySBCGH216	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH168

Example 10: Shake flask characterization of succinate production in *I. orientalis* strains ySBCGH169/ySBCGH193:

[00263] Shake flasks are used to test the ZWF1 insertion strains ySBCGH169/ySBCGH193 (Example 9). Shake flasks are inoculated with biomass harvested from seed flasks grown overnight to an OD₆₀₀ of 2 to 6. 250 mL baffled flasks (50 mL working volume) are inoculated to an OD₆₀₀ of 0.2 and fermentation occurs at 100 rpm and 30 °C. DM defined medium is used in flasks, with pH control and CO₂ provided by calcium carbonate addition at a concentration of 0.255M (1.28 g CaCO₃ per 50 ml flask). Samples are taken throughout the time course of the assay and analyzed for biomass growth via OD₆₀₀, and succinate and glucose are monitored via high performance liquid chromatography (HPLC). The resulting data shows production of greater than 40 g/L succinate by strain ySBCGH169/ySBCGH193.

Example 11: Deletion of the first and second PCK1 loci in *I. orientalis* strains ySBCGH121-144:

[00264] The first and second PCK1 loci in *I. orientalis* strains ySBCGH121-144 (Example 8) are deleted using a PCK deletion construct.

Example 11A: Construction of *I. orientalis* PCK deletion constructs:

[00265] The PCK upstream region from 432 bp upstream to the start codon is amplified by PCR. Sequence corresponding to the restriction sites NdeI/NotI/SacI is added to the 5' end of the reverse upstream primer (oKW78, SEQ ID NO:99). A PmeI restriction site is added to the 5' end of the forward upstream primer (oKW77, SEQ ID NO:98). The PCK downstream region is amplified from the stop codon to 472 bp downstream. The downstream forward primer (oKW79, SEQ ID NO:100) contained the same NdeI/NotI/SacI sequence as the reverse upstream primer. The 5' end of the reverse downstream primer also has a PmeI site (oKW80, SEQ ID NO:101). The two fragments are amplified independently using Phusion polymerase, then assembled into a full-length (926 bp) product via a two-stage PCR protocol (10 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with no primers, followed by 20 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with upstream forward and downstream reverse primers). Full-length product is gel purified and cloned into pCR-BluntII (Invitrogen) and sequenced. The plasmid confirmed to have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final construct is named pKWB20 (Figure 39).

[00266] pKWB20 is digested with NdeI and SacI and the resulting 4.4kb DNA fragment is gel purified. Plasmid pKF031 is digested with NdeI and SacI to create a fragment of 3.7kb that contains the MEL5 marker flanked by loxP sites. In the same way, pKF044 is digested to create a 4.2kb fragment containing the CYB2A marker flanked by loxP sites. Marker fragments are ligated into the digested pKWB20 plasmid to create pKWB25 (Figure 40),

containing the MEL5 marker, and pKWB30 (Figure 41), containing the CYB2A marker.

Correct constructs are confirmed by PCR and restriction digestion.

Example 11B: Deletion of PCK1 in *I. orientalis* strains ySBCGH121-ySBCGH144:

[00267] Plasmid pKWB25 is digested with PmeI to create a 5kb fragment containing the MEL5 marker surrounded by PCK1 flanking sequence. The fragment is gel purified prior to transformation. In the same way, pKWB30 is digested with PmeI to create a 5.4kb fragment containing the CYB2A marker with PCK1 flanking sequence. The fragment is gel purified prior to transformation.

[00268] The DNA fragment from pKWB30, containing the CYB2A marker, is transformed into strains ySBCGH121-ySBCGH144. Transformants are selected on YNB + lactate, and deletion of PCK at the first allele is confirmed by PCR using primers oKW77 (SEQ ID NO:98), oKW80 (SEQ ID NO:101), oGPB52 (SEQ ID NO:148), and oGPB53 (SEQ ID NO:149). The correct heterozygous strains are designated ySBCGH289-312.

[00269] Strains ySBCGH289-312 are transformed with the PmeI digestion product from pKWB30 and selected on YNB + melibiose+ x- α -gal to generate a homozygous strain with PCK deleted at both alleles. Integration is confirmed by PCR using the primers oKW77 (SEQ ID NO:98), oKW80 (SEQ ID NO:101), oGPB54 (SEQ ID NO:150), and oGPB55 (SEQ ID NO:151). The correct homozygous strains are designated ySBCGH313-336.

[00270] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH337-360.

[00271] The various PCK deletion strains generated in Example 11 are summarized in Table 13.

Table 13: *I. orientalis* PCK deletion strains:

Strain name	Description	Parent strain
ySBCGH289	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH121
ySBCGH313/ySBCGH337	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH289

ySBCGH290	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH122
ySBCGH314/ySBCGH338	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH290
ySBCGH291	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH123
ySBCGH315/ySBCGH339	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH291
ySBCGH292	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH124
ySBCGH316/ySBCGH340	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH292

ySBCGH293	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH125
ySBCGH317/ySBCGH341	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH293
ySBCGH294	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH126
ySBCGH318/ySBCGH342	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH294
ySBCGH295	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH127
ySBCGH319/ySBCGH343	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH295

ySBCGH296	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH128
ySBCGH320/ySBCGH344	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH296
ySBCGH297	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH129
ySBCGH321/ySBCGH345	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH297
ySBCGH298	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH130
ySBCGH322/ySBCGH346	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH298

ySBCGH299	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH131
ySBCGH323/ySBCGH347	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH299
ySBCGH300	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH132
ySBCGH324/ySBCGH348	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH300
ySBCGH301	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1)	ySBCGH133
ySBCGH325/ySBCGH349	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1)	ySBCGH301

ySBCGH302	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH134
ySBCGH326/ySBCGH350	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH302
ySBCGH303	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH135
ySBCGH327/ySBCGH351	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH303
ySBCGH304	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH136
ySBCGH328/ySBCGH352	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH304

ySBCGH305	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH137
ySBCGH329/ySBCGH353	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH305
ySBCGH306	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH138
ySBCGH330/ySBCGH354	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH306
ySBCGH307	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH139
ySBCGH331/ySBCGH355	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH307

ySBCGH308	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH140
ySBCGH332/ySBCGH356	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH308
ySBCGH309	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH141
ySBCGH333/ySBCGH357	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH309
ySBCGH310	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH142
ySBCGH334/ySBCGH358	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH310

ySBCGH311	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH143
ySBCGH335/ySBCGH359	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH311
ySBCGH312	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH144
ySBCGH336/ySBCGH360	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) PCK1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH312

Example 12: Deletion of the first and second MAE1 loci in *I. orientalis* strains ySBCGH121-144:

[00272] The first and second MAE1 loci in *I. orientalis* strains ySBCGH121-144 (Example 8) are deleted using an MAE1 deletion construct.

Example 12A: Construction of *I. orientalis* MAE1 deletion constructs:

[00273] The MAE1 upstream region from 370 bp upstream to the start codon is amplified by PCR. Sequence corresponding to the restriction sites NdeI/NotI/SacI is added to the 5' end of the reverse upstream primer (oKW61, SEQ ID NO:83). A PmeI restriction site is added to the 5' end of the forward upstream primer (oKW60, SEQ ID NO:82). The MAE1 downstream region is amplified from the stop codon to 392 bp downstream. The downstream forward primer (oKW62, SEQ ID NO:84) contains the same NdeI/NotI/SacI sequence as the reverse upstream primer. The 5' end of the reverse downstream primer also has a PmeI site (oKW63, SEQ ID NO:85). The two fragments are amplified independently using Phusion polymerase, then assembled into a full-length (784 bp) product via a two-stage PCR protocol

(10 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with no primers, followed by 20 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with upstream forward and downstream reverse primers). Full-length product is gel purified and cloned into pCR-BluntII (Invitrogen) and sequenced. The plasmid confirmed to have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final construct is named pKWB19 (Figure 36).

[00274] pKWB19 is digested with NdeI and SacI and the resulting 4.3kb DNA fragment is gel purified. Plasmid pKF031 is digested with NdeI and SacI to create a fragment of 3.7kb that contains the MEL5 marker flanked by loxP sites. In the same way, pKF044 is digested to create a 4.2kb fragment containing the CYB2A marker flanked by loxP sites. Marker fragments are ligated into the digested pKWB19 plasmid to create pKWB24 (Figure 37), containing the MEL5 marker, and pKWB29 (Figure 38), containing the CYB2A marker. Correct constructs are confirmed by PCR and restriction digestion.

Example 12B: Deletion of MAE1 in *I. orientalis* strains ySBCGH121-ySBCGH144:

[00275] Plasmid pKWB24 is digested with PmeI to create a 4.4kb fragment containing the MEL5 marker surrounded by MAE flanking sequence. The fragment is gel purified prior to transformation. In the same way, pKWB29 is digested with PmeI to create a 5kb fragment containing the CYB2A marker with MAE flanking sequence. The fragment is gel purified prior to transformation.

[00276] The DNA fragment containing the CYB2A marker is transformed into strains ySBCGH121-ySBCGH144. Transformants are selected on YNB + lactate, and deletion of MAE at the first allele is confirmed by PCR using primers oKW60 (SEQ ID NO:82), oKW63 (SEQ ID NO:85), oGPB52 (SEQ ID NO:148), and oGPB53 (SEQ ID NO:149). The correct heterozygous strains are designated ySBCGH217-240.

[00277] Strains ySBCGH217-240 are transformed with the PmeI digestion product from pKWB24 and selected on YNB + melibiose+ x- α -gal to generate a homozygous strain with MAE deleted at both alleles. Integration is confirmed by PCR using the primers oKW60 (SEQ ID NO:82), oKW63 (SEQ ID NO:85), oGPB54 (SEQ ID NO:150), and oGPB55 (SEQ ID NO:151). The correct homozygous strains are designated ySBCGH241-264.

[00278] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH265-288.

[00279] The various MAE deletion strains generated in Example 12 are summarized in Table 14.

Table 14: *I. orientalis* MAE deletion strains:

Strain name	Description	Parent strain
ySBCGH217	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH121
ySBCGH241/ySBCGH265	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH217
ySBCGH218	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH122
ySBCGH242/ySBCGH266	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH218
ySBCGH219	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH123
ySBCGH243/ySBCGH267	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH219

ySBCGH220	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH124
ySBCGH244/ySBCGH268	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH220
ySBCGH221	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH125
ySBCGH245/ySBCGH269	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH221
ySBCGH222	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH126
ySBCGH246/ySBCGH270	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH222

ySBCGH223	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH127
ySBCGH247/ySBCGH271	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH223
ySBCGH224	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH128
ySBCGH248/ySBCGH272	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH224
ySBCGH225	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH129
ySBCGH249/ySBCGH273	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH225

ySBCGH226	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH130
ySBCGH250/ySBCGH274	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH226
ySBCGH227	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH131
ySBCGH251/ySBCGH275	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH227
ySBCGH228	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH132
ySBCGH252/ySBCGH276	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH228

ySBCGH229	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1)	ySBCGH133
ySBCGH253/ySBCGH277	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (1)	ySBCGH229
ySBCGH230	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH134
ySBCGH254/ySBCGH278	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH230
ySBCGH231	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH135
ySBCGH255/ySBCGH279	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH231

ySBCGH232	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH136
ySBCGH256/ySBCGH280	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH1 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH232
ySBCGH233	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH137
ySBCGH257/ySBCGH281	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH233
ySBCGH234	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH138
ySBCGH258/ySBCGH282	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH234

ySBCGH235	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH139
ySBCGH259/ySBCGH283	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH235
ySBCGH236	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH140
ySBCGH260/ySBCGH284	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH236
ySBCGH237	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH141
ySBCGH261/ySBCGH285	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. mikatae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH237

ySBCGH238	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH142
ySBCGH262/ySBCGH286	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. marxianus</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH238
ySBCGH239	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH143
ySBCGH263/ySBCGH287	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>K. polyspora</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH239
ySBCGH240	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH144
ySBCGH264/ySBCGH288	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH3 insertion at ATO2 (2) <i>S. cerevisiae</i> FRD1 at ADHa (2) <i>I. orientalis</i> FUM1 at CYB2B (2)	ySBCGH240

Example 13: Insertion of PYC1 at the PDC1 locus in *I. orientalis* strains 2610 and 12506:

[00280] A PYC1 expression cassette is inserted at one or both PDC1 alleles in *I. orientalis* strains 2610 (Example 2) and 12506.

Example 13A: Construction of *I. orientalis* PYC1 expression constructs pKF043 and pKF045:

[00281] The PYC1 gene from *I. orientalis* (SEQ ID NO:7) is amplified from genomic DNA using Phusion polymerase and primers oKF245 (SEQ ID NO:134) and oKF246 (SEQ ID NO:135), which contain an MluI site and an SbfI site, respectively. After amplification, the product is gel purified, digested with MluI and SbfI, and ligated to similarly digested pKF031 and pKF044. pKF031 (Figure 11) and pKF044 (Figure 12) are constructed from pUC19 backbones, and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKF031 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKF044 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites.

[00282] The plasmids are transformed into *E. coli*, and transformants are selected on LB plates containing 100 µg/ml carbenicillin and screened using primers flanking the NotI site of pKF031 and pKF044 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)). Quickchange PCR is performed using primers oKW96 (SEQ ID NO:110) and oKW97 (SEQ ID NO:111) to eliminate an internal NdeI site (T2847C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKF043 (MEL5 marker) (Figure 15) and pKF045 (CYB2A marker) (Figure 16).

Example 13B: Construction of *S. cerevisiae* PYC1 expression constructs pKWB14 and pKWB15:

[00283] The PYC1 gene from *S. cerevisiae* (SEQ ID NO:9) is amplified from genomic DNA using Phusion polymerase and primers oKW29 (SEQ ID NO:80) and oKW30 (SEQ ID NO:81), both of which contain at their 5' end 23 bp flanking the NotI site in pKF031 and pKF044 to enable directional ligation-less cloning. After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044.

Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed using primers oKW81 (SEQ ID NO:102) and oKW82 (SEQ ID NO:103) to eliminate an internal NdeI site (T2838C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB14 (MEL5 marker) (Figure 17) and pKWB15 (CYB2A marker) (Figure 18).

Example 13C: Construction of *K. marxianus* PYC1 expression constructs pKWB16 and pKWB17:

[00284] *K. marxianus* is streaked on YPD plates, and after around 3 days the PYC1 gene (SEQ ID NO:11) is amplified from genomic DNA by colony PCR using primers oKW85 (SEQ

ID NO:106) and oKW86 (SEQ ID NO:107). After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044. Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed using primers oKW83 (SEQ ID NO:104) and oKW84 (SEQ ID NO:105) to eliminate an internal SacI site (T1446A). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB16 (contains MEL5 marker) (Figure 19) and pKWB17 (contains CYB2A marker) (Figure 20).

Example 13D: Insertion of *I. orientalis* PYC1 at the first and second PDC1 loci in *I. orientalis* strain 2610:

[00285] pKF043 and pKF045 is amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end. pKF043 is amplified using primers oKF243 (SEQ ID NO:132) and oKF244 (SEQ ID NO:133), and pKF045 is amplified using primers oKF255 (SEQ ID NO:137) and oKF244 (SEQ ID NO:133). Each of these primers contains on their 5' end 65 bp of sequence specific to the 65 bp immediately upstream and downstream of the PDC1 locus in *I. orientalis*. This recombination sequence enables double recombination and integration at the PDC1 locus.

[00286] The PCR product amplified from pKF043 is used to transform *I. orientalis* strain 2610 (Example 2). Transformants are selected on YNB + melibiose+ x-α-gal and, and integration of PYC1 at a first PDC1 allele is confirmed by PCR using primers oCM566 (SEQ ID NO:138), oKF151 (SEQ ID NO:129), oKF252 (SEQ ID NO:136), and oCM587 (SEQ ID NO:139). The correct heterozygous strain is designated 12626.

[00287] Strain 12626 is transformed with the PCR product from pKF045 amplification to generate a homozygous strain with PYC1 inserted at both PDC1 alleles. Integration is confirmed by PCR using the primers oCM566 (SEQ ID NO:138), oMM174 (SEQ ID NO:59), oCM587 (SEQ ID NO:139), and oCA397 (SEQ ID NO:71). The correct homozygous strain is designated 12629.

[00288] For marker recycling, *I. orientalis* 12629 is grown to around OD₆₀₀ of 1.0 in YP +100g/L glucose (50ml media in a 250ml flask; 30°C/250rpm). Cells are transformed with pVB32 using lithium acetate transformation, and transformants are selected on YNB + 2% sucrose plates overlaid with x-α-gal. After 4 to 5 days, white colonies are streaked to YP + 20g/L glucose plates overlaid with x-α-gal and grown at 37°C for 2 days. Genomic DNA from white colonies is screened for retention of the expression cassette at the *I. orientalis* PDC1 locus and for loss of the selectable markers using PCR primers oGPB9 (SEQ ID NO:140), oGPB10 (SEQ ID NO:141), oGPB11 (SEQ ID NO:142), and oGPB12 (SEQ ID NO:143). Positive transformants are confirmed to have lost the marker by a phenotypic

screen showing no growth on YNB + 2% lactic, 2% melibiose, or 2% sucrose. The homozygous strain with both markers removed is designated 12481.

Example 13E: Insertion of *S. cerevisiae* PYC1 at the first and second PDC1 loci in *I. orientalis* strain 12506:

[00289] pKWB14 and pKWB15 are both amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end. pKWB14 is amplified using primers oKF243 (SEQ ID NO:132) and oKF244 (SEQ ID NO:133), and pKWB15 is amplified using primers oKF255 (SEQ ID NO:137) and oKF244 (SEQ ID NO:133). The PCR product amplified from pKWB14 is used to transform *I. orientalis* strain 12506. Transformants are selected on YNB + melibiose+ x- α -gal and integration of PYC1 at a first PDC1 allele is confirmed by PCR using primers oKW70 (SEQ ID NO:92), oGPB55 (SEQ ID NO:151), oGPB54 (SEQ ID NO:150), and oKW73 (SEQ ID NO:95). The correct heterozygous strain is designated 12688.

[00290] Strain 12688 is transformed with the PCR product from pKWB15 amplification to generate a homozygous strain with PYC1 inserted at both PDC1 alleles. Integration is confirmed by PCR using the primers oKW70 (SEQ ID NO:92), oGPB53 (SEQ ID NO:149), oGPB52 (SEQ ID NO:148), and oKW73 (SEQ ID NO:95). The correct homozygous strain is designated 12694.

Example 13F: Insertion of *K. marxianus* PYC1 at the first and second PDC1 loci in *I. orientalis* strain 12506:

[00291] pKWB16 and pKWB17 are both amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end. pKWB16 is amplified using primers oKF243 (SEQ ID NO:132) and oKF244 (SEQ ID NO:133), and pKWB17 is amplified using primers oKF255 (SEQ ID NO:137) and oKF244 (SEQ ID NO:133). The PCR product amplified from pKWB14 is used to transform *I. orientalis* strain 12506. Transformants are selected on YNB + melibiose+ x- α -gal and integration of PYC1 at a first PDC1 allele is confirmed by PCR using primers oKW70 (SEQ ID NO:92), oGPB55 (SEQ ID NO:151), oGPB54 (SEQ ID NO:150), and oKW73 (SEQ ID NO:95). The correct heterozygous strain is designated 12634.

[00292] Strain 12634 is transformed with the PCR product from pKWB15 amplification to generate a homozygous strain with PYC1 inserted at both PDC1 alleles. Integration is confirmed by PCR using the primers oKW70 (SEQ ID NO:92), oGPB53 (SEQ ID NO:149), oGPB52 (SEQ ID NO:148), and oKW73 (SEQ ID NO:95). The correct homozygous strains are designated ySBCK140-ySBCK142.

[00293] The various PYC1 insertion/PDC1 deletion strains generated in Example 13 are summarized in Table 15.

Table 15: *I. orientalis* PYC1 insertion strains:

Strain name	Description	Parent strain
12506	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2)	12480 (Example 15H)
12626	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (1)	2610
12629/12481	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2)	12626
12688	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (1) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> PYC1 insertion at PDC1 (1)	12506
12694	CYB2A deletion (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>S. cerevisiae</i> PYC1 insertion at PDC1 (2)	12688
12634	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (1) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> PYC1 insertion at PDC1 (1)	12506
ySBCK140/ ySBCK141/ ySBCK142	CYB2A deletion (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2) <i>K. marxianus</i> PYC1 insertion at PDC1 (2)	12634

Example 14: Insertion of FRD1 at the ADHa locus in *I. orientalis* strain 12481:

[00294] An FRD1 expression cassette was inserted at one or both ADHa alleles of *I. orientalis* strains 12481 (Example 13D).

Example 14A: Construction of ADHa deletion constructs pGPB11, pGPB14, pGPB28, and pGPB34:

[00295] pKF044 was used as a template for quickchange mutagenesis using oligonucleotides oKW64 (SEQ ID NO:86) and oKW65 (SEQ ID NO:87) to delete an EcoRI site at nucleotide 932 of the *I. orientalis* CYB2A gene. The resulting plasmid was designated pKW49. pKW49 was digested with EcoRI and BglII and the resultant fragment ligated to EcoRI and BamHI digested pHJJ23 (Figure 21). The resulting ADHa deletion construct, designated pGPB11 (Figure 22), contains the *I. orientalis* PDC1 promoter (amplified using primers oJLJ3 (SEQ ID NO:156) and oJLJ19 (SEQ ID NO:157)) and terminator (amplified using primers oJLJ1 (SEQ ID NO:154) and oJLJ2 (SEQ ID NO:155)) and a CYB2A marker element between an 858 bp fragment corresponding to the region immediately 5' of the *I. orientalis* AHD2a open reading frame (amplified using primers oHJJ71 (SEQ ID NO:159) and oHJJ72 (SEQ ID NO:160)) and a 996 bp fragment corresponding to the region immediately 3' of the *I. orientalis* ADHa open reading frame (amplified using primer oHJJ73 (SEQ ID NO:161) and oHJJ74 (SEQ ID NO:162)).

[00296] pKF046, which contains an *S. cerevisiae* MEL5 marker gene operatively linked to a *I. orientalis* PGK promoter and an *S. cerevisiae* MEL5 terminator and flanked by LoxP sites,

was used as a template for quickchange mutagenesis using oligonucleotides oKW74 (SEQ ID NO:96) and oKW75 (SEQ ID NO:97) to delete an EcoRI site at nucleotide 2392 of the plasmid. The resulting plasmid was designated pKW50. pKW50 was digested with EcoRI and BglII and the resultant fragment ligated to EcoRI and BamHI digested pHJJ23. The resulting plasmid, designated pGPB14 (Figure 24), contains the same elements as pGPB11, but with the CYB2A selectable marker element replaced by the *S. cerevisiae* MEL5 selectable marker element.

[00297] pGPB11 and pGPB14 were each digested with EcoRI and BamHI to remove those portions of the plasmids corresponding to the PDC promoter and terminator, and each plasmid backbone was blunted with Klenow fragment and ligated to recircularize the plasmid. The plasmids were then transformed into *E. coli*. Plasmid isolated from positive colonies was designated pGPB28 (Figure 23, derived from pGPB11) and pGPB34 (Figure 25, derived from pGPB14).

Example 14B: Construction of FRD1 expression constructs pGPB20, pGPB22, pGPB25, pGPB26, pGPB36, pGPB37, pGPB39, and pGPB40:

[00298] Expression cassettes for the FRD1 gene from various sources were inserted into the ADHa deletion construct pGPB11. Sources for the FRD1 gene were *S. cerevisiae* (SEQ ID NO:25), *S. mikatae* (SEQ ID NO:27), *K. polyspora* (SEQ ID NO:29), and *K. marxianus* (SEQ ID NO:31). The latter three genes were all codon optimized to *I. orientalis*.

[00299] Plasmids containing *S. cerevisiae*, *S. mikatae*, *K. polyspora*, or *K. marxianus* FRD1 genes were digested with XbaI and PacI, and the FRD1 fragments were ligated to similarly digested pGPB11. The resulting plasmids, which contained the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and also contained the *I. orientalis* CYB2A selectable marker, were designated pGPB20 (*S. mikatae* FRD1), pGP22 (*K. marxianus* FRD1), pGPB25 (*K. polyspora* FRD1), and pGPB26 (*S. cerevisiae* FRD1) (Figure 26).

[00300] pGPB20, pGP22, pGPB25, and pGPB26 were digested with BamHI and NdeI and ligated to similarly digested pGPB14. The resulting plasmids, which contained the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and also contained the *S. cerevisiae* MEL5 selectable marker were designated pGPB36 (*S. mikatae* FRD1), pGPB37 (*K. marxianus* FRD1), pGPB39 (*K. polyspora* FRD1), and pGPB40 (*S. cerevisiae* FRD1) (Figure 27).

Example 14C: Insertion of FRD1 at first and second ADHa loci of *I. orientalis* strain 12481:

[00301] pGPB36 (*S. mikatae* FRD1), pGPB37 (*K. marxianus* FRD1), pGPB39 (*K. polyspora* FRD1), and pGPB40 (*S. cerevisiae* FRD1) are digested with SacI and ApaI and transformed into *I. orientalis* strain 12481 by lithium acetate transformation. Transformants are selected

on YNB + 2% lactic plates overlaid with x- α -gal. After around six days, blue transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. Blue colonies are picked, and genomic DNA is isolated and screened by PCR to confirm integration of the FRD1 expression cassette at the ADHa locus using primers oGPB47 (SEQ ID NO:147), oGPB56 (SEQ ID NO:187), oGPB54 (SEQ ID NO:150), and oGPB46 (SEQ ID NO:146). Strains with the correct integration of the FRD1 gene are designated ySBCG95-106.

[00302] pGPB20 (*S. mikatae* FRD1), pGPB22 (*K. marxianus* FRD1), pGPB25 (*K. polyspora* FRD1), and pGPB26 (*S. cerevisiae* FRD1) are digested with SacI and Apal and transformed into strains ySBCG95-106 by lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FRD1 expression cassette at the ADHa locus using primers oGPB47 (SEQ ID NO:147), oGPB53 (SEQ ID NO:149), oGPB52 (SEQ ID NO:148), oGPB54 (SEQ ID NO:150), and oGPB46 (SEQ ID NO:146). The resulting strains are designated ySBCG123, ySBCG124, and ySBCG126-135.

[00303] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated 12750-12753, 12763-12766, ySBCG150-ySBCG152, and ySBCG141.

[00304] The various FRD1 insertion/ADHa deletion strains generated in Example 14 are summarized in Table 16.

Table 16: *I. orientalis* FRD1 insertion strains:

Strain name	Description	Parent strain
ySBCG95/ ySBCG96/ ySBCG97	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (1)	12481
ySBCG98/ ySBCG99/ ySBCG100	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (1)	12481
ySBCG101/ ySBCG102/ ySBCG103	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (1)	12481
ySBCG104/ ySBCG105/ ySBCG106	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (1)	12481
ySBCG132/ ySBCG133/ ySBCG134/ ySBCG135	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCG95/ ySBCG97
ySBCG123/ ySBCG124	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCG98/ ySBCG99
ySBCG126/ ySBCG127/ ySBCG128	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCG101/ ySBCG102/ ySBCG103

ySBCG129/ ySBCG130/ ySBCG131	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCG104/ ySBCG105/ ySBCG106
12765/12766 ySBCG150/ ySBCG151/ ySBCG152	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2)	ySBCG132/ ySBCG133/ ySBCG134/ ySBCG135
12750/12751	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2)	ySBCG123/ ySBCG124
12752/12753 ySBCG141	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2)	ySBCG126/ ySBCG127/ ySBCG128
12763/12764	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2)	ySBCG129/ ySBCG131

Example 15: Insertion of MDH at the ATO2 locus in *I. orientalis* strains 12481, 12750-12753, 12763, and 12765:

[00305] An MDH expression cassette is inserted at one or both ATO2 alleles of *I. orientalis* strain 12481 (Example 13D) and strains 12750-12753 and 12763-12766 (Example 14C).

Example 15A: Construction of ATO2 deletion constructs pKWB18, pKWB23, pKWB28:

[00306] Upstream and downstream regions of *I. orientalis* ATO2 (SEQ ID NO:53) were amplified in order to generate ATO2 deletion constructs. The upstream and downstream regions correspond to nucleotides from 419 bp upstream to the start codon of ATO2 and from the stop codon to 625 bp downstream, respectively. Amplification of the upstream region is performed using primers oKW66 (forward, SEQ ID NO:88) and oKW67 (reverse, SEQ ID NO:89), which adds a PmeI restriction site and NdeI, NotI, and SacI restriction sites, respectively, to the product. Amplification of the downstream region is performed using primers oKW68 (forward, SEQ ID NO:90) and oKW69 (reverse, SEQ ID NO:91), which adds NdeI, NotI, and SacI restriction sites and a PmeI restriction site, respectively, to the product. The two fragments are amplified independently, then assembled into a full-length product with a two stage PCR protocol. The first stage uses 10 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with no primers, and the second stage uses 20 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with upstream forward and downstream reverse primers. The full-length product is gel purified, cloned into pCR-BluntII (Invitrogen), and sequenced. The plasmid confirmed to have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid-borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final ATO2 deletion construct is designated pKWB18 (Figure 13).

[00307] pKWB18 was digested with NdeI and SacI and ligated to like-cut pKF031 (Figure 11; *S. cerevisiae* MEL selectable marker) or pKF044 (Figure 12; CYB2A selectable marker).

The resulting ATO2 deletion constructs with MEL5 and CYB2A selectable markers are designed pKWB23 and pKWB28, respectively (Figures 42 and 43).

Example 15B: Construction of *E. coli*, *Z. rouxii*, and *R. oryzae* MDH expression constructs pGPB60, 62, 62, 64, 66, 78, and 79:

[00308] The MDH genes from *E. coli*, *Z. rouxii*, and *R. oryzae* (SEQ ID NOs:169, 167, and 171, respectively) are amplified from genomic DNA using primers designed to add a MluI restriction site to the 5' end the start codon and an SbfI restriction site to the 3' end of the stop codon. *E. coli* MDH is amplified using primers oGPB61 (SEQ ID NO:188) and oGPB62 (SEQ ID NO:189), *Z. rouxii* MDH is amplified using primers oGPB67 (SEQ ID NO:190) and oGPB68 (SEQ ID NO:191), and *R. oryzae* MDH is amplified using primers oGPB65 (SEQ ID NO:192) and oGPB66 (SEQ ID NO:193). After amplification, the products are gel purified and cloned into pZeroBluntII and transformed into *E. coli*. Transformants are selected on LB plates containing 50 µg/ml kanamycin. Correct plasmids are confirmed by sequencing, and the final constructs are designated pGPB57 (*E. coli* MDH1), pGPB58 (*Z. rouxii* MDH), and pGPB68 (*R. oryzae* MDH). pGPB57, pGPB58, and pGPB59 are digested with MfuI and SbfI to liberate the fragment containing the respective MDH gene. The fragment is gel purified and cloned into like-cut pGPB54 and pGPB55. The resulting plasmids are designated pGPB60 (*E. coli* MDH, MEL5 marker), pGPB62 (*E. coli* MDH, CYB2A marker), pGPB64 (*Z. rouxii* MDH, MEL5 marker), pGPB66 (*Z. rouxii* MDH, CYB2A marker), pGPB78 (*R. oryzae* MDH, MEL5 marker), and pGPB79 (*R. oryzae* MDH, CYB2A marker).

Example 15C: Construction of *K. marxianus* MDH3 expression constructs pGPB54 and pGPB55:

[00309] The MDH3 gene from *K. marxianus* (SEQ ID NO:23) is amplified from genomic DNA using primers designed for ligation-less cloning into the NotI site of pKF031 and pKF044. MDH3 is amplified using primers oKW104 (SEQ ID NO:116) and oKW105 (SEQ ID NO:117). After amplification, the product is gel purified and co-transformed into *E. coli* with NotI-digested pKF031 and pKF044. Transformants are selected on LB plates containing 100 µg/ml carbenicillin, and screened using primers oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109). Quickchange PCR is performed on MDH3 using primers oKW136 and oKW137 to eliminate an internal NdeI site (T18C). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKWB10 (MDH3, MEL5 marker), and pKWB13 (MDH3, CYB2A marker). pKWB10 and pKWB13 were digested with NdeI and SacI to liberate the fragment containing selectable marker, ENO promoter, *K. marxianus* MDH3, and terminator. The resulting fragments are ligated into like-cut pKWB54 containing the ATO2 deletion construct. The resulting plasmids are designated pGPB54 (*K. marxianus* MDH3, MEL5 marker) and pGPB55 (*K. marxianus* MDH3, CYB2A marker).

Example 15D: Insertion of MDH at first and second ATO2 loci in *I. orientalis* strain 12750-12753, 12763, and 12765:

[00310] pGPB64 (*Z. rouxii* MDH, MEL5 marker) is digested with PmeI and the appropriate fragments are used to transform *I. orientalis* strains 12750-12753, 12763, and 12765 (Example 14) by lithium acetate transformation. Transformants are selected by growth on YNB + melibiose and screened by PCR with primers flanking the ATO2 locus (oKW214 (SEQ ID NO:194) and oKWB155 (SEQ ID NO:195)) along with nested primers specific to the MEL5 integration cassette (oGPB55 (SEQ ID NO:151) and oGPB11 (SEQ ID NO:142)). Colonies with the correct insertion of MDH at a first ATO2 locus are designated ySBCG153-161 and ySBCG166-171.

[00311] pGPB66 (*Z. rouxii* MDH, CYB2A marker) is digested with PmeI and the appropriate fragments are used to transform *I. orientalis* strains ySBCG154-157, ySBCG160, and ySBCG166-171. Transformants are selected by growth on YNB + lactic + α -x-gal and screened by PCR with primers flanking the ATO2 locus (oKW214 (SEQ ID NO:194) and oKWB155 (SEQ ID NO:195)) along with nested primers specific to the MEL5 integration cassette (oGPB55 (SEQ ID NO:151) and the CYB2A integration cassette (oGPB52 (SEQ ID NO:148) and oGPB53 (SEQ ID NO:149)). Strains homozygous for MDH at the ATO2 loci are designated 12785-12787, 12783, 12784, and 12798-12803.

[00312] Plasmids containing an overexpression cassette with MDH from *E. coli*, *R. oryzae*, or *K. marxianus* (Examples 15B and 15C) can be integrated at the first and second ATO2 loci in *I. orientalis* strains 12750-12753, 12763, and 12765 using the same general techniques.

[00313] Marker recycling is carried out using pVB32, and homozygous *Z. rouxii* MDH strains with both markers removed are designated 12788-12792 and 12840-12846, and 12848.

Example 15E: Insertion of *K. marxianus* MDH3, *E. coli* MDH, *Z. rouxii* MDH, and null control at first and second ATO2 loci in *I. orientalis* strain 12481:

[00314] pKWB23, pGPB55, pGPB60, and pGPB64 are digested with PmeI and the appropriate fragments are used to transform *I. orientalis* strain 12481 by lithium acetate transformation. Transformants are selected by growth on YNB + melibiose or YNB + 2% lactic acid and screened by PCR with primers flanking the ATO2 locus (oKW214 (SEQ ID NO:194) and oKWB155 (SEQ ID NO:195)) along with nested primers specific to the MEL5 integration cassette (oGPB55 (SEQ ID NO:151) and oGPB11 (SEQ ID NO:142)) or the CYB2A integration cassette (oGPB53 (SEQ ID NO:149) and oGPB11 (SEQ ID NO:142)). Colonies with the correct insertion of MDH at a first ATO2 locus are designated ySBCG90-92 and ySBCG107-112. Likewise, a strain heterozygous for the ATO2 deletion is designated 12642.

[00315] pKWB28, pGPB54, pGPB62, and pGPB66 are digested with PmeI and the appropriate fragments are used to transform ySBCG90, ySBCG91, ySBCG107-109, ySBCG110, ySBCG111, and 12642. Transformants are selected by growth on YNB + melibiose + α -X-gal and screened by PCR with primers flanking the ATO2 locus (oKW214 (SEQ ID NO:194) and oKWB155 (SEQ ID NO:195)) along with nested primers specific to the MEL5 integration cassette (oGPB55 (SEQ ID NO:151) and oGPB54 (SEQ ID NO:150)) or the CYB2A integration cassette (oGPB52 (SEQ ID NO:148) and oGPB53 (SEQ ID NO:149)). Strains homozygous for MDH at the ATO2 loci are designated 12601, 12602, 12620-12622, and 12623-12625. Likewise, a strain homozygous for the ATO2 deletion is designated 12657.

[00316] Marker recycling is carried out using pVB32, and homozygous strains with both markers removed are designated 12712, 12715, and 12716.

Example 15F: Insertion of *I. orientalis* MDH1 at a first ATO2 locus in *I. orientalis* strain 12752:

[00317] A PCR product amplified with the primers oKF254 (SEQ ID NO:197) and oKF202 (SEQ ID NO:198) using pKWB2 as the template is transformed into strain 12752 (Example 14C) by lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plates overlaid with x- α -gal. Blue-colored transformants are visible after around 6 days of growth at 30°C. Transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. Blue colonies are picked, and genomic DNA is isolated and screened for correct integration of the MDH1 expression cassette at the ATO2 locus by PCR using primers oGPB55 (SEQ ID NO:151), oKW66 (SEQ ID NO:88), oKW69 (SEQ ID NO:91), and oGPB54 (SEQ ID NO:150). Sister strains with the correct integration of the MDH1 gene are designated ySBCGH471 and ySBCGH472.

Example 15G: Insertion of *I. orientalis* MDH2 at a first ATO2 locus in *I. orientalis* strains 12752 and 12481:

[00318] A PCR product amplified with the primers oKF254 (SEQ ID NO:197) and oKF202 (SEQ ID NO:198) using pKWB3 as the template is transformed into strains 12752 (Example 14C) and 12481 (Example 13D) by lithium acetate transformation, and transformants are selected on YNB + 2% melibiose plates overlaid with x- α -gal. Blue-colored transformants are visible after around 6 days of growth at 30°C. Transformants are picked and plated for single colonies on YP + 20 g/L glucose plates containing x- α -gal. Blue colonies are picked, and genomic DNA is isolated and screened for correct integration of the MDH2 expression cassette at the ATO2 locus by PCR using primers oGPB55 (SEQ ID NO:151), oKW66 (SEQ ID NO:88), oKW69 (SEQ ID NO:91), and oGPB54 (SEQ ID NO:150). Strains derived from strain 12752 with the correct integration of the MDH2 gene are designated ySBCGH473 and

ySBCGH474. The strain derived from strain 12481 with the correct integration of the MDH2 gene is designated ySBCG48.

Example 15H: Insertion of *I. orientalis* MDH2 at a second ATO2 locus in *I. orientalis* strain ySBCG48:

[00319] To generate a strain homozygous for MDH2 at ATO2, a PCR product amplified with the primers oKF254 (SEQ ID NO:197) and oKF202 (SEQ ID NO:198) using pKWB6 (Example 5B) as the template is transformed into strain ySBCG48 by lithium acetate transformation, and transformants are selected on YNB + 2% lactic acid plates. Colonies are picked, and genomic DNA is isolated and screened for correct integration of the MDH2 expression cassette at both ATO2 loci by PCR using primers oGPB53 (SEQ ID NO:149), oGPB54 (SEQ ID NO:150), oGPB55 (SEQ ID NO:151), oKW66 (SEQ ID NO:88), oKW69 (SEQ ID NO:91), and oGPB52 (SEQ ID NO:148). The strain derived from ySBCG48 with the correct integration of the MDH2 gene at both ATO2 loci is designated strain 12480.

[00320] Marker recycling is carried out on strain 12480 using pVB32, and the homozygous strain with both markers removed is designated 12506.

[00321] The various MDH insertion/ATO2 deletion strains generated in Example 15 are summarized in Table 17.

Table 17: *I. orientalis* MDH insertion strains:

Strain name	Description	Parent strain
ySBCG153/ ySBCG154/ ySBCG155	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (1)	12750/12751
ySBCG156/ ySBCG157/ ySBCG158/ ySBCG159/ ySBCG160/ ySBCG161	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (1)	12752/12753
ySBCG166/ ySBCG167/ ySBCG168	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (1)	12763
ySBCG169/ ySBCG170/ ySBCG171	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (1)	12765
12783/12784	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	ySBCG154/ ySBCG155
12785/12786/ 12787	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	ySBCG156/ ySBCG157/ ySBCG160

12798/12799/ 12800	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	ySBCG166/ ySBCG167/ ySBCG168
12801/12802/ 12803	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	ySBCG169/ ySBCG170/ ySBCG171
12788/12789	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12783/12784
12790/12791/ 12792	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12785/12786/12787
12840/12841/ 12842/12843 12844	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12798/12799/ 12800
12845/12846/ 12848	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. mikatae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12802/12803
ySBCG90/ ySBCG91/ ySBCG92	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH insertion at ATO2 (1)	12481
ySBCG107/ ySBCG108/ ySBCG109	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>E. coli</i> MDH insertion at ATO2 (1)	12481
ySBCG110/ ySBCG111/ ySBCG112	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>Z. rouxii</i> MDH insertion at ATO2 (1)	12481
12642	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) Null insertion at ATO2 (1)	12481
12601/12602	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. marxianus</i> MDH insertion at ATO2 (2)	ySBCG90/ ySBCG91
12620/12621/12622	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>E. coli</i> MDH insertion at ATO2 (2)	ySBCG107/ ySBCG108/ ySBCG109
12623/ 12624/ 12625	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	ySBCG111/ ySBCG112
12657	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) Null insertion at ATO2 (2)	12642
12712	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>E. coli</i> MDH insertion at ATO2 (2)	12622
12715/12716	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12623/12625

ySBCGH471/ ySBCGH472	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> MDH1 insertion at ATO2 (1)	12752
ySBCGH473/ ySBCGH474	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (1)	12752
ySBCG48	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (1)	12481
12480/12506	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> MDH2 insertion at ATO2 (2)	ySBCG48

Example 16: Batch fermentor characterization of *I. orientalis* strains 12657, 12601, 12620 and 12625:

[00322] Replicate batch fermentations were run to compare strain performance.

Fermentors were inoculated with biomass grown in the media outlined in Table 18. Seeds were run in 250 mL baffled flasks (50 mL working volume) at 250 rpm and 30 °C. Flasks were harvested at 15 to 20 hours incubation time with a target seed OD₆₀₀ of 2 to 5. Fermentors were inoculated from these seeds to an initial OD₆₀₀ of 0.05 to 0.1. DM defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20) was used in fermentors. pH was controlled at 4.45 with 30% Ca(OH)₂. The fermentor systems were sparged at 0.37 slpm with a blend of 69-72% pure CO₂ and 28-31% air. An agitation rate of 715 to 720 rpm was used. The sample taken at residual glucose concentration between 13 and 28 g/l was analyzed in each batch for biomass growth via OD₆₀₀, malate via gas chromatography with flame ionization detector and glucose by high performance liquid chromatography with refractive index detector. Malate is used as an indicator compound for flux through the MDH as strain 12657, 12601, 12620 and 12625 do not have the full reductive pathway to succinate. Figure 44 shows increased malate production with all three MDH's tested. Malate production increased from 2.2 to 3-fold with the expression of MDH.

Table 18: Defined media for flask cultures:

Compound	Concentration (g/kg)
C ₆ H ₁₂ O ₆	120
(NH ₂) ₂ CO	2.38
KH ₂ PO ₄	3.0
MgSO ₄ ·7H ₂ O	0.5
1000x Vitamin Solution	1
100x Trace Solution	1
C ₆ H ₁₃ NO ₄ S	3.9

Table 19: Trace element 1000x stock solution

Chemical	Concentration (g/L)
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	15.00
$ZnSO_4 \cdot 7H_2O$	4.50
$MnCl_2 \cdot 2H_2O$	1
$CoCl_2 \cdot 6H_2O$	0.30
$CuSO_4 \cdot 5H_2O$	0.30
$Na_2MoO_4 \cdot 2H_2O$	0.40
$CaCl_2 \cdot 2H_2O$	4.50
$FeSO_4 \cdot 7H_2O$	3.00
H_3BO_3	1.00
KI	0.10

Table 20: Vitamin 1000x stock solution:

Chemical	Concentration (g/L)
$C_{10}H_{16}N_2O_3S$	0.05
$C_{18}H_{32}CaN_2O_{10}$	1.00
$C_6H_5NO_2$	5
$C_6H_{12}O_6$	25.00
$C_{12}H_{18}C_{12}N_4OS \cdot xH_2O$	1.00
$C_8H_{12}ClNO_3$	1.00
$C_7H_7NO_2$	0.20

Example 17: Insertion of *I. orientalis* FUM1 genes at the CYB2B locus in *I. orientalis* strains 12788, 12789, 12790, 12791 and 12792:

[00323] *I. orientalis* FUM1 expression cassettes are inserted at both alleles of CYB2A of *I. orientalis* strains 12788, 12789, 12790, 12791 and 12792 (Example 15).

Example 17A: Construction of *I. orientalis* FUM1 expression constructs pGPB30, pGPB42, pGPB44, and pGPB47:

[00324] An expression cassette for the *I. orientalis* FUM1 gene (SEQ ID NO:1) is inserted into the ADHa deletion construct pGPB11. PCR primers oGPB38 (SEQ ID NO:144) and oGPB40 (SEQ ID NO:145) are used to amplify FUM1 using *I. orientalis* genomic DNA as the template. The 5' primer adds an XbaI site at the start site of the coding sequence and the 3' primer adds a PaeI site 3' of the stop codon. The resulting PCR product is digested with XbaI and PaeI and ligated to similarly digested pGPB11. The resulting plasmid, which contains the FUM1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and the CYB2A selectable marker, is designated pGPB30 (Figure 28).

[00325] pGPB30 is digested with BamHI and NdeI and ligated into similarly digested pGPB14. The resulting plasmid is designated pGPB44 (Figure 30).

[00326] The expression cassette from pGPB30 is excised using NotI and ligated to the NotI cut pKW22. The resulting plasmid is designated pGPB42 (Figure 29).

[00327] The expression cassette from pGPB44 is excised using NotI and ligated to the NotI cut pKW22. The resulting plasmid is designated pGPB47 (Figure 31).

Example 17B: Insertion of *I. orientalis* FUM1 at one or both *I. orientalis* CYB2B loci:

[00328] Integration of the first copy of the FUM1 expression cassette at the CYB2B locus is performed using plasmids containing the MEL5 selectable marker. pGPB47 is digested with SacI and ApaI and transformed into 12788, 12789, 12790, 12791 and 12792 using lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FUM1 expression cassette at the second CYB2B locus using primers oKW202 (SEQ ID NO:200), oGPB54 (SEQ ID NO:150), oKW195 (SEQ ID NO:199), and oGPB56 (SEQ ID NO:187). The resulting strains are designated 12824-12828.

[00329] Integration of the second copy of the FUM1 expression cassette at the CYB2B locus is performed using plasmids containing the CYB2A selectable marker. pGPB43 (Figure 67) is digested with SacI and ApaI and transformed into *I. orientalis* strains 12824-12828 using lithium acetate transformation. Transformants are screened by PCR to confirm correct integration of the FUM1 expression cassette at the first CYB2B locus using primers oKW202 (SEQ ID NO:200), oGPB54 (SEQ ID NO:150), oGPB52 (SEQ ID NO:148), oKW195 (SEQ ID NO:199), and oGPB53 (SEQ ID NO:149). The resulting strains are designated 12829-12839.

[00330] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated 12847, 12868, 12869, and 12870.

[00331] The various FUM1 insertion/CYB2B deletion strains generated in Example 17 are summarized in Table 21.

Table 21: *I. orientalis* FUM1 insertion strains:

Strain name	Description	Parent strain
12824/12825/12826	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	12790/12791/12792
12827/12828	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (1)	12788/12789
12829/12830/12831/ 12832/12833/12834/12835	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12824/12825/12826

12836/12837/12838/12839	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12827/12828
12847/12868	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12831/12833
12869/12870	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12837/12838

Example 17C: Batch fermentor characterization of *I. orientalis* strains 12791, 12824, 12826:

[00332] Replicate batch fermentations were run to compare strain performance.

Fermentors were inoculated with biomass grown in the media outlined in Table 18. Seeds were run in 250 mL baffled flasks (50 mL working volume) at 250 rpm and 30 °C. Flasks were harvested at 15 to 20 hours incubation time with a target seed OD₆₀₀ of 2 to 5. Fermentors were inoculated from these seeds to an initial OD₆₀₀ of 0.05 to 0.1. DM defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20) was used in fermentors. pH was controlled at 4.45 with 30% Ca(OH)₂. The fermentor systems were sparged at 0.37 slpm with a blend of 69-72% pure CO₂ and 28-31% air. An agitation rate of 715 to 720 rpm was used. The sample taken at residual glucose concentration between 13 and 28 g/l was analyzed in each batch for biomass growth via OD₆₀₀, succinate via gas chromatography with flame ionization detector and glucose by high performance liquid chromatography with refractive index detector.

[00333] Figure 45 shows the impact of native fumarase over-expression on succinate production. Average succinate production increased 2.75-fold over strains with only native fumarase levels.

Example 18: Insertion of ZWF1 at the GPD1 locus in *I. orientalis* strain 12868 and 12869:

[00334] A ZWF1 expression cassette is inserted at one or both GPD1 alleles in *I. orientalis* strains 12868 and 12869 (Example 17B).

Example 18A: Construction of *I. orientalis* ZWF1 expression constructs pKF033 and pGPB56:

[00335] The ZWF1 gene from *I. orientalis* (SEQ ID NO:33) is amplified from genomic DNA using Phusion polymerase and primers oKF168 (SEQ ID NO:131) and oKF163 (SEQ ID NO:130), which contain an MluI site and an SbfI site, respectively. After amplification, the product is gel purified, digested with MluI and SbfI, and ligated to similarly digested pKF031 and pKF044. pKF031 (Figure 32) and pKF044 (Figure 33) are constructed from pUC19

backbones, and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKF031 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKF044 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites.

[00336] The plasmids are transformed into *E. coli*, and transformants are selected on LB plates containing 100 µg/ml carbenicillin and screened using primers flanking the NotI site of pKF031 and pKF044 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)). Correct plasmids are confirmed by sequencing, and the final constructs are designated pKF033 (MEL5 marker) (Figure 34) and pGPB056 (CYB2A marker) (Figure 35).

Example 18B: Insertion of *I. orientalis* ZWF1 at the first and second GPD1 loci in *I. orientalis* strains 12868 and 12869:

[00337] pKF033 and pGPB56 are both amplified from the loxP site on the 5' end to the GAL10 terminator on the 3' end using primers oGPBH1 (SEQ ID NO:163) and oGPBH2 (SEQ ID NO:164). Each of these primers contains on their 5' end 65 bp of sequence specific to the 65 bp immediately upstream and downstream of the GPD1 locus in *I. orientalis*. This recombination sequence enables double recombination and integration at the GPD1 locus.

[00338] The PCR product amplified from pKF033 is used to transform *I. orientalis* strains 12868 and 12869. Transformants are selected on YNB + melibiose+ x-α-gal, and integration of ZWF1 at a first GPD1 allele is confirmed by PCR using primers oGPBH3 (SEQ ID NO:165), oGPBH4 (SEQ ID NO:166), oGPB55 (SEQ ID NO:151), and oGPB11 (SEQ ID NO:142). The correct heterozygous strains are designated ySBCGH464 and ySBCGH465.

[00339] To generate homozygous strains with ZWF1 inserted at both GPD1 alleles, strains ySBCGH464 and ySBCGH465 are transformed with the PCR product amplified from pGPB56. Transformants are selected on YNB + 2% lactic acid + x-α-gal, and integration of ZWF1 is confirmed by PCR using the primers oGPBH3 (SEQ ID NO:165), oGPBH4 (SEQ ID NO:166), oGPB53 (SEQ ID NO:149), and oGPB52 (SEQ ID NO:148). The correct homozygous strains are designated ySBCGH466 and ySBCGH467.

[00340] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated ySBCGH468 and ySBCGH469.

[00341] The various ZWF1 insertion/GPD1 deletion strains generated in Example 18 are summarized in Table 22.

Table 22: *I. orientalis* ZWF1 insertion strains:

Strain name	Description	Parent strain
ySBCGH464	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (1)	12868
ySBCGH465	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	12869
ySBCGH466/ ySBCGH468	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH464
ySBCGH467/ ySBCGH469	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>I. orientalis</i> ZWF1 at GPD1 (2)	ySBCGH465

Example 18C: Shake flask characterization of succinate production in *I. orientalis* strains ySBCGH193 and ySBCG194:

[00342] Shake flasks are used to test the ZWF1 insertion strains ySBCGH468 and ySBCG469. Shake flasks are inoculated with biomass harvested from seed flasks grown overnight to an OD₆₀₀ of 2 to 6. 250 mL baffled flasks (50 mL working volume) are inoculated to an OD₆₀₀ of 0.2 and fermentation occurs at 100 rpm and 30 °C. DM defined medium is used in flasks, with pH control and CO₂ provided by calcium carbonate addition at a concentration of 0.255M (1.28 g CaCO₃ per 50 ml flask). Samples are taken throughout the time course of the assay and analyzed for biomass growth via OD₆₀₀, and succinate and glucose are monitored via high performance liquid chromatography (HPLC). The resulting data shows production of greater than 40 g/L succinate by strain ySBCGH468 and ySBCGH469.

Example 19: Deletion of the first and second PCK1 loci in *I. orientalis* strains 12868 and 12869:

[00343] The first and second PCK1 loci in *I. orientalis* strains 12868 and 12869 (Example 17B) are deleted using a PCK deletion construct.

Example 19A: Construction of *I. orientalis* PCK deletion constructs:

[00344] The PCK upstream region from 432 bp upstream to the start codon is amplified by PCR. Sequence corresponding to the restriction sites NdeI/NotI/SacI is added to the 5' end of the reverse upstream primer (oKW78 (SEQ ID NO:99)). A PmeI restriction site is added to the 5' end of the forward upstream primer (oKW77 (SEQ ID NO:98)). The PCK downstream region is amplified from the stop codon to 472 bp downstream. The downstream forward primer (oKW79 (SEQ ID NO:100)) contained the same NdeI/NotI/SacI sequence as the reverse upstream primer. The 5' end of the reverse downstream primer also has a PmeI site (oKW80 (SEQ ID NO:101)). The two fragments are amplified independently using Phusion polymerase, then assembled into a full-length (926 bp) product via a two-stage PCR protocol (10 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with no primers, followed by 20 cycles (98 °C 10s, 55 °C 20s, 72 °C 60s) with upstream forward and downstream reverse primers). Full-length product is gel purified and cloned into pCR-BluntII (Invitrogen) and sequenced. The plasmid confirmed to have correct sequence is subjected to quickchange PCR using Phusion polymerase to eliminate the plasmid borne SacI site. Correct plasmids are confirmed by digestion with SacI and sequencing. The final construct is named pKWB20 (Figure 39).

[00345] pKWB20 is digested with NdeI and SacI and the resulting 4.4kb DNA fragment is gel purified. Plasmid pKF031 is digested with NdeI and SacI to create a fragment of 3.7kb that contains the MEL5 marker flanked by loxP sites. In the same way, pKF044 is digested to create a 4.2kb fragment containing the CYB2A marker flanked by loxP sites. Marker fragments are ligated into the digested pKWB20 plasmid to create pKWB25 (Figure 40), containing the MEL5 marker, and pKWB30 (Figure 41), containing the CYB2A marker. Correct constructs are confirmed by PCR and restriction digestion.

Example 19B: Deletion of PCK1 in *I. orientalis* strains 12868 and 12869:

[00346] Plasmid pKWB25 is digested with PmeI to create a 5kb fragment containing the MEL5 marker surrounded by PCK1 flanking sequence. The fragment is gel purified prior to transformation. In the same way, pKWB30 is digested with PmeI to create a 5.4kb fragment containing the CYB2A marker with PCK1 flanking sequence. The fragment is gel purified prior to transformation.

[00347] The DNA fragment from pKWB30, containing the CYB2A marker, is transformed into strains 12868 and 12869. Transformants are selected on YNB + lactate, and deletion of PCK at the first allele is confirmed by PCR using primers oKW77 (SEQ ID NO:98), oKW80 (SEQ ID NO:101), oGPB52 (SEQ ID NO:148), and oGPB53 (SEQ ID NO:149). The correct heterozygous strains are designated ySBCGH475 and ySBCGH476.

[00348] Strains ySBCGH475 and ySBCGH476 are transformed with the PmeI digestion product from pKWB30 and selected on YNB + melibiose+ x- α -gal to generate a homozygous strain with PCK deleted at both alleles. Integration is confirmed by PCR using the primers oKW77 (SEQ ID NO:98), oKW80 (SEQ ID NO:101), oGPB54 (SEQ ID NO:150), and oGPB55 (SEQ ID NO:151). The correct homozygous strains are designated ySBCGH477 and ySBCGH478.

[00349] The various PCK deletion strains generated in Example 19 are summarized in Table 23.

Table 23: *I. orientalis* PCK deletion strains:

Strain name	Description	Parent strain
ySBCGH475	PCK1 deletion (1) <i>I. CYB2A</i> deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12868
ySBCGH476	PCK1 deletion (1) <i>CYB2A</i> deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	12869
ySBCGH477	PCK1 deletion (2) <i>I. CYB2A</i> deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH475
ySBCGH478	PCK1 deletion (2) <i>I. CYB2A</i> deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>K. polyspora</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2)	ySBCGH476

Example 20: Deletion of RIOR43690 in *I. orientalis* strain 12868:

[00350] An RIOR43690 deletion cassette is inserted at one or both RIOR43690 alleles in *I. orientalis* strain 12868 (Example 17B).

Example 20A: Synthesis of the RIOR43690 deletion construct pVMB54:

[00351] The upstream 180bp and downstream 180bp regions of the RIOR43690 gene (SEQ ID NOs:205 and 206, respectively) are ordered from IDT (Coralville, IA) in the vector pIDTSmart. The upstream and downstream regions are flanked by PmeI sites, and contain internal NdeI and SacI restriction sites. The construct is named pVMB54 (Figure 46).

Example 20B: Construction of RIOR43690::MEL5 deletion construct pKWB86:

[00352] pVMB54 is cut with NdeI/SacI and the 2281bp fragment is gel purified. pKF031 (Figure 11) is digested with NdeI/SacI to liberate the 3694bp fragment containing the MEL5 marker cassette, ENO promoter, and GAL10 terminator. The fragment is gel purified, and ligated into purified cut pVMB54 using T4 DNA ligase, and transformed into *E. coli*. Transformants are selected on LB + kanamycin, and colonies are screened with M13F (SEQ ID NO:152) and oGPB55 (SEQ ID NO:151) primers to identify those containing the correct insert. A colony having the desired insert is miniprep, sequenced and named pKWB86 (Figure 47).

Example 20C: Construction of RIOR43690::CYB2A deletion construct pKWB87:

[00353] pVMB54 is cut with NdeI/SacI and the 2281bp fragment is gel purified. pKF044 (Figure 12) is digested with NdeI/SacI to liberate the 4198bp fragment containing the CYB2A marker cassette, ENO promoter, and GAL10 terminator. The fragment is gel purified, and ligated into purified cut pVMB54 using T4 DNA ligase, and transformed into *E. coli*. Transformants are selected on LB + kanamycin, and colonies are screened with M13F (SEQ ID NO:152) and oGPB53 (SEQ ID NO:149) primers to identify those containing the correct insert. A colony having the desired insert is miniprep, sequenced and named pKWB87 (Figure 48).

Example 20D: Construction of RIOR43690::MEL5 deletion strain 13054:

[00354] pKWB86 is digested with PmeI, and the 4065bp fragment corresponding to RIOR43690::MEL5 deletion cassette is gel purified. This fragment is then transformed into *I. orientalis* strain 12868. Transformants are selected on YNB + melibiose + x-gal and screened by PCR using flanking primers oKB86 (SEQ ID NO:203) and oKB87 (SEQ ID NO:204) and nested primers oGPB55 (SEQ ID NO:151) and oGPB54 (SEQ ID NO:150) to verify correct insertion at the RIOR43690 locus. A heterozygous strain with one copy of the RIOR43690 gene deleted is designated 13054.

Example 20E: Construction of double RIOR43690 deletion strain 13055:

[00355] pKWB87 is digested with *PmeI*, and the 4569bp fragment corresponding to RIOR43690::CYB2A deletion cassette is gel purified. This fragment is then transformed into *I. orientalis* strain 13054. Transformants are selected on YNB + lactic and screened by PCR using flanking primers oKB86 (SEQ ID NO:203) and oKB87 (SEQ ID NO:204) and nested primers oGPB53 (SEQ ID NO:149) and oGPB52 (SEQ ID NO:148) to verify correct insertion at the RIOR43690 locus. A homozygous strain with both copies of the RIOR43690 gene deleted is designated 13055.

[00356] The various RIOR43690 deletion strains generated in Example 20 are summarized in Table 24.

Table 24: *I. orientalis* RIOR43690 deletion strains:

Strain name	Description	Parent strain
13054	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) RIOR43690 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. cerevisiae</i> FRD insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	12868
13055	CYB2A deletion (2) GPD1 deletion (2) CYB2B deletion (2) RIOR43690 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. cerevisiae</i> FRD insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2)	13054

Example 20F: Growth characterization of RIOR43690 deletion strain 13055:

[00357] Plates are prepared having succinate as the sole carbon source at a final concentration of 2g/L YNB agar is prepared, and a filter sterilized solution of succinate is added after autoclaving. The pH of the medium is adjusted to near 5 prior to pouring plates and allowed to drift up to near 6 as the plates cool.

[00358] Strain 12868 is streaked onto a YNB-succinate plate together with strains 13054 and 13055. After 3 days incubation at 30 °C, growth is observed for strains 12868 and 13054. No growth is observed on the portion of the plate where strain 13055 is streaked indicating this gene is responsible for consumption of succinate (Figure 49).

Example 21: Insertion of *S. pombe* MAE at the putative RIOR43690 locus in *I. orientalis* strain 12868:

[00359] An *S. pombe* MAE expression cassette was inserted at one or both RIOR43690 loci of *I. orientalis* strain 12868 (Example 17B).

Example 21A: Construction of *S. pombe* MAE bipartite integration constructs pKWB95, pKWB96 and pKWB97:

[00360] The malic anion exporter (MAE) gene from *S. pombe* (SEQ ID NO:181) was synthesized such that the coding sequence was segmented into five fragments with overlapping regions of homology. Fragments 2-5 (SEQ ID NO:207) were joined into one larger fragment via multi-fragment primerless PCR. The resulting fragment, which encoded the last 1154bp of the *S. pombe* transporter, was topo cloned and sequenced for verification. Fragment 1 (SEQ ID NO:208) was topo cloned separately and encoded the first 384bp of the *S. pombe* transporter. Each of the fragments was flanked by MluI and SbfI restriction sites. After restriction digest, the fragments were gel purified, and ligated to similarly digested pKW086 and pKW087. pKW086 and pKW087 are constructed from pVMB54 backbones,

and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKW086 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKW087 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites.

[00361] The plasmids were transformed into *E. coli*, and transformants were selected on LB plates containing 50 µg/ml Kanamycin and screened using primers flanking the NotI site of pKW087 and pKW086 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)). Correct plasmids were designated pKWB95 (fragment 1 with MEL5 marker) (Figure 50), pKWB96 (fragment 1 with CYB2A marker) (Figure 51) and pKWB97 (Fragment 2 with MEL5 marker) (Figure 52).

Example 21B: Construction of *A. oryzae* MAE expression constructs pVMB108 and pVMB109:

[00362] The putative dicarboxylic acid transporter gene from *A. oryzae* was codon optimized and synthesized by DNA 2.0 flanked by a 5' MluI restriction site and a 3' SbfI restriction site (SEQ ID NO:183). The resulting fragment was cloned into pJ201 containing the Kanamycin resistance marker. This plasmid was digested with MluI and SbfI and the fragment containing the *A. oryzae* MAE gene was gel purified and ligated into both pKF31 and pKF44 which had been similarly digested. pKF031 and pKF044 are constructed from pUC backbones, and both contain a multiple cloning site containing MluI, NotI, and SbfI sites operatively linked to the *I. orientalis* ENO promoter and the *S. cerevisiae* GAL10 terminator. pKWB31 also contains a selection marker cassette comprising the *S. cerevisiae* MEL5 gene operatively linked to the *I. orientalis* PGK promoter. This selection marker cassette is flanked by loxP sites. pKWB44 contains an expression cassette comprising the *I. orientalis* CYB2A promoter, gene, and terminator. This expression cassette is flanked by loxP sites. The plasmids were transformed into *E. coli*, and transformants were selected on LB plates containing 100 µg/ml ampicillin and screened using primers flanking the cloning site of pKF031 and pKF044 (oKW93 (SEQ ID NO:108) and oKW95 (SEQ ID NO:109)). Correct plasmids were designated pVMB108 (MEL5 marker) (Figure 53) and pVMB109 (CYB2A marker) (Figure 54).

Example 21C: Construction of *A. oryzae* MAE integration constructs pVMB116 and pVMB117

[00363] *A. oryzae* MAE expression constructs were amplified from plasmid DNA using Phusion polymerase and primers uVMB4 (SEQ ID NO:209) and uVMB5 (SEQ ID NO:210). uVMB4 contains 100bp of sequence identity to the upstream flanking region of the

RIOR43960 locus, uVMB5 contains 100bp of sequence identity to the downstream flanking region of the RIOR43690 locus. After amplification, the product is gel purified and used as a template for amplification using primers oVMB145 (SEQ ID NO:218) and oVMB146 (SEQ ID NO:219) which serves to add PmeI sites to the 5' and 3' end of the preceding fragment. This fragment is topo cloned. The plasmids were transformed into *E. coli* and transformants are selected on LB plates containing 50 µg/ml kanamycin, and screened using diagnostic digests involving PmeI, NdeI, and SbfI. Correct plasmids are confirmed by sequencing, and the final constructs are designated pVMB116 (MEL5 marker) (Figure 55) and pVMB117 (CYB2A marker) (Figure 56).

Example 21D: Integration of *S. pombe* MAE at CB1 RIOR43690 in *I. orientalis* strain 12868

[00364] Plasmids pKWB95 and pKWB96 were digested with PmeI and SbfI and gel purified. Each of these digests served to liberate a DNA fragment containing the upstream flanking region, the selective marker and fragment 1 from the vector backbone. pKWB097 was digested with PmeI and MluI. This digest liberated fragment 2 through the GAL10 terminator and the downstream flanking region from the vector backbone. Strain 12868 (Example 17B) was transformed with the fragments purified from both pKWB95 and pKWB97 simultaneously. This transformation was designed such that integration of both fragments reconstitutes the intact coding sequence intracellularly to produce a functional transport protein. Crossover events occur between the flanking regions of the fragments and genomic DNA as well as the overlapping regions of the *S. pombe* MAE gene. Transformants are selected on YNB + melibiose+ x-α-gal and, and integration of *S. pombe* MAE at a first RIOR43690 locus is confirmed by PCR. The correct heterozygous strain is designated strain 13050.

[00365] A second integration targeting the second RIOR43690 locus is performed using the fragment purified from pKW096 and pKW097. Transformants are selected on YNB + lactic + x-α-gal and, and integration of *S. pombe* MAE at the second RIOR43690 locus is confirmed by PCR. The correct heterozygous strain is designated strain 13051.

[00366] Marker recycling is carried out with plasmid pVB32. The correct homozygous strains with both markers removed are designated 13053.

Example 21E: Integration of *A. oryzae* MAE at CB1 RIOR43690 in 12868

[00367] Integration of one copy of *A. oryzae* MAE at the RIOR43690 locus is performed using plasmids containing the CYB2A selectable marker. Strain 12868 was transformed with lithium acetate transformation using PmeI and MluI purified digest from pVMB117. Transformants were selected on YNB + lactic and integration of *A. oryzae* MAE at a first RIOR43690 locus is confirmed by PCR. The correct heterozygous strain was designated strain 13125.

[00368] The various MAE C4 transporter strains generated in Example 21 are summarized in Table 25.

Table 25: *I. orientalis* C4 transporter strains:

Strain name	Description	Parent strain
13050	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. pombe</i> MAE insertion at RIOR43690 (1)	12868
13051/13053	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. pombe</i> MAE insertion at RIOR43690 (2)	13050
13125	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>A. oryzae</i> MAE insertion at RIOR43690 (1)	12868

Example 21F: Shake flask characterization of succinate production in *I. orientalis* strains 13125, 13051, and 12868:

[00369] Shake flasks were used to test the strains. Shake flasks were inoculated with biomass harvested from yeast peptone glucose plates (YPD). 250 mL baffled flasks (50 mL working volume) were inoculated to an OD₆₀₀ of 0.2. Incubation conditions were 100 rpm and 30 °C. DM defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20) was used in flasks, with pH control and CO₂ provided by calcium carbonate addition at a concentration of 0.255M (1.28 g CaCO₃ per 50 ml culture). Samples from triplicate flasks were taken at 96 hours and averaged for biomass growth via OD₆₀₀, succinate via gas chromatography with flame ionization detector and glucose by high performance liquid chromatography with refractive index detector.

[00370] Results are shown in Figure 57. Expression of one copy of *S. pombe* MAE or *A. oryzae* MAE resulted in a 7 to 10-fold increase in succinate production.

Example 22: Deletion of MAE1 in *I. orientalis* strain 13053:

[00371] An MAE1 deletion cassette is inserted at one or both MAE1 alleles in *I. orientalis* strain 13053 (Example 21D).

Example 22A: Construction of MAE::CYB2A deletion strain 13126:

[00372] pKWB29 (Figure 38) was digested with PmeI, and the 4955bp fragment corresponding to the MAE::CYB2A deletion cassette was gel purified. This purified fragment was then transformed into *I. orientalis* strain 13053. Transformants were selected on YNB +

lactate and screened by PCR using flanking primers oGPB1 (SEQ ID NO:201) and oGPB4 (SEQ ID NO:202) and nested primers oGPB52 (SEQ ID NO:148) and oGPB53 (SEQ ID NO:149) to verify correct insertion at the MAE1 locus. A heterozygous strain with one copy of the MAE1 gene deleted was designated strain 13126.

Example 22B: Construction of double MAE1 deletion strain 13221:

[00373] pKWB24 was digested with PmeI, and the 4451bp fragment corresponding to the MAE::MEL5 deletion cassette was gel purified. This purified fragment was then transformed into *I. orientalis* strain 13126. Transformants were selected on YNB + melibiose +x-gal and screened by PCR using flanking primers oGPB1 (SEQ ID NO:201) and oGPB4 (SEQ ID NO:202) and nested primers oGPB54 (SEQ ID NO:150) and oGPB55 (SEQ ID NO:151) to verify correct insertion at the MAE1 locus. A homozygous strain with both copies of the MAE1 gene deleted was designated strain 13221.

[00374] The various MAE1 deletion strains generated in Example 22 are summarized in Table 26.

Table 26: *I. orientalis* MAE deletion strains:

Strain name	Description	Parent strain
13126	CYB2A deletion (2) MAE1 deletion (1) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. cerevisiae</i> FRD insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>S. pombe</i> MAE insertion at RIOR43690 (2)	13053
13221	CYB2A deletion (2) MAE1 deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>S. cerevisiae</i> FRD insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>S. pombe</i> MAE insertion at RIOR43690 (2)	13216

Example 22C: Shake flask characterization of malate production in *I. orientalis* strains 13053 and 13221:

[00375] Shake flasks were used to test the parental control (13053) and MAE deletion (13221) strains. Shake flasks were inoculated with biomass harvested from yeast peptone glucose plates (YPD). 250 mL baffled flasks (50 mL working volume) were inoculated to an OD₆₀₀ of 0.3. Incubation conditions were 150 rpm and 30°C. DM defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20) was used in flasks, with pH control and CO₂ provided by calcium carbonate addition at a concentration of 0.355M (1.33 g CaCO₃ per 50 ml culture). Samples were taken at 24 to 66 hours and analyzed for biomass growth via OD₆₀₀. Malate and glucose are detected by high performance liquid chromatography with refractive index detector.

[00376] As malate is the compound directly impacted by this enzyme, increased malate production was used as an indicator of an improved strain. Malate production results are shown in Figure 58. A greater than 3g/L increase in malate production was observed with the deletion of MAE1 from strain 13221 compared to the parental strain 13053 after 66 hours.

Example 23: Insertion of *T. brucei*, *T. cruzi*, *L. braziliensis*, or *L. mexicana* FRD1 at the ADHb locus in *I. orientalis* strain 13053:

[00377] An FRD1 expression cassette is inserted at one or both ADHb alleles of *I. orientalis* strain 13053 (Example 21D).

Example 23A: Construction of ADHb deletion construct pGPB119:

[00378] pGPB26 was digested with NotI and the resultant fragment ligated to NotI digested pHJJ77 (Figure 59). The resulting ADHb deletion construct, designated pGPB119 (Figure 60), contains the *I. orientalis* PDC1 promoter (amplified using primers oJLJ3 (SEQ ID NO:156) and oJLJ19 (SEQ ID NO:157)) and terminator (amplified using primers oJLJ1 (SEQ ID NO:154) and oJLJ2 (SEQ ID NO:155)) and a CYB2A marker element between a 770 bp fragment corresponding to the region immediately 5' of the *I. orientalis* AHD2b open reading frame (amplified using primers oHJJ124 (SEQ ID NO:211) and oHJJ125 (SEQ ID NO:212)) and a 615 bp fragment corresponding to the region immediately 3' of the *I. orientalis* ADHb open reading frame (amplified using primer oHJJ126 (SEQ ID NO:213) and oHJJ127 (SEQ ID NO:214)).

Example 23B: Construction of FRD expression constructs pGPB126, pGPB127, pGPB159, pGPB160, pGPB161, and ADHb null constructs pGPB148 and pGPB168:

[00379] Expression cassettes for FRD1 genes were inserted into the ADHb deletion construct pGPB119: the FRD1 genes from *T. brucei* (SEQ ID NO:173), *T. cruzi* (SEQ ID NO:175), *L. braziliensis* (SEQ ID NO:177), and *L. mexicana* (SEQ ID NO:179), were codon optimized to *I. orientalis*, and lacked the C-terminal glyoxysomal targeting sequence from the native gene.

[00380] Plasmids containing the codon optimized FRD1 genes were digested with XbaI and PacI, and the FRD1 fragments were ligated to similarly digested pGPB119. The resulting plasmids, containing the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and the *I. orientalis* CYB2A selectable marker, were designated pGPB126 (*T. brucei*), pGPB159 (*T. cruzi*), pGPB160 (*L. braziliensis*), and pGPB161 (*L. mexicana*) (Figure 61).

[00381] pGPB126 was digested with NdeI and a partial NotI digest to remove the CYB2A selectable marker, and the resulting vector was ligated to the NdeI and NotI fragment from pGPB14, which contained the MEL5 selectable marker. The resulting plasmid, which

contained the FRD1 coding sequence flanked by the *I. orientalis* PDC1 promoter and terminator and the *S. cerevisiae* MEL5 selectable marker was designated pGPB127 (Figure 62).

[00382] pGPB126 (CYB2A) and pGPB127 (MEL5) were each digested with NdeI and BamHI to remove those portions of the plasmids corresponding to the PDC promoter, *T. brucei* FRD, and PDC terminator, and each plasmid backbone was blunted with Klenow fragment and ligated to recircularize the plasmid. The plasmids were then transformed into *E. coli*. Plasmids isolated from positive colonies were designated pGPB168 (Figure 63, derived from pGPB126) and pGPB148 (Figure 64, derived from pGPB127).

Example 23C: Insertion of FRD1 at first and second ADHb loci of *I. orientalis* strain 13053:

[00383] pGPB126, pGPB159, pGPB160, pGPB161 and pGPB148 were digested with SacI and Apal and transformed into *I. orientalis* strain 13053 by lithium acetate transformation. Transformants were selected on YNB + 2% lactic plates or YNB + 2% melibiose plates (pGPB148). After around six days, transformants were picked and plated for single colonies on YP + 20 g/L glucose plates. Colonies were picked, and genomic DNA was isolated and screened by PCR to confirm integration of the FRD1 expression cassette at the ADHb locus using primers oGPB106 (SEQ ID NO:215), oGPB56 (SEQ ID NO:187), oGPB52 (SEQ ID NO:148), oGPB54 (SEQ ID NO:150), oGPB55 (SEQ ID NO:151), and oGPB107 (SEQ ID NO:196). Strains with the correct integration of the FRD1 gene were designated ySBCG261, 13171, ySBCG263 (*T. brucei* FRD); ySBCG283, 13256, ySBCG285 (*T. cruzi* FRD); ySBCG286, 13257, ySBCG288 (*L. braziliensis* FRD); and ySBCG289, 13258, ySBCG291 (*L. mexicana* FRD). Strains with a single ADHb allele deleted are designated ySBCG280, 13255, and ySBCG282.

[00384] pGPB127 and pGPB168 were digested with SacI and Apal and transformed into *I. orientalis* strains ySBCG261, 13171, ySBCG263, ySBCG280, 13255, and ySBCG282 by lithium acetate transformation. Transformants were selected on either YNB + 2% lactic plates overlaid with α -x-gal or YNB + 2% melibiose plates overlaid with α -x-gal. After around seven days, transformants were picked and plated for single colonies on YP + 20 g/L glucose plates. Colonies are picked, and genomic DNA is isolated and screened by PCR to confirm integration of the FRD1 expression cassette at the ADHb locus using primers oGPB106 (SEQ ID NO:215), oGPB54 (SEQ ID NO:150), oGPB52 (SEQ ID NO:148), oGPB53 (SEQ ID NO:149), oGPB55 (SEQ ID NO:151), and oGPB107 (SEQ ID NO:196). Strains with the correct integration of the FRD1 gene were designated 13143, 13144, and ySBCG270. Strains with a homozygous knockout of the ADHb allele were designated ySBCGH460, ySBCGH461, and ySBCGH462.

[00385] The various FRD1 insertion/ADHb deletion strains generated in Example 23 are summarized in Table 27.

Table 27: *I. orientalis* FRD1 insertion strains:

Strain name	Description	Parent strain
ySBCG261/13171/ ySBCG263	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>T. brucei</i> FRD1 insertion at ADHb (1)	13053
ySBCG283/13256/ ySBCG285	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>T. cruzi</i> FRD1 insertion at ADHb (1)	13053
ySBCG286/13257/ ySBCG288	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>L. braziliensis</i> FRD1 insertion at ADHb (1)	13053
ySBCG289/13258/ ySBCG291	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>L. mexicana</i> FRD1 insertion at ADHb (1)	13053
ySBCG280/13255/ ySBCG282	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) Null insertion at ADHb (1)	13053
13143/13144/ ySBCG270	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) <i>T. brucei</i> FRD1 insertion at ADHb (2)	ySBCG261/13171/ ySBCG263
ySBCGH460/ySBCGH461/ ySBCGH462	CYB2A deletion (2) <i>I. orientalis</i> PYC1 insertion at PDC1 (2) <i>S. cerevisiae</i> FRD1 insertion at ADHa (2) <i>Z. rouxii</i> MDH insertion at ATO2 (2) <i>I. orientalis</i> FUM1 insertion at CYB2B (2) Null insertion at ADHb (2)	ySBCG280/13255/ ySBCG282

Example 23D: Shake flask characterization of malate production in *I. orientalis* strains 13053, ySBCG261/13171/ySBCG263, ySBCG283/13256/ySBCG285, ySBCG286/13257/ySBCG288, ySBCG289/13258/ySBCG291, and ySBCG280/13255/ySBCG282:

[00386] Shake flasks are used to test the parental control (13053), FRD expression strains (ySBCG261/13171/ySBCG263, ySBCG283/13256/ySBCG285, ySBCG286/13257/ySBCG288, and ySBCG289/13258/ySBCG291), and site controls (ySBCG280/13255/ySBCG282). Shake flasks are inoculated with biomass harvested from yeast peptone glucose plates (YPD). 250 mL baffled flasks (50 mL working volume) were inoculated to an OD₆₀₀ of 0.3. Incubation conditions were 150 rpm and 30 °C. DM defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20) was used in flasks, with pH control and CO₂ provided by calcium carbonate addition at a concentration of 0.355M (1.33 g CaCO₃ per 50 ml culture). Samples were taken at 68 hours and analyzed for biomass growth via OD₆₀₀. Succinate and glucose were detected by high performance liquid chromatography with refractive index detector.

[00387] Results are shown in Figure 65. Increased succinate production was observed with gene insertion.

Example 24: Succinate production by *I. orientalis* strain 13171:

[00388] *I. orientalis* strain 13171 (Example 23C) was run in fermentors to test succinic acid production. Fermentors are inoculated with biomass grown in defined medium (adapted from Verduyn et al. Yeast 8:501-517 (1992); see Tables 18-20). Seeds are run in 1L baffled flasks (250 mL working volume) at 250 rpm and 30 °C. The contents of the flasks are harvested at approximately 24 hours incubation time with 10% v/v inoculum used to start fermentors. Fermentor initial working volume is 1.2 L for fed-batch glucose and 1.5 L for straight batch glucose. Fermentor media is outlined in Tables 18-20. Glucose was provided by either controlling feed addition to the fermentor, with feed maintained at <10 g/l residual glucose, or by the addition of 115 g/l at the start of the batch (straight batch). Straight batch is used in this organism as a preferred mode of operation, due to the lack of a Crabtree positive phenotype. This mode offers simplicity in industrial operation.

[00389] pH is controlled at 3.0 with 5 N KOH. The fermentor systems are sparged at 0.24 slpm with a blend of pure CO₂ and air to yield 10% CO₂ in the inlet gas stream. Different oxygen uptake rates are applied to the vessels by changing vessel agitation rate. These fermentations are operated such that oxygen limitation was maintained (e.g. dissolved oxygen <5%).

[00390] Samples are taken at 90 hour batch time and analyzed for biomass growth via OD_{600} , succinate via gas chromatography with flame ionization detector and glucose by high performance liquid chromatography with refractive index detector.

[00391] Figure 66 illustrates succinic acid production under the conditions outline above in both fed-batch and straight batch glucose. A previous experiment (WO2010/0032728, p. 9, lines 15-18) reported that “an Oxygen Uptake Rate (OUR) above 5 mmol/l/h resulted in lower succinic acid production”. Surprisingly, we found improving production above 5 mmol/l/h with succinic acid titer increases with increasing OUR such that the no decrease in succinic production was observed even at the highest OUR tested, 20 mmol/l/h.

What is claimed is:

1. A genetically modified yeast cell having an active succinate fermentation pathway from phosphoenolpyruvate or pyruvate to succinate.
2. The genetically modified yeast cell of claim 1, wherein the active succinate fermentation pathway includes the reactions:
 - (a) pyruvate to oxaloacetate;
 - (b) oxaloacetate to malate;
 - (c) malate to fumarate; and
 - (d) fumarate to succinate.
3. The genetically modified yeast cell of claim 2, wherein the active succinate fermentation pathway further includes:
 - (e) export of succinate from inside the cell to the extracellular environment.
4. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises one or more endogenous genes that encode an enzyme that catalyzes any of reactions (a) through (e).
5. The genetically modified yeast cell of claim 4, wherein the cell comprises one or more copies of one or more endogenous genes selected from the group consisting of pyruvate carboxylase, malate dehydrogenase, fumarase, fumarate reductase, and succinate exporter genes.
6. The genetically modified yeast cell of claim 5, wherein one or more of said endogenous genes are operatively linked to an exogenous regulatory element selected from the group consisting of an exogenous promoter and an exogenous terminator.
7. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises one or more exogenous genes that encode an enzyme that catalyzes any of reactions (a) through (e).
8. The genetically modified yeast cell of claim 7, wherein the cell comprises one or more copies of one or more exogenous genes selected from the group consisting of pyruvate carboxylase, malate dehydrogenase, fumarase, fumarate reductase, and succinate exporter genes.
9. The genetically modified yeast cell of claim 8, wherein said exogenous pyruvate carboxylase gene is derived from a yeast pyruvate carboxylase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast pyruvate carboxylase source gene.
10. The genetically modified yeast cell of claim 9, wherein said exogenous pyruvate carboxylase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12.

11. The genetically modified yeast cell of claim 10, wherein said exogenous pyruvate carboxylase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11.

12. The genetically modified yeast cell of claim 8, wherein said exogenous pyruvate carboxylase gene is derived from a fungal pyruvate carboxylase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the fungal pyruvate carboxylase source gene, wherein the fungal pyruvate carboxylase source gene is not derived from *R. oryzae*.

13. The genetically modified yeast cell of claim 8, wherein said exogenous malate dehydrogenase gene is derived from a yeast malate dehydrogenase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast malate dehydrogenase source gene.

14. The genetically modified yeast cell of claim 13, wherein said exogenous malate dehydrogenase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:168, SEQ ID NO:20, SEQ ID NO:22, or SEQ ID NO:24.

15. The genetically modified yeast cell of claim 14, wherein said exogenous malate dehydrogenase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:167, SEQ ID NO:19, SEQ ID NO:21, or SEQ ID NO:23.

16. The genetically modified yeast cell of claim 8, wherein said exogenous malate dehydrogenase gene is derived from a bacterial malate dehydrogenase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the bacterial malate dehydrogenase source gene.

17. The genetically modified yeast cell of claim 16, wherein said exogenous malate dehydrogenase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:170.

18. The genetically modified yeast cell of claim 17, wherein said exogenous malate dehydrogenase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:169.

19. The genetically modified yeast cell of claim 8, wherein said exogenous malate dehydrogenase gene is derived from a fungal malate dehydrogenase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the fungal malate dehydrogenase source gene.

20. The genetically modified yeast cell of claim 19, wherein said exogenous malate dehydrogenase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:172.

21. The genetically modified yeast cell of claim 20, wherein said exogenous malate dehydrogenase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:171.

22. The genetically modified yeast cell of claim 8, wherein said exogenous fumarase gene is derived from a yeast fumarase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast fumarase source gene.

23. The genetically modified yeast cell of claim 22, wherein said exogenous fumarase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:2.

24. The genetically modified yeast cell of claim 23, wherein said exogenous fumarase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1.

25. The genetically modified yeast cell of claim 8, wherein said exogenous fumarate reductase gene is derived from a yeast fumarate reductase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast fumarate reductase source gene.

26. The genetically modified yeast cell of claim 25, wherein said exogenous fumarate reductase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:32.

27. The genetically modified yeast cell of claim 26, wherein said exogenous fumarate reductase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, or SEQ ID NO:31.

28. The genetically modified yeast cell of claim 8, wherein said exogenous fumarate reductase gene is derived from a protozoan fumarate reductase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the protozoan fumarate reductase source gene.

29. The genetically modified yeast cell of claim 28, wherein said exogenous fumarate reductase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:174, SEQ ID NO:176, SEQ ID NO:178, or SEQ ID NO:180.

30. The genetically modified yeast cell of claim 29, wherein said exogenous fumarate reductase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:173, SEQ ID NO:175, SEQ ID NO:177, or SEQ ID NO:178.

31. The genetically modified yeast cell of claim 8, wherein said exogenous succinate exporter gene is derived from a fungal succinate exporter source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the fungal succinate exporter source gene.

32. The genetically modified yeast cell of claim 31, wherein said exogenous succinate exporter gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:182 or SEQ ID NO:184.

33. The genetically modified yeast cell of claim 32, wherein said exogenous succinate exporter gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:181 or SEQ ID NO:183.

34. The genetically modified yeast cell of claim 2, wherein the active succinate fermentation pathway comprises the reaction phosphoenolpyruvate to oxaloacetate in lieu of reaction (a).

35. The genetically modified yeast cell of claim 2 or 3, wherein the active succinate fermentation pathway comprises the reaction phosphoenolpyruvate to oxaloacetate in addition to reaction (a).

36. The genetically modified yeast cell of claim 34 or 35, wherein the cell comprises one or more copies of one or more endogenous phosphoenolpyruvate carboxylase genes.

37. The genetically modified yeast cell of claim 36, wherein one or more of said endogenous phosphoenolpyruvate carboxylase genes are operatively linked to an exogenous regulatory element selected from the group consisting of an exogenous promoter and an exogenous terminator.

38. The genetically modified yeast cell of claim 34 or 35, wherein the cell comprises one or more copies of one or more exogenous phosphoenolpyruvate carboxylase genes.

39. The genetically modified yeast cell of claim 38, wherein said exogenous phosphoenolpyruvate carboxylase gene is derived from a bacterial phosphoenolpyruvate carboxylase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the bacterial phosphoenolpyruvate carboxylase source gene.

40. The genetically modified yeast cell of claim 39, wherein said exogenous phosphoenolpyruvate carboxylase gene encodes a polypeptide comprising an amino acid

sequence with at least 95% sequence identity to an amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ ID NO:4 or SEQ ID NO:6.

41. The genetically modified yeast cell of claim 40, wherein said exogenous phosphoenolpyruvate carboxylase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:5.

42. The genetically modified yeast cell of claim 2 or 3, wherein the cell has an active reduction pathway from glucose-6-phosphate to ribulose 5-phosphate.

43. The genetically modified yeast cell of claim 42, wherein the active reduction pathway includes the reactions:

(f) glucose 6-phosphate to 6-phosphogluconolactone;

(g) 6-phosphogluconolactone to 6-phosphogluconate; and

(h) 6-phosphogluconate to ribulose 5-phosphate.

44. The genetically modified yeast cell of claim 43, wherein the cell comprises one or more endogenous genes that encode an enzyme that catalyzes any of reactions (e) through (g).

45. The genetically modified yeast cell of claim 44, wherein the cell comprises one or more copies of one or more endogenous genes selected from the group consisting of glucose 6-phosphate dehydrogenase, gluconolactonase, and 6-phosphogluconate dehydrogenase genes.

46. The genetically modified yeast cell of claim 45, wherein one or more of said endogenous genes are operatively linked to an exogenous regulatory element selected from the group consisting of an exogenous promoter and an exogenous terminator.

47. The genetically modified yeast cell of claim 43, wherein the cell comprises one or more exogenous genes that encode an enzyme that catalyzes any of reactions (e) through (g).

48. The genetically modified yeast cell of claim 47, wherein the cell comprises one or more copies of one or more exogenous genes selected from the group consisting of glucose 6-phosphate dehydrogenase, gluconolactonase, and 6-phosphogluconate dehydrogenase genes.

49. The genetically modified yeast cell of claim 48, wherein said exogenous glucose 6-phosphate dehydrogenase gene is derived from a yeast glucose 6-phosphate dehydrogenase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast glucose 6-phosphate dehydrogenase source gene.

50. The genetically modified yeast cell of claim 49, wherein said exogenous glucose 6-phosphate dehydrogenase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:34.

51. The genetically modified yeast cell of claim 50, wherein said exogenous glucose 6-phosphate dehydrogenase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:33.

52. The genetically modified yeast cell of claim 48, wherein said exogenous gluconolactonase gene is derived from a yeast gluconolactonase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast gluconolactonase source gene.

53. The genetically modified yeast cell of claim 52, wherein said exogenous gluconolactonase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:36.

54. The genetically modified yeast cell of claim 53, wherein said exogenous gluconolactonase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:35.

55. The genetically modified yeast cell of claim 48, wherein said exogenous 6-phosphogluconate dehydrogenase gene is derived from a yeast 6-phosphogluconate dehydrogenase source gene and encodes a polypeptide with at least 95% sequence identity to a polypeptide encoded by the yeast 6-phosphogluconate dehydrogenase source gene

56. The genetically modified yeast cell of claim 55, wherein said exogenous 6-phosphogluconate dehydrogenase gene encodes a polypeptide comprising an amino acid sequence with at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:38.

57. The genetically modified yeast cell of claim 56, wherein said exogenous 6-phosphogluconate dehydrogenase gene comprises a nucleotide sequence with at least 95% sequence identity to the nucleotide sequence set forth in SEQ ID NO:37.

58. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises a deletion or disruption of an endogenous pyruvate carboxykinase gene.

59. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises a deletion or disruption of an endogenous malic enzyme gene.

60. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises a deletion or disruption of an endogenous pyruvate decarboxylase gene.

61. The genetically modified yeast cell of claim 2 or 3, wherein the cell comprises a deletion or disruption of an endogenous succinate importer gene.

62. The genetically modified yeast cell of claim 61, wherein said endogenous succinate importer gene is RIOR43690.

63. The genetically modified yeast cell of any of claims 1-62, wherein the yeast cell belongs to a genus selected from the group consisting of *Issatchenkia*, *Candida*, *Pichia*,

Zygosaccharomyces, *Kluyveromyces*, *Saccharomyces*, *Debaryomyces*, and *Saccharomycopsis*.

64. The genetically modified yeast cell of claim 63, wherein the yeast cell is a species selected from the group consisting of *Issatchenkia orientalis*, *Candida lambica*, *Candida sorboxylosa*, *Candida zemplinina*, *Candida geochares*, *Pichia membranifaciens*, *Zygosaccharomyces kombuchaensis*, *Candida sorbosivorans*, *Kluyveromyces marxianus*, *Candida vanderwaltii*, *Candida sorbophila*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces lentus*, *Saccharomyces bayanus*, *Saccharomyces bulderi*, *Debaryomyces castellii*, *Candida boidinii*, *Candida etchellsii*, *Kluyveromyces lactis*, *Pichia jadinii*, *Pichia anomala*, and *Saccharomycopsis crataegensis*.

65. The genetically modified yeast cell of any of claims 1-62, wherein the yeast cell is from the *Pichia fermentans*/*Issatchenkia orientalis* clade.

66. The genetically modified yeast cell of any of claims 1-65, wherein the yeast cell is succinate resistant.

67. A method of producing succinate comprising culturing the genetically modified yeast cell of any of claims 1-66 in the presence of at least one carbon source and isolating succinate from the culture.

68. The method of claim 67, wherein said carbon source is selected from the group consisting of glucose, xylose, arabinose, sucrose, fructose, cellulose, glucose oligomers, and glycerol.

69. A process for converting succinate produced by the method of claim 67 to a product selected from the group consisting of poly-butylene succinate (PBS), other polymers, cosmetics, foodstuff, feed, and pharmaceuticals.

Figure 1

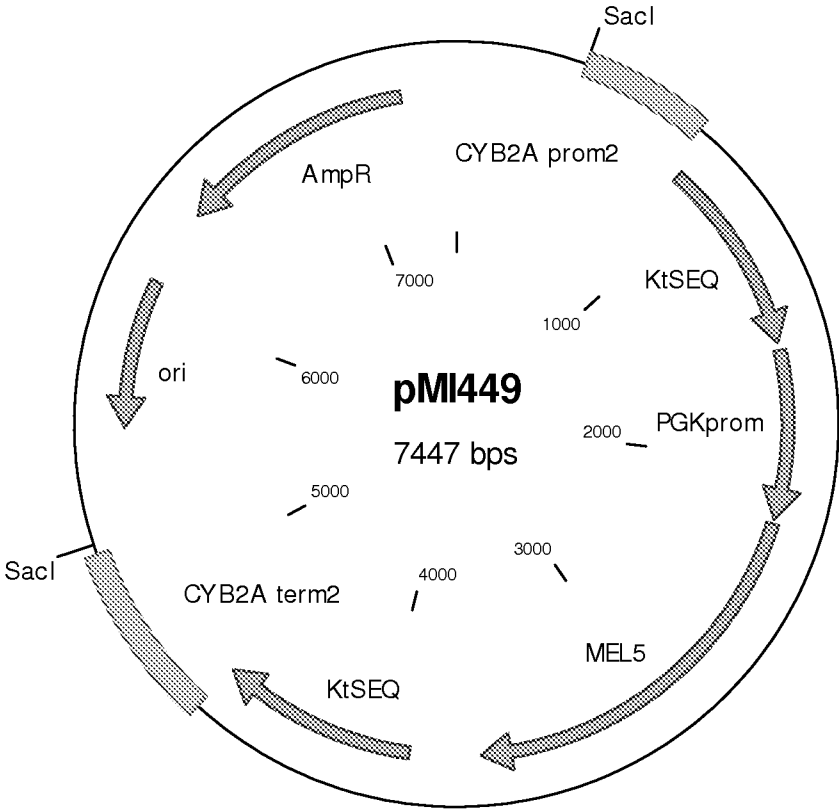


Figure 2

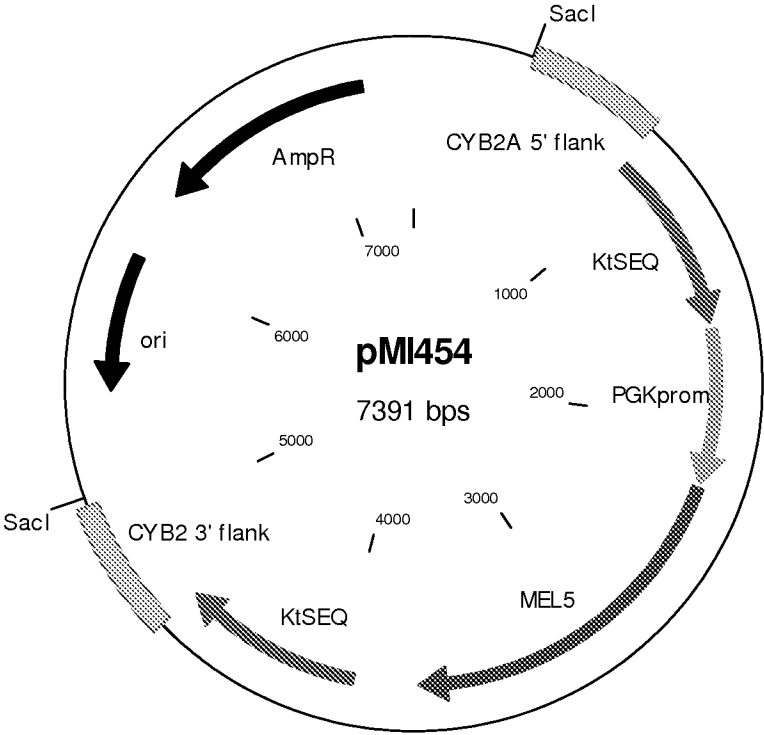


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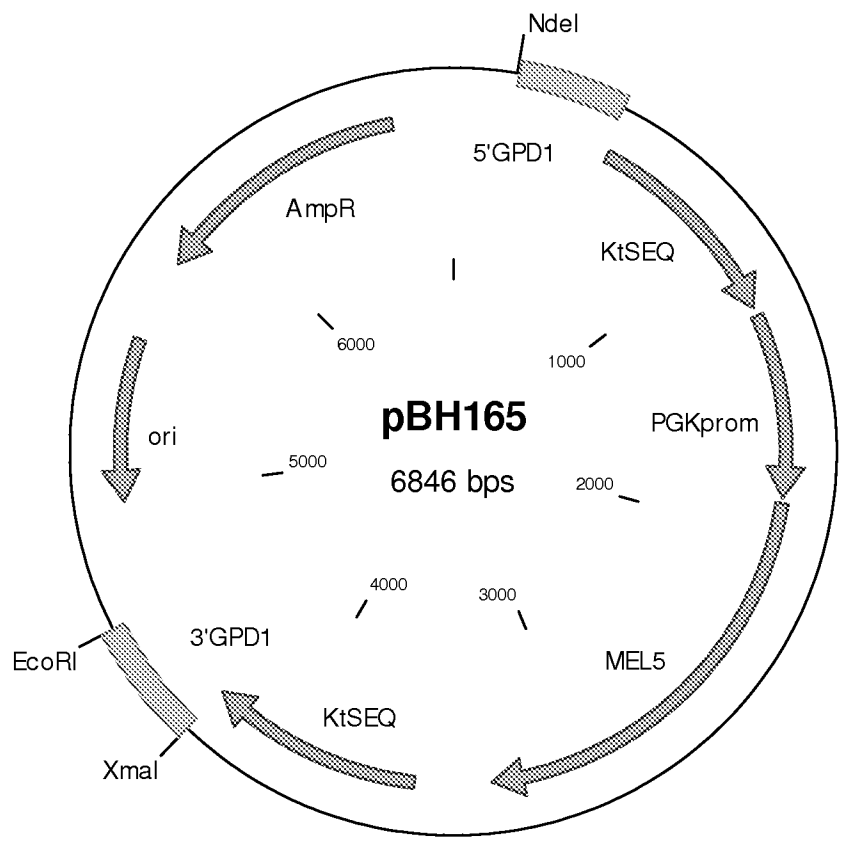


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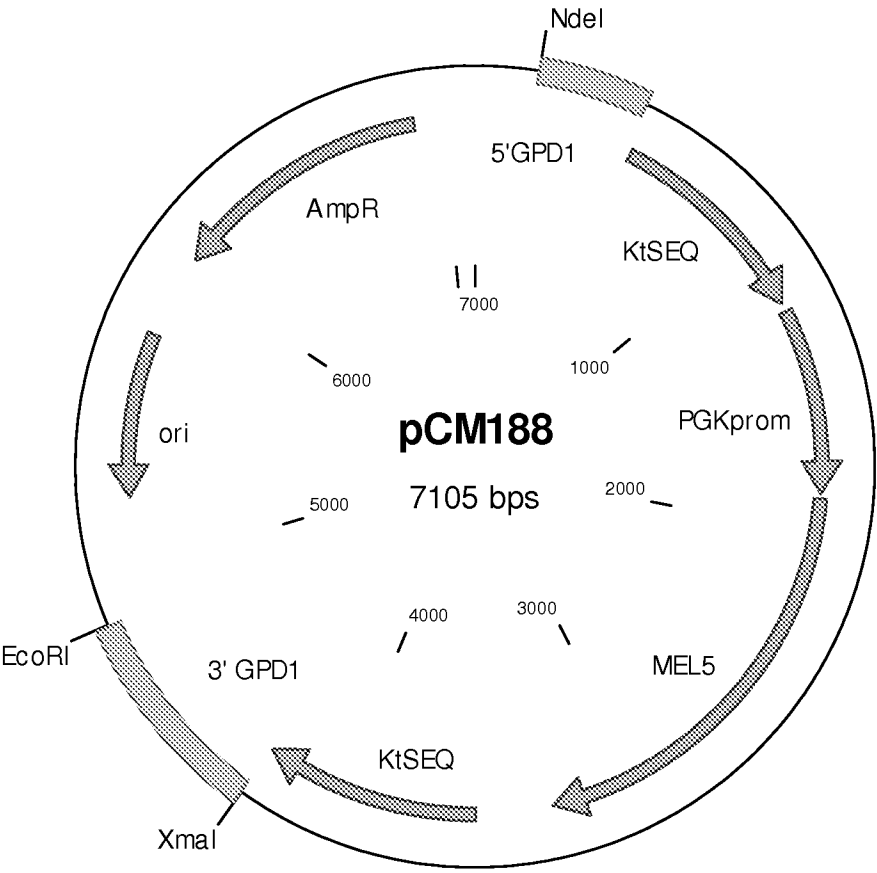


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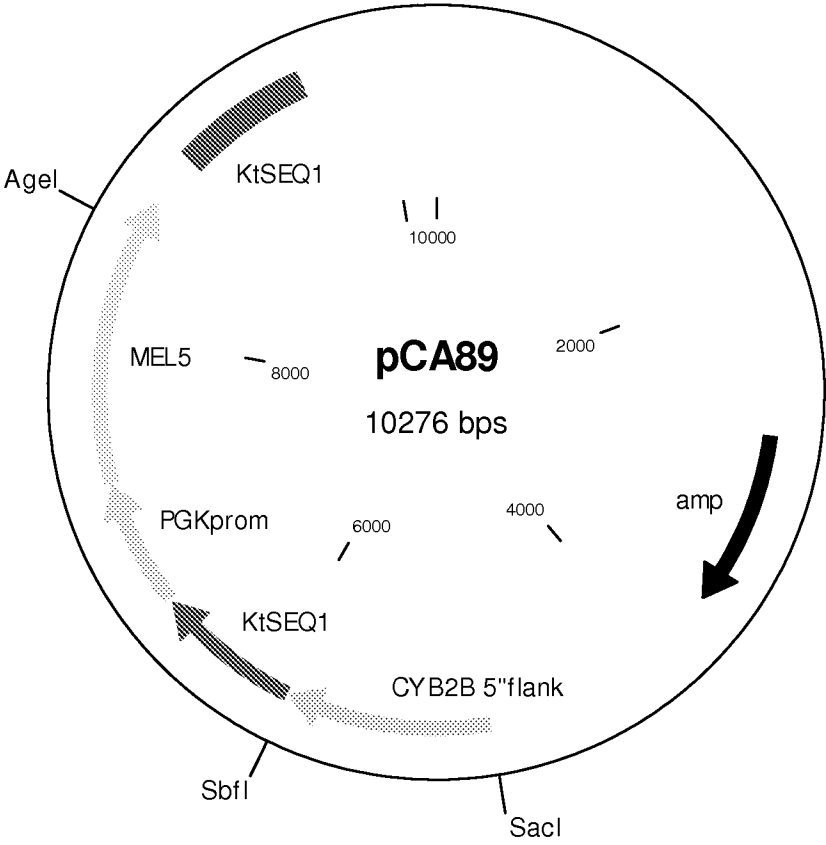


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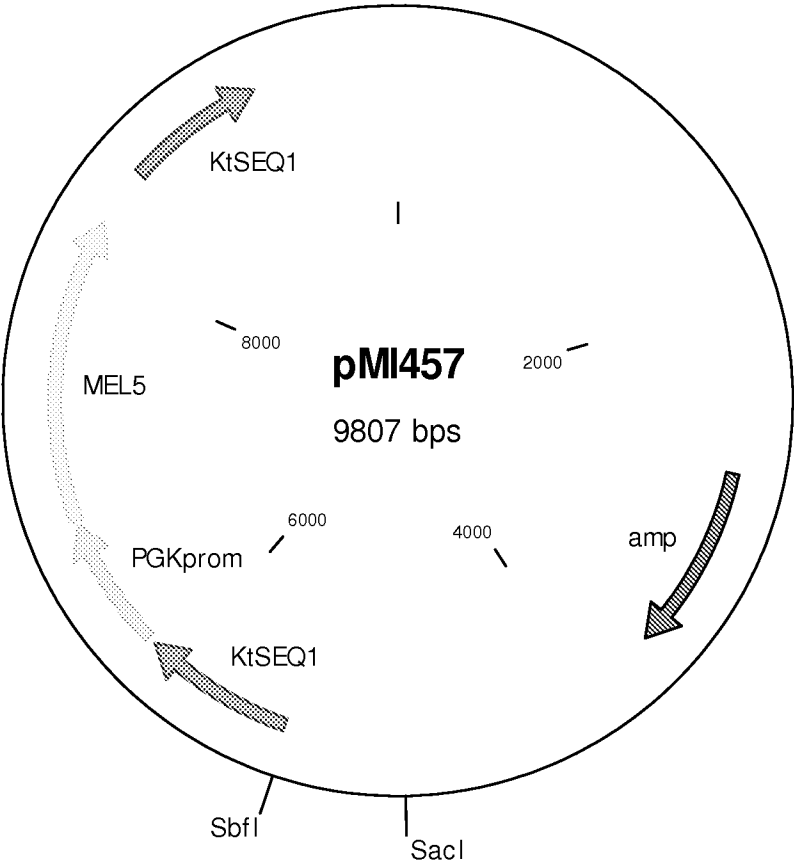


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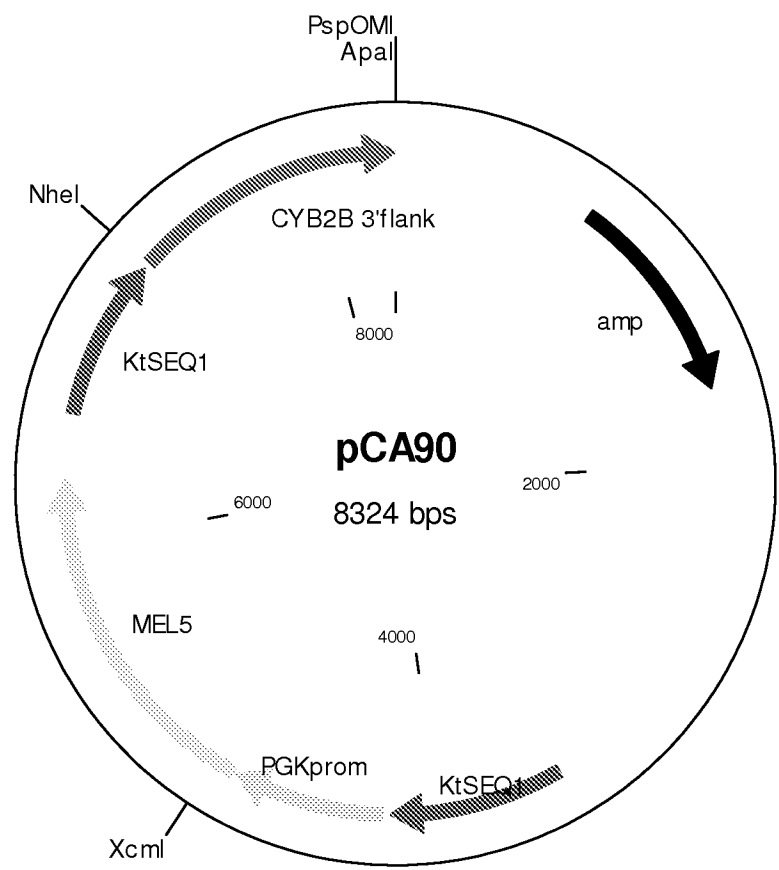


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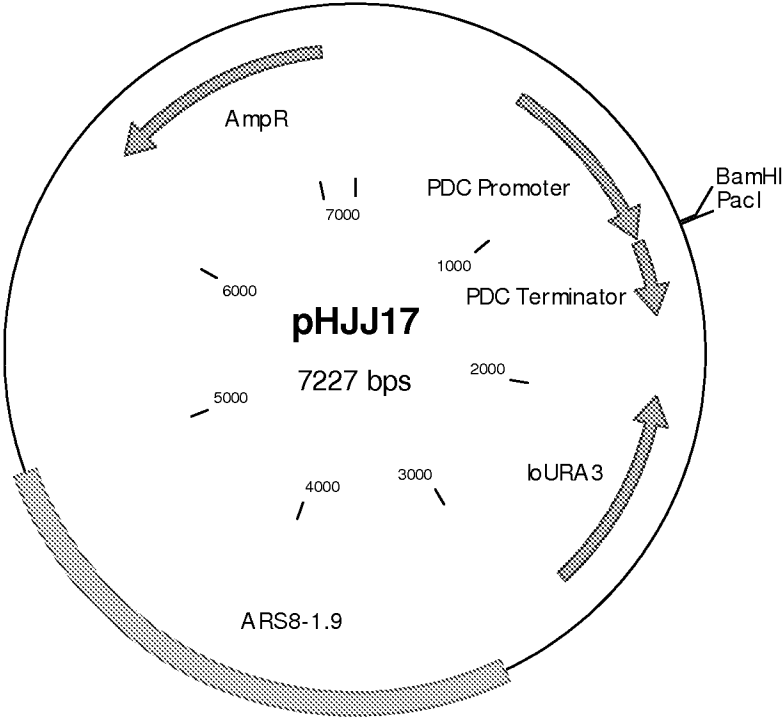


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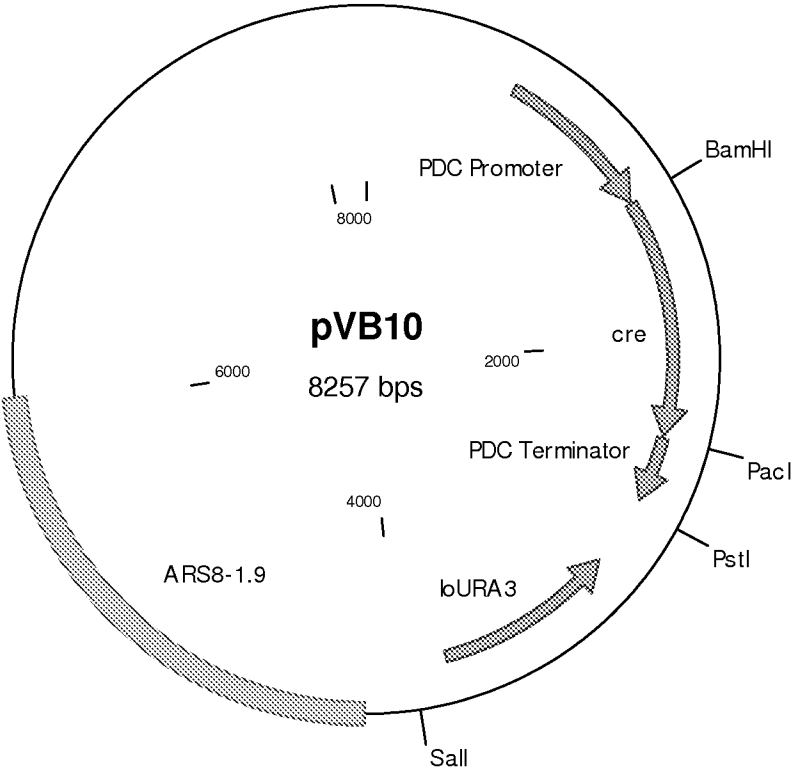
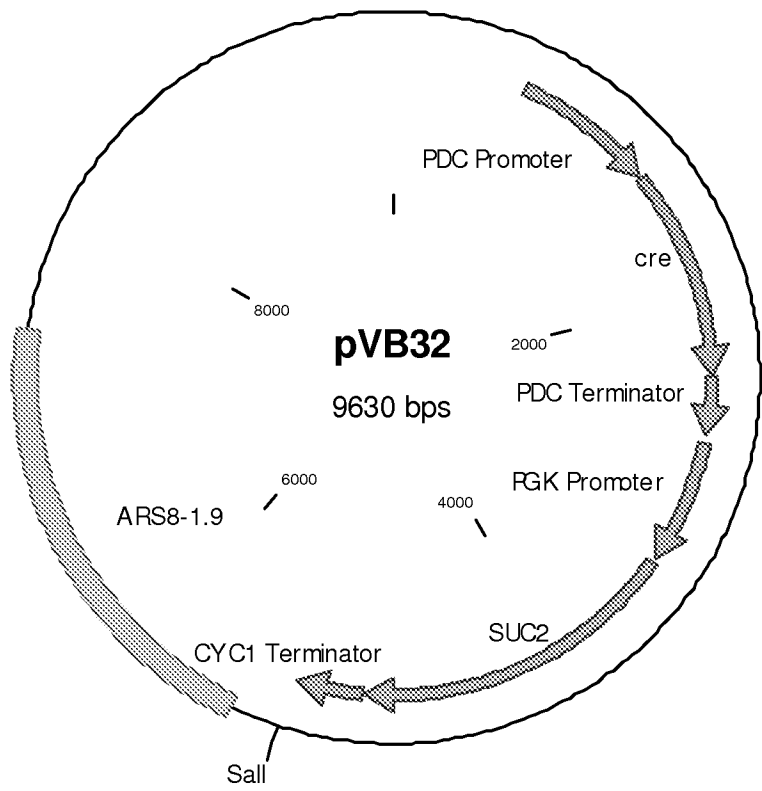
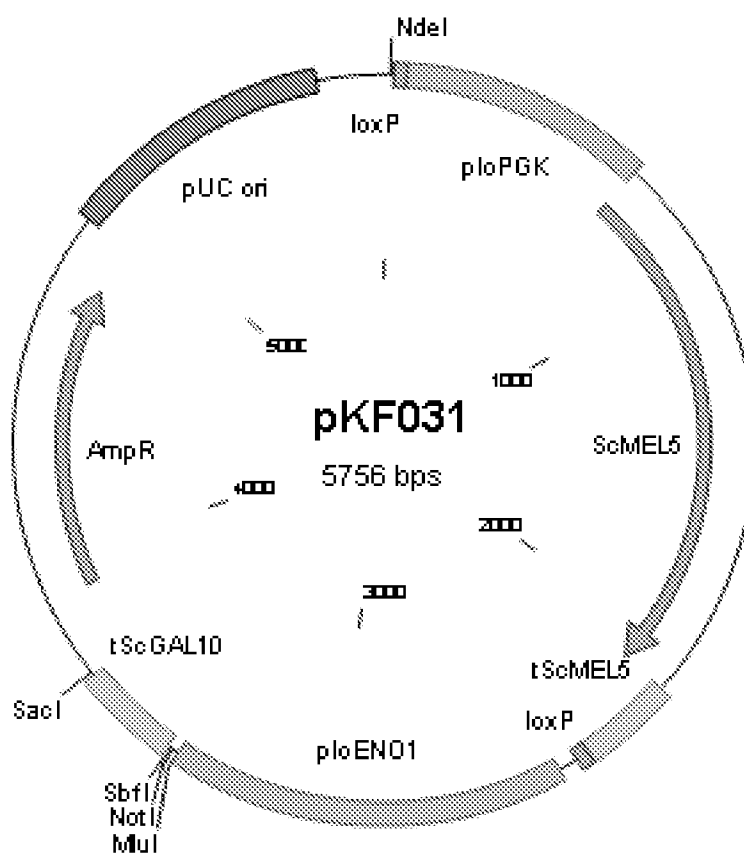


Figure 10



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Figure 11



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Figure 12

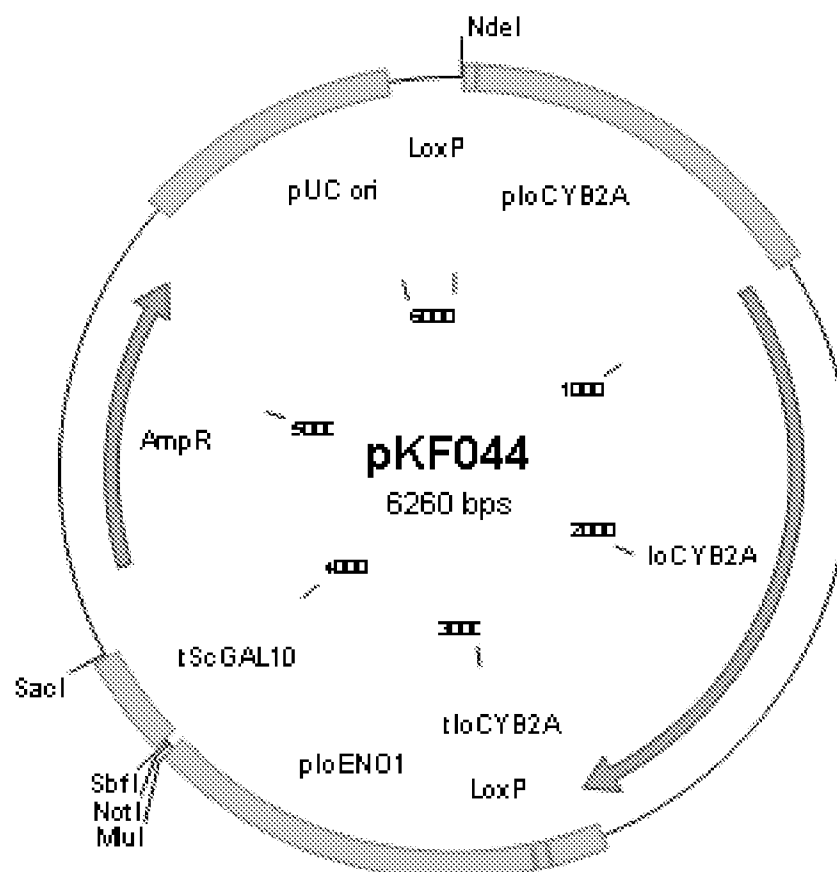


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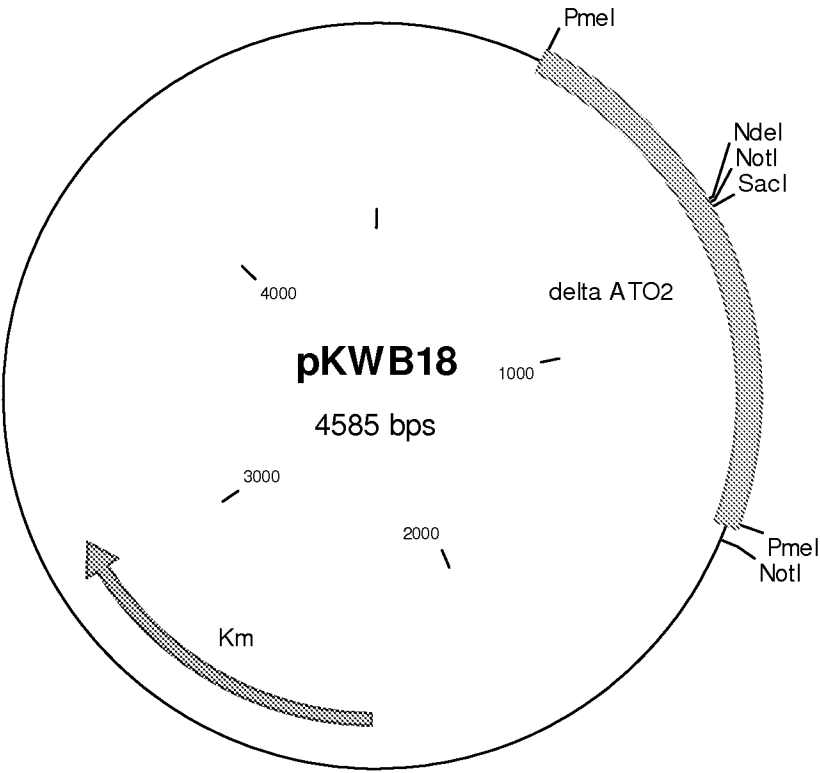
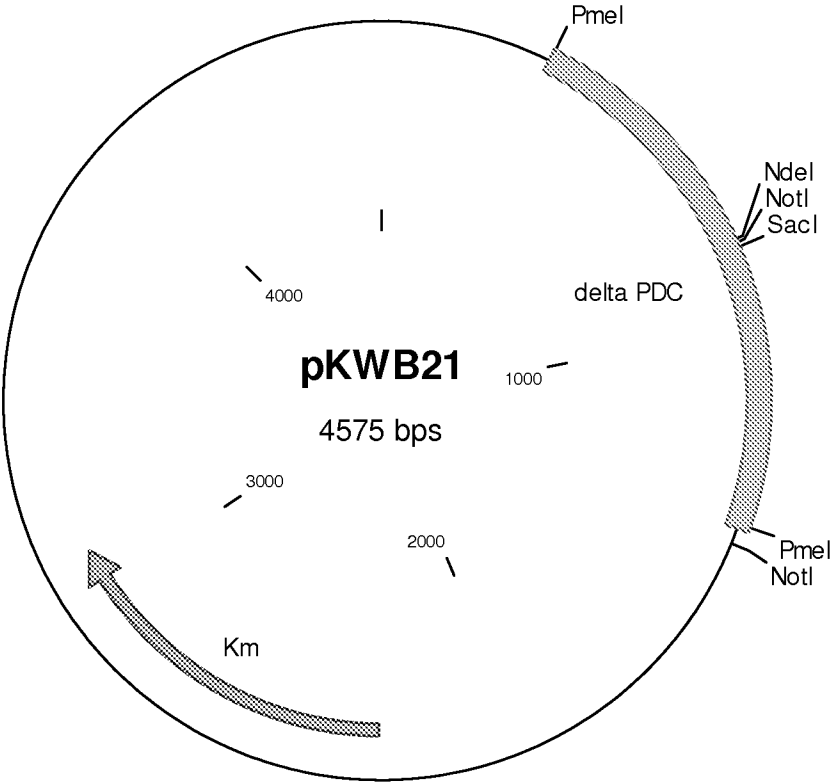
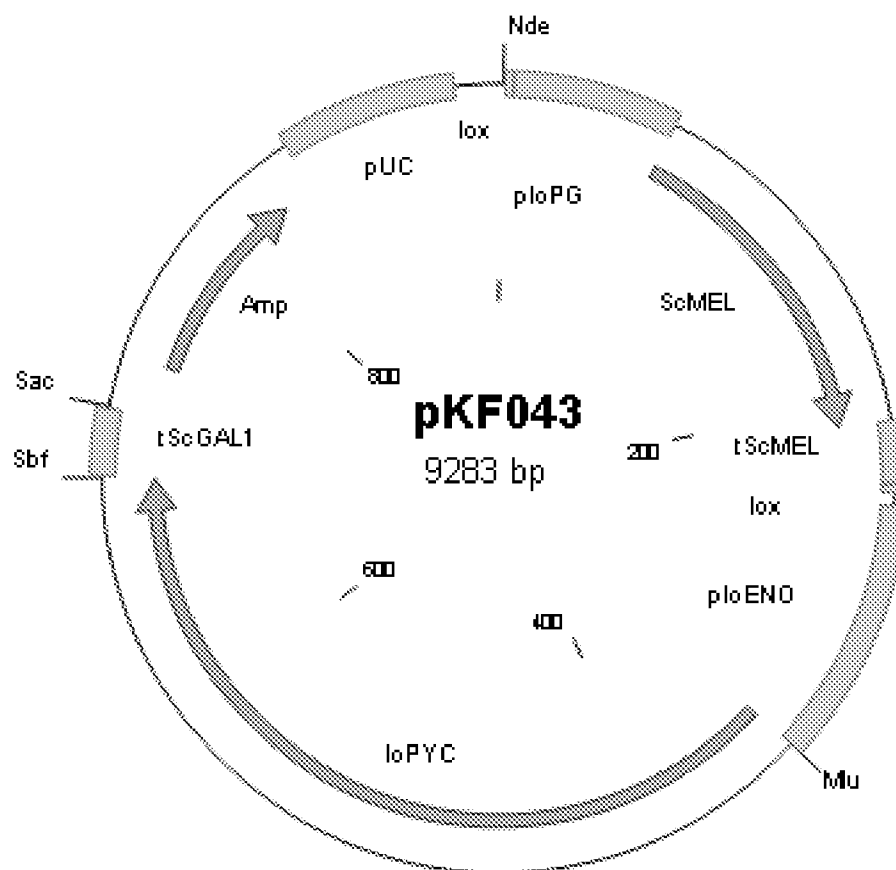


Figure 14



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Figure 15



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Figure 16

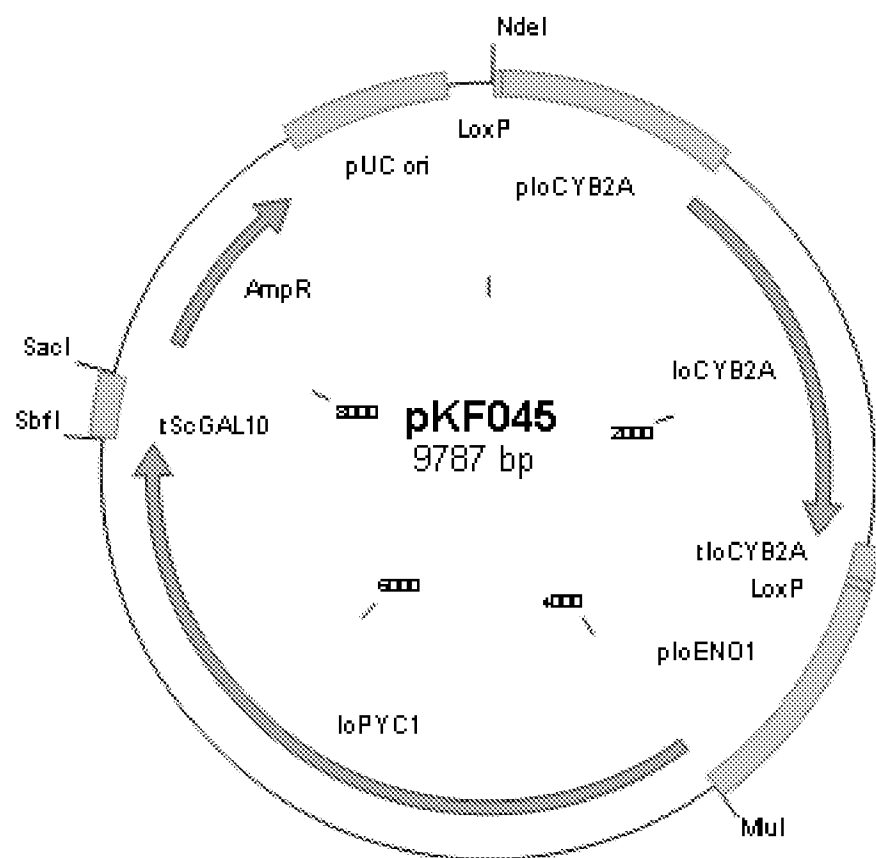


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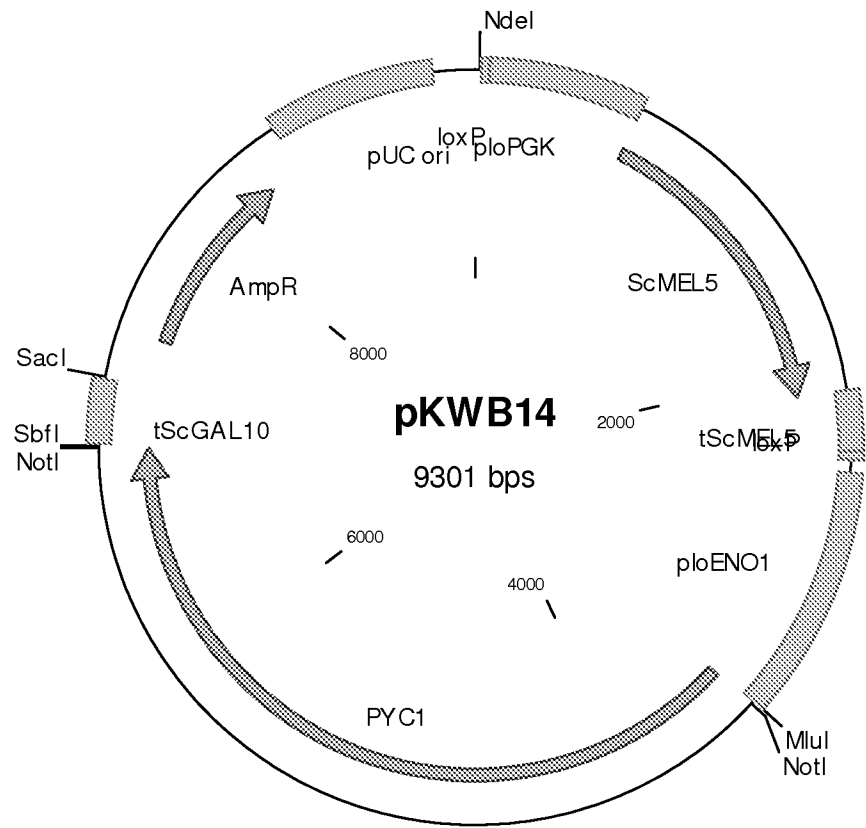


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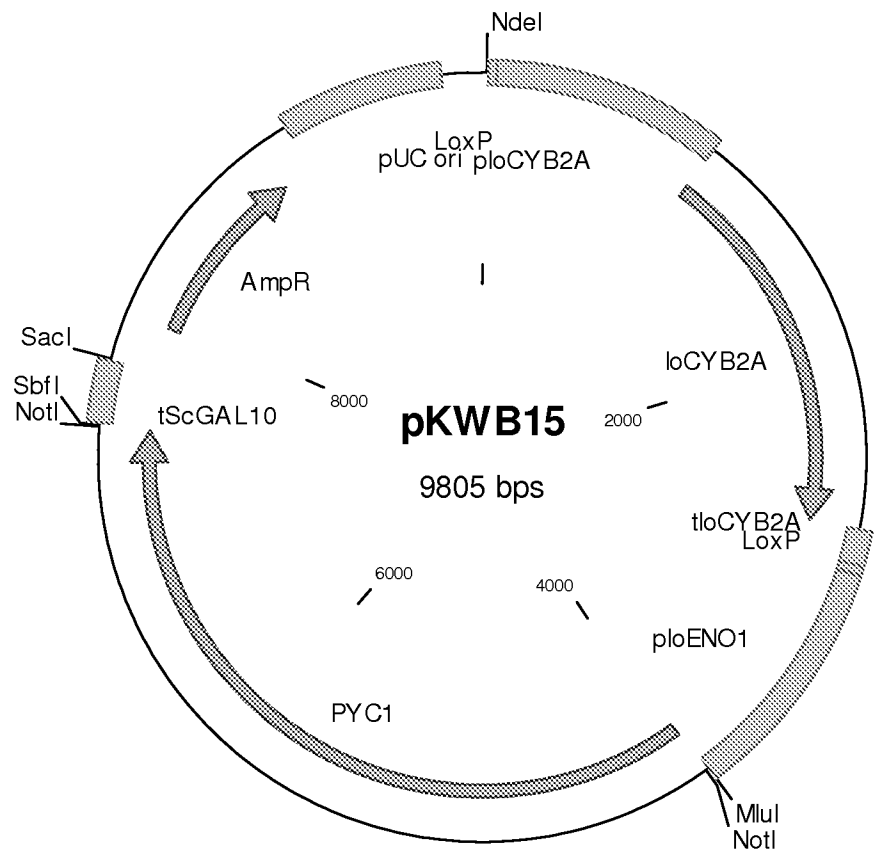


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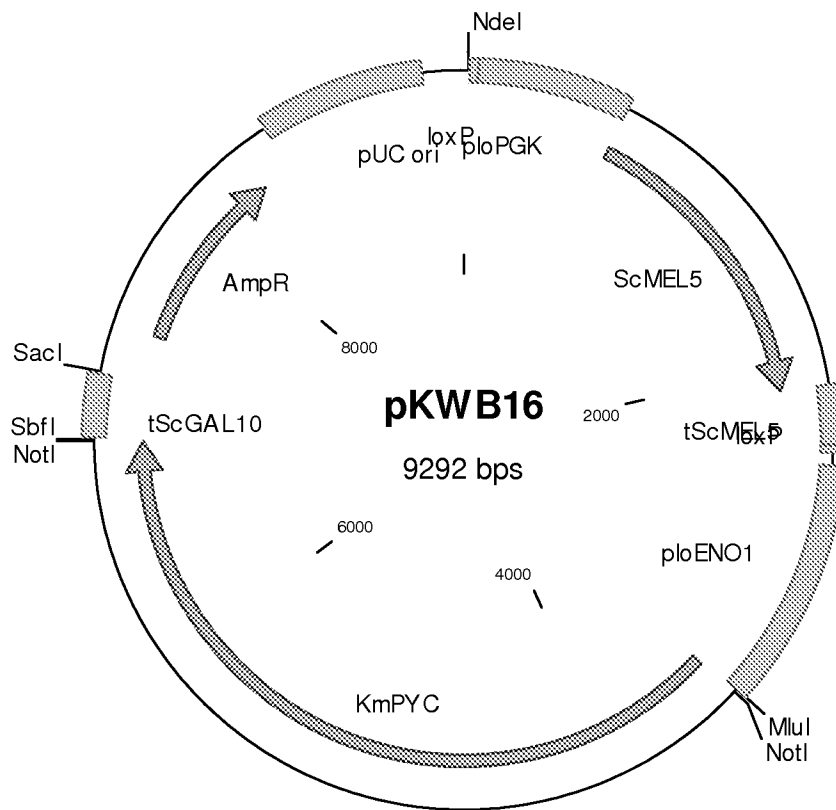


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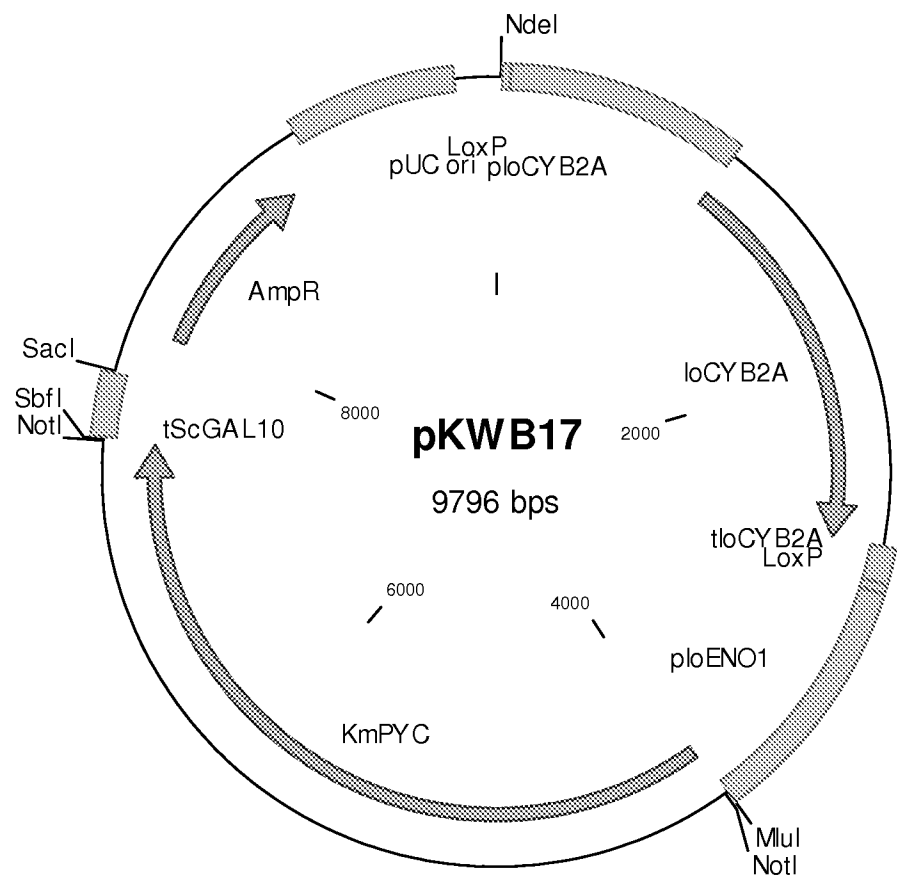


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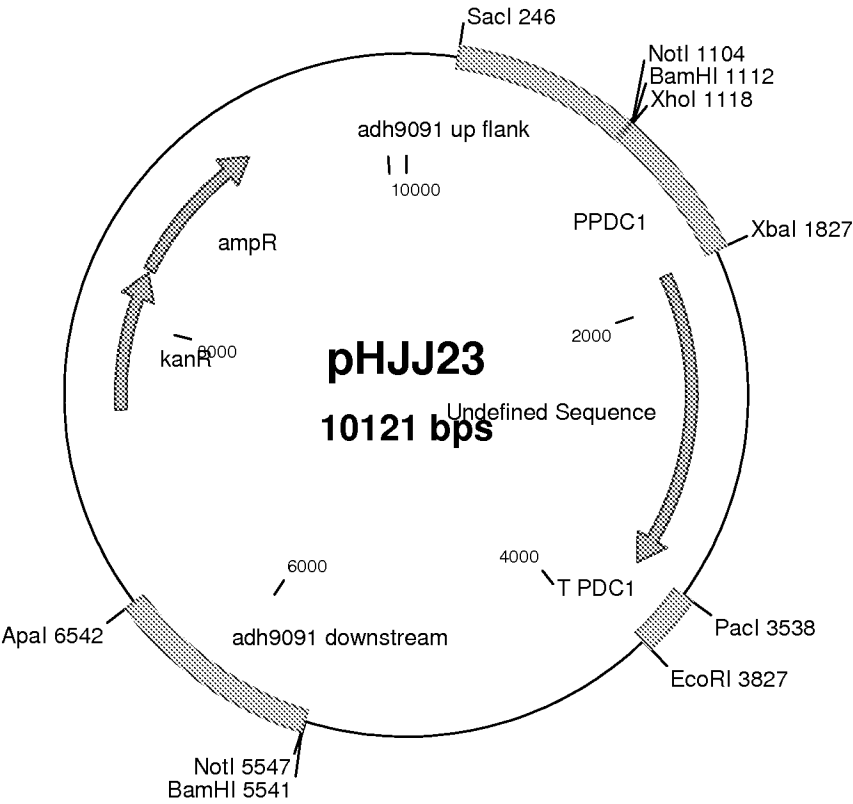


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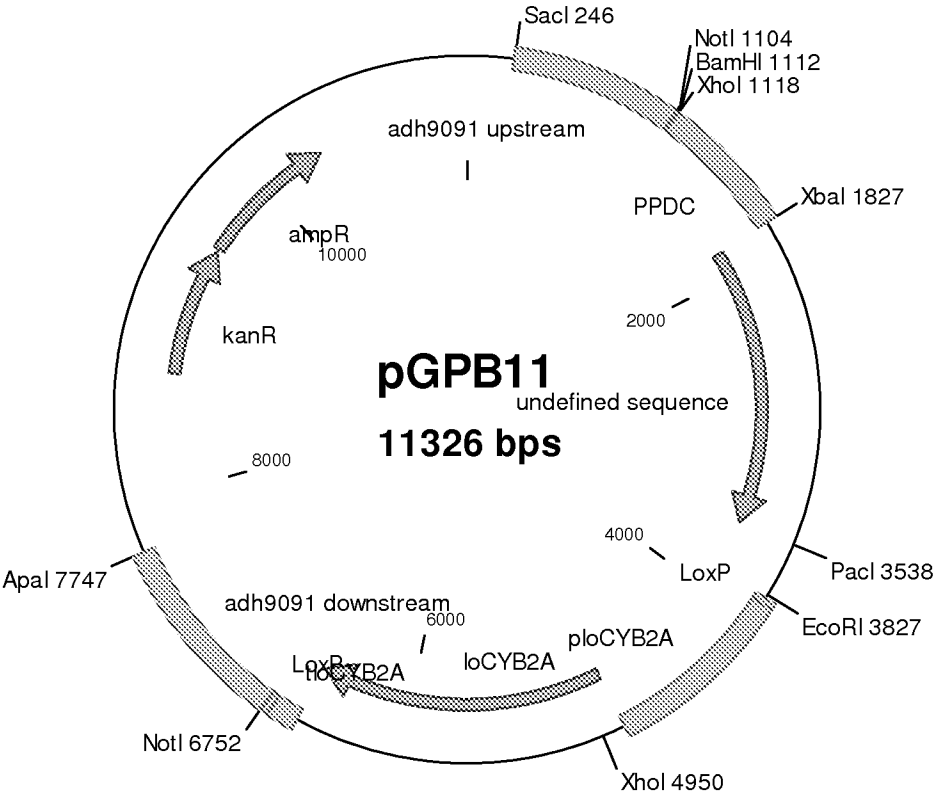


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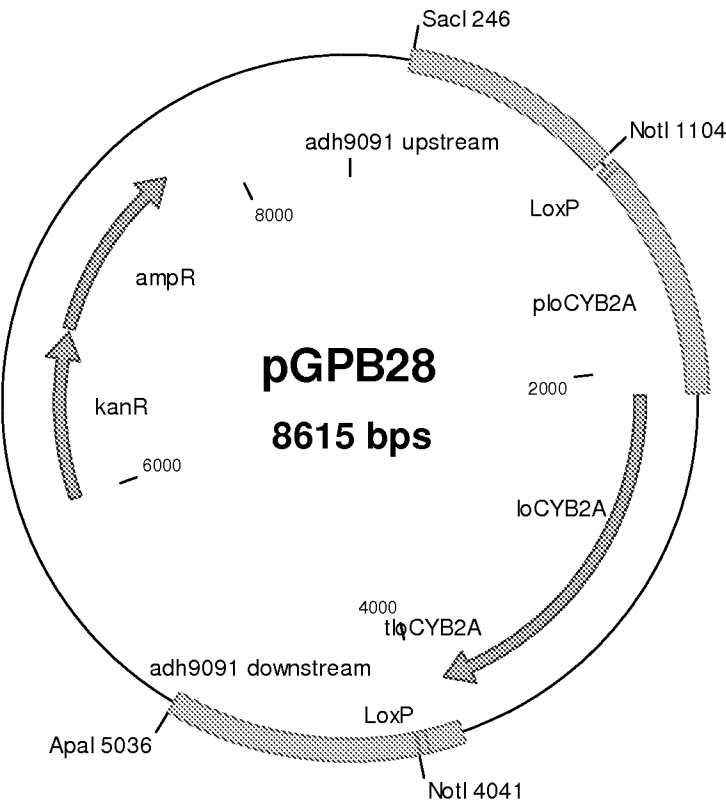


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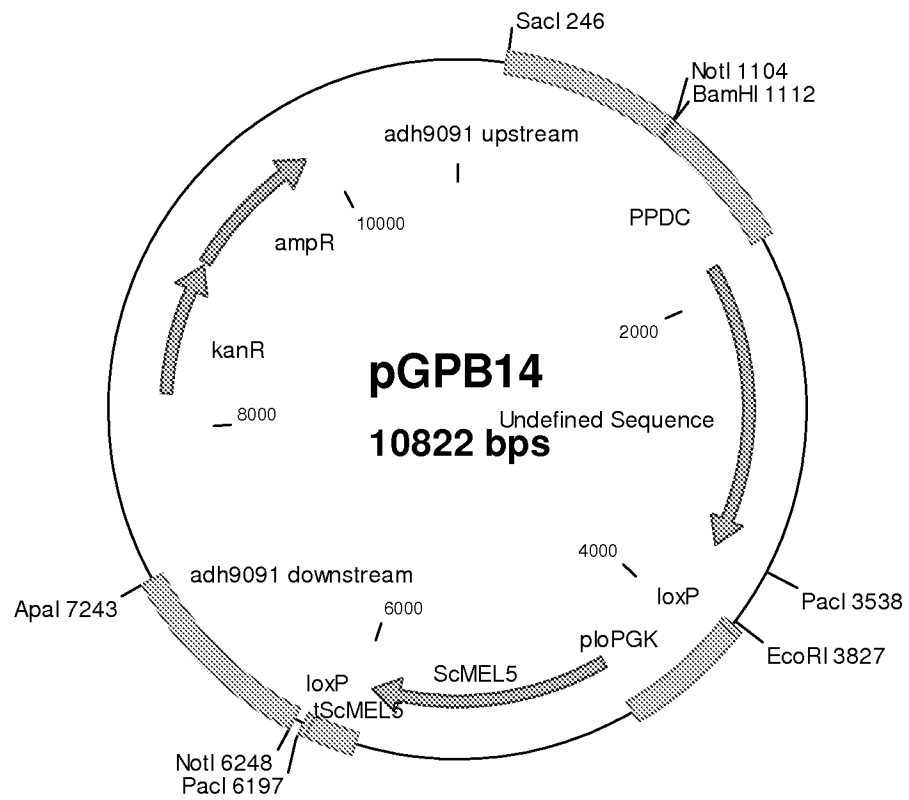


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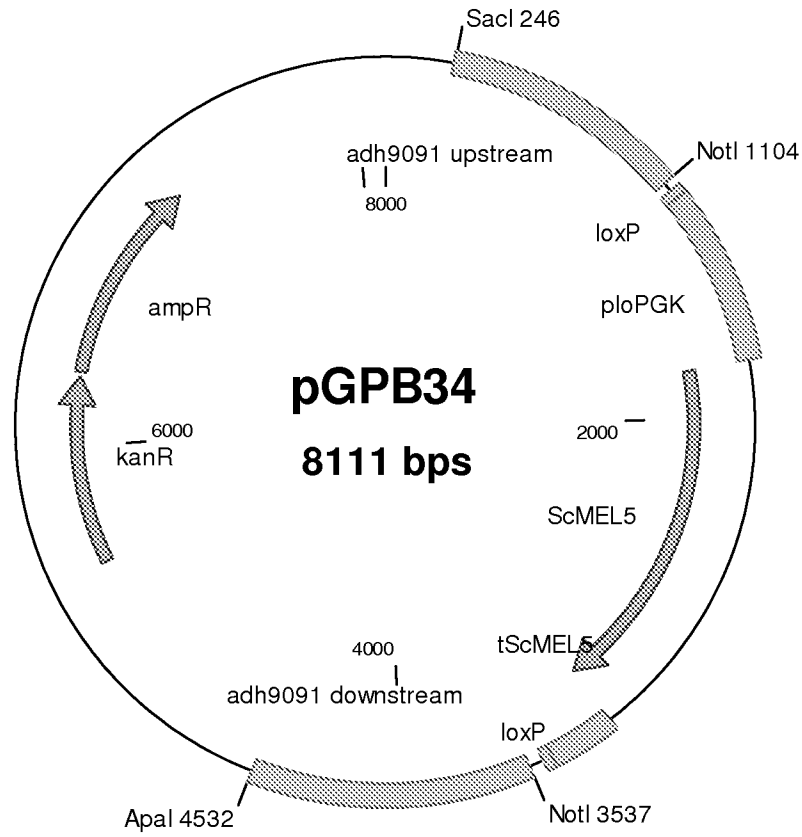


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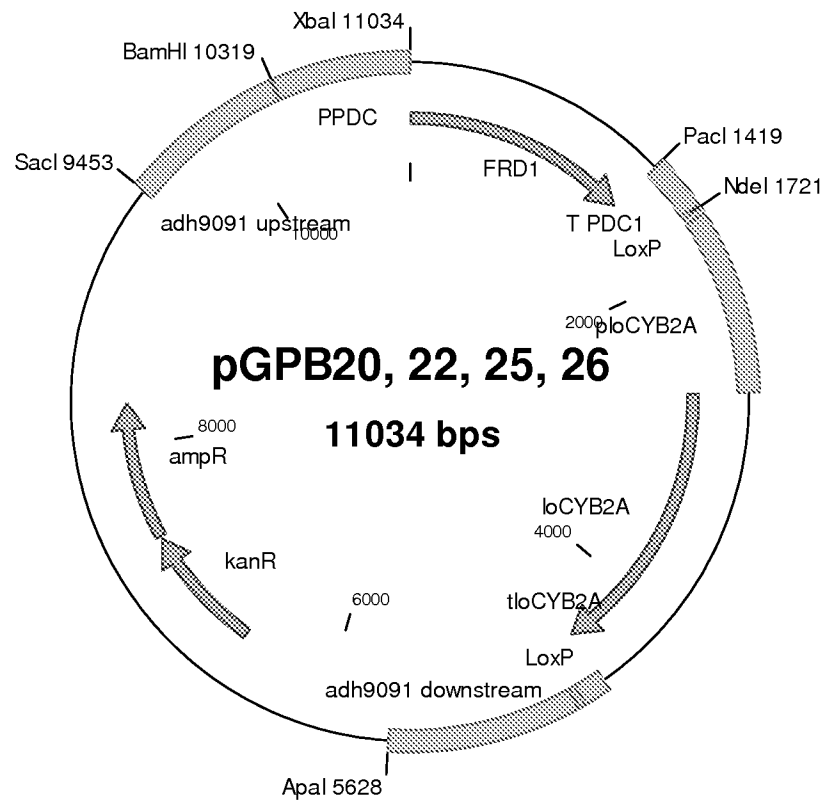


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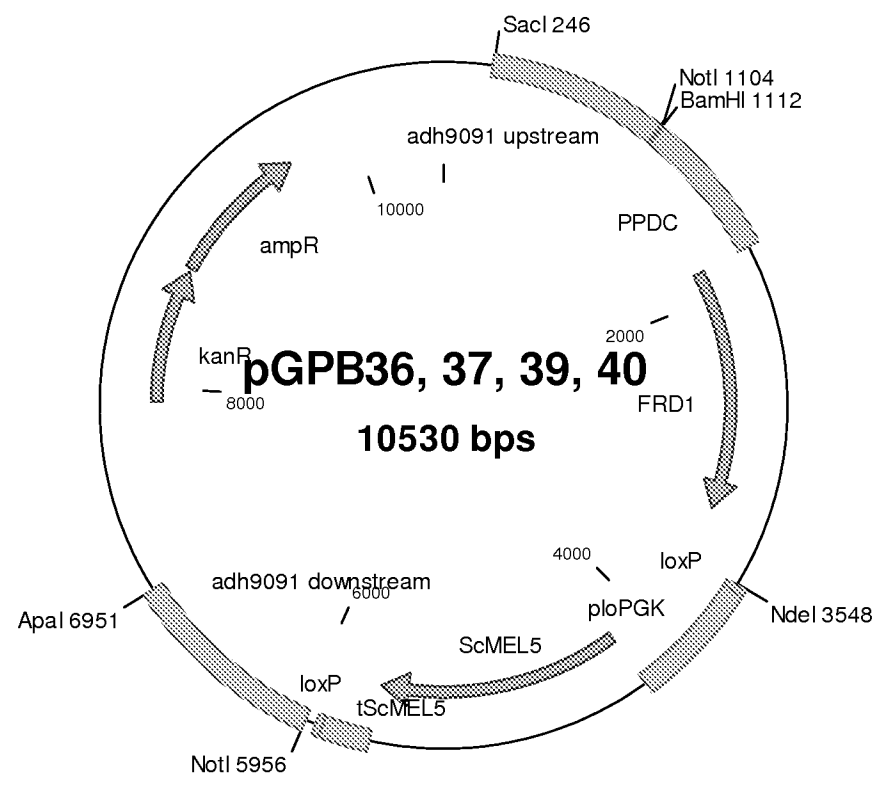


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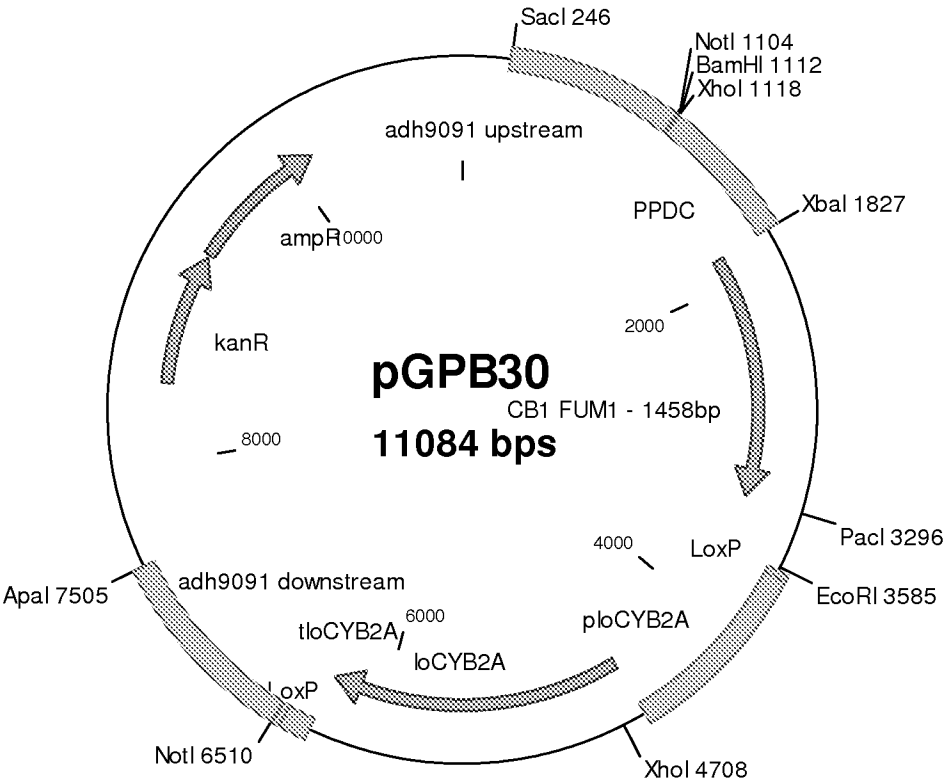


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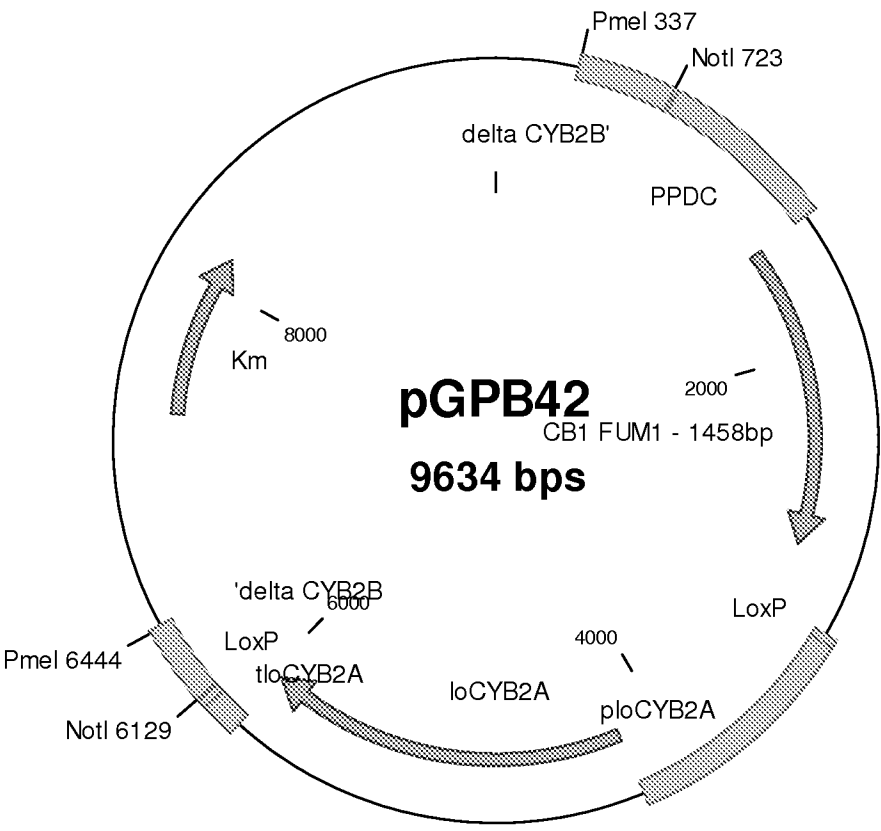


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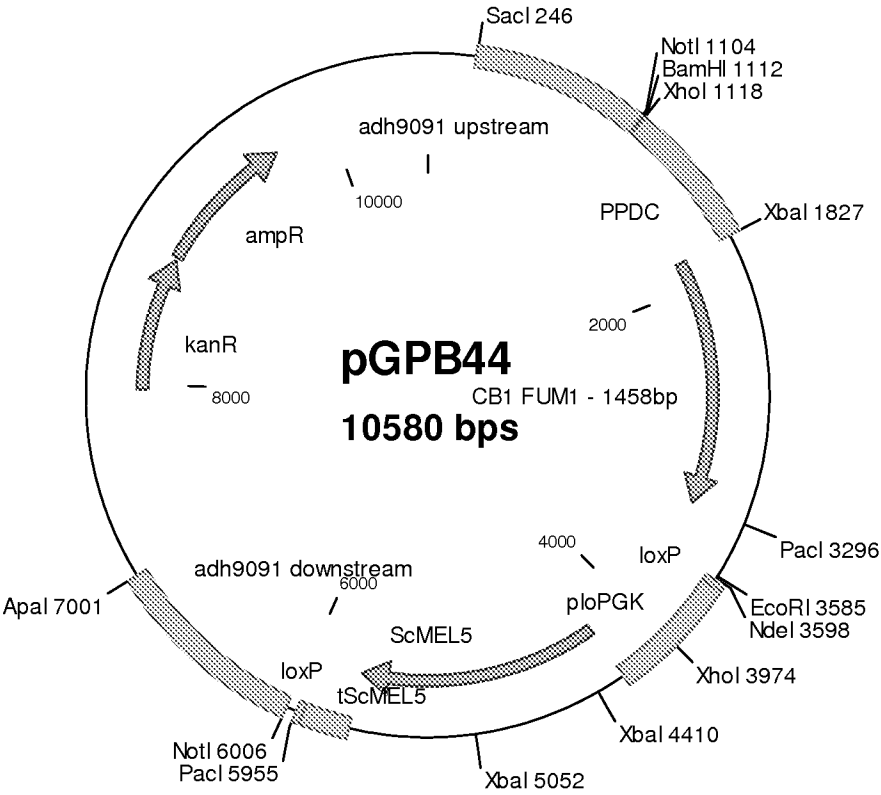


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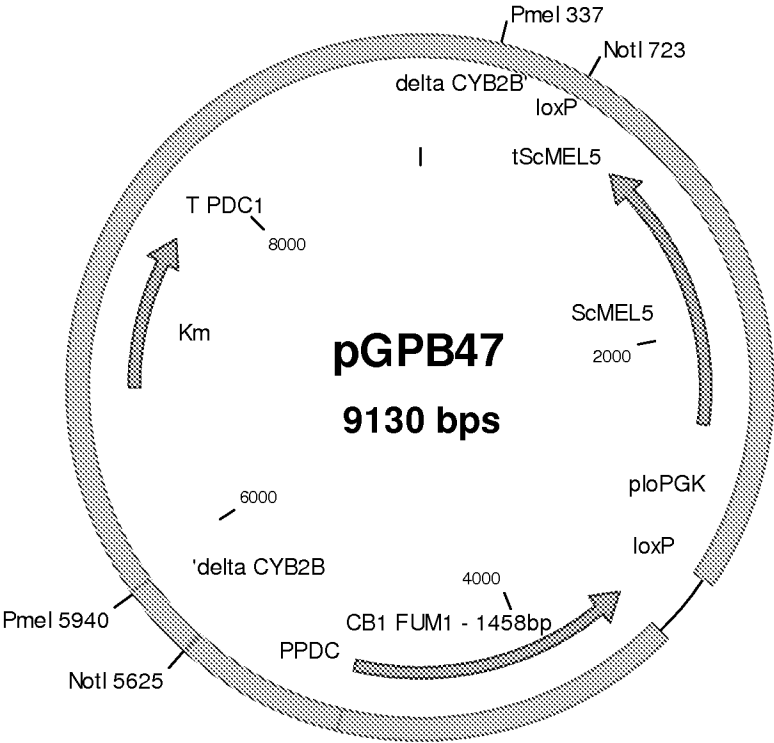


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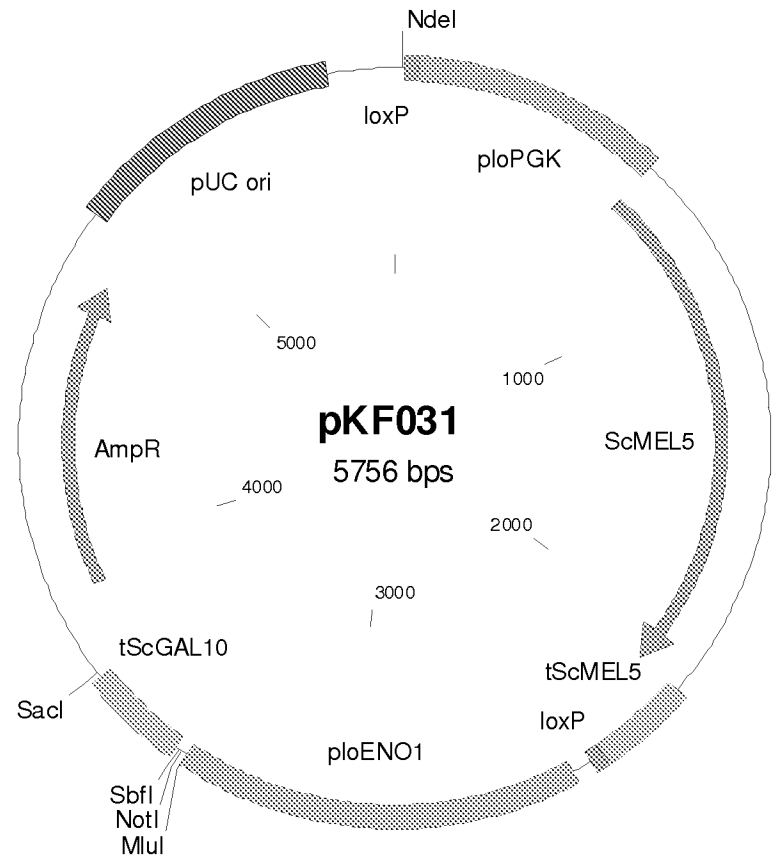


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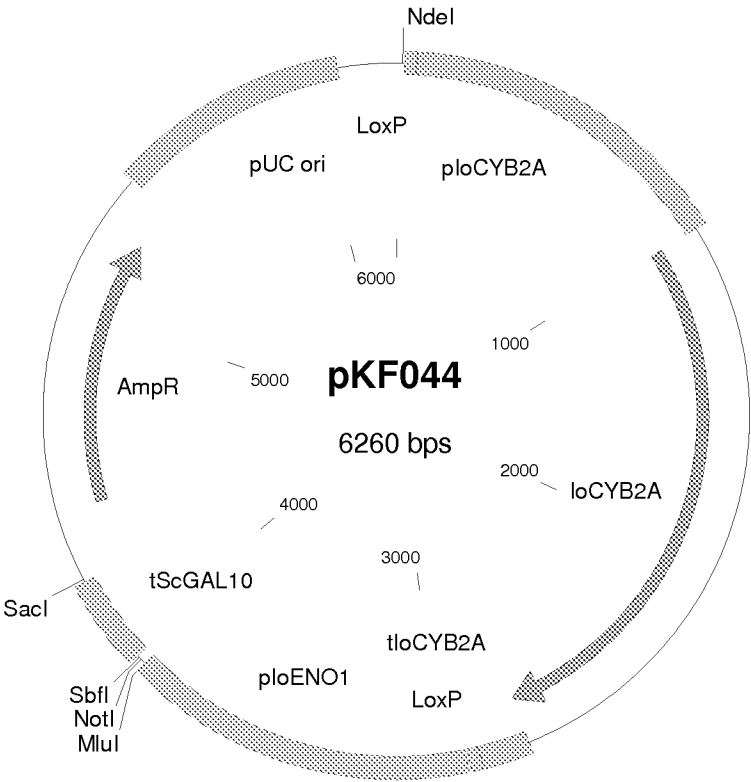


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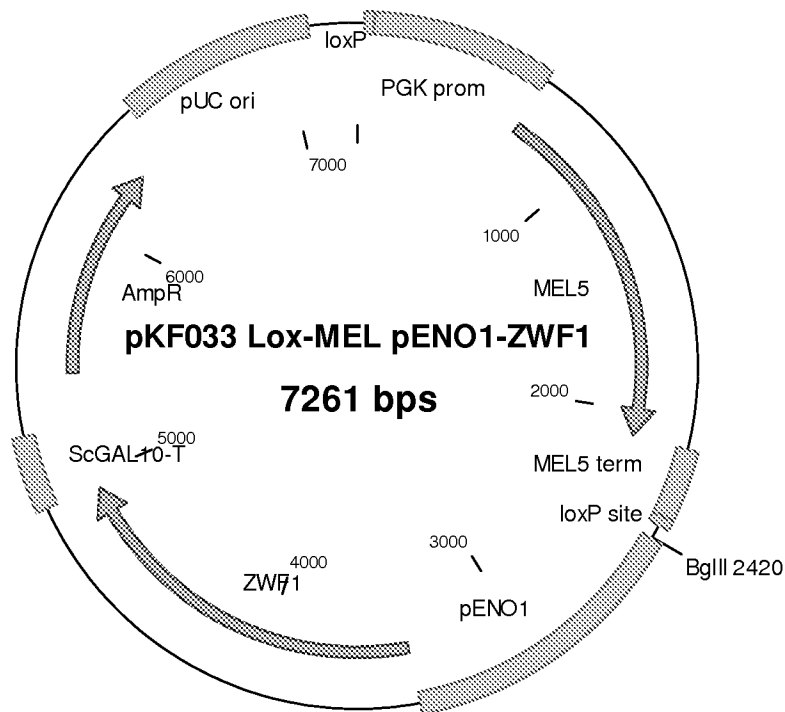


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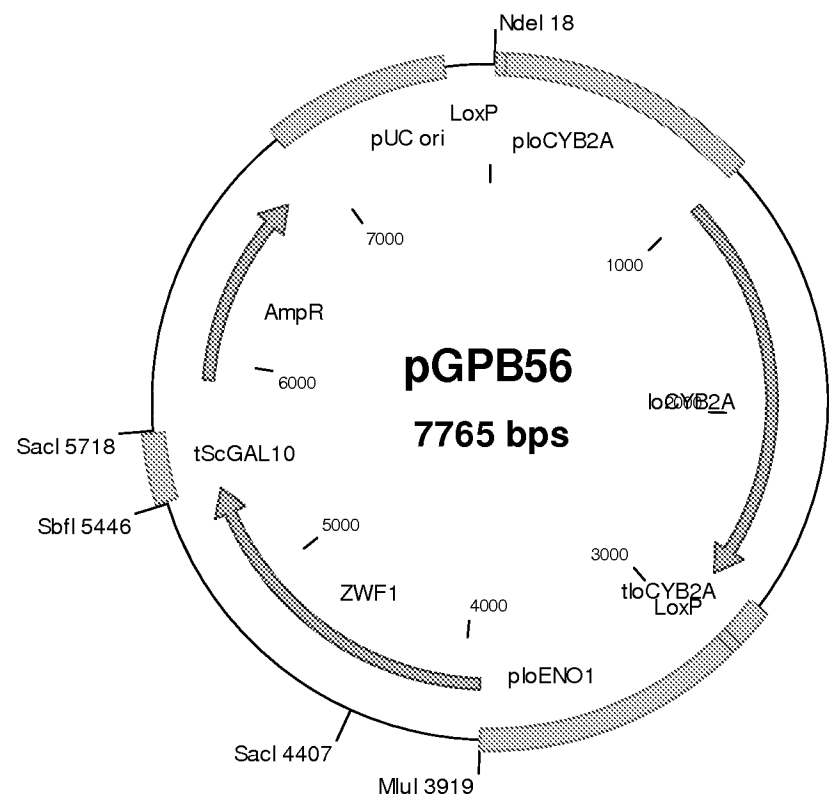


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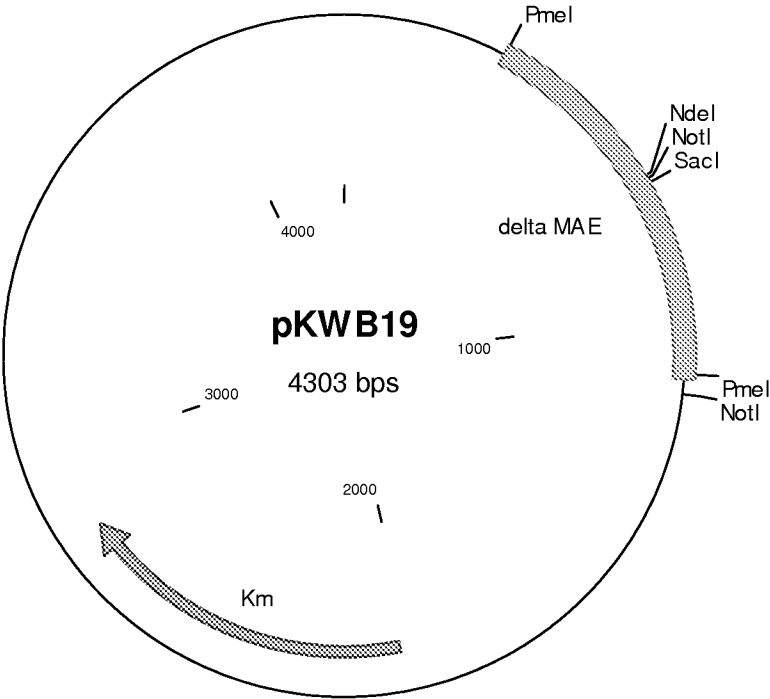


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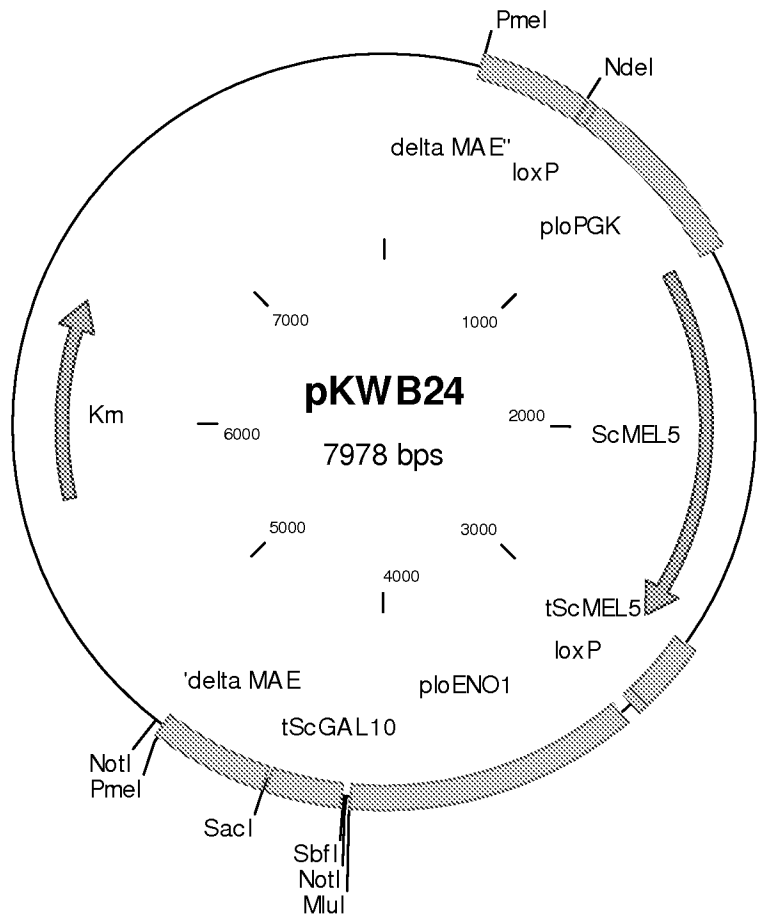


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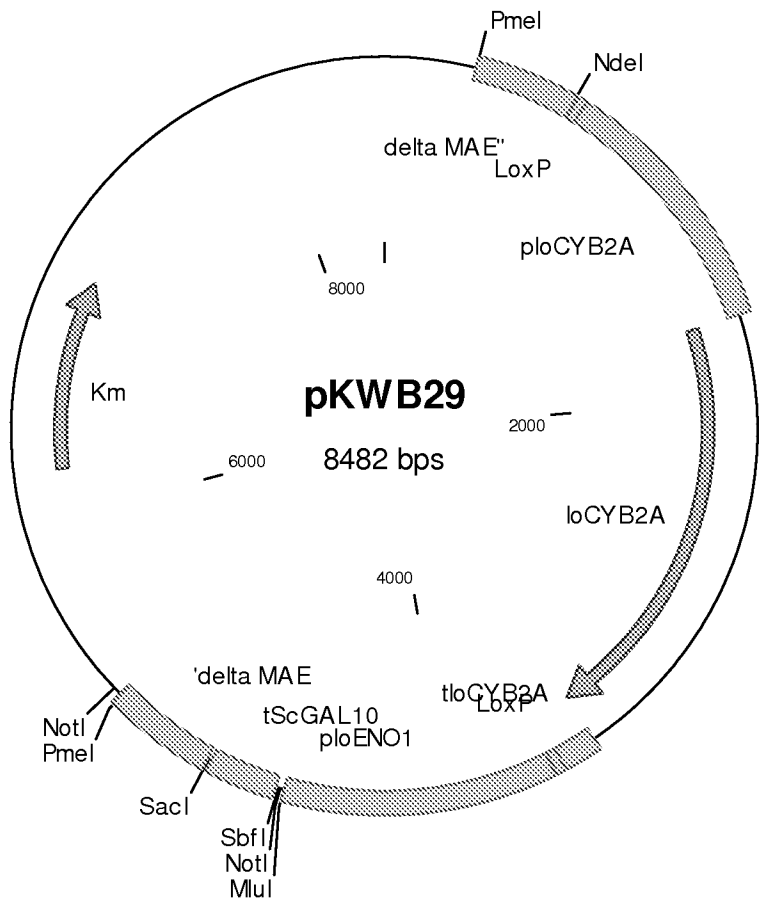


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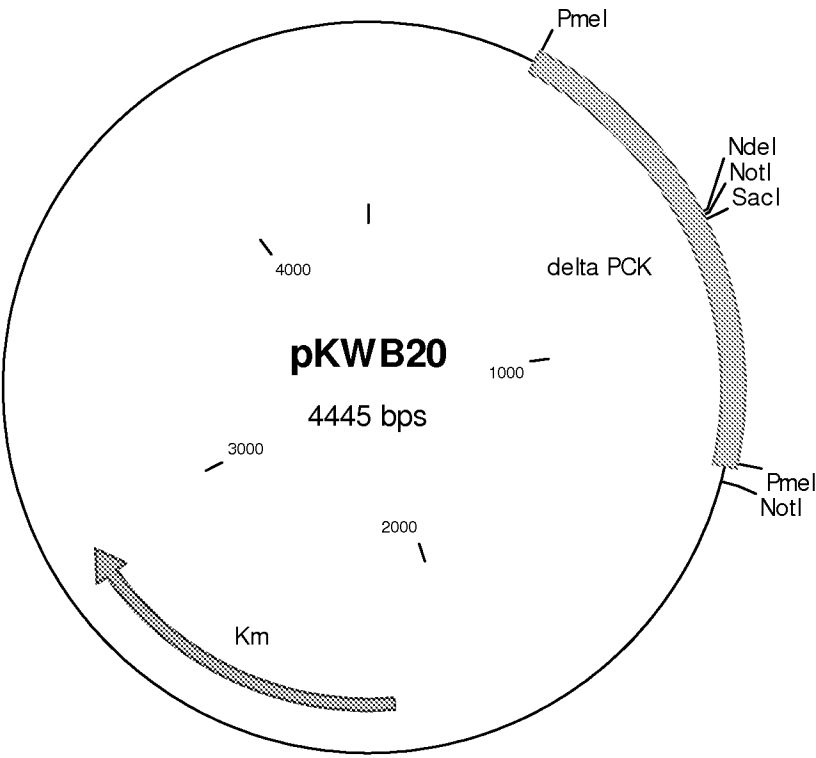


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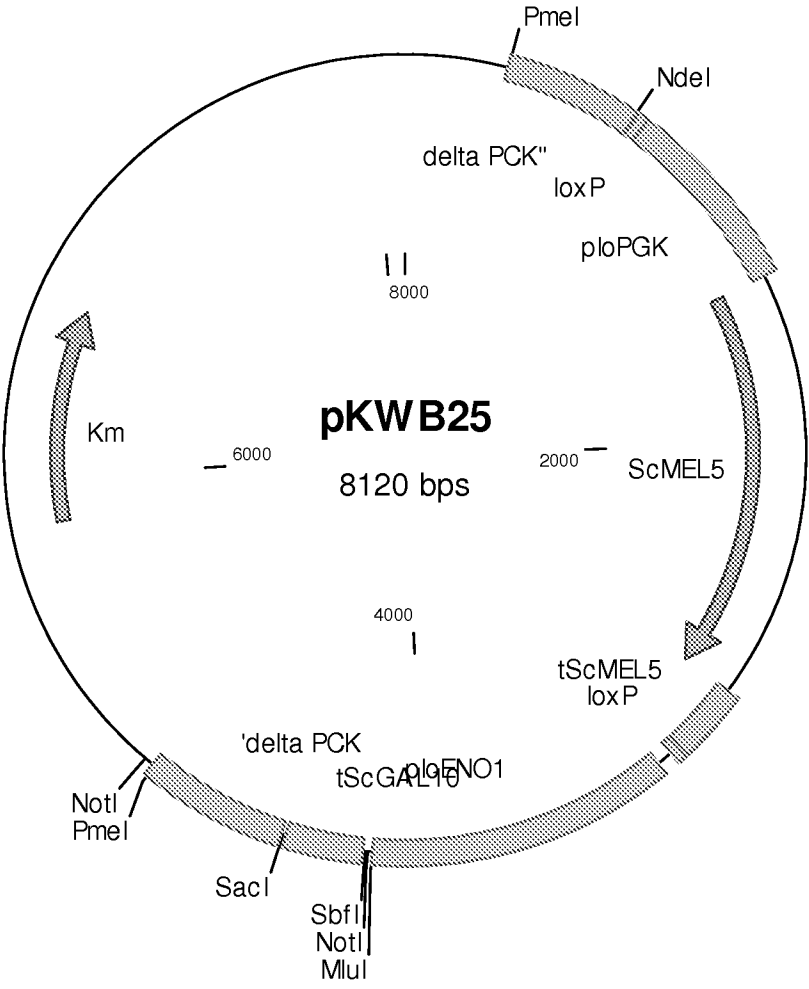


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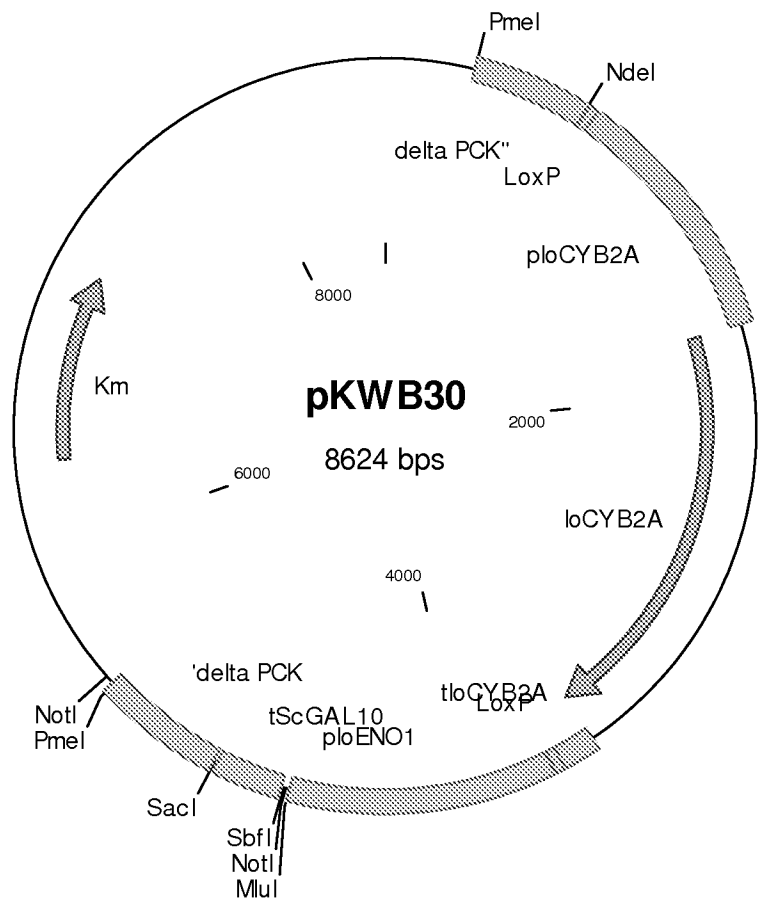


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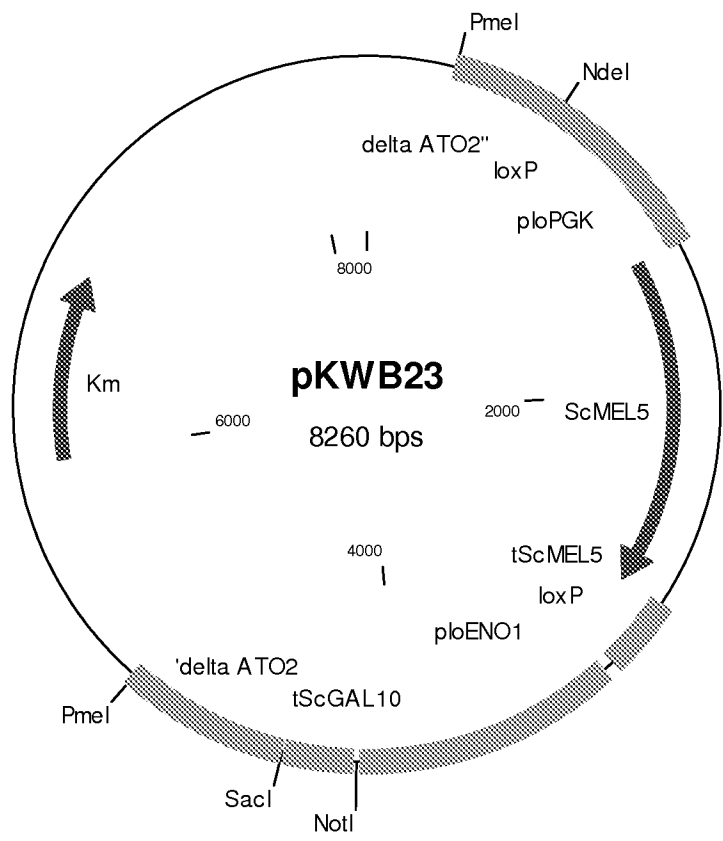
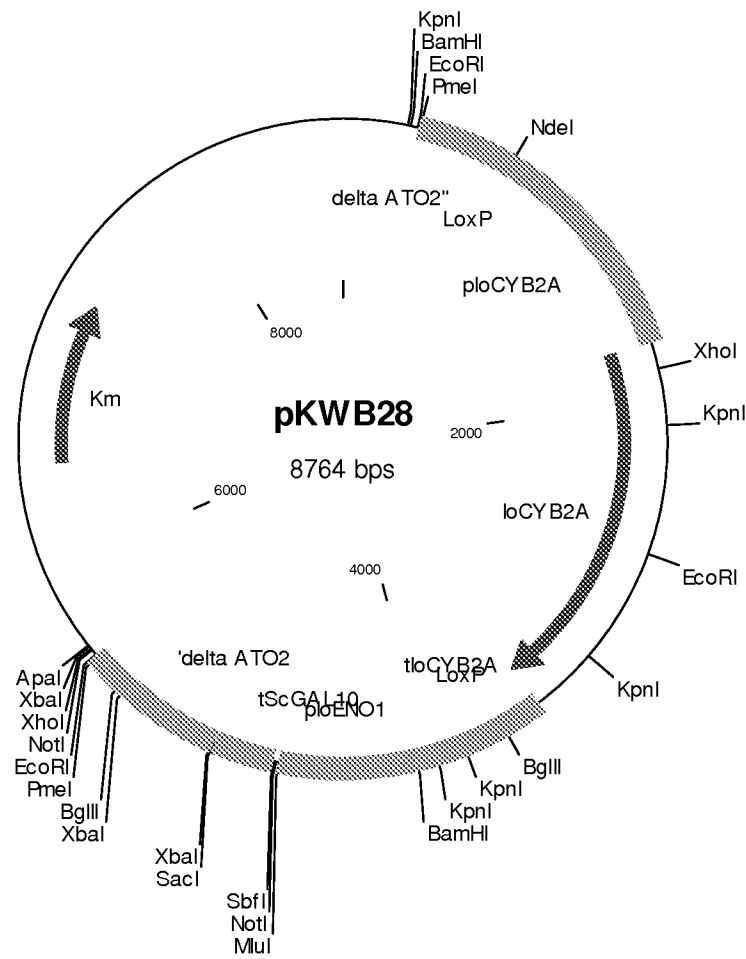
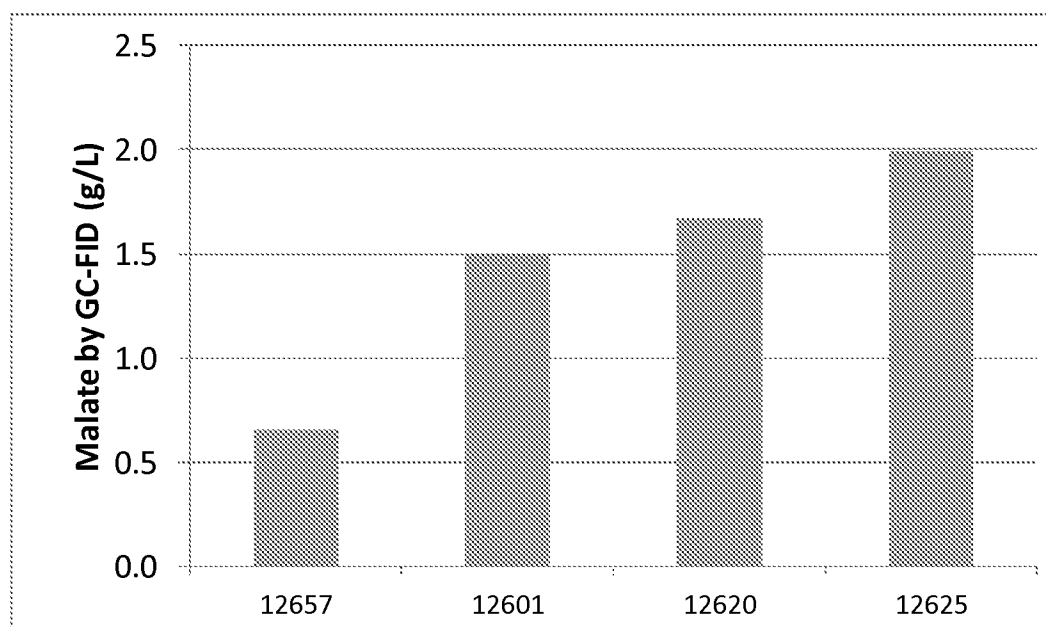


Figure 43



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Figure 44



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Figure 45

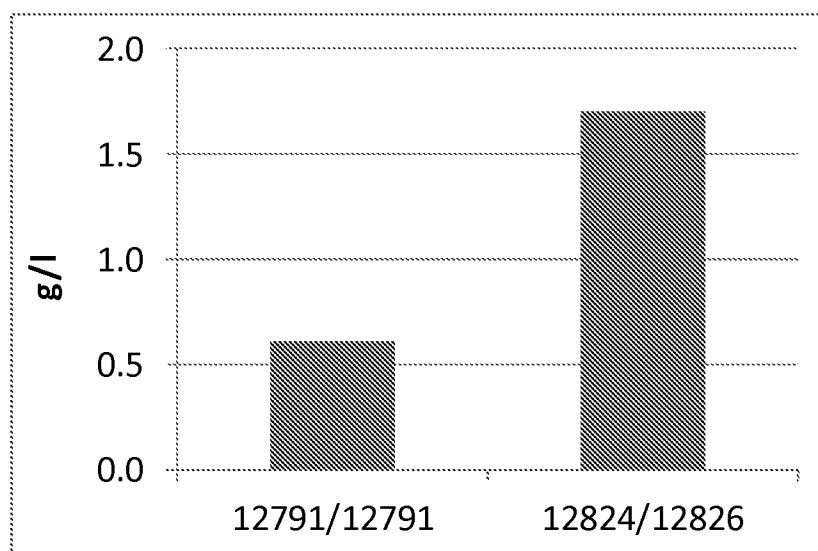


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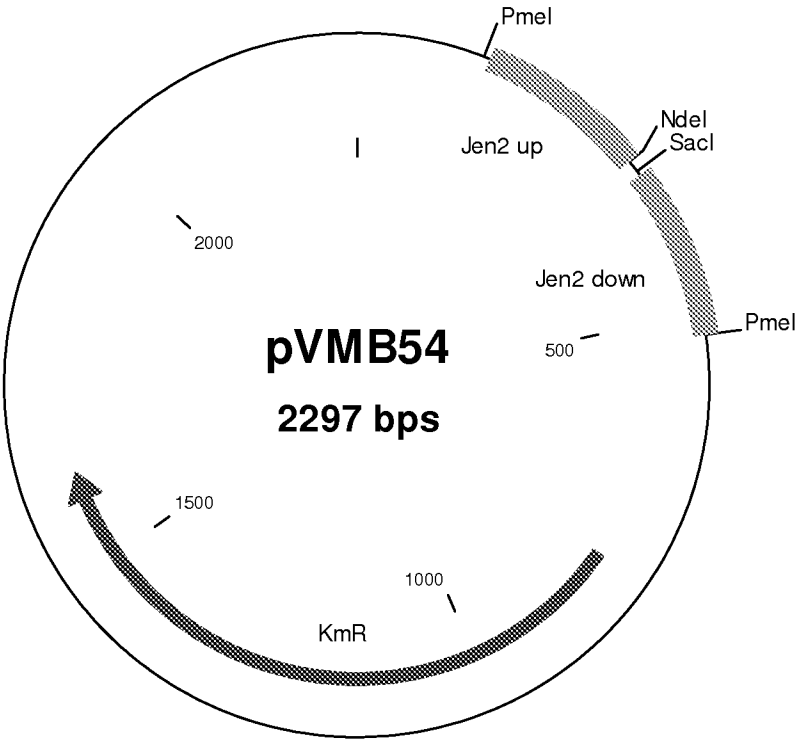
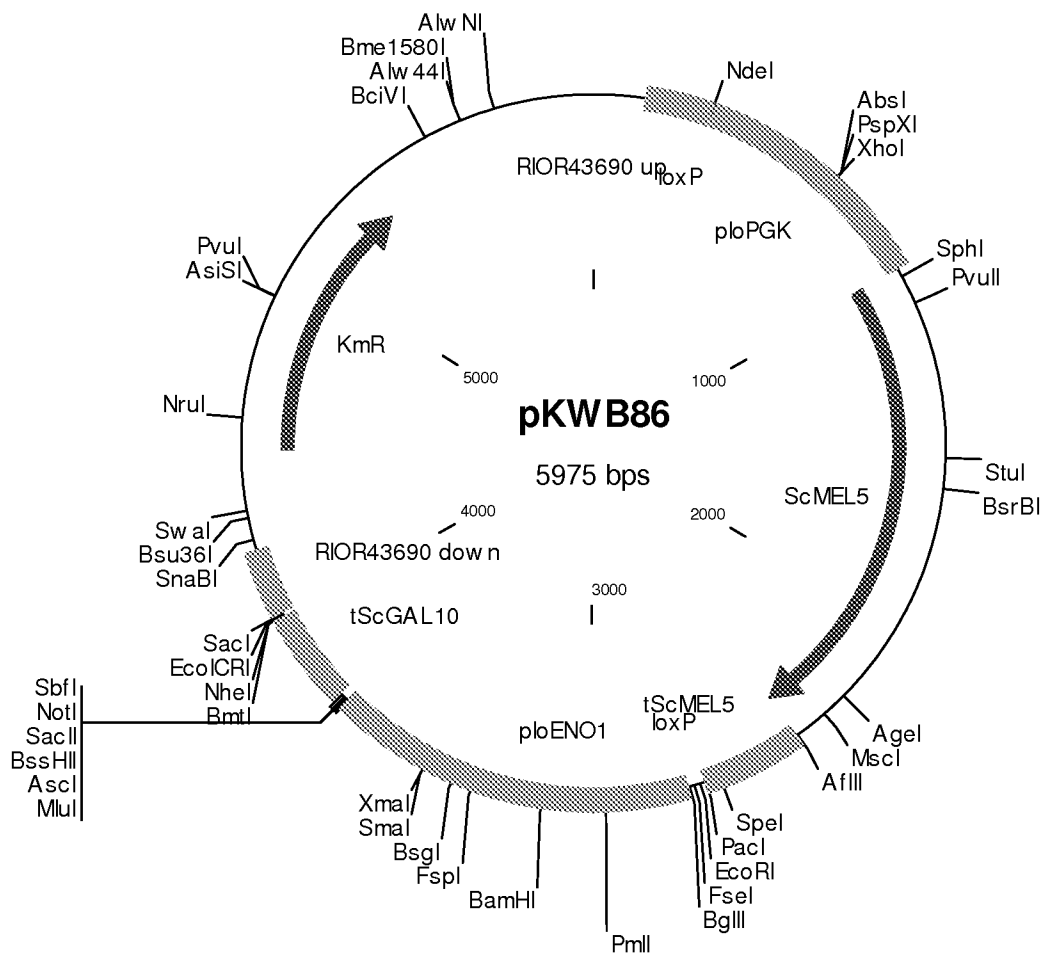
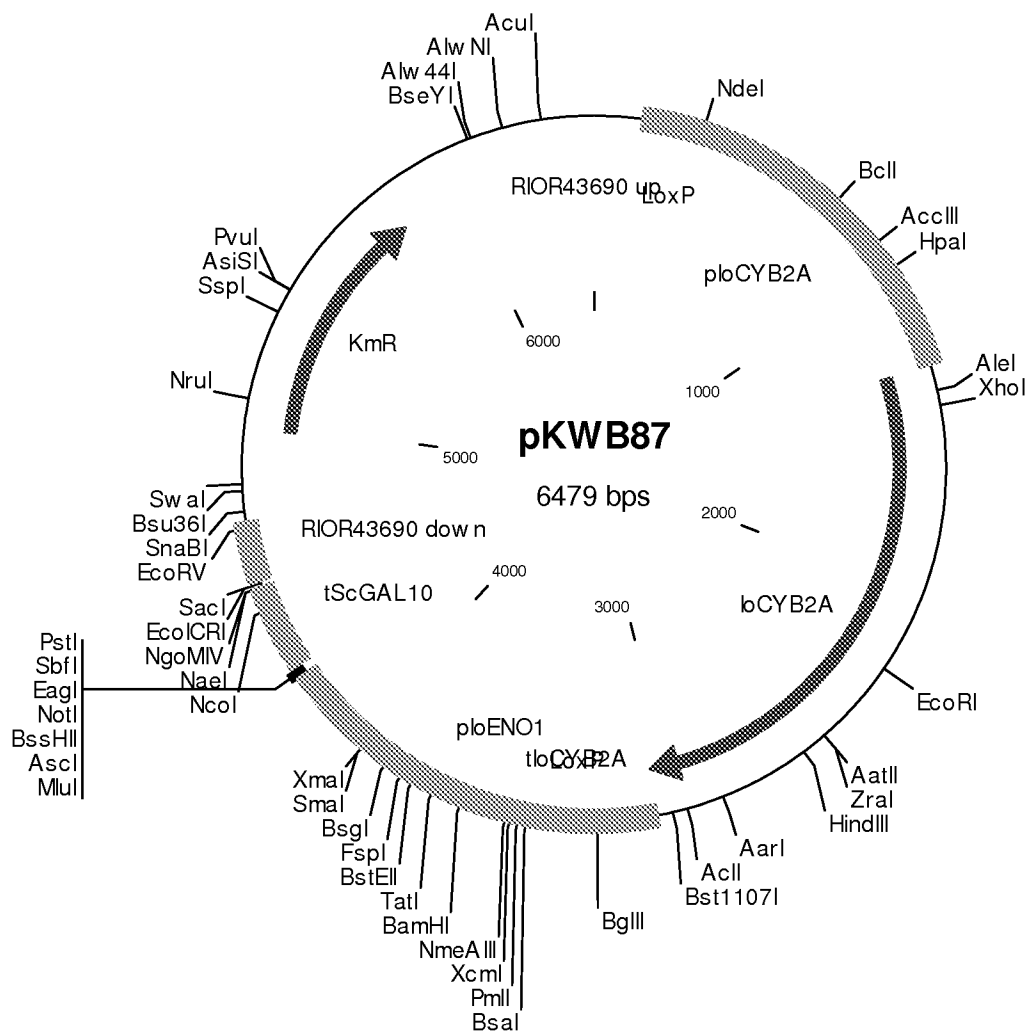


Figure 47



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Figure 48



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Figure 49

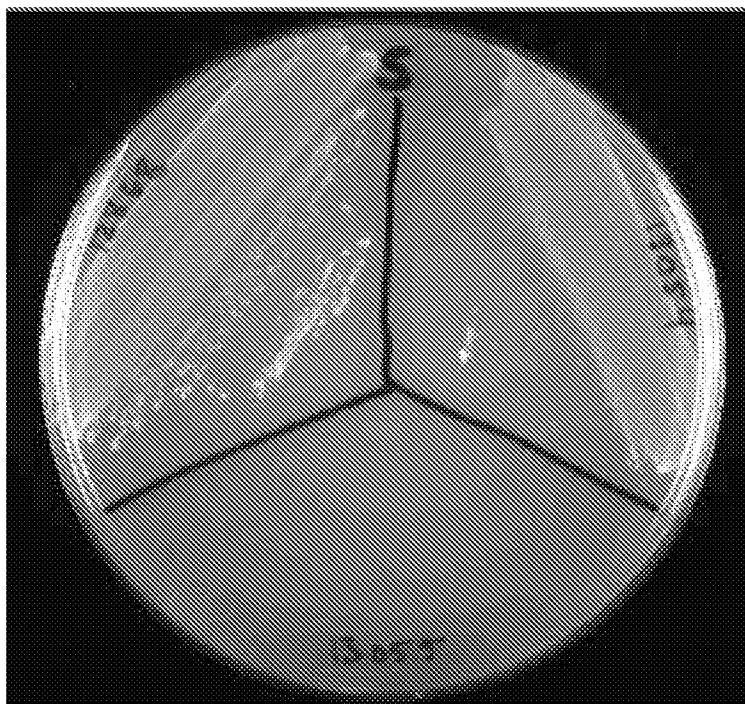


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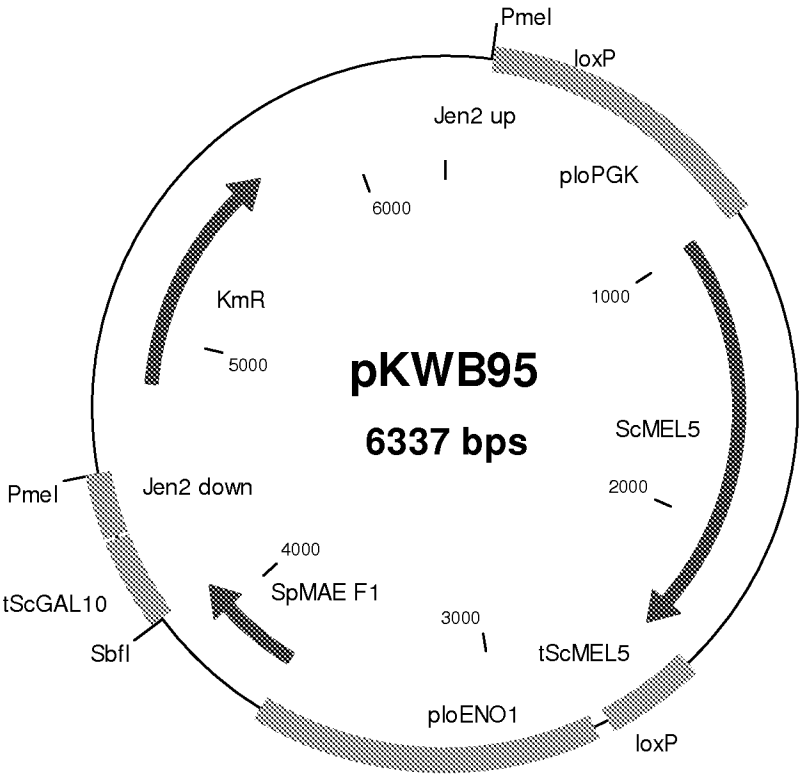


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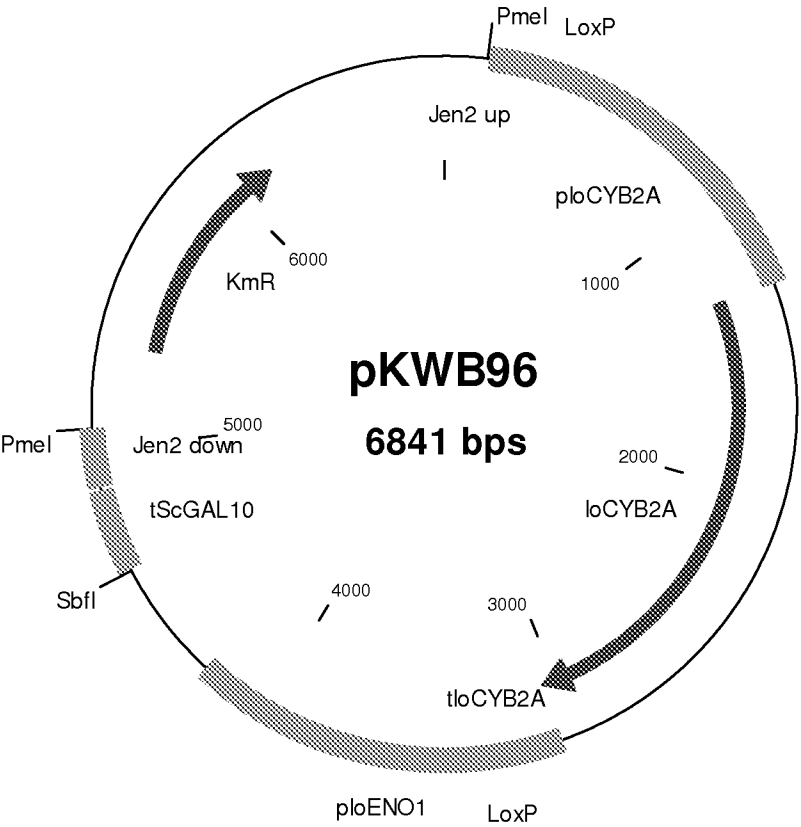


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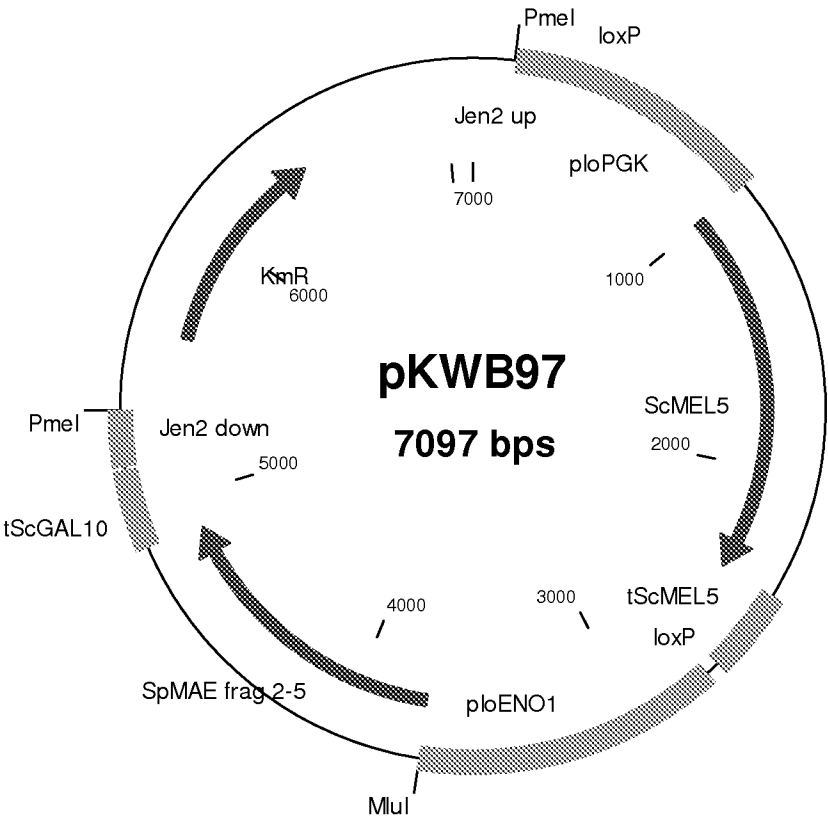


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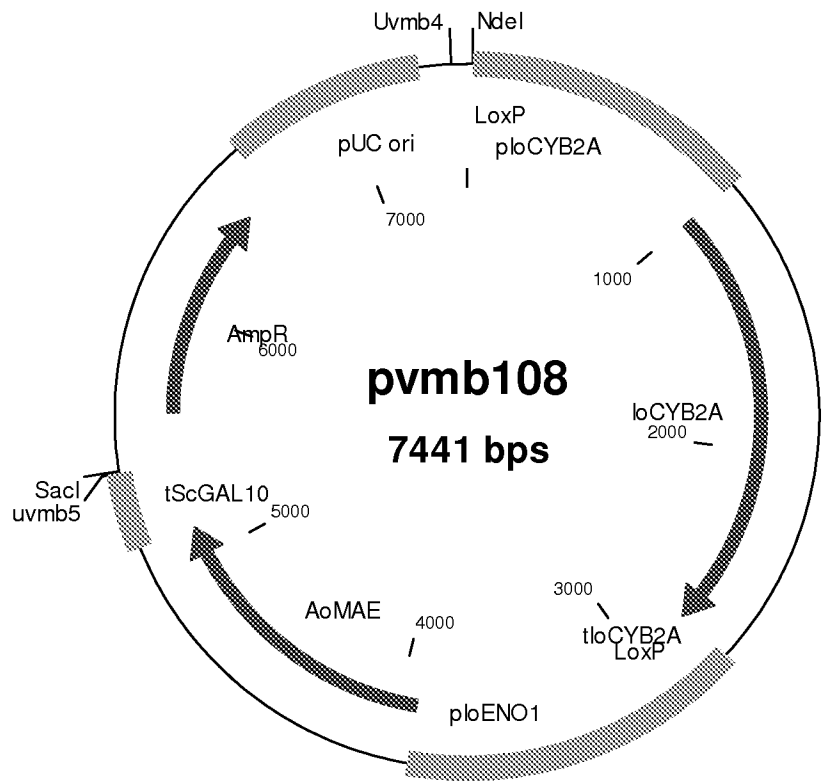


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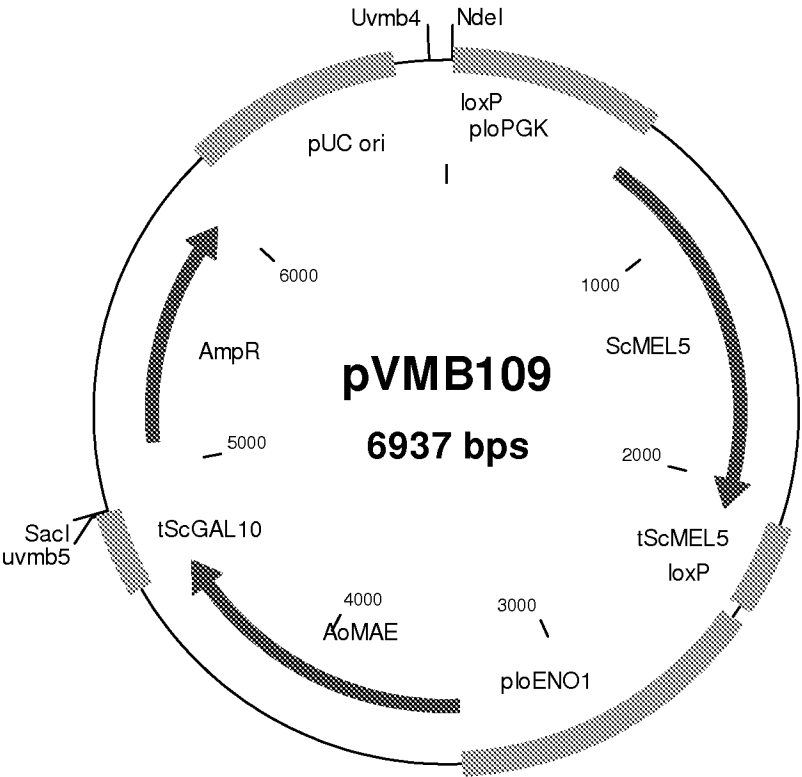


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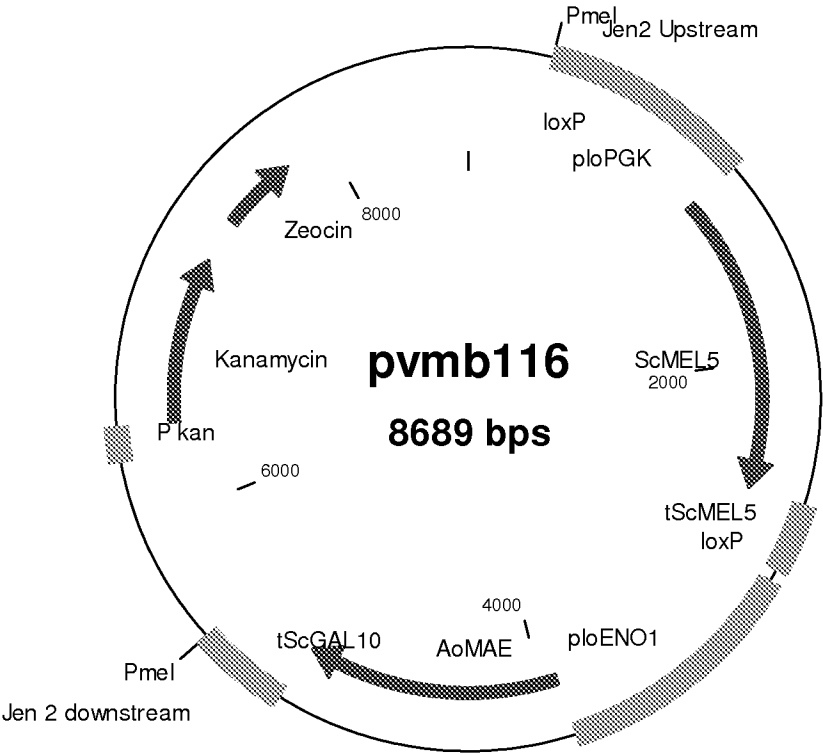
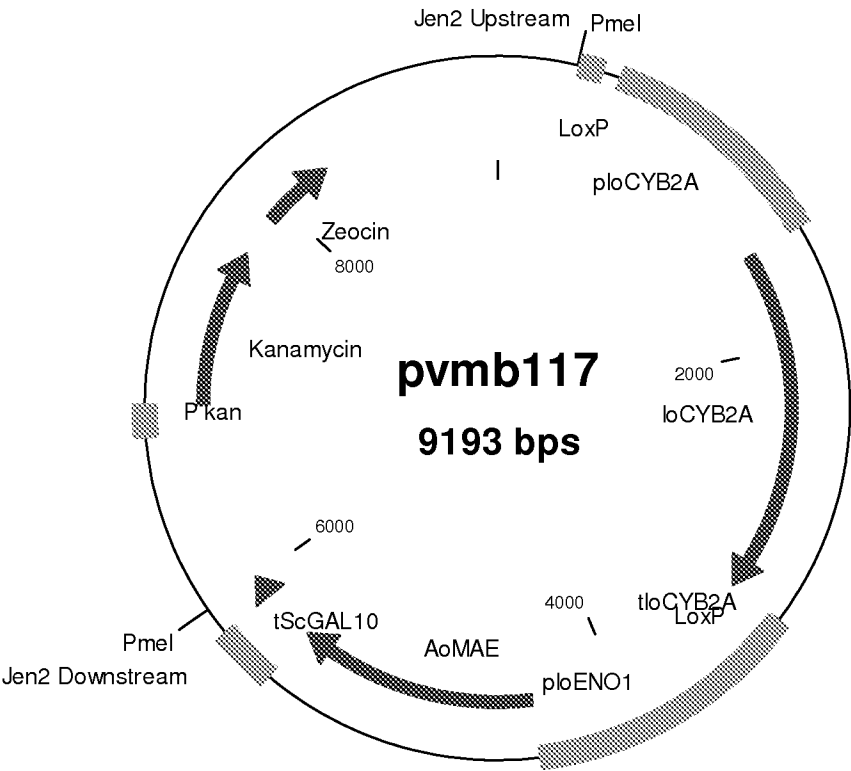
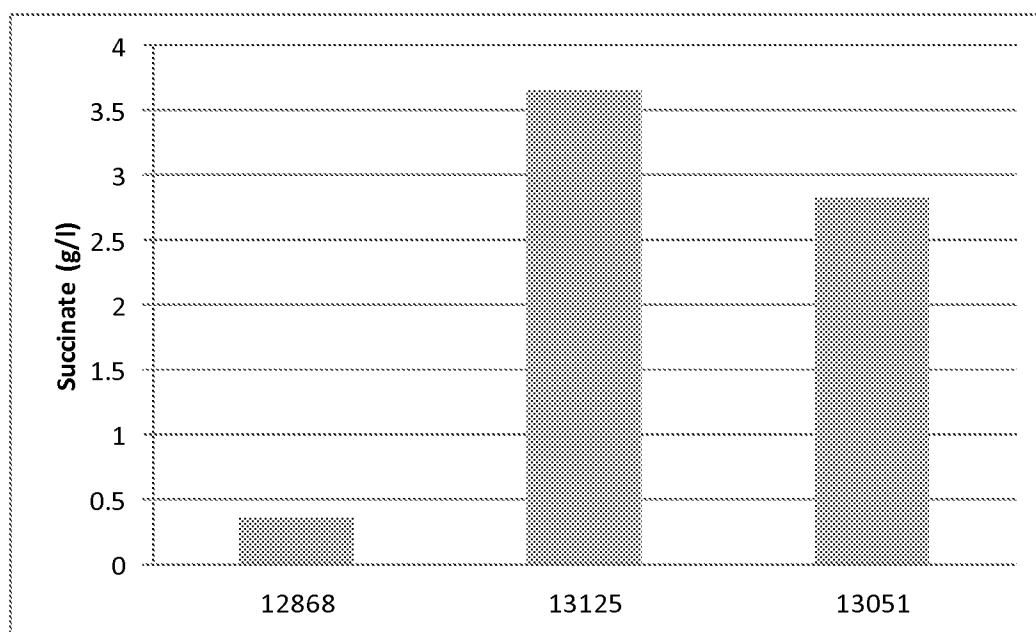


Figure 56



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Figure 57



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Figure 58

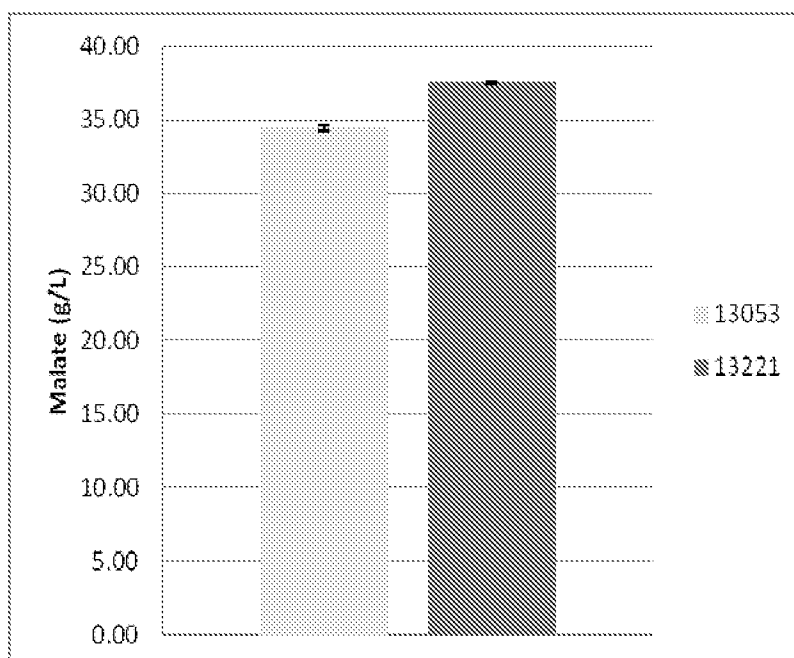
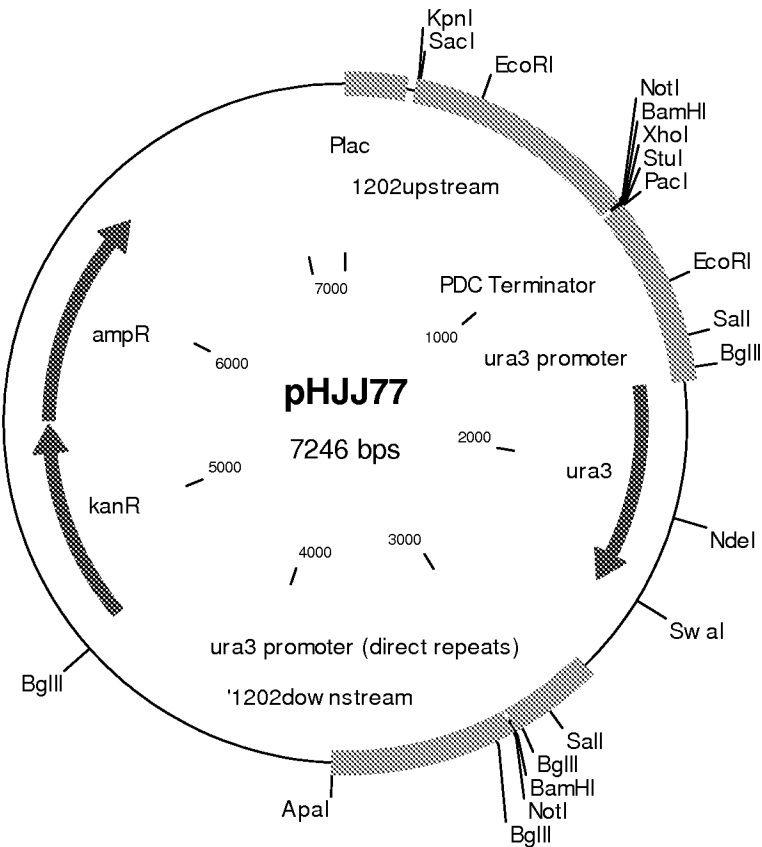


Figure 59



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Figure 60

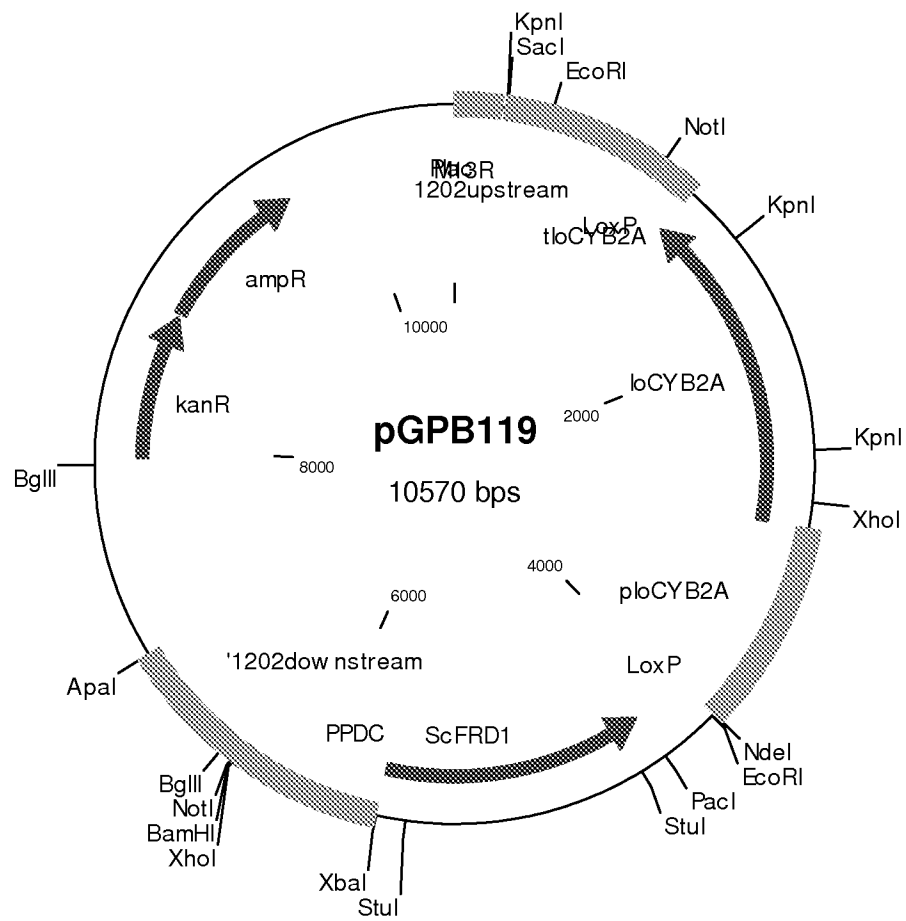


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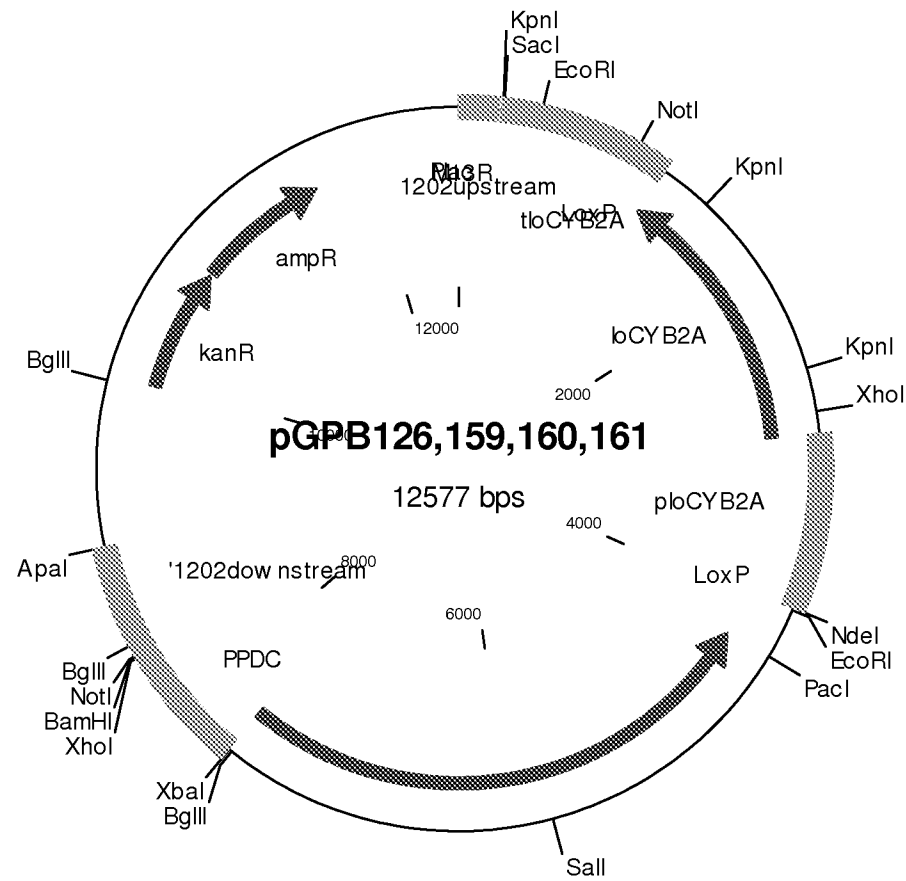


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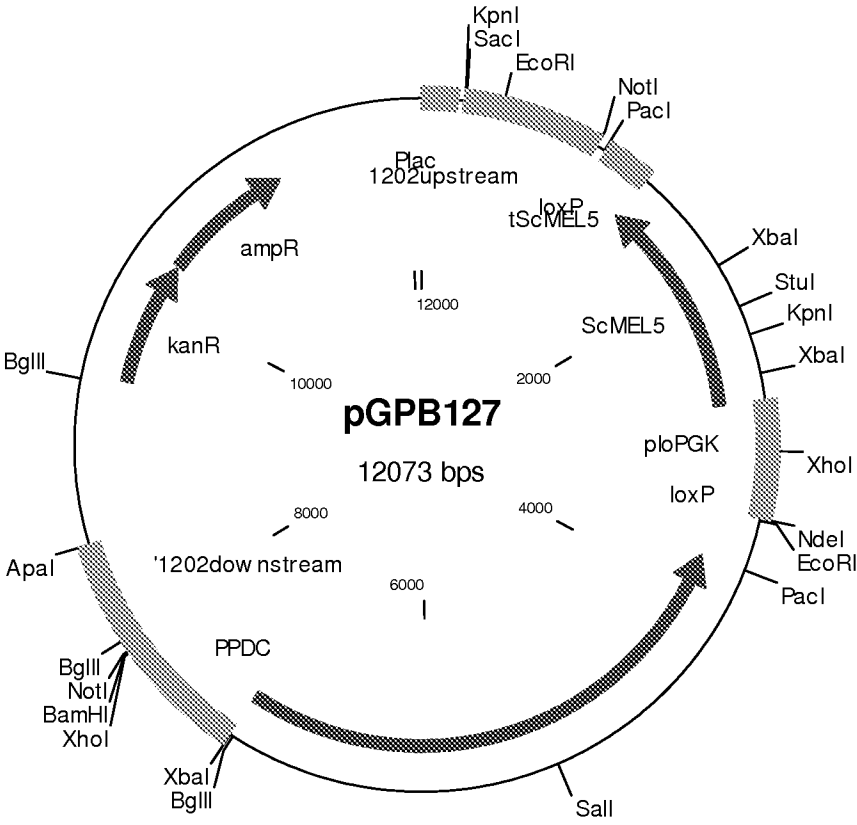


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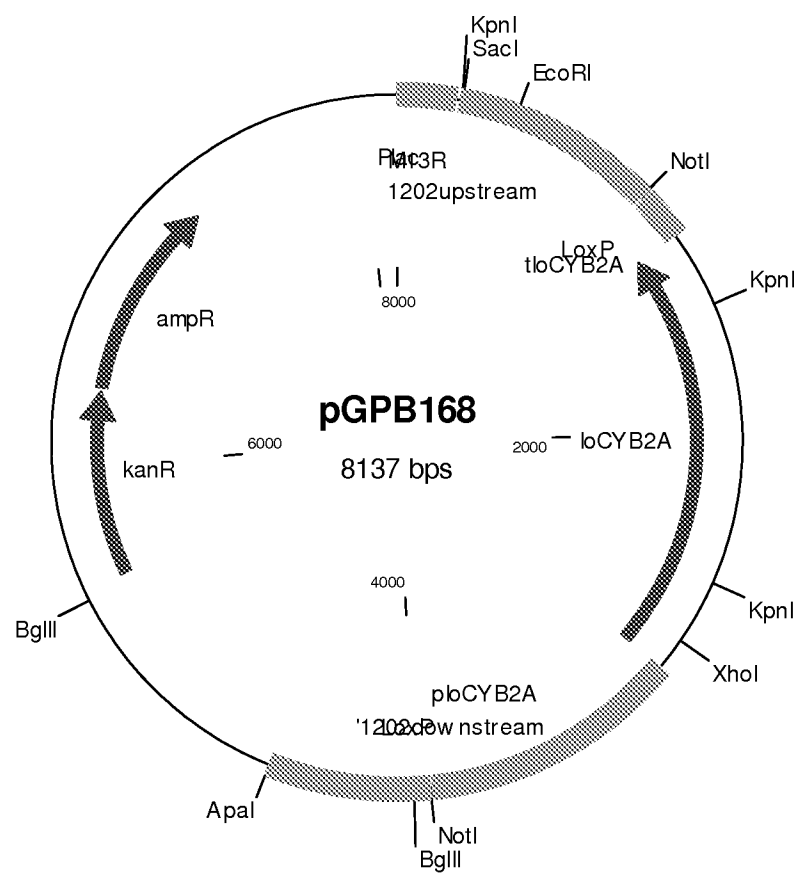
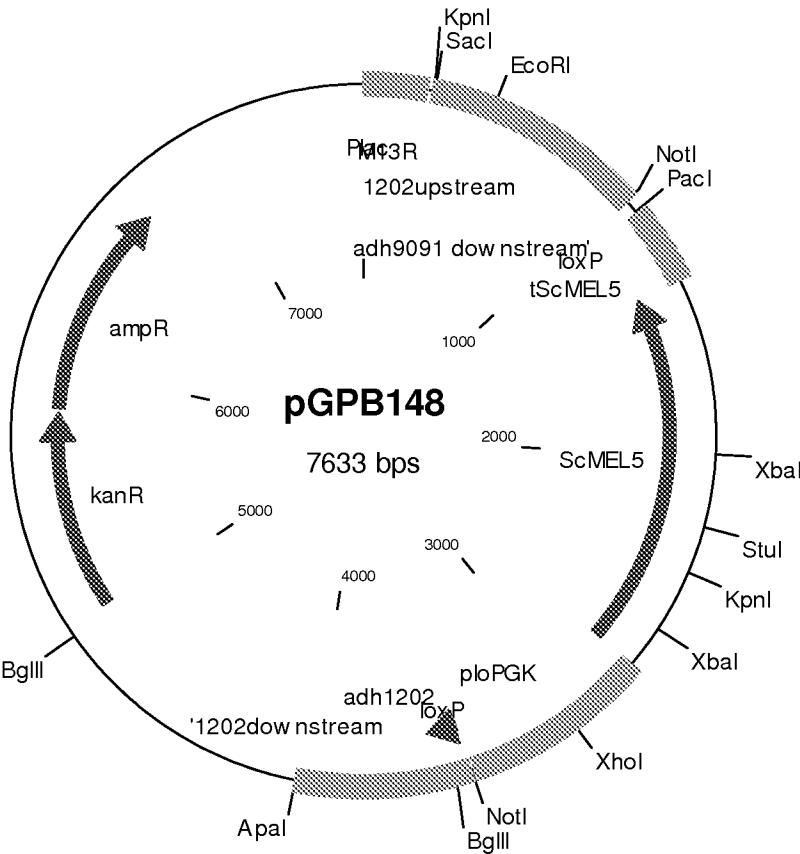
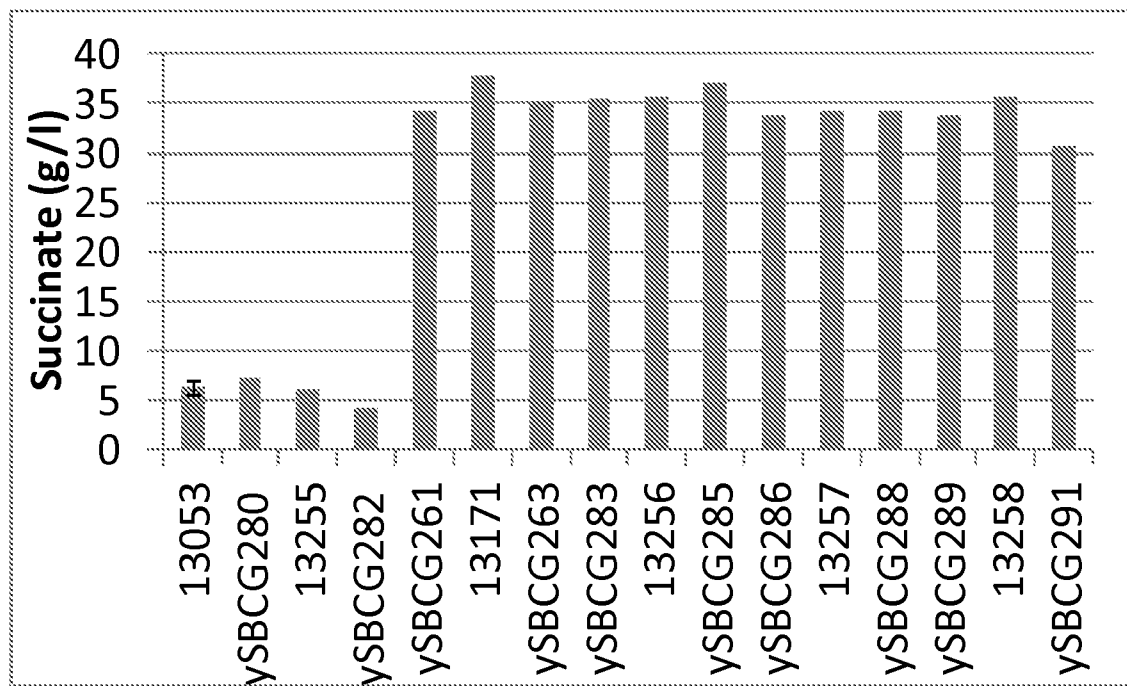


Figure 64



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Figure 65



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Figure 66

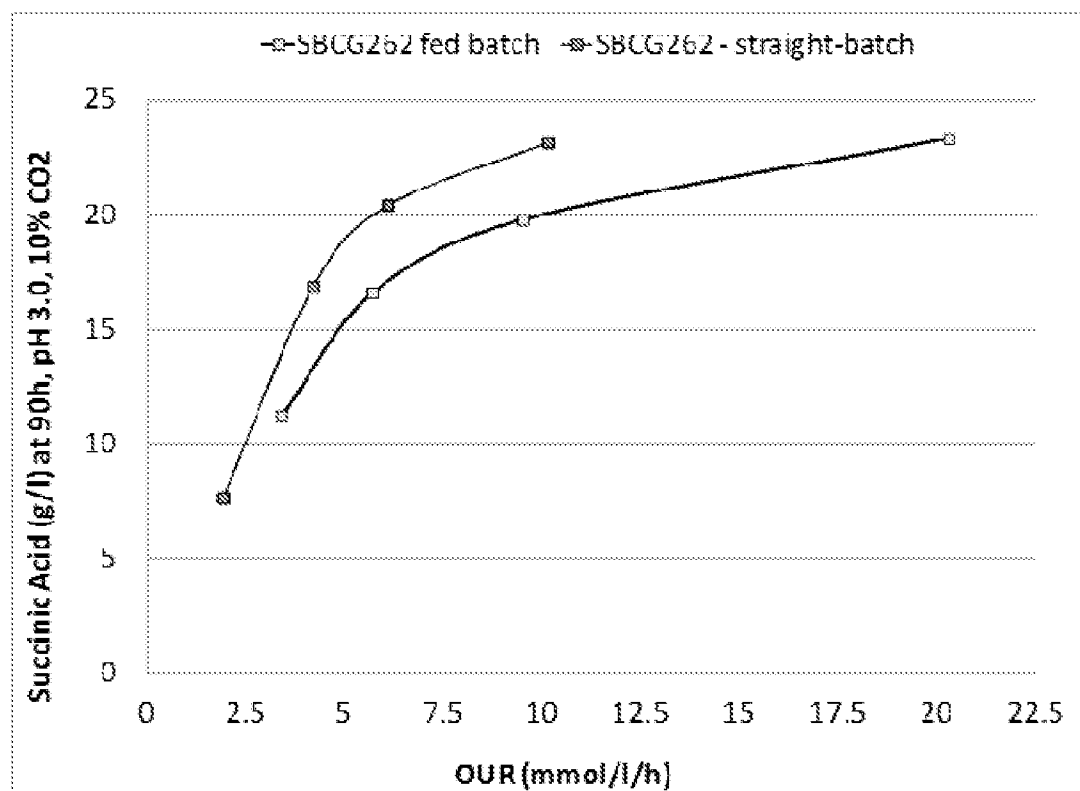


Figure 67

