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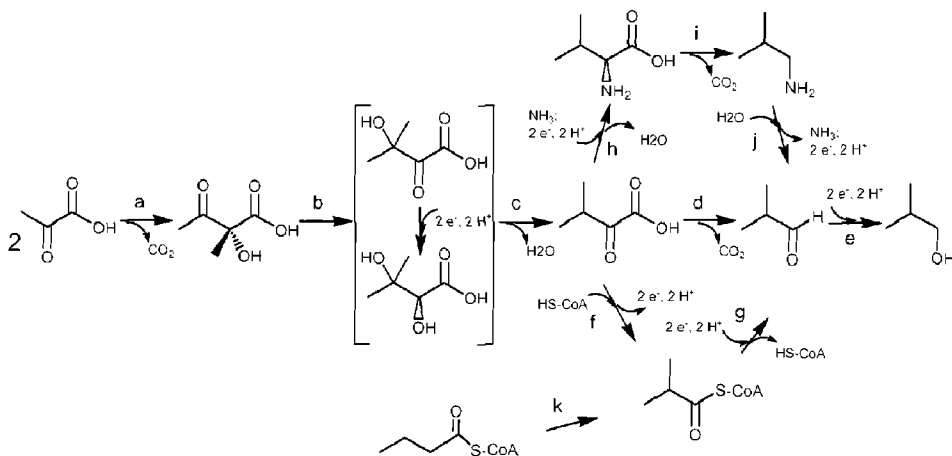
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(54) Title: HOST CELLS AND METHODS FOR PRODUCTION OF ISOBUTANOL



(57) Abrégé/Abstract:

Provided herein are recombinant yeast host cells and methods for their use for production of isobutanol. Yeast host cells provided comprise an isobutanol biosynthetic pathway and at least one of reduced or eliminated aldehyde dehydrogenase activity, reduced

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or eliminated acetolactate reductase activity; or a heterologous polynucleotide encoding a polypeptide having ketol-acid reductoisomerase activity.

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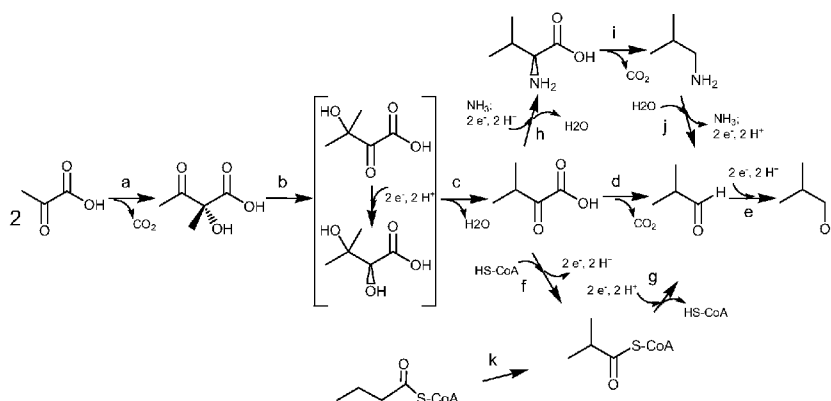
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[Continued on next page]

(54) Title: HOST CELLS AND METHODS FOR PRODUCTION OF ISOBUTANOL

Figure 1



(57) Abstract: Provided herein are recombinant yeast host cells and methods for their use for production of isobutanol. Yeast host cells provided comprise an isobutanol biosynthetic pathway and at least one of reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated acetolactate reductase activity; or a heterologous polynucleotide encoding a polypeptide having ketol-acid reductoisomerase activity.

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TITLE

HOST CELLS AND METHODS FOR PRODUCTION OF ISOBUTANOL

5

FIELD OF THE INVENTION

10       The invention relates to recombinant host cells and methods for  
fermentative production of isobutanol.

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BACKGROUND OF THE INVENTION

30       Butanol is an important industrial chemical, useful as a fuel  
additive, as a feedstock chemical in the plastics industry, and as a food  
grade extractant in the food and flavor industry. Each year 10 to 12 billion  
pounds of butanol are produced by petrochemical means and the need  
for this commodity chemical will likely increase in the future.

Methods for the chemical synthesis of isobutanol are known, such as oxo synthesis, catalytic hydrogenation of carbon monoxide (Ullmann's Encyclopedia of Industrial Chemistry, 6th edition, 2003, Wiley-VCH Verlag GmbH and Co., Weinheim, Germany, Vol. 5, pp. 716-719) and Guerbet  
5 condensation of methanol with n-propanol (Carlini et al., J. Molec. Catal. A. Chem. 220:215-220, 2004). These processes use starting materials derived from petrochemicals, are generally expensive, and are not environmentally friendly. The production of isobutanol from plant-derived raw materials would minimize green house gas emissions and would  
10 represent an advance in the art.

Isobutanol is produced biologically as a by-product of yeast fermentation. It is a component of "fusel oil" that forms as a result of the incomplete metabolism of amino acids by fungi. Isobutanol is specifically produced from catabolism of L-valine. After the amine group of L-valine is  
15 harvested as a nitrogen source, the resulting  $\alpha$ -keto acid is decarboxylated and reduced to isobutanol by enzymes of the so-called Ehrlich pathway (Dickinson et al., J. Biol. Chem. 273:25752-25756, 1998).

Improvements and alternatives for the biosynthesis of butanol directly from sugars would improve economic viability and would  
20 represent an advance in the art.

#### SUMMARY OF THE INVENTION

Provided herein are recombinant yeast host cells and methods for the production of isobutanol.

In some embodiments, a recombinant host cell comprises an  
25 engineered isobutanol production pathway and (a) at least one of (i) a heterologous polypeptide with ketol-acid reductoisomerase (KARI) activity selected from the group consisting of (1) a polypeptide having at least about 90% identity to a KARI enzyme derived from *Bifidobacterium angulatum*, *Bifidobacterium dentium*, *Zymomonas mobilis*, *Clostridium*  
30 *beijerinckii* or *Anaerostipes caccae*, or an active fragment thereof (2) a polypeptide having at least about 90% identity or at least about 95% identity to SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65; or (ii) a heterologous polynucleotide

encoding the heterologous polypeptide with KARI activity of (a); and (b) at least one host cell modification that enhances performance of the engineered isobutanol production pathway. In some embodiments, the combination of (a) and (b) results is a synergistic increase in isobutanol  
 5 production pathway performance.

In some embodiments, a recombinant host cell comprises an isobutanol biosynthetic pathway and (a) a heterologous polypeptide with ketol-acid reductoisomerase (KARI) activity selected from the group consisting of (i) a polypeptide having at least about 90% identity to a KARI  
 10 enzyme derived from *Bifidobacterium angulatum*, *Bifidobacterium dentium*, *Zymomonas mobilis*, *Clostridium beijerinckii* or *Anaerostipes caccae*, or an active fragment thereof, (ii) a polypeptide having at least about 90% identity or at least about 95% identity to SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65, (iii) a  
 15 polypeptide having at least about 90% identity or at least about 95% identity to a KARI enzyme derived from *Bifidobacterium angulatum*, *Bifidobacterium dentium*, *Enterococcus gallinarum*, *Streptococcus thermophiles*, *Zymomonas mobilis*, *Clostridium beijerinckii*, *Anaerostipes caccae*, or *Lactococcus lactis subsp. cremoris* MG1363 or an active  
 20 fragment thereof, wherein the polypeptide has a  $K_M$  for NADH less than about 50, (iv) a polypeptide having at least about 90% identity or at least about 95% identity to a KARI enzyme derived from *Staphylococcus capitis* SK14, *Staphylococcus epidermidis* M23864-W1, *Staphylococcus hominis* SK119, *Staphylococcus aureus subsp. aureus* TCH130, *Staphylococcus warneri* L37603, *Staphylococcus epidermidis* W23144, *Staphylococcus saprophyticus subsp. Saprophyticus* ATCC15305, *Staphylococcus carnosus subsp. Carnosus* TM300, *Listeria monocytogenes* EGD-e, *Listeria grayi* DSM 20601, *Enterococcus casseliflavus* EC30, *Enterococcus gallinarum* EG2, *Macroccoccus caseolyticus* JCSC5402,  
 25 *Streptococcus vestibularis*, *Streptococcus mutans* UA159, *Streptococcus gordonii str. cgakkus* sybstr. CH1, *Streptococcus suis* 89/1591, *Streptococcus infantarius subsp. infantarius* ATCC BAA-102, *Lactococcus lactis subsp. cremoris* MG1363, *Lactococcus lactis*, *Leuconostoc*

- mesenteroides* subsp *mesenteroides* ATCC8293, *Lactobacillus buchneri* ATCC 11577, *Staphylococcus haemolyticus* JCSC1435, *Staphylococcus epidermidis* ATCC12228, *Streptococcus pneumoniae* CGSP14, *Streptococcus pneumoniae* TIGR4, *Streptococcus sanguinis* SK36,
- 5 *Streptococcus salivarius* SK126, *Streptococcus thermophilus* LMD-9, *Streptococcus pneumoniae* CCRI 1974M2, *Lactococcus lactis* subsp. *lactis* Il1403, *Leuconostoc mesenteroides* subsp *cremoris* ATCC19254, *Leuconostoc mesenteroides* subsp *cremoris*, *Lactobacillus brevis* subsp. *gravesensis* ATCC27305, or *Lactococcus lactis* subsp *lactis* NCDO2118
- 10 or an active fragment thereof, wherein the heterologous polypeptide has a  $K_M$  for NADH less than about 50, (v) a heterologous polypeptide with KARI activity having at least about 90% identity or at least about 95% identity to SEQ ID NO: 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127,
- 15 129, 131, 133, or 135 or an active fragment thereof, wherein the heterologous polypeptide has a  $K_M$  for NADH less than about 50; (b) a heterologous polynucleotide encoding the heterologous polypeptide with KARI activity of (a); (c) reduced or eliminated aldehyde dehydrogenase activity; (d) reduced or eliminated aldehyde oxidase activity; (e) reduced or
- 20 eliminated acetolactate reductase activity; or (f) a combination thereof.

In one embodiment, the recombinant host cell comprises reduced or eliminated aldehyde dehydrogenase expression activity and reduced or eliminated acetolactate reductase expression or activity.

- In another embodiment, the recombinant host cell comprises (i)
- 25 reduced or eliminated aldehyde dehydrogenase expression or activity or reduced or eliminated acetolactate reductase expression or activity and (ii) a heterologous polynucleotide encoding a polypeptide having KARI activity and  $K_M$  for NADH less than 300  $\mu$ M.

- In another embodiment, the recombinant host comprises a
- 30 heterologous polypeptide with KARI activity that has at least about 90% or at least about 95% identity to SEQ ID NO: 27, 29, 141, 143, 275, or 277.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that comprises substitutions in

amino acids corresponding to S56 and S58 of SEQ ID NO: 27. In some embodiments, the polypeptide with KARI activity further comprises a substitution of one or more of the amino acids corresponding to I86, N87, T131, or T191 of SEQ ID NO: 27. In some embodiments, the polypeptide  
5 with KARI activity having at least 90% identity or at least 95% identity to SEQ ID NO: 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65.

In some embodiments, the recombinant host cell has an effective isobutanol productivity of at least about 3, at least about 4, or at least  
10 about 5 grams per gram of cells after about 48 hours, wherein at least the last about 24 of the 48 hours are under anaerobic conditions.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that has a  $K_M$  for NADH less than about 350, less than about 100, less than about 50, or less than  
15 about 10  $\mu M$  at pH 6.8.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that has at least about 90% identity or at least about 95% identity to SEQ ID NO: 376, 382, 378, or  
20 275.

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity comprises an amino acid substitution at one or more of the positions corresponding to amino acids A41, S56, S58, I87, T131, T191, R227, or Q246 of a KARI enzyme derived from *Anaerostipes caccae* (SEQ ID NO:27).  
25

In some embodiments, the recombinant host cell comprises a heterologous polypeptide with KARI activity that comprises SEQ ID NO: 33 or SEQ ID NO:35 or an active fragment thereof.  
30

In another embodiment, the recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity. In some  
embodiments, the polypeptide having aldehyde dehydrogenase activity catalyzes the conversion of isobutyraldehyde to isobutyric acid. In some  
embodiments, the polypeptide having aldehyde dehydrogenase activity

corresponds to Enzyme Commission Number EC 1.21.3, EC 1.2.1.4, and/or EC1.2.1.5. In some embodiments, the host cell is *S. cerevisiae* and the polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, ALD6 or a homolog thereof. In some embodiments,

5 the host cell is *K. lactis* and the polypeptide having aldehyde dehydrogenase activity is KLLA0F00440, KLLA0E23057, KLLA0D10021, or KLLA0D09999G. In some embodiments, the host cell is *P. stipitis* and the polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, or ALD7. In some embodiments, the host

10 cell is *Lactobacillus plantarum* and said polypeptide having aldehyde dehydrogenase activity is AldH. In some embodiments, the host cell is *E. coli* and the polypeptide having aldehyde dehydrogenase activity is aldA, aldB, or aldH.

In another embodiment, the host cell comprises a deletion,

15 mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde oxidase activity. In some embodiments, the polypeptide having aldehyde oxidase activity catalyzes the conversion of isobutyraldehyde to isobutyric acid. In some embodiments, the polypeptide having aldehyde oxidase activity corresponds to Enzyme

20 Commission Number EC 1.2.3.1. In some embodiments, the polypeptide having aldehyde oxidase activity is AOX1 and/or AOX2.

In another embodiment, the host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity. In some embodiments,

25 the polypeptide having acetolactate reductase activity comprises a polypeptide encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 676, SEQ ID NO: 678, SEQ ID NO: 680, SEQ ID NO: 682, SEQ ID NO: 684, SEQ ID NO: 686, SEQ ID NO: 688, SEQ ID NO: 690, SEQ ID NO: 692, SEQ ID NO: 694, SEQ ID NO: 696, SEQ ID

30 NO: 702, SEQ ID NO: 704, SEQ ID NO: 706, SEQ ID NO: 708, SEQ ID NO: 710, SEQ ID NO: 712, SEQ ID NO: 714, SEQ ID NO: 716, SEQ ID NO: 718, SEQ ID NO: 720, SEQ ID NO: 722, SEQ ID NO: 724, SEQ ID

NO:726 , SEQ ID NO:728, and SEQ ID NO: 730. In some embodiments, the polypeptide having acetolactate reductase activity is YMR226C.

In another embodiment, the recombinant host cell is a yeast host cell. In some embodiments, the yeast is selected from the group  
5 consisting of *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, or *Pichia*. In some embodiments, the host cell is *Saccharomyces cerevisiae*.

In another embodiment, the host cell is a bacterial cell. In some  
embodiments, the bacterial cell is a *Clostridium*, *Zymomonas*, *Escherichia*,  
10 *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, or *Streptococcus* cell. In some embodiment, the bacterial cell is not *E. coli*.

15 In some embodiments, the engineered isobutanol production pathway of the recombinant host cell comprises the following substrate to product conversions: (a) pyruvate to acetolactate, (b) acetolactate to 2,3-dihydroxyisovalerate, (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate, (d)  
20 2-ketoisovalerate to isobutyraldehyde, and (e) isobutyraldehyde to isobutanol and more than one of the substrate to product conversions is catalyzed by an enzyme that is heterologous to the host cell. In some embodiments, all of the substrate to product conversions are catalyzed by enzymes heterologous to the host cell. In some embodiments, at least  
25 one heterologous polynucleotide encoding an enzyme heterologous to the host cell is chromosomally integrated into the host cell. In some embodiments, the substrate to product conversions are catalyzed by enzymes substantially localized to the cytosol. In some embodiments, the substrate to product conversion for isobutyraldehyde to isobutanol is catalyzed by an alcohol dehydrogenase enzyme that utilizes NADH as a  
30 cofactor. In some embodiments, the conversion of acetolactate to 2,3-dihydroxyisovalerate is catalyzed by a KARI that can use NADH as a cofactor.

In some embodiments, the host cell comprises the plasmid pLH702 or pLH701 or a plasmid having the same coding regions. In some embodiments, the host cell comprises the plasmid pBP915 or a plasmid having the same coding regions. In some embodiments, the host cell  
5 comprises the plasmid pYZ067 $\Delta$ kivD $\Delta$ hADH or a plasmid having the same coding regions.

In some embodiments, the host cell comprises reduced, disrupted, or eliminated ability to convert acetolactate to 2,3-dihydroxy-2-methylbutyrate.

10 In some embodiments, the host cell is yeast and has reduced or eliminated pyruvate decarboxylase expression or activity. In some embodiments, the host cell has reduced or eliminated PDC1, PDC5, or PDC6 activity or a combination thereof.

In some embodiments, the host cell has reduced or eliminated  
15 NAD-dependent glycerol-3-phosphate dehydrogenase expression or activity. In some embodiments, the host cell has reduced GPD2 activity.

In some embodiments, the host cell has reduced or eliminated FRA2 expression or activity.

In some embodiments, the host cell produces isobutanol under  
20 anaerobic conditions and the molar ratio of isobutanol to glycerol is greater than 1.

In some embodiments, the polypeptide having ketol-acid reductoisomerase activity matches the profile HMM given provided in Table Z with a profile HMM E value of  $<10^{-3}$ .

25 In some embodiments, the host cell produces isobutanol at a yield greater than about 25%, about 50%, about 75%, or about 90% of theoretical yield.

In some embodiments, isobutanol and ethanol are produced.

Methods for producing isobutanol include methods comprising  
30 providing a recombinant host cell as described above and contacting the host cell with a carbon substrate under conditions whereby isobutanol is produced. In some embodiments, at least a portion of the contacting occurs under anaerobic conditions. In some embodiments, the contacting



occurs in the presence of an extractant. In some embodiments, the contacting occurs in the presence of a sufficient quantity of organic extractant to form a two-phase system comprising an aqueous phase and an organic phase. In some embodiments, one or more of the effective

5 rate, effective titer, or effective yield of isobutanol is increased as compared to methods using a recombinant host cell that does not comprise a heterologous polypeptide with KARI activity, a heterologous polynucleotide encoding a polypeptide with KARI activity, reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated

10 aldehyde oxidase activity, reduced or eliminated acetolactate reductase activity, or a combination thereof. In some embodiments, one or more of the effective rate, effective titer, or effective yield of isobutanol is increased as compared to methods using a recombinant host cell that does not comprise (i) a heterologous polypeptide with KARI activity or a

15 heterologous polynucleotide encoding a polypeptide with KARI activity and (ii) at least one modification that enhances performance of the engineered isobutanol production pathway. In some embodiments, DHMB production, isobutyric acid production, or both is reduced as compared to methods using a recombinant host cell that does not comprise a

20 heterologous polypeptide with KARI activity, a heterologous polynucleotide encoding a polypeptide with KARI activity, reduced or eliminated aldehyde dehydrogenase activity, reduced or eliminated aldehyde oxidase activity, reduced or eliminated acetolactate reductase activity, or a combination thereof. In some embodiments, DHMB production, isobutyric

25 acid production, or both is reduced as compared to methods using a recombinant host cell that does not comprise (i) a heterologous polypeptide with KARI activity or a heterologous polynucleotide encoding a polypeptide with KARI activity and (ii) at least one modification that enhances performance of the engineered isobutanol production pathway.

30 In some embodiments, the molar ratio of isobutanol to glycerol is greater than 1.

Methods for producing isobutanol also comprise providing a recombinant host cell that produces isobutanol and contacting the host cell

with a carbon substrate under conditions whereby isobutanol is produced, wherein at least a portion of the contacting occurs under anaerobic conditions, and wherein the ratio of isobutanol to glycerol produced is greater than 1.

- 5           Methods for producing isobutanol also comprise growing a recombinant yeast comprising a biosynthetic pathway capable of converting pyruvate to acetolactate under conditions whereby butanol is produced and removing DHMB from the culture.

Compositions produced by such methods are also provided herein.

- 10          In some embodiments, the composition comprises isobutanol and a recombinant host cell provided above. In some embodiments, the composition comprises butanol and no more than about 0.5 mM DHMB.

- Fermentative compositions are also provided herein. In some embodiments, a fermentative composition comprises the host cell and  
15          isobutanol produced according to the methods provided above.

Compositions comprising i) a recombinant yeast capable of producing butanol, ii) butanol, and iii) no more than about 0.5 mM DHMB are also provided.

- Methods for producing a recombinant host cell are also provided.  
20          Such methods can comprise (a) providing a recombinant host cell comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity or aldehyde oxidase activity; and (b) transforming the host cell with a polynucleotide encoding a polypeptide of an isobutanol biosynthetic pathway.

- 25           Methods for reducing or eliminating the conversion of isobutyraldehyde to isobutyric acid are also provided. Such methods can comprise (a) providing the recombinant host cell of any one of claims 1-52; and (b) subjecting the host cell to conditions wherein the conversion of isobutyraldehyde to isobutyric acid is reduced or eliminated compared to  
30          methods using a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity.

Certain polypeptides are also provided herein. In some embodiments, the polypeptides comprise at least about 90% identity or at

least about 95% identity or at least about 99% identity to SEQ ID NO: 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 417, 419, 421, 423, 425, 427, 429, 431, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 5 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 10 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 624, 626, 628, 630, 632 or an active fragment thereof and have ketol-acid reductoisomerase activity. In some embodiments, the polypeptides comprise at least about 90% identity or at least about 95% identity or at least about 99% identity to SEQ ID NO: 417, 419, 421, 423, 425, or 427 or 15 an active fragment thereof and have ketol-acid reductoisomerase activity. In some embodiments, a polypeptide comprises a sequence with at least about 90% identity, at least about 95% identity, or at least about 99% identity to SEQ ID NO: 927, 928, 196, 266, 267, 389, 405, 637, 781, 782, 783, 835, 853, 854, 855, 856, 857, or 859.

20 Polynucleotides encoding such polypeptides and host cells comprising such polynucleotides and polypeptides are also provided.

Methods of converting acetolactate to 2,3-dihydroxyisovalerate are also provided. For example, such methods can comprise (a) providing a polypeptide described above, and (b) contacting the polypeptide with 25 acetolactate under conditions wherein 2,3-dihydroxyisovalerate is produced.

Recombinant yeast cells are also provided herein. In some embodiments, a recombinant yeast comprises a biosynthetic pathway capable of converting pyruvate to acetolactate, and the yeast produces 30 less than 0.01 moles 2,3-dihydroxy-2-methyl butyrate (DHMB) per mole of sugar consumed. In some embodiments, a recombinant yeast comprises capable of converting pyruvate to acetolactate, and the yeast produces DHMB at a rate of less than about 1.0 mM/hour. In some embodiments, a

recombinant yeast comprises a biosynthetic pathway capable of converting pyruvate to acetolactate, and the yeast produces an amount of 2,3-dihydroxy-3-isovalerate (DHIV) that is at least about 1.5 times the amount of DHMB produced.

- 5           Methods of identifying a gene involved in DHMB production are also provided. In some embodiments, the methods comprise (i) providing a collection of yeast strains comprising at least two or more gene deletions; (ii) measuring the amount of DHMB produced by individual yeast strains; (iii) selecting a yeast strain that produces no more than  
10   about 1.0 mM DHMB/hour; and (iv) identifying the gene that is deleted in the selected yeast strain. In some embodiments, the methods comprise (i) providing a collection of yeast strains that over-express at least two or more genes; (ii) measuring the amount of DHMB produced by individual yeast strains; (iii) selecting a yeast strain that produces at least about 1.0  
15   mM DHMB; and (iv) identifying the gene that is over-expressed in the selected yeast strain.

- Methods for the production of butanol are also provided. In some embodiments, the methods comprise (a) growing a recombinant yeast comprising a biosynthetic pathway capable of converting pyruvate to  
20   acetolactate under conditions whereby butanol is produced; and b) measuring DHIV and/or DHMB concentration. In some embodiments, the growing and measuring can be performed simultaneously or sequentially and in any order. In some embodiments, the measuring comprises liquid chromatography-mass spectrometry.

- 25           Methods for increasing ketol-acid reductoisomerase (KARI) activity are also provided. In some embodiments, the methods comprise (a) providing a composition comprising acetolactate, a KARI enzyme, and an acetolactate reductase enzyme and (b) decreasing DHMB levels. In some  
30   embodiments, decreasing DHMB levels is achieved by decreasing acetolactate reductase enzyme activity. In some embodiments, decreasing DHMB levels is achieved by removing DHMB from the composition.

In some embodiments, increasing KARI enzyme productivity in a host cell can comprises culturing a host cell, wherein the host cell comprises a heterologous KARI enzyme and at least one genetic modification that reduces, disrupts, or eliminates acetolactate reductase expression or activity, and wherein the KARI enzyme activity is decreased in the presence of DHMB. In some embodiments, the KARI has at least about 90%, at least about 95%, or at least about 99% identity to *E. coli* or *L. lactis* KARI. In some embodiments, the reduced, disrupted, or eliminated acetolactate reductase expression or activity substantially reduces the presence of DHMB.

Methods for increasing dihydroxyacid dehydratase (DHAD) activity are also provided. In some embodiments, the methods comprise (a) providing a composition comprising dihydroxyisovalerate (DHIV) and a DHAD enzyme and (b) decreasing DHMB levels.

15

#### BRIEF DESCRIPTION OF THE FIGURES AND INCORPORATION OF THE TABLE FILED ELECTRONICALLY HEREWITH

The invention can be more fully understood from the following detailed description, the Figures, and the accompanying sequence descriptions, which form part of this application.

20

FIGURE 1- Shows four different isobutanol biosynthetic pathways. The steps labeled "a", "b", "c", "d", "e", "f", "g", "h", "i", "j" and "k" represent the substrate to product conversions described below.

FIGURE 2 depicts an alignment of the amino acid sequences of the KARI from *Pseudomonas fluorescens* ("PF5"; SEQ ID NO: 5) and KARI from *Anaerostipes caccae* ("K9"; SEQ ID NO: 27). The bolded positions are targeted for mutagenesis as described herein.

25

FIGURE 3A, 3B and 3C depict an alignment of the amino acid sequences of KARI enzymes from *Bifidobacterium angulatum* DSM 20098 ("K1"; SEQ ID NO: 141), *Bifidobacterium dentium* ATCC 27678 ("K2"; SEQ ID NO: 143), *Clostridium beijerinckii* NCIMB 8052 ("K7"; SEQ ID NO: 275), *Anaerostipes caccae* DSM 14662 ("K9"; SEQ ID NO: 27), *Enterococcus gallinarum* EG2 ("K25" SEQ ID NO: 376), *Streptococcus*

30

*thermophilus* LMD-9 ("K26" SEQ ID NO: 121), *Lactococcus lactis* subsp. cremoris MG1363 ("K29"; SEQ ID NO: 382), *Zymomonas mobilis* ("S2"; SEQ ID NO: 277), and *Lactococcus lactis* ("LTS"; SEQ ID NO: 380). The bolded positions are targeted for mutagenesis as described herein.

5       FIGURE 4 is a plasmid map of pLH556 (pHR81-Pilv5-Pf5.KARI) vector (SEQ ID NO: 138).

FIGURE 5 shows the specific rate of isobutanol production,  $Q_p$ , of the two strains, PNY1910 and PNY2242.

10       FIGURE 6 shows the accumulation of DHIV + DHMB in the culture supernatant during the fermentation time course with PNY1910 (triangles) and PNY2242 (diamonds). (DHMB and DHIV are not distinguished by the HPLC method used.)

FIGURE 7 shows the yield of glycerol, pyruvic acid, 2,3-butanediol (BDO), DHIV/DHMB,  $\alpha$ -ketoisovalerate (aKIV), and isobutyric acid (iBuAc).  
15       DHIV and DHMB are shown together as these are not distinguished by the HPLC method used.

FIGURE 8 shows a summary of  $V_{max}/K_M$  values for K9G9 variants as described in Example 16.

20       FIGURES 9A, B, and C show isobutanol to glycerol molar yield ratios, isobutanol molar yields, and isobutanol titers for K9 variants as described in Example 19.

FIGURE 10 shows an isobutanol biosynthetic pathway. Step "a" represents the conversion of pyruvate to acetolactate. Step "b" represents the conversion of acetolactate to DHIV. Step "c" represents the  
25       conversion of DHIV to KIV. Step "d" represents the conversion of KIV to isobutyraldehyde. Step "e" represents the conversion of isobutyraldehyde to isobutanol. Step "f" represents the conversion of acetolactate to DHMB.

FIGURE 11 shows a phylogenetic tree of YMR226c homologs from species of ascomycete yeast. A filamentous fungi (*Neurospora crassa*)  
30       sequence is included as an outgroup.

FIGURE 12 shows a multiple sequence alignment (MSF Format) of nucleotide sequences of ORFs with homology to YMR226C. The gene

names shown correspond to the accession numbers and SEQ ID NOs.  
given in Table 7. The alignment was produced by AlignX (Vector NTI).

FIGURE 13 shows a graph of the molar yield of DHMB over time.

FIGURE 14 depicts the production of isobutanol and isobutyric acid

5 in yeast strain NYLA84.

Table Z- is a table

of the Profile HMM of experimentally verified KARI enzymes

listed in Table 1 and as described in US App. Pub. Nos. 2010/0197519

10 and 2009/0163376 .

Table 1. Experimentally verified KARI enzymes.

| GI Number | Accession      | SEQ ID NO: | Microorganism                                             |
|-----------|----------------|------------|-----------------------------------------------------------|
| 70732562  | YP_262325.1    | 5          | <i>Pseudomonas fluorescens</i> Pf-5                       |
| 15897495  | NP_342100.1    | 1          | <i>Sulfolobus solfataricus</i> P2                         |
| 18313972  | NP_560639.1    | 2          | <i>Pyrobaculum aerophilum</i> str. IM2                    |
| 76801743  | YP_326751.1    | 7          | <i>Naatronomonas pharaonis</i> DSM 2160                   |
| 16079881  | NP_390707.1    | 8          | <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168  |
| 19552493  | NP_600495.1    | 9          | <i>Corynebacterium glutamicum</i> ATCC 13032              |
| 6225553   | O32414         | 10         | <i>Phaeosprillum molischianum</i>                         |
| 17546794  | NP_520196.1    | 3          | <i>Ralstonia solanacearum</i> GM1000                      |
| 56552037  | YP_102070.1    | 11         | <i>Zymomonas mobilis</i> subsp. <i>mobilis</i> ZM4        |
| 114319705 | YP_741388.1    | 12         | <i>Alkalilimnicola ehrlichi</i> MLHE-1                    |
| 57240359  | ZP_00368308.1  | 13         | <i>Campylobacter lari</i> RM2100                          |
| 120553816 | YP_958167.1    | 14         | <i>Marinobacter aquaeolei</i> VT8                         |
| 71065099  | YP_263826.1    | 15         | <i>Psychrobacter arcticus</i> 273-4                       |
| 83648555  | YP_436990.1    | 16         | <i>Hahella chejuensis</i> KCTC 2396                       |
| 74318007  | YP_315747.1    | 17         | <i>Thiobacillus denitrificans</i> ATCC 25259              |
| 67159493  | ZP_00420011.1  | 18         | <i>Azotobacter vinelandii</i> AvOP                        |
| 66044103  | YP_233944.1    | 19         | <i>Pseudomonas syringae</i> pv. <i>syringae</i> B728a     |
| 28868203  | NP_790822.1    | 20         | <i>Pseudomonas syringae</i> pv. <i>tomato</i> str. DC3000 |
| 26991362  | NP_746787.1    | 21         | <i>Pseudomonas putida</i> KT2440                          |
| 104783656 | YP_610154.1    | 22         | <i>Pseudomonas entomophila</i> L48                        |
| 146306044 | YP_001186509.1 | 23         | <i>Pseudomonas mendocina</i> ymp                          |
| 15599888  | NP_253382.1    | 4          | <i>Pseudomonas aeruginosa</i> PAO1                        |

|          |             |    |                                   |
|----------|-------------|----|-----------------------------------|
| 42780593 | NP_977840.1 | 24 | <i>Bacillus cereus</i> ATCC 10987 |
| 42781005 | NP_978252.1 | 25 | <i>Bacillus cereus</i> ATCC 10987 |
| 266346   | Q01292      | 6  | <i>Spinacia oleracea</i>          |

The eleven positions in the profile HMM representing the columns in the alignment which correspond to the eleven cofactor switching positions in *Pseudomonas fluorescens* Pf-5 KARI are identified as

5 positions 24, 33, 47, 50, 52, 53, 61, 80, 115, 156, and 170. Table Z is submitted herewith electronically .

The sequences provided in the sequence listing filed herewith (Name: 20120323\_CL5367USNA\_SEQLIST.txt; Size 2,003,893 bytes; Date of Creation March 23, 2012) .

10 Consistent with the World Intellectual Property Organization (WIPO) Standard ST.25 (2009), certain primers given in the sequence listing and herein use N to represent nucleotides a or g or c or t. K is used to represent g or t. M is used to represent a or c.

#### 15 DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application including the definitions will control. Also, unless

20 otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

25

Although methods and materials similar or equivalent to those disclosed herein can be used in practice or testing of the present invention, suitable methods and materials are disclosed below. The

30 materials, methods and examples are illustrative only and are not intended



to be limiting. Other features and advantages of the invention will be apparent from the detailed description and from the claims.

The last step in the biosynthesis of isobutanol via a pyruvate-utilizing biosynthetic pathway is the conversion of isobutyraldehyde to isobutanol (Figure 1). A side reaction in this pathway is the conversion of isobutyraldehyde to isobutyric acid which results in reduced amounts of isobutyraldehyde available to convert into isobutanol and reduced isobutanol yield. For an efficient biosynthetic process, there is a need to prevent the conversion of isobutyraldehyde to isobutyric acid such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

Aldehyde dehydrogenases are a family of enzymes that catalyze the oxidation (dehydrogenation) of aldehydes (Wang *et al.*, *J. Bacteriol.* 180:822-30, 1998; Navarro-Avino *et al.*, *Yeast* 15:829-42, 1999; and Saint-Prix *et al.*, *Microbiology* 150:2209-20, 2004). There is a need to identify suitable aldehyde dehydrogenases that can be modified to reduce or eliminate aldehyde dehydrogenase activity, and can reduce or eliminate the conversion of isobutyraldehyde to isobutyric acid, such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

Aldehyde oxidases are a family of enzymes that catalyze the production of carboxylic acids from aldehydes (Nomura *et al.*, *Biosci. Biotechnol. Biochem.* 62:1134-7, 1998; and Johnson *et al.*, *Genetics* 151:1379-1391, 1999). There is a need to identify suitable aldehyde oxidases that can be modified to reduce or eliminate aldehyde oxidase activity and can reduce or eliminate the conversion of isobutyraldehyde to isobutyric acid, such that increased amounts of isobutyraldehyde are available for conversion to isobutanol and isobutanol yields are increased.

The biosynthesis pathway for the production of butanol in genetically engineered yeast includes the conversion of acetolactate to 2,3-dihydroxy-3-isovalerate (DHIV), which is subsequently converted to butanol. See Figure 10. However, a side reaction in this pathway, which decreases the overall production of butanol, is the conversion of

acetolactate to 2,3-dihydroxy-2-methylbutyrate (DHMB). In fact, Applicants have discovered that DHMB has inhibitory effects on enzymes (dihydroxyacid dehydratase and ketol-acid reductoisomerase) in an isobutanol production pathway. For an efficient biosynthetic process,

5 there is a need to prevent the conversion of acetolactate to DHMB.

Applicants have solved the stated problems by providing recombinant yeast host cells comprising an isobutanol biosynthetic pathway; and at least one of: i) reduced or eliminated aldehyde dehydrogenase activity ii) reduced or eliminated aldehyde oxidase activity  
10 iii) reduced or eliminated acetolactate reductase activity; iv) a heterologous polynucleotide encoding a polypeptide having ketol-acid reductoisomerase activity; and v) a heterologous polypeptide having ketol-acid reductoisomerase activity. Further, Applicants provide methods of producing butanol utilizing such host cells. Such recombinant host cells  
15 can be used to increase the production of a product of a biosynthetic pathway (e.g., isobutanol, 1-butanol, or 2-butanol) and/or reduce or eliminate the conversion of pathway intermediates to undesirable byproducts. Applicants have also provided a suitable screening strategy for evaluating various candidate enzymes. The identified enzymes can be  
20 altered to enhance the production of a product of a biosynthetic pathway (e.g., isobutanol, 1-butanol, or 2-butanol) and/or reduce or eliminate the conversion of pathway intermediates to undesirable byproducts.

In order to further define this invention, the following terms, abbreviations and definitions are provided.

25 It will be understood that "derived from" with reference to polypeptides disclosed herein encompasses sequences synthesized based on the amino acid sequences of the KARIs present in the indicated organisms as well as those cloned directly from the organism's genetic material.

30 As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains" or "containing," or any other variation thereof, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or

group of integers and are intended to be non-exclusive or open-ended.

For example, a composition, a mixture, a process, a method, an article, or an apparatus that comprises a list of elements is not necessarily limited to only those elements but can include other elements not expressly listed or

5 inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and  
10 both A and B are true (or present).

As used herein, the term "consists of," or variations such as "consist of" or "consisting of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, but that no additional integer or group of integers can be added to the  
15 specified method, structure, or composition.

As used herein, the term "consists essentially of," or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited integer or group of integers, and the optional inclusion of any recited integer or group  
20 of integers that do not materially change the basic or novel properties of the specified method, structure or composition. See M.P.E.P. § 2111.03.

Also, the indefinite articles "a" and "an" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances, *i.e.*, occurrences of the element or component.  
25 Therefore "a" or "an" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

The term "invention" or "present invention" as used herein is a non-limiting term and is not intended to refer to any single embodiment of the  
30 particular invention but encompasses all possible embodiments as described in the claims as presented or as later amended and supplemented, or in the specification.

As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or to carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, or within 5% of the reported numerical value.

As used herein, "synergistic" refers to a greater-than-additive effect produced by a combination (i.e., an effect that is greater than the sum of individual effects) or an additive effect when the individual effects are not expected to be additive. The term also refers to the addition of one compound which results in less of another compound being required.

The term "butanol biosynthetic pathway" as used herein refers to the enzymatic pathway to produce 1-butanol, 2-butanol, or isobutanol. For example, isobutanol biosynthetic pathways are disclosed in U.S. Patent Application Publication No. 2007/0092957.

The term "isobutanol biosynthetic pathway" refers to the enzymatic pathway to produce isobutanol. Certain isobutanol biosynthetic pathways are illustrated in Figure 1 and described herein. From time to time "isobutanol biosynthetic pathway" is used synonymously with "isobutanol production pathway".

The term "butanol" as used herein refers to 2-butanol, 1-butanol, isobutanol or mixtures thereof. Isobutanol is also known as 2-methyl-1-propanol.

A recombinant host cell comprising an "engineered alcohol production pathway" (such as an engineered butanol or isobutanol

production pathway) refers to a host cell containing a modified pathway that produces alcohol in a manner different than that normally present in the host cell. Such differences include production of an alcohol not typically produced by the host cell, or increased or more efficient

5 production.

The term "heterologous biosynthetic pathway" as used herein refers to an enzyme pathway to produce a product in which at least one of the enzymes is not endogenous to the host cell containing the biosynthetic pathway.

10 The term "extractant" as used herein refers to one or more organic solvents which can be used to extract butanol from a fermentation broth.

The term "effective isobutanol productivity" as used herein refers to the total amount in grams of isobutanol produced per gram of cells.

15 The term "effective titer" as used herein, refers to the total amount of a particular alcohol (e.g. butanol) produced by fermentation per liter of fermentation medium. The total amount of butanol includes: (i) the amount of butanol in the fermentation medium; (ii) the amount of butanol recovered from the organic extractant; and (iii) the amount of butanol recovered from the gas phase, if gas stripping is used.

20 The term "effective rate" as used herein, refers to the total amount of butanol produced by fermentation per liter of fermentation medium per hour of fermentation.

25 The term "effective yield" as used herein, refers to the amount of butanol produced per unit of fermentable carbon substrate consumed by the biocatalyst.

The term "separation" as used herein is synonymous with "recovery" and refers to removing a chemical compound from an initial mixture to obtain the compound in greater purity or at a higher concentration than the purity or concentration of the compound in the  
30 initial mixture.

The term "aqueous phase," as used herein, refers to the aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant. In an embodiment of a

process described herein that includes fermentative extraction, the term "fermentation broth" then specifically refers to the aqueous phase in biphasic fermentative extraction.

The term "organic phase," as used herein, refers to the non-  
5 aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant.

The terms "PDC-," "PDC knockout," or "PDC-KO" as used herein refer to a cell that has a genetic modification to inactivate or reduce expression of a gene encoding pyruvate decarboxylase (PDC) so that the  
10 cell substantially or completely lacks pyruvate decarboxylase enzyme activity. If the cell has more than one expressed (active) PDC gene, then each of the active PDC genes can be inactivated or have minimal expression thereby producing a PDC- cell.

The term "polynucleotide" is intended to encompass a singular  
15 nucleic acid as well as plural nucleic acids, and refers to a nucleic acid molecule or construct, e.g., messenger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide can contain the nucleotide sequence of the full-length cDNA sequence, or a fragment thereof, including the untranslated 5' and 3' sequences and the coding sequences. The polynucleotide can  
20 be composed of any polyribonucleotide or polydeoxyribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-  
25 stranded regions, hybrid molecules comprising DNA and RNA that can be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. "Polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

A polynucleotide sequence can be referred to as "isolated," in  
30 which it has been removed from its native environment. For example, a heterologous polynucleotide encoding a polypeptide or polypeptide fragment having dihydroxy-acid dehydratase activity contained in a vector is considered isolated for the purposes of the present invention. Further

examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated polynucleotides or nucleic acids according to the present invention further include such

5 molecules produced synthetically. An isolated polynucleotide fragment in the form of a polymer of DNA can be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "NAD(P)H consumption assay" refers to an enzyme assay for the determination of the specific activity of the KARI enzyme, involving

10 measuring the disappearance of the KARI cofactor, NAD(P)H, from the enzyme reaction. Such assays are described in Aulabaugh and Schloss, *Biochemistry* 29: 2824-2830, 1990 .

The term "NAD(P)H" refers to either NADH or NADPH.

15 "KARI" is the abbreviation for the enzyme ketol-acid reductoisomerase.

The term "close proximity" when referring to the position of various amino acid residues of a KARI enzyme with respect to the adenosyl 2'-phosphate of NADPH means amino acids in the three-dimensional model

20 for the structure of the enzyme that are within about 4.5 Å of the phosphorus atom of the adenosyl 2'-phosphate of NADPH bound to the enzyme.

The term "ketol-acid reductoisomerase" (abbreviated "KARI"), and "acetohydroxy acid isomeroreductase" will be used interchangeably and

25 refer to enzymes capable of catalyzing the reaction of (S)-acetolactate to 2,3-dihydroxyisovalerate, classified as EC number EC 1.1.1.86 (*Enzyme Nomenclature* 1992, Academic Press, San Diego). As used herein the term "Class I ketol-acid reductoisomerase enzyme" means the short form that typically has between 330 and 340 amino acid residues, and is

30 distinct from the long form, called class II, that typically has approximately 490 residues. These enzymes are available from a number of sources, including, but not limited to *E. coli* (amino acid SEQ ID NO: 942; GenBank Accession Number NC\_000913 REGION: 3955993.3957468), *Vibrio*

*cholerae* (GenBank Accession Number NC\_002505 REGION: 157441.158925), *Pseudomonas aeruginosa*, (GenBank Accession Number NC\_002516, REGION: 5272455.5273471), and *Pseudomonas fluorescens* (amino acid SEQ ID NO: 943; GenBank Accession Number NC\_004129 REGION: 6017379.6018395). KARI enzymes are described for example, in U.S. Patent Nos. 7,910,342 and 8,129,162 and U.S. Pub. App, No.2010/0197519 .

KARI is found in a variety of organisms and amino acid sequence comparisons across species have revealed that there are 2 types of this enzyme: a short form (class I) found in fungi and most bacteria, and a long form (class II) typical of plants. Class I KARIs typically have between 330-340 amino acid residues. The long form KARI enzymes have about 490 amino acid residues. However, some bacteria such as *Escherichia coli* possess a long form, where the amino acid sequence differs appreciably from that found in plants. KARI is encoded by the *ilvC* gene and is an essential enzyme for growth of *E. coli* and other bacteria in a minimal medium. Class II KARIs generally consist of a 225-residue N-terminal domain and a 287-residue C-terminal domain. The N-terminal domain, which contains the NADPH-binding site, has an  $\alpha\beta$  structure and resembles domains found in other pyridine nucleotide-dependent oxidoreductases. The C-terminal domain consists almost entirely of  $\alpha$ -helices.

Ketol-acid reductoisomerase (KARI) enzymes are useful in pathways for the production of isobutanol using engineered microorganisms (US Patents 7,851,188 and 7,993,889).

A KARI that can utilize NADH can capitalize on the NADH produced by the existing glycolytic and other metabolic pathways in most commonly used microbial cells and can result in improved isobutanol production. Rane et al. (Arch. Biochem. Biophys., 338: 83-89, 1997) discusses cofactor switching of a ketol acid reductoisomerase isolated from *E. coli*. US Appl. Pub. Nos. 2009/0163376 and 2010/0197519



describe the generation of  
KARI enzymes which can use NADH. US Appl. Pub. No. 2010/0143997  
describes *E. coli*  
variants with improved  $K_M$  values for NADH.

- 5       The terms "ketol-acid reductoisomerase activity" and "KARI activity" refer to the ability to catalyze the substrate to product conversion (S)-acetolactate to 2,3-dihydroxyisovalerate.

The term "acetolactate synthase" refers to an enzyme that catalyzes the conversion of pyruvate to acetolactate and  $\text{CO}_2$ .

- 10       Acetolactate has two stereoisomers ((R) and (S)); the enzyme prefers the (S)- isomer, which is made by biological systems. Certain acetolactate synthases are known by the EC number 2.2.1.6 (*Enzyme Nomenclature* 1992, Academic Press, San Diego). These enzymes are available from a number of sources, including, but not limited to, *Bacillus subtilis* (GenBank  
15       Nos: CAB15618, Z99122, NCBI (National Center for Biotechnology Information) amino acid sequence, NCBI nucleotide sequence, respectively), *Klebsiella pneumoniae* (GenBank Nos: AAA25079, M73842 and *Lactococcus lactis* (GenBank Nos: AAA25161, L16975).

- 20       The term "acetohydroxy acid dehydratase" refers to an enzyme that catalyzes the conversion of 2,3-dihydroxyisovalerate to  $\alpha$ -ketoisovalerate. Certain acetohydroxy acid dehydratases are known by the EC number 4.2.1.9. These enzymes are available from a vast array of microorganisms, including, but not limited to, *E. coli* (GenBank Nos: YP\_026248, NC\_000913, *S. cerevisiae* (GenBank Nos: NP\_012550,  
25       NC\_001142), *M. maripaludis* (GenBank Nos: CAF29874, BX957219), *B. subtilis* (GenBank Nos: CAB14105, Z99115), *Lactococcus lactis* (SEQ ID NO: 926), and *Streptococcus mutans* (SEQ ID NO: 939).

- 30       The term "branched-chain  $\alpha$ -keto acid decarboxylase" refers to an enzyme that catalyzes the conversion of  $\alpha$ -ketoisovalerate to isobutyraldehyde and  $\text{CO}_2$ . Certain branched-chain  $\alpha$ -keto acid decarboxylases are known by the EC number 4.1.1.72 and are available from a number of sources, including, but not limited to, *Lactococcus lactis* (GenBank Nos: AAS49166, AY548760; CAG34226, AJ746364,

*Salmonella typhimurium* (GenBank Nos: NP-461346, NC-003197),  
*Clostridium acetobutylicum* (GenBank Nos: NP-149189, NC-001988),  
*Macrococcus caseolyticus* (SEQ ID NO: 940), and *Listeria grayi* (SEQ ID  
 NO: 941).

- 5           The term "branched-chain alcohol dehydrogenase" refers to an  
 enzyme that catalyzes the conversion of isobutyraldehyde to isobutanol.  
 Certain branched-chain alcohol dehydrogenases are known by the EC  
 number 1.1.1.265, but can also be classified under other alcohol  
 dehydrogenases (specifically, EC 1.1.1.1 or 1.1.1.2). These enzymes  
 10   utilize NADH (reduced nicotinamide adenine dinucleotide) and/or NADPH  
 as electron donor and are available from a number of sources, including,  
 but not limited to, *S. cerevisiae* (GenBank Nos: NP\_010656, NC\_001136;  
 NP\_014051, NC\_001145), *E. coli* (GenBank No: NP\_417484), *C.*  
*acetobutylicum* (GenBank Nos: NP\_349892, NC\_003030), *B. indica*  
 15   (amino acid SEQ ID NO: 945), *A. xylosoxidans* (amino acid SEQ ID NO:  
 944).

- The term "branched-chain keto acid dehydrogenase" refers to an  
 enzyme that catalyzes the conversion of  $\alpha$ -ketoisovalerate to isobutyryl-  
 CoA (isobutyryl-cofactor A), using NAD<sup>+</sup> (nicotinamide adenine  
 20   dinucleotide) as electron acceptor. Certain branched-chain keto acid  
 dehydrogenases are known by the EC number 1.2.4.4. These branched-  
 chain keto acid dehydrogenases comprise four subunits, and sequences  
 from all subunits are available from a vast array of microorganisms,  
 including, but not limited to, *B. subtilis* (GenBank Nos: CAB14336,  
 25   Z99116; CAB14335, Z99116; CAB14334, Z99116; and CAB14337,  
 Z99116) and *Pseudomonas putida* (GenBank Nos: AAA65614, M57613;  
 AAA65615, M57613; AAA65617, M57613; and AAA65618, M57613).

- As used herein, "aldehyde dehydrogenase activity" refers to any  
 polypeptide having a biological function of an aldehyde dehydrogenase,  
 30   including the examples provided herein. Such polypeptides include a  
 polypeptide that catalyzes the oxidation (dehydrogenation) of aldehydes.  
 Such polypeptides include a polypeptide that catalyzes the conversion of  
 isobutyraldehyde to isobutyric acid. Such polypeptides also include a

polypeptide that corresponds to Enzyme Commission Numbers EC 1.2.1.3, EC 1.2.1.4 or EC 1.2.1.5. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "aldehyde oxidase activity" refers to any

5 polypeptide having a biological function of an aldehyde oxidase, including the examples provided herein. Such polypeptides include a polypeptide that catalyzes carboxylic acids from aldehydes. Such polypeptides include a polypeptide that catalyzes the conversion of isobutyraldehyde to isobutyric acid. Such polypeptides also include a polypeptide that

10 corresponds to Enzyme Commission Number EC 1.2.3.1. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "pyruvate decarboxylase activity" refers to the activity of any polypeptide having a biological function of a pyruvate

15 decarboxylase enzyme, including the examples provided herein. Such polypeptides include a polypeptide that catalyzes the conversion of pyruvate to acetaldehyde. Such polypeptides also include a polypeptide that corresponds to Enzyme Commission Number 4.1.1.1. Such polypeptides can be determined by methods well known in the art and

20 disclosed herein. A polypeptide having pyruvate decarboxylate activity can be, by way of example, PDC1, PDC5, PDC6, or any combination thereof.

As used herein, "acetolactate reductase activity" refers to the activity of any polypeptide having the ability to catalyze the conversion of

25 acetolactate to DHMB. Such polypeptides can be determined by methods well known in the art and disclosed herein.

As used herein, "DHMB" refers to 2,3-dihydroxy-2-methyl butyrate. DHMB includes "fast DHMB," which has the 2S, 3S configuration, and "slow DHMB," which has the 2S, 3R configurate. See Kaneko et al.,

30 Phytochemistry 39: 115-120 (1995),

and refers to fast DHMB as angliceric acid and slow DHMB as tigliceric acid.

As used herein, "reduced activity" refers to any measurable decrease in a known biological activity of a polypeptide when compared to the same biological activity of the polypeptide prior to the change resulting in the reduced activity. Such a change can include a modification of a polypeptide or a polynucleotide encoding a polypeptide as described herein. A reduced activity of a polypeptide disclosed herein can be determined by methods well known in the art and disclosed herein.

As used herein, "eliminated activity" refers to the complete abolishment of a known biological activity of a polypeptide when compared to the same biological activity of the polypeptide prior to the change resulting in the eliminated activity. Such a change can include a modification of a polypeptide or a polynucleotide encoding a polypeptide as described herein. An eliminated activity includes a biological activity of a polypeptide that is not measurable when compared to the same biological activity of the polypeptide prior to the change resulting in the eliminated activity. An eliminated activity of a polypeptide disclosed herein can be determined by methods well known in the art and disclosed herein.

The term "carbon substrate" or "fermentable carbon substrate" refers to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof. Non-limiting examples of carbon substrates are provided herein and include, but are not limited to, monosaccharides, oligosaccharides, polysaccharides, ethanol, lactate, succinate, glycerol, carbon dioxide, methanol, glucose, fructose, sucrose, xylose, arabinose, dextrose, or mixtures thereof. Other carbon substrates can include ethanol, lactate, succinate, or glycerol.

"Fermentation broth" as used herein means the mixture of water, sugars (fermentable carbon sources), dissolved solids (if present), microorganisms producing alcohol, product alcohol and all other constituents of the material held in the fermentation vessel in which product alcohol is being made by the reaction of sugars to alcohol, water and carbon dioxide (CO<sub>2</sub>) by the microorganisms present. From time to

time, as used herein the term "fermentation medium" and "fermented mixture" can be used synonymously with "fermentation broth".

"Biomass" as used herein refers to a natural product containing a hydrolysable starch that provides a fermentable sugar, including any  
5 cellulose or lignocellulosic material and materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides, disaccharides, and/or monosaccharides. Biomass can also comprise additional components, such as protein and/or lipids. Biomass can be derived from a single source, or biomass can comprise a  
10 mixture derived from more than one source. For example, biomass can comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood, and forestry waste. Examples  
15 of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and  
20 bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

"Feedstock" as used herein means a product containing a fermentable carbon source. Suitable feedstock include, but are not limited to, rye, wheat, corn, sugar cane, and mixtures thereof.

The term "aerobic conditions" as used herein means growth  
25 conditions in the presence of oxygen.

The term "microaerobic conditions" as used herein means growth conditions with low levels of oxygen (i.e., below normal atmospheric oxygen levels).

The term "anaerobic conditions" as used herein means growth  
30 conditions in the absence of oxygen.

The term "specific activity" as used herein is defined as the units of activity in a given amount of protein. Thus, the specific activity is not directly measured but is calculated by dividing 1) the activity in units/ml of

the enzyme sample by 2) the concentration of protein in that sample, so the specific activity is expressed as units/mg, where an enzyme unit is defined as moles of product formed/minute. The specific activity of a sample of pure, fully active enzyme is a characteristic of that enzyme. The  
5 specific activity of a sample of a mixture of proteins is a measure of the relative fraction of protein in that sample that is composed of the active enzyme of interest.

The terms " $k_{cat}$ " and " $K_M$ " are known to those skilled in the art and are described in Enzyme Structure and Mechanism, 2<sup>nd</sup> ed. (Ferst; W.H. Freeman Press, NY, 1985; pp 98-120).  $K_M$ , the Michaelis constant, is the  
10 concentration of substrate that leads to half-maximal velocity. The term " $k_{cat}$ ", often called the "turnover number", is defined as the maximum number of substrate molecules converted to products per active site per unit time, or the number of times the enzyme turns over per unit time.  $k_{cat}$   
15 =  $V_{max}/[E]$ , where  $[E]$  is the enzyme concentration (Ferst, *supra*). The terms "total turnover" and "total turnover number" are used herein to refer to the amount of product formed by the reaction of a KARI enzyme with substrate.

The term "catalytic efficiency" is defined as the  $k_{cat}/K_M$  of an  
20 enzyme. Catalytic efficiency is used to quantify the specificity of an enzyme for a substrate.

The term "isolated nucleic acid molecule", "isolated nucleic acid fragment" and "genetic construct" will be used interchangeably and will mean a polymer of RNA or DNA that is single- or double-stranded,  
25 optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA can be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "amino acid" refers to the basic chemical structural unit of  
30 a protein or polypeptide. The following abbreviations are used herein to identify specific amino acids:

| <u>Amino Acid</u> | <u>Three-Letter Abbreviation</u> | <u>One-Letter Abbreviation</u> |
|-------------------|----------------------------------|--------------------------------|
| Alanine           | Ala                              | A                              |
| Arginine          | Arg                              | R                              |
| Asparagine        | Asn                              | N                              |
| Aspartic acid     | Asp                              | D                              |
| Cysteine          | Cys                              | C                              |
| Glutamine         | Gln                              | Q                              |
| Glutamic acid     | Glu                              | E                              |
| Glycine           | Gly                              | G                              |
| Histidine         | His                              | H                              |
| Leucine           | Leu                              | L                              |
| Lysine            | Lys                              | K                              |
| Methionine        | Met                              | M                              |
| Phenylalanine     | Phe                              | F                              |
| Proline           | Pro                              | P                              |
| Serine            | Ser                              | S                              |
| Threonine         | Thr                              | T                              |
| Tryptophan        | Trp                              | W                              |
| Tyrosine          | Tyr                              | Y                              |
| Valine            | Val                              | V                              |

The term "gene" refers to a nucleic acid fragment that is capable of being expressed as a specific protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene can comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of a microorganism. A "foreign" gene refers to a gene not normally found in the host

microorganism, but that is introduced into the host microorganism by gene transfer. Foreign genes can comprise native genes inserted into a non-native microorganism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

5       As used herein, "native" refers to the form of a polynucleotide, gene, or polypeptide as found in nature with its own regulatory sequences, if present.

As used herein the term "coding sequence" or "coding region" refers to a DNA sequence that encodes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences can include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

10       

15       

As used herein, "endogenous" refers to the native form of a polynucleotide, gene or polypeptide in its natural location in the organism or in the genome of an organism. "Endogenous polynucleotide" includes a native polynucleotide in its natural location in the genome of an organism. "Endogenous gene" includes a native gene in its natural location in the genome of an organism. "Endogenous polypeptide" includes a native polypeptide in its natural location in the organism transcribed and translated from a native polynucleotide or gene in its natural location in the genome of an organism.

20       

25       

The term "heterologous" when used in reference to a polynucleotide, a gene, or a polypeptide refers to a polynucleotide, gene, or polypeptide not normally found in the host organism. "Heterologous" also includes a native coding region, or portion thereof, that is reintroduced into the source organism in a form that is different from the corresponding native gene, e.g., not in its natural location in the organism's genome. The heterologous polynucleotide or gene can be

30



introduced into the host organism by, e.g., gene transfer. A heterologous gene can include a native coding region with non-native regulatory regions that is reintroduced into the native host. For example, a heterologous gene can include a native coding region that is a portion of a chimeric  
5 gene including non-native regulatory regions that is reintroduced into the native host. "Heterologous polypeptide" includes a native polypeptide that is reintroduced into the source organism in a form that is different from the corresponding native polypeptide.

A "transgene" is a gene that has been introduced into the genome  
10 by a transformation procedure.

As used herein, the term "modification" refers to a change in a polynucleotide disclosed herein that results in reduced or eliminated activity of a polypeptide encoded by the polynucleotide, as well as a change in a polypeptide disclosed herein that results in reduced or  
15 eliminated activity of the polypeptide. Such changes can be made by methods well known in the art, including, but not limited to, deleting, mutating (e.g., spontaneous mutagenesis, random mutagenesis, mutagenesis caused by mutator genes, or transposon mutagenesis), substituting, inserting, down-regulating, altering the cellular location,  
20 altering the state of the polynucleotide or polypeptide (e.g., methylation, phosphorylation or ubiquitination), removing a cofactor, introduction of an antisense RNA/DNA, introduction of an interfering RNA/DNA, chemical modification, covalent modification, irradiation with UV or X-rays, homologous recombination, mitotic recombination, promoter replacement  
25 methods, and/or combinations thereof. Guidance in determining which nucleotides or amino acid residues can be modified, can be found by comparing the sequence of the particular polynucleotide or polypeptide with that of homologous polynucleotides or polypeptides, e.g., yeast or bacterial, and maximizing the number of modifications made in regions of  
30 high homology (conserved regions) or consensus sequences.

The term "recombinant genetic expression element" refers to a nucleic acid fragment that expresses one or more specific proteins, including regulatory sequences preceding (5' non-coding sequences) and

following (3' termination sequences) coding sequences for the proteins. A chimeric gene is a recombinant genetic expression element. The coding regions of an operon can form a recombinant genetic expression element, along with an operably linked promoter and termination region.

5        "Regulatory sequences" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences can include promoters,  
10    enhancers, operators, repressors, transcription termination signals, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing site, effector binding site and stem-loop structure.

      The term "promoter" refers to a nucleic acid sequence capable of  
15    controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters can be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleic acid segments. It is  
20    understood by those skilled in the art that different promoters can direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive  
25    promoters". "Inducible promoters," on the other hand, cause a gene to be expressed when the promoter is induced or turned on by a promoter-specific signal or molecule. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths can have identical  
30    promoter activity. For example, it will be understood that "FBA1 promoter" can be used to refer to a fragment derived from the promoter region of the FBA1 gene.

The term "terminator" as used herein refers to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The  
5 polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence. It is recognized that since in most cases the exact boundaries of regulatory sequences have not  
10 been completely defined, DNA fragments of different lengths can have identical terminator activity. For example, it will be understood that "CYC1 terminator" can be used to refer to a fragment derived from the terminator region of the CYC1 gene.

The term "operably linked" refers to the association of nucleic acid  
15 sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be  
20 operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression can also refer to translation of mRNA into a polypeptide.

25 The term "overexpression," as used herein, refers to expression that is higher than endogenous expression of the same or related gene. A heterologous gene is overexpressed if its expression is higher than that of a comparable endogenous gene. The term overexpression refers to an increase in the level of nucleic acid or protein in a host cell. Thus,  
30 overexpression can result from increasing the level of transcription or translation of an endogenous sequence in a host cell or can result from the introduction of a heterologous sequence into a host cell.

Overexpression can also result from increasing the stability of a nucleic acid or protein sequence.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host microorganism, resulting  
5 in genetically stable inheritance. Host microorganisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" microorganisms.

The terms "plasmid", "vector" and "cassette" refer to an extra  
10 chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements can be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide  
15 sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign  
20 gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The term "site-saturation library" refers to a library which contains  
25 random substitutions at a specific amino acid position with up to and including all 20 possible amino acids at once.

The term "error-prone PCR" refers to adding random copying errors by imposing imperfect or 'sloppy' PCR reaction conditions which generate randomized libraries of mutations in a specific nucleotide sequence.

30 As used herein the term "codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in

usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

5       The term "codon-optimized" as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA. Such optimization includes  
10 replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that organism.

Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in  
15 the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). The "genetic code" which shows which codons  
20 encode which amino acids is reproduced herein as Table 2A. As a result, many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary  
25 over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

**Table 2A. The Standard Genetic Code**

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

Many organisms display a bias for use of particular codons to code for insertion of a particular amino acid in a growing peptide chain. Codon preference, or codon bias, differences in codon usage between

- 5 organisms, is afforded by degeneracy of the genetic code, and is well documented among many organisms. Codon bias often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, *inter alia*, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules.
- 10 The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization.

- 15 Given the large number of gene sequences available for a wide variety of animal, plant and microbial species, it is possible to calculate the relative frequencies of codon usage. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at [www.kazusa.or.jp/codon/](http://www.kazusa.or.jp/codon/) (visited March 20, 2008), and these tables can be adapted in a number of ways. See Nakamura, Y., *et al. Nucl. Acids*
- 20 *Res.* 28:292 (2000). Codon usage tables for yeast, calculated from GenBank Release 128.0 [15 February 2002], are reproduced below as

Table 2B. This table uses mRNA nomenclature, and so instead of thymine (T) which is found in DNA, the tables use uracil (U) which is found in RNA. Table 2B has been adapted so that frequencies are calculated for each amino acid, rather than for all 64 codons.

5 **Table 2B. Codon Usage Table for *Saccharomyces cerevisiae***

| Amino Acid | Codon | Number | Frequency per thousand |
|------------|-------|--------|------------------------|
| Phe        | UUU   | 170666 | 26.1                   |
| Phe        | UUC   | 120510 | 18.4                   |
| Leu        | UUA   | 170884 | 26.2                   |
| Leu        | UUG   | 177573 | 27.2                   |
| Leu        | CUU   | 80076  | 12.3                   |
| Leu        | CUC   | 35545  | 5.4                    |
| Leu        | CUA   | 87619  | 13.4                   |
| Leu        | CUG   | 68494  | 10.5                   |
| Ile        | AUU   | 196893 | 30.1                   |
| Ile        | AUC   | 112176 | 17.2                   |
| Ile        | AUA   | 116254 | 17.8                   |
| Met        | AUG   | 136805 | 20.9                   |
| Val        | GUU   | 144243 | 22.1                   |
| Val        | GUC   | 76947  | 11.8                   |
| Val        | GUA   | 76927  | 11.8                   |
| Val        | GUG   | 70337  | 10.8                   |
| Ser        | UCU   | 153557 | 23.5                   |
| Ser        | UCC   | 92923  | 14.2                   |
| Ser        | UCA   | 122028 | 18.7                   |
| Ser        | UCG   | 55951  | 8.6                    |
| Ser        | AGU   | 92466  | 14.2                   |
| Ser        | AGC   | 63726  | 9.8                    |
| Pro        | CCU   | 88263  | 13.5                   |
| Pro        | CCC   | 44309  | 6.8                    |
| Pro        | CCA   | 119641 | 18.3                   |
| Pro        | CCG   | 34597  | 5.3                    |
| Thr        | ACU   | 132522 | 20.3                   |
| Thr        | ACC   | 83207  | 12.7                   |
| Thr        | ACA   | 116084 | 17.8                   |
| Thr        | ACG   | 52045  | 8.0                    |
| Ala        | GCU   | 138358 | 21.2                   |
| Ala        | GCC   | 82357  | 12.6                   |
| Ala        | GCA   | 105910 | 16.2                   |
| Ala        | GCG   | 40358  | 6.2                    |

| Amino Acid | Codon | Number | Frequency per thousand |
|------------|-------|--------|------------------------|
| Tyr        | UAU   | 122728 | 18.8                   |
| Tyr        | UAC   | 96596  | 14.8                   |
| His        | CAU   | 89007  | 13.6                   |
| His        | CAC   | 50785  | 7.8                    |
| Gln        | CAA   | 178251 | 27.3                   |
| Gln        | CAG   | 79121  | 12.1                   |
| Asn        | AAU   | 233124 | 35.7                   |
| Asn        | AAC   | 162199 | 24.8                   |
| Lys        | AAA   | 273618 | 41.9                   |
| Lys        | AAG   | 201361 | 30.8                   |
| Asp        | GAU   | 245641 | 37.6                   |
| Asp        | GAC   | 132048 | 20.2                   |
| Glu        | GAA   | 297944 | 45.6                   |
| Glu        | GAG   | 125717 | 19.2                   |
| Cys        | UGU   | 52903  | 8.1                    |
| Cys        | UGC   | 31095  | 4.8                    |
| Trp        | UGG   | 67789  | 10.4                   |
| Arg        | CGU   | 41791  | 6.4                    |
| Arg        | CGC   | 16993  | 2.6                    |
| Arg        | CGA   | 19562  | 3.0                    |
| Arg        | CGG   | 11351  | 1.7                    |
| Arg        | AGA   | 139081 | 21.3                   |
| Arg        | AGG   | 60289  | 9.2                    |
| Gly        | GGU   | 156109 | 23.9                   |
| Gly        | GGC   | 63903  | 9.8                    |
| Gly        | GGA   | 71216  | 10.9                   |
| Gly        | GGG   | 39359  | 6.0                    |
| Stop       | UAA   | 6913   | 1.1                    |
| Stop       | UAG   | 3312   | 0.5                    |
| Stop       | UGA   | 4447   | 0.7                    |

- By utilizing this or similar tables, one of ordinary skill in the art can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide, but which uses codons optimal for a given species.
- 5

Randomly assigning codons at an optimized frequency to encode a given polypeptide sequence, can be done manually by calculating codon frequencies for each amino acid, and then assigning the codons to the



- polypeptide sequence randomly. Additionally, various algorithms and computer software programs are readily available to those of ordinary skill in the art. For example, the "EditSeq" function in the Lasergene Package, available from DNASTar, Inc., Madison, WI, the backtranslation function in the VectorNTI Suite, available from InforMax, Inc., Bethesda, MD, and the "backtranslate" function in the GCG-Wisconsin Package, available from Accelrys, Inc., San Diego, CA. In addition, various resources are publicly available to codon-optimize coding region sequences, e.g., the "backtranslation" function
- 5 (see Entelechon GmbH, Regensburg, Germany website) and the "backtranseq" function (see NRC Saskatoon Bioinformatics, Saskatoon, Saskatchewan, Canada website) Constructing a rudimentary algorithm to assign codons based on a given frequency can also easily be accomplished with basic mathematical
- 15 functions by one of ordinary skill in the art.
- Codon-optimized coding regions can be designed by various methods known to those skilled in the art including software packages such as "synthetic gene designer" (see University of Maryland, Baltimore, Maryland website).
- 20 A polynucleotide or nucleic acid fragment is "hybridizable" to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA molecule, when a single-stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing
- 25 conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989), particularly Chapter 11 and Table 11.1 therein.
- The conditions of temperature and ionic strength determine
- 30 the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments (such as homologous sequences from distantly related organisms), to highly similar fragments (such as genes that duplicate functional enzymes from closely related

organisms). Post hybridization washes determine stringency conditions. One set of conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45 °C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50 °C for 30 min. Another set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60 °C. Another set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65 °C. An additional set of stringent conditions include hybridization at 0.1X SSC, 0.1% SDS, 65 °C and washes with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS, for example.

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

As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to  
5 any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" can be used  
10 instead of, or interchangeably with any of these terms. A polypeptide can be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

15 By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host  
20 cells are considered isolated for purposes of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

As used herein, the terms "variant" and "mutant" are synonymous and refer to a polypeptide differing from a specifically recited polypeptide  
25 by one or more amino acid insertions, deletions, mutations, and substitutions, created using, e.g., recombinant DNA techniques, such as mutagenesis. Guidance in determining which amino acid residues can be replaced, added, or deleted without abolishing activities of interest, can be found by comparing the sequence of the particular polypeptide with that of  
30 homologous polypeptides, e.g., yeast or bacterial, and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions) or by replacing amino acids with consensus sequences.

"Engineered polypeptide" as used herein refers to a polypeptide that is synthetic, i.e., differing in some manner from a polypeptide found in nature.

Alternatively, recombinant polynucleotide variants encoding these  
5 same or similar polypeptides can be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as silent changes which produce various restriction sites, can be introduced to optimize cloning into a plasmid or viral vector for expression. Mutations in the polynucleotide sequence can be reflected in the  
10 polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide. For example, mutations can be used to reduce or eliminate expression of a target protein and include, but are not limited to, deletion of the entire gene or a portion of the gene, inserting a DNA fragment into the gene (in either the  
15 promoter or coding region) so that the protein is not expressed or expressed at lower levels, introducing a mutation into the coding region which adds a stop codon or frame shift such that a functional protein is not expressed, and introducing one or more mutations into the coding region to alter amino acids so that a non-functional or a less enzymatically active  
20 protein is expressed.

Amino acid "substitutions" can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements, or they can be the result of replacing one amino acid with an amino acid having different  
25 structural and/or chemical properties, i.e., non-conservative amino acid replacements. "Conservative" amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine,  
30 isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged

(acidic) amino acids include aspartic acid and glutamic acid. Alternatively, "non-conservative" amino acid substitutions can be made by selecting the differences in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of any of these amino acids. "Insertions" or

5 "deletions" can be within the range of variation as structurally or functionally tolerated by the recombinant proteins. The variation allowed can be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant

10 variants for activity.

A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one

15 skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Altschul, S. F., *et al.*, *J. Mol. Biol.*, 215:403-410 (1993)). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as

20 homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides can be used in sequence-dependent methods of gene identification (*e.g.*, Southern hybridization) and isolation (*e.g.*, *in situ* hybridization of bacterial colonies or bacteriophage plaques).

25 In addition, short oligonucleotides of 12-15 bases can be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence.

30 The instant specification teaches the complete amino acid and nucleotide sequence encoding particular proteins. The skilled artisan, having the benefit of the sequences as reported herein, can now use all or a substantial portion of the disclosed sequences for purposes known to

those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

5       The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenine is complementary to thymine and cytosine is complementary to guanine, and with respect to RNA, adenine is complementary to uracil and cytosine is complementary to guanine.

10       The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as  
15       determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: 1.) Computational Molecular Biology (Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) Biocomputing: Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY  
20       (1993); 3.) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., Eds.) Humana: NJ (1994); 4.) Sequence Analysis in Molecular Biology (von Heinje, G., Ed.) Academic (1987); and 5.) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).

25       Methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations can be performed using the MegAlign™ program of the LASERGENE bioinformatics computing suite  
30       (DNASTAR Inc., Madison, WI). Multiple alignments of the sequences are performed using the "Clustal method of alignment" which encompasses several varieties of the algorithm including the "Clustal V method of alignment" corresponding to the alignment method labeled Clustal V

(described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D.G. *et al.*, *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). For multiple alignments, the default values correspond

5 to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and

10 DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp,

15 *CABIOS*. 5:151-153 (1989); Higgins, D.G. *et al.*, *Comput. Appl. Biosci.* 8:189-191(1992)) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5,

20 Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB ). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

It is well understood by one skilled in the art that many levels of

25 sequence identity are useful in identifying polypeptides, such as from other species, wherein such polypeptides have the same or similar function or activity, or in describing the corresponding polynucleotides. Useful examples of percent identities include, but are not limited to: 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from

30 55% to 100% can be useful in describing the present invention, such as 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98% or 99%. Suitable polynucleotide fragments not only have the above homologies but typically comprise a polynucleotide having at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, or at least 250 nucleotides.

- 5 Further, suitable polynucleotide fragments having the above homologies encode a polypeptide having at least 50 amino acids, at least 100 amino acids, at least 150 amino acids, at least 200 amino acids, or at least 250 amino acids.

The term "sequence analysis software" refers to any computer  
10 algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" can be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group  
15 (GCG), Madison, WI); 2.) BLASTP, BLASTN, BLASTX (Altschul *et al.*, *J. Mol. Biol.*, 215:403-410 (1990)); 3.) DNASTAR (DNASTAR, Inc. Madison, WI); 4.) Sequencher (Gene Codes Corporation, Ann Arbor, MI); and 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994),  
20 Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any  
25 set of values or parameters that originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Second Edition,  
30 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1984); and by Ausubel, F. M. *et al.*,



- Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987). Additional methods used here are in *Methods in Enzymology*, Volume 194, *Guide to Yeast Genetics and Molecular and Cell Biology* (Part A, 2004, Christine Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA). Other molecular tools and techniques are known in the art and include splicing by overlapping extension polymerase chain reaction (PCR) (Yu, et al. (2004) *Fungal Genet. Biol.* 41:973-981), positive selection for mutations at the *URA3* locus of *Saccharomyces cerevisiae* (Boeke, J.D. et al. (1984) *Mol. Gen. Genet.* 197, 345-346; M A Romanos, et al. *Nucleic Acids Res.* 1991 January 11; 19(1): 187), the cre-lox site-specific recombination system as well as mutant lox sites and FLP substrate mutations (Sauer, B. (1987) *Mol Cell Biol* 7: 2087-2096; Senecoff, et al. (1988) *Journal of Molecular Biology*, Volume 201, Issue 2, Pages 405-421; Albert, et al. (1995) *The Plant Journal*. Volume 7, Issue 4, pages 649-659), "seamless" gene deletion (Akada, et al. (2006) *Yeast*;23(5):399-405), and gap repair methodology (Ma et al., *Genetics* 58:201-216; 1981).

The genetic manipulations of a recombinant host cell disclosed herein can be performed using standard genetic techniques and screening and can be made in any host cell that is suitable to genetic manipulation (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202).

In embodiments, a recombinant host cell disclosed herein can be any yeast or fungi host useful for genetic modification and recombinant gene expression including those yeast mentioned elsewhere herein, such as in Table 7. In other embodiments, a recombinant host cell can be a member of the genera *Issatchenkia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Dekkera*, *Torulopsis*, *Brettanomyces*, *Torulaspora*, *Hanseniaspora*, *Kluveromyces*, *Yarrowia*, and some species of *Candida*.

#### Polypeptides with KARI Activity

In some embodiments, the recombinant host cells and methods provided herein address a need that arises in the microbial production of

isobutanol where the KARI enzyme performs a vital role. In the isobutanol biosynthetic pathway shown in Figure 1, the substrate to product conversion of acetolactate to dihydroxyisovalerate (DHIV) is catalyzed by the KARI enzyme. Disclosed in US Appl. Publication No.

- 5 US2011/0244536 , are polypeptides having ketol-acid reductoisomerase activity that are members of the SLSL Clade of KARIs. Polypeptides having KARI activity disclosed therein were found to be effective for isobutanol production. The SLSL Clade of KARIs include those KARI enzymes listed in Table 3.

10 Table 3. Effective KARIs

| Description                                                               | SEQ ID NO:<br>Nucleic acid | SEQ ID NO:<br>Amino acid |
|---------------------------------------------------------------------------|----------------------------|--------------------------|
| <i>Staphylococcus capitis</i> SK14                                        | 66                         | 67                       |
| <i>Staphylococcus epidermidis</i> M23864-W1                               | 68                         | 69                       |
| <i>Staphylococcus hominis</i> SK119                                       | 134                        | 135                      |
| <i>Staphylococcus aureus</i> subsp. <i>aureus</i> TCH130                  | 70                         | 71                       |
| <i>Staphylococcus warneri</i> L37603                                      | 72                         | 73                       |
| <i>Staphylococcus epidermidis</i> W23144                                  | 74                         | 75                       |
| <i>Staphylococcus saprophyticus</i> subsp. <i>Saprophyticus</i> ATCC15305 | 76                         | 77                       |
| <i>Staphylococcus carnosus</i> subsp. <i>Carnosus</i> TM300               | 78                         | 79                       |
| <i>Listeria monocytogenes</i> EGD-e                                       | 80                         | 81                       |
| <i>Listeria grayi</i> DSM 20601                                           | 82                         | 83                       |
| <i>Enterococcus casseliflavus</i> EC30                                    | 84                         | 85                       |
| <i>Enterococcus gallinarum</i> EG2                                        | 86                         | 87                       |
| <i>Macrococcus caseolyticus</i> JCSC5402                                  | 88                         | 89                       |
| <i>Streptococcus vestibularis</i>                                         | 90                         | 91                       |
| <i>Streptococcus mutans</i> UA159                                         | 92                         | 93                       |
| <i>Streptococcus gordonii</i> str. <i>cgakkus</i> sybstr. CH1             | 94                         | 95                       |
| <i>Streptococcus suis</i> 89/1591                                         | 96                         | 97                       |
| <i>Streptococcus infantarius</i> subsp. <i>infantarius</i> ATCC BAA-102   | 98                         | 99                       |
| <i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363                   | 100                        | 101                      |

|                                                                  |     |     |
|------------------------------------------------------------------|-----|-----|
| <i>Lactococcus lactis</i>                                        | 102 | 103 |
| <i>Leuconostoc mesenteroides subsp mesenteroides</i><br>ATCC8293 | 104 | 105 |
| <i>Lactobacillus buchneri</i> ATCC 11577                         | 106 | 107 |
| <i>Staphylococcus haemolyticus</i> JCSC1435                      | 108 | 109 |
| <i>Staphylococcus epidermidis</i> ATCC12228                      | 110 | 111 |
| <i>Streptococcus pneumoniae</i> CGSP14                           | 112 | 113 |
| <i>Streptococcus pneumoniae</i> TIGR4                            | 114 | 115 |
| <i>Streptococcus sanguinis</i> SK36                              | 116 | 117 |
| <i>Streptococcus salivarius</i> SK126                            | 118 | 119 |
| <i>Streptococcus thermophilus</i> LMD-9                          | 120 | 121 |
| <i>Streptococcus pneumoniae</i> CCRI 1974M2                      | 122 | 123 |
| <i>Lactococcus lactis subsp. lactis</i> II1403                   | 124 | 125 |
| <i>Leuconostoc mesenteroides subsp cremoris</i><br>ATCC19254     | 126 | 127 |
| <i>Leuconostoc mesenteroides subsp cremoris</i>                  | 128 | 129 |
| <i>Lactobacillus brevis subsp. gravesensis</i><br>ATCC27305      | 130 | 131 |
| <i>Lactococcus lactis subsp lactis</i> NCDO2118                  | 132 | 133 |

As described and demonstrated herein, Applicants have discovered additional KARI enzymes and variants of the additional KARIs that result in isobutanol production comparable to and/or exceeding that observed with the KARI from *Lactococcus lactis*. Such KARI enzymes and variants result in comparable or higher isobutanol titer and/or higher effective isobutanol productivity when compared to that observed with *Lactococcus lactis* KARI in the same conditions. Accordingly, in embodiments, polypeptides having KARI activity that function in an isobutanol production pathway have isobutanol titer and/or effective isobutanol productivity comparable to or better than that with the *Lactococcus lactis* KARI (SEQ ID NO: 380).

Such polypeptides having KARI activity may thus be suitable for isobutanol production. It will be appreciated that using a combination of structural and sequence information available in the art, polypeptides

comprising KARI activity and less than 100% identity to the exemplified sequences can be constructed for use in isobutanol biosynthetic pathways. For example, crystal structures of the *E. coli* KARI enzyme at 2.6 Å resolution have been solved (Tyagi, et al., Protein Sci., 14: 3089-3100, 2005) as has the structure of the *P. aeruginosa* KARI (Ahn, et al., J. Mol. Biol., 328: 505-515, 2003) and the KARI enzyme from spinach (Biou V., et al. The EMBO Journal, 16: 3405-3415, 1997). Furthermore, described herein is a Profile HMM (provided herein; Table Z) prepared using amino acid sequences of 25 KARI proteins with experimentally verified function as outlined in Table 1. The KARIs were from *Pseudomonas fluorescens* Pf-5, *Sulfolobus solfataricus* P2, *Pyrobaculum aerophilum* str. IM2, *Natronomonas pharaonis* DSM 2160, *Bacillus subtilis* subsp. *subtilis* str. 168, *Corynebacterium glutamicum* ATCC 13032, *Phaeosporium molischianum*, *Ralstonia solanacearum* GM1000, *Zymomonas mobilis* subsp. *mobilis* ZM4, *Alkalilimnicola ehrlichei* MLHE-, *Carnipyllobacter lari* RM2100, *Marinobacter aquaeolei* VT8, *Psychrobacter arcticus* 273-4, *Hahella chejuensis* KCTC 2396, *Thiobacillus denitrificans* ATCC 25259, *Azotobacter vinelandii* AvOP, *Pseudomonas syringae* pv. *syringae* B728a, *Pseudomonas syringae* pv. *tomato* str. DC3000, *Pseudomonas putida* KT2440, *Pseudomonas entomophila* L48, *Pseudomonas mendocina* ymp, *Pseudomonas aeruginosa* PAO1, *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 10987, and *Spinacia oleracea*. Any protein that matches the Profile HMM with an E value of  $<10^{-3}$  using hmmsearch program in the HMMER package is expected to be a functional KARI.

Production of isobutanol is believed to utilize the glycolysis pathway present in the host microorganism. During the production of two molecules of pyruvate from glucose during glycolysis, there is net production of two molecules of NADH from NAD<sup>+</sup> by the glyceraldehyde-3-phosphate dehydrogenase reaction. During the further production of one molecule of isobutanol from two molecules of pyruvate, there is net consumption of one molecule of NAD(P)H, by the KARI reaction, and one molecule of NAD(P)H by the isobutanol dehydrogenase reaction. The

interconversion of NADH with NADPH is generally slow and inefficient in yeast; thus, NADPH to be consumed is generated by metabolism (for example, by the pentose phosphate pathway) consuming substrate in the process. Meanwhile, the cell strives to maintain homeostasis in the

5 NAD<sup>+</sup>/NADH ratio, leading to the excess NADH produced in isobutanol production being consumed in wasteful reduction of other metabolic intermediates; e.g., by the production of glycerol (Bakker, *et al.*, 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25:15-37.). Thus, an

10 imbalance between NADH produced and NADPH consumed by the isobutanol pathway can lead to a reduction in the molar yield of isobutanol produced from glucose in two ways: 1) unnecessary operation of metabolism to produce NADPH, and 2) wasteful reaction of metabolic intermediates to maintain NAD<sup>+</sup>/NADH homeostasis. Polypeptides having

15 KARI activity that function well in an isobutanol pathway and have a low  $K_M$  for NADH can be used to improve the production of isobutanol.

Also disclosed herein are substitutions to the KARI enzyme sequences provided in Table 3 and in Table 10 to produce variants with varying ability to utilize NADH as a cofactor. Such variants provide

20 alternatives that may be employed to optimize the efficiency of a biosynthetic pathway utilizing KARI, such as an isobutanol biosynthetic pathway, for particular production conditions. Demonstrated in the Examples is isobutanol production under conditions switched from aerobic to anaerobic for variants of the K9 KARI enzyme derived from

25 *Anaerostipes caccae* with differing abilities to utilize NADH. Thus, equipped with this disclosure, one of skill in the art will be able to produce recombinant host cells comprising a SLSL Clade KARI enzyme, or a an *Enterococcus gallinarum*, *Streptococcus thermophilus* *Lactococcus lactis* subsp. *cremoris* MG1363, *Bifidobacterium angulatum*, *Bifidobacterium*

30 *dentium*, or *Anaerostipes caccae*, *Lactococcus lactis* KARI enzyme or a variant or active fragment thereof suited for a range of production conditions.

In some embodiments, provided herein is a polypeptide having KARI activity and having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to a KARI enzyme of Table 3 or Table 10, or Examples 16, 17, 21 and having a  $K_M$  for NADH less than about 300  $\mu$ M, 100  $\mu$ M, 50  $\mu$ M, 20  $\mu$ M, 10  $\mu$ M, or 5  $\mu$ M. In some embodiments, provided herein is an engineered polypeptide having KARI activity and having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to a KARI enzyme of Table 3, Table 10 or Examples 16, 17, 21. In some embodiments, such polypeptides have a  $K_M$  for NADH less than that of the corresponding native enzyme. In some embodiments, the ratio of  $K_M$  for NADH to  $K_M$  for NADPH is less than 0.1, in some embodiments less than 1, in some embodiments less than 2, in some embodiments, less than 4.

KARI enzymes and variants thereof that are particularly suitable for isobutanol production include, but are not limited to, variants of a ketol-acid reductoisomerase from *Anaerostipes caccae* DSM 14662 (SEQ ID NO: 643): "K9G9" (SEQ ID NO: 644) and "K9D3" (SEQ ID NO: 645) which have  $K_M$  for NADH lower than that of the native enzyme (SEQ ID NO: 643).

Host cells provided herein may comprise a polypeptide having ketol-acid reductoisomerase activity. In embodiments, such polypeptides have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to SEQ ID NO: 643, an active variant thereof; or a KARI derived from *Anaerostipes caccae* DSM 14662, or an active variant thereof. In embodiments, the polypeptides have at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to SEQ ID NO: 645 or 644. In embodiments, the polypeptides comprise SEQ ID NO: 645 or 644.

In some embodiments, polypeptides having KARI activity comprise at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to the amino acid

sequence of SEQ ID NO: 419 [JB4P], 427 [SB2], and all those variants listed in Tables 25 and 26. Such variants provide alternatives for optimizing the efficiency of the isobutanol biosynthetic pathway for particular production conditions. Demonstrated in the Examples is  
5 isobutanol production under conditions.

Identification of additional polypeptides having KARI activity

Described in Example 1 is a biodiversity screen of KARI-encoding genes from various bacterial and fungal species which revealed suitable  
10 KARIs for isobutanol production. Equipped with this disclosure, one of skill in the art will be readily able to identify additional suitable polypeptides having KARI activity.

The sequences of other polynucleotides, genes and/or polypeptides can be identified in the literature and in bioinformatics databases well  
15 known to the skilled person using sequences disclosed herein and available in the art. For example, such sequences can be identified through BLAST searching of publicly available databases with polynucleotide or polypeptide sequences provided herein. In such a method, identities can be based on the Clustal W method of alignment  
20 using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

Additionally, polynucleotide or polypeptide sequences disclosed herein can be used to identify other KARI homologs in nature. For example, each of the KARI encoding nucleic acid fragments disclosed  
25 herein can be used to isolate genes encoding homologous proteins. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various  
30 uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Patent No. 4,683,202; ligase chain reaction (LCR), Tabor et al., Proc. Acad. Sci. USA 82:1074 (1985); or strand displacement amplification (SDA), Walker et al., Proc. Natl. Acad.

Sci. U.S.A., 89:392 (1992)]; and (3) methods of library construction and screening by complementation.

It will be appreciated that one of ordinary skill in the art, equipped with this disclosure, can generate active fragments of polypeptides provided herein, for example, by truncating polypeptides provided herein based on sequence alignments at the N-terminus and confirming KARI activity. In embodiments, *Anaerostipes caccae* KARIs and variants thereof provided herein are truncated at the N-terminus. In one embodiment, up to and including the first five amino acids are truncated from a polypeptide provided herein. In embodiments, the polypeptide is SEQ ID NO: 27 or a variant thereof. In one embodiment, a polypeptide having KARI activity comprises SEQ ID NO: 635, 637 (encoded by polynucleotide sequences SEQ ID NO: 636 and 638, respectively), K9\_Annabel\_SH (SEQ ID NO: 862, protein SEQ ID NO: 863) and K9\_Zeke\_SH (SEQ ID NO: 860, protein SEQ ID NO: 861), or any variant listed in Table 40.

#### Lowering $K_M$ for NADH

As shown in Figure 2 and Examples, mutations in the positions corresponding to 50, 52 and 53, and optionally 47, of the *Pseudomonas fluorescens* KARI in the KARI enzyme from *Anaerostipes caccae* result in KARIs with lowered  $K_M$  for NADH as compared to wild-type, verifying that mutations in these positions produce NADH accepting variants of highly effective KARIs. Further mutations of *Anaerostipes caccae* KARI, revealed positions which further lower the  $K_M$  for NADH.

As demonstrated herein (see Examples), substitution of amino acids in the phosphate binding region, particularly in two or more positions corresponding to positions 47, 50, 52, and 53 of PF5 KARI (SEQ ID NO: 5) results in lowered  $K_M$  for NADH. Therefore, provided herein are polypeptides derived from an organism listed herein, for example, in Tables 3 and 10 having KARI activity and comprising substitutions at at least two of the four positions corresponding to positions 47, 50, 52, and 53 of PF5 KARI as compared to the native amino acid sequence.



Provided herein are polypeptides having KARI activity and comprising substitutions in the phosphate binding region. Provided herein are polypeptides having KARI activity and comprising substitutions at positions corresponding to S56 and S58 of K9 KARI (SEQ ID NO: 27). In some

5   embodiments the substitution at the position corresponding to S56 is A. In some embodiments, the substitution at the position corresponding to S58 is D or E. In some embodiments, the substitution at the position corresponding to S53 is Q, E, P, or A. In some embodiments, the substitution at the position corresponding to S56 is V or D. In some

10   embodiments, the substitution at the position corresponding to S58 is D or Q. In embodiments, the polypeptides further comprise a substitution at one or more positions corresponding to I86, N87, N107, T131, or T191 of K9 KARI (SEQ ID NO: 27). In some embodiments, the polypeptides comprise a substitution at at least 2, at least 3, at least 4, or all of the

15   indicated positions. In some embodiments, the substitution at the position corresponding to I86 is T or V. In some embodiments, the substitution at the position corresponding to N87 is P. In some embodiments, the substitution at the position corresponding to N107 is S. In some embodiments, the substitution at the position corresponding to T131 is C,

20   L, A, M or V. In some embodiments, the substitution at the position corresponding to T191 is A, S, D, C, or G.

In embodiments, the polypeptides comprise fewer than 10, 15, or 20 substitutions with respect to the wild-type sequence. In embodiments, the polypeptides match the Profile HMM based on experimentally verified

25   KARIs and given in Table Z with an E value less than  $< 10^{-3}$ . Sequences can be compared to the profile HMM given in Table Z using hmmsearch (HMMER software package available from Janelia Farm Research Campus, Ashburn, VA).

Additional polypeptides having KARI activity and lowered  $K_M$  for

30   NADH can be obtained using methods described and demonstrated herein. For example, a polypeptide having KARI activity can be employed in the construction of a site-saturation gene library as described herein. Kits for construction of such gene libraries are commercially available (for

example, from USB Corporation, Cleveland, OH, #78480.) Site-directed mutagenesis can also be carried out using commercially available kits (for example, the QuickChange II XL site directed mutagenesis kit, Catalog # 200524, Stratagene, La Jolla, CA). Primer design for target sites for  
5 mutagenesis is well-known in the art, and multiple sequence alignment to identify the target sites is likewise well-known.

Once variants have been generated, KARI activity with NADH or NADPH can be readily assessed using methods known in the art and/or disclosed herein. For example, KARI activity can be determined by  
10 measuring the disappearance of the NADPH or NADH from the reaction at 340 nm or by determination of the Michaelis constant via measurement of formation of 2,3-dihydroxyisovalerate using HPLC/MS. Likewise, isobutanol production from a strain comprising variants can be confirmed.

15 Cofactor specificity

To determine cofactor specificity,  $V_{\max}/K_M$  ratios can be calculated for each cofactor at saturating acetolactate; those variants with a higher ratio for NADH will react at a higher rate with NADH than NADPH under conditions of equal-molar concentrations of the two cofactors and  
20 saturating acetolactate.  $V_{\max}$  and  $K_M$  values for NADH and NADPH can be determined using methods known in the art and/or provided herein (see Example 16). For example, to determine  $V_{\max}$  and  $K_M$  values for NADH and NADPH, the partially purified proteins can be assayed at various concentrations of NADH and NADPH.

25 As demonstrated herein (see Examples 16 and 18 and Figure 8), substitution of additional amino acids in K9G9 results in variants having increased specificity for NADH. Thus, provided herein are polypeptides comprising substitution at one or more or all of the positions corresponding to K57, Y53, and E74 of K9 KARI (SEQ ID NO: 27). Also provided herein  
30 are polypeptides comprising substitutions at one or more or all of the positions corresponding to Y53, K57, E74, N87 and K90. In embodiments, the substitution at the position corresponding to Y53 is F. In embodiments, the substitution at the position corresponding to K57 is E.

In embodiments, the substitution at the position corresponding to E74 is G.  
 In embodiments, the substitution at the position corresponding to N87 is P.  
 In embodiments, the substitution at the position corresponding to K90 is M  
 or L. In embodiments, the variants comprise substitutions of at least one  
 5 position corresponding to S56 or S58 of SEQ ID NO: 27 and further  
 comprise at least one, at least two, at least three, or more than three  
 further substitution(s) corresponding to positions of SEQ ID NO: 27  
 identified herein.

In embodiments, the polypeptides comprise fewer than 2, 3, 4, 5,  
 10 10, 15, or 20 substitutions with respect to the wild-type sequence. In  
 embodiments, the polypeptides match the Profile HMM based on  
 experimentally verified KARIs and given in Table Z with an E value less  
 than  $< 10^{-3}$ .

As demonstrated in the Examples, variants of K9SB2 (SEQ ID NO:  
 15 427) were generated and screened for variants with reduced NADPH  
 affinity, revealing additional positions for substitution. Thus, in  
 embodiments, polypeptides further comprise substitutions at one or more  
 positions corresponding to F53, G55, A56, W59, F67, I84, L85, Q91, M94,  
 and P135 of SEQ ID NO: 427. In embodiments, the substitution at  
 20 position G55 is D or C, the substitution at position Q91 is L, the  
 substitution at position A56 is T or V, the substitution at P135 is S, the  
 substitution at position F53 is L, the substitution at position M94 is I, the  
 substitution at position F67 is L or I, the substitution at position W59 is C,  
 the substitution at position I84 is L, and the substitution at position L85 is  
 25 M.

#### KARI Structure

Structural information useful in the identification and modification of  
 polypeptides having KARI activity is provided in art, such as in the  
 30 references described here as well as in the Profile HMM provided herewith  
 in Table Z and in US App. Pub. Nos. 20100197519 and 20090163376 .

It was reported that phosphate p2' oxygen atoms of NADPH form hydrogen bonds with side chains of Arg162, Ser165 and Ser167 of spinach KARI (Biou V., et al. The EMBO Journal, 16: 3405-3415, 1997). Studies by Ahn et al., (J. Mol. Biol., 328: 505-515, 2003) had identified  
 5 three NADPH phosphate binding sites (Arg47, Ser50 and Thr52) for *Pseudomonas aeruginosa* (PAO-KARI) following comparing its structure with that of the spinach KARI. The structure of PF5-KARI with bound NADPH, acetolactate and magnesium ions was built based on the crystal structure of *P. aeruginosa* PAO1-KARI (PDB ID 1NP3, Ahn H. J. et al., J.  
 10 Mol. Biol., 328: 505-515, 2003) which has 92% amino acid sequence homology to PF5 KARI. PAO1-KARI structure is a homo-dodecamer and each dodecamer consists of six homo-dimers with extensive dimer interface. The active site of KARI is located in this dimer interface. The biological assembly is formed by six homo-dimers positioned on the edges  
 15 of a tetrahedron resulting in a highly symmetrical dodecamer of 23 point group symmetry.

The model of PF5-KARI dimer was built based on the coordinates of monomer A and monomer B of PAO1-KARI and sequence of PF5-KARI using DeepView/Swiss PDB viewer (Guex, N. and Peitsch, M.C.,  
 20 Electrophoresis, 18: 2714-2723, 1997). This model was then imported to program O (Jones, T.A. et al, Acta Crystallogr. A 47: 110-119, 1991) on a Silicon Graphics system for further modification.

The structure of PAO1-KARI has no NADPH, substrate or inhibitor or magnesium in the active site. Therefore, the spinach KARI structure  
 25 (PDB ID 1yve, Biou V. et al., The EMBO Journal, 16: 3405-3415, 1997.), which has magnesium ions, NADPH and inhibitor (N-Hydroxy-N-isopropylloxamate) in the acetolactate binding site, was used to model these molecules in the active site. The plant KARI has very little sequence homology to either PF5- or PAO1 KARI (<20% amino acid identity),  
 30 however the structures in the active site region of these two KARI enzymes are very similar. To overlay the active site of these two KARI structures, commands LSQ\_ext, LSQ\_improve, LSQ\_mol in the program O were used to line up the active site of monomer A of spinach KARI to

the monomer A of PF5 KARI model. The coordinates of NADPH, two magnesium ions and the inhibitor bound in the active site of spinach KARI were extracted and incorporated to molecule A of PF5 KARI. A set of the coordinates of these molecules were generated for monomer B of PF5 KARI by applying the transformation operator from monomer A to monomer B calculated by the program.

Because there is no NADPH in the active site of PAO1 KARI crystal structure, the structures of the phosphate binding loop region in the NADPH binding site (residues 44-45 in PAO1 KARI, 157-170 in spinach KARI) are very different between the two. To model the NADPH bound form, the model of the PF5-KARI phosphate binding loop (44-55) was replaced by that of 1yve (157-170). Any discrepancy of side chains between these two was converted to those in the PF5-KARI sequence using the mutate\_replace command in program O, and the conformations of the replaced side-chains were manually adjusted. The entire NADPH/Mg/ inhibitor bound dimeric PF5-KARI model went through one round of energy minimization using program CNX (ACCELRYS San Diego CA, Burnger, A.T. and Warren, G.L., Acta Crystallogr., D 54: 905-921, 1998) after which the inhibitor was replaced by the substrate, acetolactate (AL), in the model.

#### Isobutanol Production

Host cells provided herein can comprise a polypeptide having ketol-acid reductoisomerase activity. As described and demonstrated herein, Applicants have discovered additional KARI enzymes and variants of the additional KARIs that result in isobutanol production comparable to and/or exceeding that observed with the KARI from *Lactococcus lactis* (see Examples). Accordingly, in embodiments, polypeptides having KARI activity that function in an isobutanol production pathway have effective isobutanol productivity and/or produce isobutanol at a titer comparable to or better than that with the *Lactococcus lactis* KARI (SEQ ID NO: 380). Such polypeptides are thus considered to be useful for isobutanol production, particularly in cells comprising isobutanol production pathways

described herein. In embodiments, polypeptides provided herein have effective isobutanol productivity and/or produce isobutanol at a titer greater than or about equal to that observed with the the *Lactococcus lactis* KARI (SEQ ID NO: 380) under the same conditions. In

5       embodiments, polypeptides provided herein have effective isobutanol productivity greater than about 3 grams per gram of cells, greater than about 4, greater than about 5, or greater than about 6 grams per gram of cells after about 48 hours wherein at least the last about 24 hours of the 48 hours are under anaerobic conditions.

10       Furthermore, Applicants have discovered that variants of the polypeptides having KARI activity described above, including those with  $K_M$  for NADH lower than that of the unsubstituted polypeptide, provide advantages for isobutanol production under anaerobic conditions. While not wishing to be bound by theory, it is believed that such variants provide

15       improved isobutanol production due to more effective use of NADH as reducing equivalents. In embodiments, isobutanol production employing such a variant provides reduced glycerol accumulation. In embodiments, the molar ratio of isobutanol to glycerol is increased for a variant of a polypeptide having KARI activity described above with  $K_M$  for NADH lower

20       than that of the unsubstituted polypeptide. In embodiments, the molar ratio of isobutanol to glycerol is greater than 1. In embodiments, the molar ratio of isobutanol to glycerol is greater than 2. In embodiments, the molar ratio is greater than 3. In embodiments, the molar ratio is greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater than

25       9, greater than 10, greater than 12, or greater than 14. In embodiments, the molar ratio is in the range of about 1 to 5, about 1 to 10, about 2 to 8, about 5 to 10, about 5 to 15 about 10 to 15 or about 12 to 15.

As demonstrated in the Examples herein, as the biochemical specificity for the NADH cofactor, as defined by  $(\text{NADH } V_{\max}/K_M)/(\text{NADPH } V_{\max}/K_M)$  increases, there is an observed increase in the

30       isobutanol/glycerol ratio, suggesting that the altered cofactor specificity led to diminished NADPH utilization and by-product formation.

Modification of Aldehyde Dehydrogenase

In embodiments of the invention, a recombinant host cell can comprise reduced or eliminated aldehyde dehydrogenase activity and an isobutanol biosynthetic pathway wherein the host cell produces butanol.

- 5 In other embodiments, the recombinant host cell can comprise an isobutanol or a 1-butanol biosynthetic pathway as described further herein. In other embodiments, the isobutanol biosynthetic pathway can comprise a polynucleotide encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of: (a) pyruvate to
- 10 acetolactate; (b) acetolactate to 2,3-dihydroxyisovalerate; (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate; (d) 2-ketoisovalerate to isobutyraldehyde; and (e) isobutyraldehyde to isobutanol. In other embodiments, the isobutanol biosynthetic pathway can comprise polynucleotides encoding polypeptides having acetolactate synthase, keto
- 15 acid reductoisomerase, dihydroxy acid dehydratase, ketoisovalerate decarboxylase, and alcohol dehydrogenase activity. In other embodiments, the recombinant cell comprises a 1-butanol biosynthetic pathway. In other embodiments, the 1-butanol biosynthetic pathway comprises a polynucleotide encoding a polypeptide that catalyzes a
- 20 substrate to product conversion selected from the group consisting of: (a) acetyl-CoA to acetoacetyl-CoA; (b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA; (c) 3-hydroxybutyryl-CoA to crotonyl-CoA; (d) crotonyl-CoA to butyryl-CoA; (e) butyryl-CoA to butyraldehyde; (f) butyraldehyde to 1-
- 25 butanol. In other embodiments, the 1-butanol biosynthetic pathway can comprise polynucleotides encoding polypeptides having activity.

- In embodiments of the invention, a recombinant host cell can comprise a modification or disruption of a polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity or a modification or
- 30 disruption of a polypeptide having aldehyde dehydrogenase activity. Many methods for genetic modification and disruption of target genes to reduce or eliminate expression are known to one of ordinary skill in the art and can be used to create a recombinant host cell disclosed herein. In other embodiments, the recombinant host cell can comprise a deletion,

mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity or in an endogenous polypeptide having aldehyde dehydrogenase activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can  
5 result in aldehyde dehydrogenase activity that is reduced or eliminated. Modifications that can be used include, but are not limited to, deletion of the entire gene or a portion of the gene encoding an aldehyde dehydrogenase protein, inserting a DNA fragment into the encoding gene (in either the promoter or coding region) so that the protein is not  
10 expressed or expressed at lower levels, introducing a mutation into the coding region which adds a stop codon or frame shift such that a functional protein is not expressed, and introducing one or more mutations into the coding region to alter amino acids so that a non-functional or a less active protein is expressed. In other embodiments, expression of a  
15 target gene can be blocked by expression of an antisense RNA or an interfering RNA, and constructs can be introduced that result in cosuppression. In other embodiments, the synthesis or stability of the transcript can be lessened by mutation. In embodiments, the efficiency by which a protein is translated from mRNA can be modulated by mutation.  
20 All of these methods can be readily practiced by one skilled in the art making use of the known or identified sequences encoding target proteins.

In other embodiments, DNA sequences surrounding a target aldehyde dehydrogenase coding sequence are also useful in some modification procedures and are available, for example, for yeasts such as  
25 *Saccharomyces cerevisiae* in the complete genome sequence coordinated by Genome Project ID9518 of Genome Projects coordinated by NCBI (National Center for Biotechnology Information) with identifying GPID #13838. An additional non-limiting example of yeast genomic sequences is that of *Candida albicans*, which is included in GPID #10771, #10701  
30 and #16373. Other yeast genomic sequences can be readily found by one of skill in the art in publicly available databases.

In other embodiments, DNA sequences surrounding a target aldehyde dehydrogenase coding sequence can be useful for modification



methods using homologous recombination. In a non-limiting example of this method, aldehyde dehydrogenase gene flanking sequences can be placed bounding a selectable marker gene to mediate homologous recombination whereby the marker gene replaces the aldehyde dehydrogenase gene. In another non-limiting example, partial aldehyde dehydrogenase gene sequences and aldehyde dehydrogenase gene flanking sequences bounding a selectable marker gene can be used to mediate homologous recombination whereby the marker gene replaces a portion of the target aldehyde dehydrogenase gene. In embodiments, the selectable marker can be bounded by site-specific recombination sites, so that following expression of the corresponding site-specific recombinase, the resistance gene is excised from the aldehyde dehydrogenase gene without reactivating the latter. In embodiments, the site-specific recombination leaves behind a recombination site which disrupts expression of the aldehyde dehydrogenase protein. In other embodiments, the homologous recombination vector can be constructed to also leave a deletion in the aldehyde dehydrogenase gene following excision of the selectable marker, as is well known to one skilled in the art.

In other embodiments, deletions can be made to an aldehyde dehydrogenase target gene using mitotic recombination as described by Wach et al. (Yeast, 10:1793-1808; 1994). Such a method can involve preparing a DNA fragment that contains a selectable marker between genomic regions that can be as short as 20 bp, and which bound a target DNA sequence. In other embodiments, this DNA fragment can be prepared by PCR amplification of the selectable marker gene using as primers oligonucleotides that hybridize to the ends of the marker gene and that include the genomic regions that can recombine with the yeast genome. In embodiments, the linear DNA fragment can be efficiently transformed into yeast and recombined into the genome resulting in gene replacement including with deletion of the target DNA sequence (as disclosed, for example, in Methods in Enzymology, Volume 194, Guide to Yeast Genetics and Molecular and Cell Biology (Part A, 2004, Christine

Guthrie and Gerald R. Fink (Eds.), Elsevier Academic Press, San Diego, CA)).

Moreover, promoter replacement methods can be used to exchange the endogenous transcriptional control elements allowing  
5 another means to modulate expression such as described by Mnaimneh et al., ((2004) Cell 118(1):31-44).

In other embodiments, the aldehyde dehydrogenase target gene encoded activity can be disrupted using random mutagenesis, which can then be followed by screening to identify strains with reduced or  
10 substantially eliminated activity. In this type of method, the DNA sequence of the target gene encoding region, or any other region of the genome affecting carbon substrate dependency for growth, need not be known. In embodiments, a screen for cells with reduced aldehyde dehydrogenase activity, or other mutants having reduced aldehyde dehydrogenase  
15 activity, can be useful for recombinant host cells of the invention.

Methods for creating genetic mutations are common and well known in the art and can be applied to the exercise of creating mutants. Commonly used random genetic modification methods (reviewed in  
20 Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) include spontaneous mutagenesis, mutagenesis caused by mutator genes, chemical mutagenesis, irradiation with UV or X-rays, or transposon mutagenesis.

Chemical mutagenesis of host cells can involve, but is not limited to, treatment with one of the following DNA mutagens: ethyl  
25 methanesulfonate (EMS), nitrous acid, diethyl sulfate, or N-methyl-N'-nitro-N-nitroso-guanidine (MNNG). Such methods of mutagenesis have been reviewed in Spencer et al. (Mutagenesis in Yeast, 1996, Yeast Protocols: Methods in Cell and Molecular Biology. Humana Press, Totowa, NJ). In  
30 embodiments, chemical mutagenesis with EMS can be performed as disclosed in Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Irradiation with ultraviolet (UV) light or X-rays can also be used to produce random mutagenesis in yeast cells. The primary effect of mutagenesis by UV irradiation is the formation

of pyrimidine dimers which disrupt the fidelity of DNA replication.

Protocols for UV-mutagenesis of yeast can be found in Spencer et al.

(Mutagenesis in Yeast, 1996, Yeast Protocols: Methods in Cell and

Molecular Biology. Humana Press, Totowa, NJ). In embodiments, the

5 introduction of a mutator phenotype can also be used to generate random

chromosomal mutations in host cells. In embodiments, common mutator

phenotypes can be obtained through disruption of one or more of the

following genes: PMS1, MAG1, RAD18 or RAD51. In other embodiments,

restoration of the non-mutator phenotype can be obtained by insertion of

10 the wildtype allele. In other embodiments, collections of modified cells

produced from any of these or other known random mutagenesis

processes can be screened for reduced or eliminated aldehyde

dehydrogenase activity.

Genomes have been completely sequenced and annotated and are

15 publicly available for the following yeast strains: *Ashbya gossypii* ATCC

10895, *Candida glabrata* CBS 138, *Kluyveromyces lactis* NRRL Y-1140,

*Pichia stipitis* CBS 6054, *Saccharomyces cerevisiae* S288c,

*Schizosaccharomyces pombe* 972h-, and *Yarrowia lipolytica* CLIB122.

Typically BLAST (described above) searching of publicly available

20 databases with known aldehyde dehydrogenase polynucleotide or

polypeptide sequences, such as those provided herein, is used to identify

aldehyde dehydrogenase-encoding sequences of other host cells, such as

yeast cells.

In other embodiments, a polypeptide having aldehyde

25 dehydrogenase activity can catalyze the conversion of isobutyraldehyde to

isobutyric acid. In other embodiments, the conversion of isobutyraldehyde

to isobutyric acid in a recombinant host cell is reduced or eliminated. In

still other embodiments, a polynucleotide, gene or polypeptide having

aldehyde dehydrogenase activity can correspond to Enzyme Commission

30 Number EC 1.2.1.3, EC 1.2.1.4, and/or EC 1.2.1.5.

In embodiments, a recombinant host cell of the invention can be *S.*

*cerevisiae*, and a polypeptide having aldehyde dehydrogenase activity can

be ALD2, ALD3, ALD4, ALD5, ALD6, or combinations thereof. In other

embodiments, a recombinant host cell can be *Kluyveromyces lactis*, and a polypeptide having aldehyde dehydrogenase activity can be KLLA0F00440, KLLA0E23057, KLLA0D10021, KLLA0D09999G, or combinations thereof. In other embodiments, a recombinant host cell can be *Pichia stipitis*, and a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD7, or combinations thereof. In other embodiments, a recombinant host cell can be *Lactobacillus plantarum*, and a polypeptide having aldehyde dehydrogenase activity can be AldH. In other embodiments, a recombinant host cell can be *E. coli*, and a polypeptide having aldehyde dehydrogenase activity can be aldA, aldB, aldH, or combinations thereof.

In embodiments of the invention, a recombinant host cell can be *S. cerevisiae*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD6, or combinations thereof. In embodiments of the invention, a recombinant host cell can be *S. cerevisiae*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD6. In other embodiments, a recombinant host cell can be *Kluyveromyces lactis*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be KLLA0F00440, KLLA0E23057, KLLA0D10021, KLLA0D09999G, or combinations thereof. In other embodiments, a recombinant host cell can be *Pichia stipitis*, and an endogenous polynucleotide or gene encoding a polypeptide having aldehyde dehydrogenase activity can be ALD2, ALD3, ALD4, ALD5, ALD7, or combinations thereof. In embodiments, the polypeptide having aldehyde dehydrogenase activity is a homolog of ALD6 from *Saccharomyces cerevisiae*. *S. cerevisiae* deletion strains containing aldehyde dehydrogenase gene deletions with a kanMX cassette are commercially available from American Type Culture Collection [catalog #4000753].

In other embodiments, a recombinant host cell can be *Lactobacillus plantarum*, and an endogenous polynucleotide encoding a polypeptide

having aldehyde dehydrogenase activity can be AldH. In other embodiments, a recombinant host cell can be *E. coli*, and an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity can be aldA, aldB, aldH, or combinations thereof.

- 5           Examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, those of the following Table 4.

10           Table 4. Aldehyde dehydrogenase target gene coding regions and proteins.

|                                                 | Nucleic acid SEQ<br>ID NO: | Amino acid SEQ<br>ID NO: |
|-------------------------------------------------|----------------------------|--------------------------|
| ALD2 from <i>S. cerevisiae</i>                  | 732                        | 733                      |
| ALD3 from <i>S. cerevisiae</i>                  | 734                        | 735                      |
| ALD4 from <i>S. cerevisiae</i>                  | 736                        | 737                      |
| ALD5 from <i>S. cerevisiae</i>                  | 738                        | 739                      |
| ALD6 from <i>S. cerevisiae</i>                  | 740                        | 741                      |
| KLLA0F00440 from<br><i>Kluyveromyces lactis</i> | 742                        | 743                      |
| KLLA0E23057 from<br><i>Kluyveromyces lactis</i> | 744                        | 745                      |
| KLLA0D10021 from<br><i>Kluyveromyces lactis</i> | 746                        | 747                      |
| KLLA0D09999 from<br><i>Kluyveromyces lactis</i> | 748                        | 749                      |
| ALD2 from <i>Pichia stipitis</i>                | 750                        | 751                      |
| ALD3 from <i>Pichia stipitis</i>                | 752                        | 753                      |
| ALD4 from <i>Pichia stipitis</i>                | 754                        | 755                      |
| ALD5 from <i>Pichia stipitis</i>                | 756                        | 757                      |
| ALD7 from <i>Pichia stipitis</i>                | 758                        | 759                      |
| aldA from<br><i>E. coli</i>                     | 760                        | 761                      |
| aldB from<br><i>E. coli</i>                     | 762                        | 763                      |
| aldH<br>(puuC) from<br><i>E. coli</i>           | 764                        | 765                      |

Other examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, aldehyde dehydrogenase polynucleotides, genes and/or

5 polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 4, wherein such a

10 polynucleotide or gene encodes, or such a polypeptide has, aldehyde dehydrogenase activity. Still other examples of aldehyde dehydrogenase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 4, wherein such a polynucleotide or

15 gene encodes, or such a polypeptide has, aldehyde dehydrogenase activity.

In embodiments, the sequences of other aldehyde dehydrogenase polynucleotides, genes and/or polypeptides can be identified in the literature and in bioinformatics databases well known to the skilled person

20 using sequences disclosed herein and available in the art. For example, such sequences can be identified through BLAST searching of publicly available databases with known aldehyde dehydrogenase-encoding polynucleotide or polypeptide sequences. In such a method, identities can be based on the Clustal W method of alignment using the default

25 parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

Additionally, the aldehyde dehydrogenase polynucleotide or polypeptide sequences disclosed herein or known the art can be used to identify other aldehyde dehydrogenase homologs in nature. For example,

30 each of the aldehyde dehydrogenase encoding nucleic acid fragments disclosed herein can be used to isolate genes encoding homologous proteins. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent

protocols include, but are not limited to (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis *et al.*, U.S. Patent No. 4,683,202; 5 ligase chain reaction (LCR), Tabor *et al.*, *Proc. Acad. Sci. USA* 82:1074 (1985); or strand displacement amplification (SDA), Walker *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 89:392 (1992)]; and (3) methods of library construction and screening by complementation.

Accordingly, it is within the scope of the invention to provide 10 aldehyde dehydrogenase polynucleotides, genes and polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any of the aldehyde dehydrogenase polynucleotides or polypeptides disclosed herein 15 (e.g., SEQ ID NOs: 732-765 of Table 4) Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of aldehyde dehydrogenase in a recombinant host 20 cell disclosed herein to reduce or eliminate aldehyde dehydrogenase activity can be confirmed using methods known in the art. For example, disruption of a particular aldehyde dehydrogenase could be confirmed with PCR screening using primers internal and external to the aldehyde dehydrogenase gene or by Southern blot using a probe designed to the 25 aldehyde dehydrogenase gene sequence. Alternatively, one could utilize gas chromatography-mass spectroscopy or liquid chromatography to screen strains exposed to isobutyraldehyde for decreased formation of isobutyric acid. Accordingly, provided herein is a method of screening for strains with decreased isobutyric acid formation comprising: a) providing a 30 strain comprising a modification in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity and/or a modification in a polynucleotide encoding a polypeptide having aldehyde oxidase activity; b) contacting the cell with isobutyraldehyde; and c) measuring

isobutyric acid formation; wherein isobutyric acid formation is reduced as compared to a control strain without the modification. In some embodiments, the modification is a deletion, mutation, and/or substitution. In some embodiments, the measuring is carried out using gas chromatography-mass spectroscopy. In some embodiments, isobutyric acid is reduced by at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In some embodiments, isobutyric acid formation is substantially eliminated.

10 Modification of Aldehyde Oxidase

In embodiments of the invention, a recombinant host cell disclosed herein can have a modification or disruption of a polynucleotide, gene or polypeptide encoding aldehyde oxidase. In embodiments, the recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having aldehyde oxidase activity, or in an endogenous polypeptide having aldehyde oxidase activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in aldehyde oxidase activity that is reduced or eliminated.

20 In embodiments of the invention, a polypeptide having aldehyde oxidase activity can catalyze the conversion of isobutyraldehyde to isobutyric acid. In other embodiments, the conversion of isobutyraldehyde to isobutyric acid in a recombinant host cell is reduced or eliminated. In other embodiments, a polynucleotide, gene or polypeptide having aldehyde oxidase activity can correspond to Enzyme Commission Number EC 1.2.3.1.

In embodiments, a recombinant host cell of the invention can be *Pichia stipitis* and a polynucleotide, gene or polypeptide having aldehyde oxidase activity can be AOX1 and/or AOX2.

30 Examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, those of the following Table 5.



Table 5. Aldehyde oxidase target gene coding regions and proteins.

|                                  | Nucleic acid SEQ ID NO: | Amino acid SEQ ID NO: |
|----------------------------------|-------------------------|-----------------------|
| AOX1 from <i>Pichia stipitis</i> | 864                     | 866                   |
| AOX2 from <i>Pichia stipitis</i> | 867                     | 868                   |

Other examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, aldehyde oxidase polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 5, wherein such a polynucleotide or gene encodes a polypeptide having, or such a polypeptide has, aldehyde oxidase activity. Still other examples of aldehyde oxidase polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 5, wherein such a polynucleotide or gene encodes, or such a polypeptide has, aldehyde oxidase activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding an aldehyde oxidase sequence disclosed herein or known in the art can be modified, as disclosed above for acetolactate reductase or aldehyde dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding aldehyde oxidase can be used to identify another aldehyde oxidase polynucleotide, gene and/or polypeptide sequence and/or can be used to identify an aldehyde oxidase homolog in other cells, as disclosed above for aldehyde dehydrogenase. Such aldehyde oxidase encoding sequences can be identified, for example, in the literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of an aldehyde oxidase encoding sequence in another cell type using bioinformatics can be accomplished through BLAST (as disclosed above) searching of publicly available

databases with a known hexose kinase encoding DNA and polypeptide sequence, such as any of those provided herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of aldehyde oxidase in a recombinant host cell disclosed herein to reduce or eliminate aldehyde oxidase activity can be confirmed using methods known in the art. For example, disruption of a particular aldehyde oxidase could be confirmed with PCR screening using primers internal and external to the aldehyde oxidase gene or by Southern blot using a probe designed to the aldehyde oxidase gene sequence. Alternatively, one could utilize gas chromatography or other analytical methods to screen strains exposed to isobutyraldehyde for decreased formation of isobutyric acid (as described and demonstrated in the Examples). In some embodiments, isobutyric acid is reduced by at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In some embodiments, isobutyric acid formation is substantially eliminated.

Applicants have provided recombinant host cells comprising reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity. In embodiments, a recombinant host cell disclosed herein can further comprise a modification in a polynucleotide encoding a polypeptide having pyruvate decarboxylase activity and/or a modification in a polynucleotide encoding a polypeptide having hexokinase 2 activity. In embodiments, a recombinant host cell of the invention can produce a production of a biosynthetic pathway (e.g., isobutanol), and can comprise a polynucleotide encoding a polypeptide that catalyzes a substrate to product conversion selected from the group consisting of: (a) pyruvate to acetolactate; (b) acetolactate to 2,3-dihydroxyisovalerate; (c) 2,3-dihydroxyisovalerate to 2-ketoisovalerate; (d) 2-ketoisovalerate to isobutyraldehyde; and (e) isobutyraldehyde to isobutanol. In other embodiments, such a recombinant host cell can produce a product of a biosynthetic pathway (e.g., isobutanol) at a yield or amount that is greater

than the yield or amount of the same product produced by a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase activity and/or aldehyde oxidase activity. In other embodiments, a recombinant host cell of the invention can reduce or  
5 eliminate the conversion of isobutyraldehyde to isobutyric acid, and can be used for screening candidate polypeptides having aldehyde dehydrogenase and/or aldehyde oxidase activity. As such, Applicants have also provided methods of increasing the yield or titer of a product of a biosynthetic pathway (e.g., isobutanol), methods for reducing or  
10 eliminating the conversion of isobutyraldehyde to isobutyric acid, and methods for screening candidate polypeptides having aldehyde dehydrogenase and/or aldehyde oxidase activity.

In embodiments of the invention, methods of producing a recombinant host cell are provided which comprise (a) providing a  
15 recombinant host cell disclosed herein; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of a biosynthetic pathway (e.g., an isobutanol biosynthetic pathway). In other embodiments, methods of producing a recombinant host cell are provided which comprise (a) providing a recombinant host cell comprising a modification  
20 in a polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity or in a polypeptide having aldehyde dehydrogenase activity; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of a biosynthetic pathway (e.g., an isobutanol biosynthetic pathway). In other embodiments, methods of  
25 producing a recombinant host cell are provided which comprise (a) providing a recombinant host cell comprising a modification in a polynucleotide encoding a polypeptide having aldehyde oxidase activity or in a polypeptide having aldehyde oxidase activity; and (b) transforming said host cell with a polynucleotide encoding a polypeptide of an  
30 isobutanol biosynthetic pathway.

In embodiments, methods for reducing or eliminating the conversion of isobutyraldehyde to isobutyric acid are provided which comprise (a) providing a recombinant host cell disclosed herein; and (b)

growing said host cell under conditions wherein the conversion of isobutyraldehyde to isobutyric acid is reduced or eliminated compared to a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity. The conversion of isobutyraldehyde to isobutyric acid of a recombinant host cell disclosed herein can be measured by methods known in the art (see, e.g., Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and/or described herein.

10 Reduction of DHMB

The production of DHMB in a host cell comprising an isobutanol biosynthetic pathway indicates that not all of the pathway substrates are being converted to the desired product. Thus, yield is lowered. In addition, DHMB can have inhibitory effects on product production. For example, DHMB can decrease the activity of enzymes in the biosynthetic pathway or have other inhibitory effects on yeast growth and/or productivity during fermentation. Thus, the methods described herein provide ways of reducing DHMB during fermentation. The methods include both methods of decreasing the production of DHMB and methods of removing DHMB from fermenting compositions.

Decreasing DHMB Production

In some embodiments described herein, a recombinant host cell can comprise reduced or eliminated ability to convert acetolactate to DHMB. The ability of a host cell to convert acetolactate to DHMB can be reduced or eliminated, for example, by a modification or disruption of a polynucleotide or gene encoding a polypeptide having acetolactate reductase activity or a modification or disruption of a polypeptide having acetolactate reductase activity. In other embodiments, the recombinant host cell can comprise a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having acetolactate reductase activity or in an endogenous polypeptide having acetolactate reductase. Such modifications, disruptions, deletions,

mutations, and/or substitutions can result in acetolactate reductase activity that is reduced, substantially eliminated, or eliminated. In some embodiments of the invention, the product of the biosynthetic pathway is produced at a greater yield or amount compared to the production of the same product in a recombinant host cell that does not comprise reduced or eliminated ability to convert acetolactate to DHMB.

Thus, the product can be a composition comprising butanol that is substantially free of, or free of DHMB. In some embodiments, the composition comprising butanol contains no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM DHMB, or about 0.2 mM DHMB.

The product can also be a composition comprising 2,3-butanediol (BDO) that is substantially free of, or free of DHMB. In some embodiments, the composition comprising BDO contains no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM DHMB, or about 0.2 mM DHMB.

Any product of a biosynthetic pathway that involves the conversion of acetolactate to a substrate other than DHMB can be produced with greater effectiveness in a recombinant host cell disclosed herein having the described modification of acetolactate reductase activity. Such products include, but are not limited to, butanol, e.g., isobutanol, 2-butanol, and BDO, and branched chain amino acids.

In some embodiments, the host cell comprises at least one deletion, mutation, and/or substitution in at least one endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity. In some embodiments, the host cell comprises at least one deletion, mutation, and/or substitution in each of at least two endogenous polynucleotides encoding polypeptides having acetolactate reductase activity.

In some embodiments, a polypeptide having acetolactate reductase activity can catalyze the conversion of acetolactate to DHMB. In some embodiments, a polypeptide having acetolactate reductase activity is

capable of catalyzing the reduction of acetolactate to 2S,3S-DHMB (fast DHMB) and/or 2S,3R-DHMB (slow DHMB).

Table 6

Polypeptides and polynucleotides having acetolactate reductase

5 activity in *Saccharomyces cerevisiae*

| Gene                   | SEQ ID NO:<br>(nucleic acid,<br>amino acid) |
|------------------------|---------------------------------------------|
| YMR226C                | 676, 677                                    |
| YIL074C (Chr 9)        | 678, 679                                    |
| YIR036C (Chr 9)        | 680, 681                                    |
| YPL061W (ALD6)(Chr 16) | 682, 683                                    |
| YPL088W(Chr 16)        | 684, 685                                    |
| YCR105W (ADH7)(Chr 3)  | 686, 687                                    |
| YDR541C(Chr 4)         | 688, 689                                    |
| YER081 (SER3)(Chr 5)   | 690, 691                                    |
| YPL275W (FDH2)(Chr 16) | 692, 693                                    |
| YBR006W (UGA5)(Chr2)   | 694, 695                                    |
| YOL059W (Chr 15)       | 696, 697                                    |
| YER081W (Chr 5)        | 869, 870                                    |
| YOR375C (Chr 15)       | 871, 872                                    |

In some embodiments, the conversion of acetolactate to DHMB in a recombinant host cell is reduced, substantially eliminated, or eliminated.

In some embodiments, the polypeptide having acetolactate reductase activity is selected from the group consisting of: YMR226C, YER081W,

10 YIL074C, YBR006W, YPL275W, YOL059W, YIR036C, YPL061W, YPL088W, YCR105W, YOR375C, and YDR541C. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide comprising a sequence listed in Table 6 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at  
15 least about 90%, at least about 95%, or at least about 99% identical to a polypeptide sequence listed in Table 6. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide encoded by a polynucleotide sequence listed in Table 6 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least  
20 about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polynucleotide sequence listed in Table 6.

Table 7: Example YMR226C Yeast Homologs

| Species                                                                            | Accession #  | SEQ ID NO: (nucleic acid, amino acid)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|------------------------------------------------------------------------------------|--------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Saccharomyces paradoxus</i>                                                     | AABY01000127 | 698, 699                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Saccharomyces bayanus</i>                                                       | AACA01000631 | 700, 701<br>MSQGRKAAERLANKTVLITGASA<br>GIGKATALEYLEASNGNMKLILAA<br>RRLEKLEELKKTIDEEFPNAKVH<br>VGQLDITQAEKIKPFIENLPEAFK<br>DIDILINNAGKALGSEVRGEIATQ<br>DIQDVFDNTVNTALINVTQAVLPIF<br>QAKNSGDIVNLGLGGRQRRIPH<br>RLHLLCFQVCRRCVH*QFEKGT<br>DQHEDQSYLDRAGAG*DRVLTG<br>QIQR**GTS*KRLQGHYAVDGRR<br>RG*LNRIHFHQAEHRGCRHPDL<br>PHQPSLALPHLSRL* (SEQ ID<br>NO:701)<br>The sequence came from a<br>comparative genomics study using<br>"draft" genome sequences with 7-<br>fold coverage (Kellis et al, Nature<br>423:241-254 (2003)). |
| <i>Saccharomyces castellii</i>                                                     | AACF01000116 | 702, 703                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Saccharomyces mikatae</i>                                                       | AACH01000019 | 704, 705                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Ashbya gossypii</i>                                                             | AE016819     | 706, 707                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Candida glabrata</i>                                                            | CR380959     | 708, 709                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Debaryomyces hansenii</i>                                                       | CR382139     | 710, 711                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Scheffersomyces stipitis</i><br>(formerly <i>Pichia stipitis</i> )              | XM_001387479 | 712, 713                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Meyerozyma guilliermondii</i><br>(formerly <i>Pichia guilliermondii</i> )       | XM_001482184 | 714, 715                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Vanderwaltozyma polyspora</i><br>(formerly <i>Kluyveromyces polysporus</i> )    | XM_001645671 | 716, 717                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Candida dubliniensis</i>                                                        | XM_002419771 | 718, 719                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Zygosaccharomyces rouxii</i>                                                    | XM_002494574 | 720, 721                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Lachancea thermotolerans</i><br>(formerly <i>Kluyveromyces thermotolerans</i> ) | XM_002553230 | 722, 723                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Kluyveromyces lactis</i>                                                        | XM_451902    | 724, 725                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Saccharomyces kluyveri</i>                                                      | SAKL0H04730  | 726, 727                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Yarrowia lipolytica</i>                                                         | XM_501554    | 728, 729                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| <i>Schizosaccharomyces pombe</i>                                                   | NM_001018495 | 730, 731                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |

In some embodiments, a polypeptide having acetolactate reductase activity is YMR226C or a homolog of YMR226C. Thus, in some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide comprising a sequence listed in Table 7 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polypeptide sequence listed in Table 7. In some embodiments, the polypeptide having acetolactate reductase activity is a polypeptide encoded by a polynucleotide sequence listed in Table 7 or a sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identical to a polynucleotide sequence listed in Table 7. Acetolactate reductases capable of converting acetolactate to DHMB can be identified, for example, by screening genetically altered yeast for changes in acetolactate consumption, changes in DHMB production, changes in DHIV production, or changes in other downstream product (e.g., butanol) production.

One way of identifying a gene involved in DHMB production comprises measuring the amount of DHMB produced by individual yeast strains in a yeast knock-out library. Knock-out libraries are available, for example, from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, MA). In this method, a decrease in DHMB production indicates that the gene that has been knocked-out functions to increase DHMB production, and an increase in DHMB production indicates that the gene that has been knocked-out functions to decrease DHMB production.

Two ways that a knockout ("KO") library can be used to identify candidate genes for involvement in DHMB synthesis include: (1) DHMB and DHIV accumulated in the culture during growth from endogenous substrates (acetolactate and NADPH or NADH) can be analyzed in samples from cultures. These samples can be placed in a hot (80-100 °C) water bath for 10-20 min, or diluted into a solution such as 2% formic acid that will kill and permeabilize the cells. After either treatment, small



molecules will be found in the supernatant after centrifugation (5 min, 1100 x g). The DHMB/DHIV ratio of a control strain (e.g., BY4743) can be compared to that of the different KO derivatives, and the gene(s) missing from any strain(s) with exceptionally low DHMB/DHIV ratios can encode  
5 acetolactate reductase (ALR). (2) DHMB and/or DHIV formation rates in vitro from exogenous substrates (acetolactate and NADH and/or NADPH) can be measured in timed samples taken from a suspension of permeabilized cells, and inactivated in either of the ways described above. Since the substrates for DHMB and DHIV synthesis are the same, this  
10 allows one to measure the relative levels of ALR and KARI activity in the sample.

Another way of identifying a gene involved in DHMB production comprises measuring the amount of DHMB produced by individual yeast strains in a yeast overexpression library. Overexpression libraries are  
15 available, for example, from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, MA). In this method, a decrease in DHMB production indicates that the overexpressed gene functions to decrease DHMB production, and an increase in DHMB production indicates that the overexpressed gene functions to increase DHMB production.

20 Another way of identifying a gene involved in DHMB production is to biochemically analyze a DHMB-producing yeast strain. For example, DHMB-producing cells can be disrupted. This disruption can be performed at low pH and cold temperatures. The cell lysates can be separated into fractions, e.g., by adding ammonium sulfate or other  
25 techniques known to those of skill in the art, and the resulting fractions can be assayed for enzymatic activity. For example, the fractions can be assayed for the ability to convert acetolactate to DHMB. Fractions with enzymatic activity can be treated by methods known in the art to purify and concentrate the enzyme (e.g., dialysis and chromatographic  
30 separation). When a sufficient purity and concentration is achieved, the enzyme can be sequenced, and the corresponding gene encoding the acetolactate reductase capable of converting acetolactate to DHMB can be identified.

Furthermore, since the reduction of acetolactate to DHMB occurs in yeast, but does not occur to the same extent in *E. coli*, acetolactate reductases that are expressed in yeast, but not expressed in *E. coli*, can be selected for screening. Selected enzymes can be expressed in yeast  
5 or other protein expression systems and screened for the capability to convert acetolactate to DHMB.

Enzymes capable of catalyzing the conversion of acetolactate to DHMB can be screened by assaying for acetolactate levels, by assaying for DHMB levels, by assaying for DHIV levels, or by assaying for any of  
10 the downstream products in the conversion of DHIV to butanol, including isobutanol.

DHMB can be measured using any technique known to those of skill in the art. For example, DHMB can be separated and quantified by methods known to those of skill in the art and techniques described in the  
15 Examples provided herein. For example, DHMB can be separated and quantified using liquid chromatography-mass spectrometry, liquid chromatography-nuclear magnetic resonance (NMR), thin-layer chromatography, and/or HPLC with UV/Vis detection.

In embodiments, selected acetolactate reductase polynucleotides, genes and/or polypeptides disclosed herein can be modified or disrupted.  
20 Many suitable methods are known to those of ordinary skill in the art and include those described for aldehyde dehydrogenase (above):

The modification of acetolactate reductase in a recombinant host cell disclosed herein to reduce or eliminate acetolactate reductase activity  
25 can be confirmed using methods known in the art. For example, the presence or absence of an acetolactate reductase-encoding polynucleotide sequence can be determined using PCR screening. A decrease in acetolactate reductase activity can also be determined based on a reduction in conversion of acetolactate to DHMB. A decrease in  
30 acetolactate reductase activity can also be determined based on a reduction in DHMB production. A decrease in acetolactate reductase activity can also be determined based on an increase in butanol production.

Thus, in some embodiments, a yeast that is capable of producing butanol produces no more than about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.9 mM, about 0.8 mM., about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM or about 0.3 mM DHMB. In  
5 some embodiments, a yeast producing butanol produces no more than about about 5 mM, about 4 mM, about 3 mM, about 2 mM, about 1 mM, about 0.9 mM, about 0.8 mM., about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM or about 0.3 mM DHMB. In some embodiments, a yeast producing butanol produces no more than about 0.2 mM or 0.2 mM  
10 DHMB.

In some embodiments, a yeast capable of producing butanol produces no more than about about 10 mM DHMB when cultured under fermentation conditions for at least about 50 hours. In some  
embodiments, a yeast capable of producing butanol produces no more  
15 than about about 5 mM DHMB when cultured under fermentation conditions for at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some embodiments, a yeast capable of producing butanol produced no more than about 3 mM DHMB  
20 when cultured under fermentation conditions for at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some embodiments, a yeast capable of producing butanol produced no  
25 more than about 1 mM DHMB when cultured under fermentation conditions for at least about 1 hour, at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours. In some  
30 embodiments, a yeast capable of producing butanol produced no more than about 0.5 mM DHMB when cultured under fermentation conditions for at least about 1 hour, at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at

least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours.

- In some embodiments, a yeast comprising at least one deletion, mutation, and/or substitution in an endogenous polynucleotide encoding an acetolactate reductase produces no more than about 0.5 times, about 0.4 times, about 0.3 times, about 0.2 times, about 0.1 times, about 0.05 times the amount of DHMB produced by a yeast containing the endogenous polynucleotide encoding an acetolactate reductase when cultured under fermentation conditions for the same amount of time.
- 10 In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about 5 mM, at least about 6 mM, at least about 7 mM, at least about 8 mM, at least about 9 mM, or at least about 10 mM.

- In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about the amount of DHMB produced. In some embodiments, a yeast that is capable of producing butanol produces an amount of DHIV that is at least about twice, about three times, about five times, about ten times, about 15 times, about 20 times, about 25 times, about 30 times, about 35 times, about 40 times, about 45 times, or about 50 times the amount of DHMB produced.
- 20 In some embodiments, a yeast that is capable of producing butanol produces DHIV at a rate that is at least about equal to the rate of DHMB production. In some embodiments, a yeast that is capable of producing butanol produces DHIV at a rate that is at least about twice, about three times, about five times, about ten times, about 15 times, about 20 times, about 25 times, about 30 times, about 35 times, about 40 times, about 45 times, or about 50 times the rate of DHMB production.

- In some embodiments, a yeast that is capable of producing butanol produces less than 0.010 moles of DHMB per mole of glucose consumed.
- 30 In some embodiments, a yeast produces less than about 0.009, less than about 0.008, less than about 0.007, less than about 0.006, or less than about 0.005 moles of DHMB per mole of glucose consumed. In some embodiments, a yeast produces less than about 0.004, less than about

0.003, less than about 0.002, or less than about 0.001 moles of DHMB per mole of glucose consumed.

In some embodiments, acetolactate reductase activity is inhibited by chemical means. For example, acetolactate reductase could be inhibited using other known substrates such as those listed in Fujisawa et al. including L-serine, D-serine, 2-methyl-DL-serine, D-threonine, L-allo-threonine, L-3-hydroxyisobutyrate, D-3-hydroxyisobutyrate, 3-hydroxypropionate, L-3-hydroxybutyrate, and D-3-hydroxybutyrate. *Biochimica et Biophysica Acta* 1645:89-94 (2003).

#### DHMB Removal

In other embodiments described herein, a reduction in DHMB can be achieved by removing DHMB from a fermentation. Thus, fermentations with reduced DHMB concentrations are also described herein. Removal of DHMB can result, for example, in a product of greater purity, or a product requiring less processing to achieve a desired purity. Therefore, compositions comprising products of biosynthetic pathways such as ethanol or butanol with increased purity are also provided.

DHMB can be removed during or after a fermentation process and can be removed by any means known in the art. DHMB can be removed, for example, by extraction into an organic phase or reactive extraction.

In some embodiments, the fermentation broth comprises less than about 0.5 mM DHMB. In some embodiments, the fermentation broth comprises less than about 1.0 mM DHMB after about 5 hours, about 10 hours, about 15 hours, about 20 hours, about 25 hours, about 30 hours, about 35 hours, about 40 hours, about 45 hours, or about 50 hours of fermentation. In some embodiments, the fermentation broth comprises less than about 5.0 mM DHMB after about 20 hours, about 25 hours, about 30 hours, about 35 hours, about 40 hours, about 45 hours, or about 50 hours of fermentation.

#### Butanol Biosynthetic Pathways

Certain suitable isobutanol biosynthetic pathways are disclosed in U.S. Patents 7,851,188 and 7,993,889 .

A diagram of the disclosed isobutanol biosynthetic pathways is provided in Figure 1. As described in U.S. Patent 7,851,188 ,  
5 steps in an example isobutanol biosynthetic pathway include conversion of:

- pyruvate to acetolactate (see Fig. 1, pathway step a therein), as catalyzed for example by acetolactate synthase (ALS),
- acetolactate to 2,3-dihydroxyisovalerate (see Fig. 1, pathway step b therein) as catalyzed for example by acetohydroxy acid isomeroreductase (KARI);
- 2,3-dihydroxyisovalerate to 2-ketoisovalerate (see Fig. 1, pathway step c therein) as catalyzed for example by acetohydroxy acid dehydratase, also called dihydroxy-acid dehydratase (DHAD);
- 15 - 2-ketoisovalerate to isobutyraldehyde (see Fig. 1, pathway step d therein) as catalyzed for example by branched-chain 2-keto acid decarboxylase; and
- isobutyraldehyde to isobutanol (see Fig. 1, pathway step e therein) as catalyzed for example by branched-chain alcohol dehydrogenase.

20 In another embodiment, the isobutanol biosynthetic pathway comprises the following substrate to product conversions:

- pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;
- acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed,  
25 for example, by ketol-acid reductoisomerase;
- 2,3-dihydroxyisovalerate to  $\alpha$ -ketoisovalerate, which may be catalyzed, for example, by dihydroxyacid dehydratase;
- $\alpha$ -ketoisovalerate to valine, which may be catalyzed, for example, by transaminase or valine dehydrogenase;
- 30 - valine to isobutylamine, which may be catalyzed, for example, by valine decarboxylase;
- isobutylamine to isobutyraldehyde, which may be catalyzed by, for example, omega transaminase; and,

– isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

In another embodiment, the isobutanol biosynthetic pathway comprises the following substrate to product conversions:

5       – pyruvate to acetolactate, which may be catalyzed, for example, by acetolactate synthase;

– acetolactate to 2,3-dihydroxyisovalerate, which may be catalyzed, for example, by acetohydroxy acid reductoisomerase;

10       – 2,3-dihydroxyisovalerate to  $\alpha$ -ketoisovalerate, which may be catalyzed, for example, by acetohydroxy acid dehydratase;

– $\alpha$ -ketoisovalerate to isobutyryl-CoA, which may be catalyzed, for example, by branched-chain keto acid dehydrogenase;

–isobutyryl-CoA to isobutyraldehyde, which may be catalyzed, for example, by acetylating aldehyde dehydrogenase; and,

15       – isobutyraldehyde to isobutanol, which may be catalyzed, for example, by a branched-chain alcohol dehydrogenase.

In another embodiment, the isobutanol biosynthetic pathway comprises the substrate to product conversions shown as steps k, g, and e in Figure 1.

20       Genes and polypeptides that can be used for the substrate to product conversions described above as well as those for additional isobutanol pathways, are described in U.S. Patent Appl. Pub. No. 2007/0092957 and PCT Pub. No. WO 2011/019894 .

US Appl. Pub. Nos. 2011/019894, 2007/0092957,  
25   2010/0081154, describe dihydroxyacid dehydratases including those from *Lactococcus lactis* and *Streptococcus mutans*. Ketoisovalerate decarboxylases include those derived from *Lactococcus lactis*, *Macrococcus caseolyticus* (SEQ ID NO: 542) and *Listeria grayi* (SEQ ID NO: 543). U.S. Patent Appl. Publ. No. 2009/0269823 and U.S. Appl. Publ. No. 2011/0269199,

30       describe alcohol dehydrogenases, including those that utilize NADH as a cofactor. Alcohol dehydrogenases include SadB from *Achromobacter xylosoxidans*. Additional alcohol dehydrogenases include

horse liver ADH and *Beijerinikia indica* ADH. Alcohol dehydrogenases include those that utilize NADH as a cofactor. In one embodiment a butanol biosynthetic pathway comprises a) a ketol-acid reductoisomerase that has a  $K_M$  for NADH less than about 300  $\mu\text{M}$ , less than about 100  $\mu\text{M}$ ,  
 5 less than about 50  $\mu\text{M}$ , less than about 20  $\mu\text{M}$  or less than about 10  $\mu\text{M}$ ; b) an alcohol dehydrogenase that utilizes NADH as a cofactor; or c) both a) and b).

WO 2011/019894 and US Appl. Pub. Nos. 2011/019894, 2007/0092957, 2010/0081154.

10 describe suitable dihydroxyacid dehydratases. Methods of increasing DHAD activity are described, for example, in U.S. Patent Application Publication No. 2010/0081173 and U.S. Patent Application No. 13/029,558, filed February 17, 2011.

15 Suitable ketol-acid reductoisomerase (KARI) enzymes are described in U.S. Patent Appl. Pub. Nos. 2008/0261230 A1, 2009/0163376, 2010/0197519, 2010/0143997 and 2011/0244536.

#### Examples of

KARIs disclosed therein are those from *Vibrio cholerae*, *Pseudomonas*  
 20 *aeruginosa* PAO1, and *Pseudomonas fluorescens* PF5. In some embodiments, the KARI enzyme has a specific activity of at least about 0.1 micromoles/min/mg, at least about 0.2 micromoles/min/mg, at least about 0.3 micromoles/min/mg, at least about 0.4 micromoles/min/mg, at least about 0.5 micromoles/min/mg, at least about 0.6 micromoles/min/mg,  
 25 at least about 0.7 micromoles/min/mg, at least about 0.8 micromoles/min/mg, at least about 0.9 micromoles/min/mg, at least about 1.0 micromoles/min/mg, or at least about 1.1 micromoles/min/mg.

Suitable polypeptides to catalyze the substrate to product conversion acetolactate to 2,3-dihydroxyisovalerate include those that have a  $K_M$   
 30 for NADH less than about 300  $\mu\text{M}$ , less than about 100  $\mu\text{M}$ , less than about 50  $\mu\text{M}$ , less than about 25  $\mu\text{M}$  or less than about 10  $\mu\text{M}$ .

In some embodiments, the KARI utilizes NADPH. Methods of measuring NADPH consumption are known in the art. For example, US



Published Application No. 2008/0261230 ,

provides methods of measuring NADPH

consumption. In some embodiments, an NADPH consumption assay is a method that measures the disappearance of the cofactor, NADPH, during  
5 the enzymatic conversion of acetolactate to  $\alpha$ - $\beta$ -dihydroxy-isovalerate at 340 nm. The activity is calculated using the molar extinction coefficient of  $6220 \text{ M}^{-1}\text{cm}^{-1}$  for NADPH and is reported as  $\mu\text{mole}$  of NADPH consumed per min per mg of total protein in cell extracts (see Aulabaugh and Schloss, Biochemistry 29: 2824-2830, 1990).

10 In some embodiments, the KARI is capable of utilizing NADH. In some embodiments, the KARI is capable of utilizing NADH under anaerobic conditions. KARI enzymes using NADH are described, for example, in U.S. Patent Application Publication No. 2009/0163376 .

15 Additional genes that can be used can be identified by one skilled in the art through bioinformatics or using methods well-known in the art.

Additionally described in U.S. Patent Application Publication No. US 2007/0092957 A1, are

construction of chimeric genes and genetic engineering of bacteria and  
20 yeast for isobutanol production using the disclosed biosynthetic pathways.

Biosynthetic pathways for the production of 1-butanol that may be used include those described in U.S. Appl. Pub. No. 2008/0182308, .

In one embodiment, the 1-butanol biosynthetic pathway comprises the following substrate to product  
25 conversions:

– a) acetyl-CoA to acetoacetyl-CoA, which may be catalyzed, for example, by acetyl-CoA acetyl transferase;

– b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which may be catalyzed, for example, by 3-hydroxybutyryl-CoA dehydrogenase;

30 – c) 3-hydroxybutyryl-CoA to crotonyl-CoA, which may be catalyzed, for example, by crotonase;

– d) crotonyl-CoA to butyryl-CoA, which may be catalyzed, for example, by butyryl-CoA dehydrogenase;

– e) butyryl-CoA to butyraldehyde, which may be catalyzed, for example, by butyraldehyde dehydrogenase; and,

– f) butyraldehyde to 1-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

- 5        Biosynthetic pathways for the production of 2-butanol that may be used include those described in U.S. Appl. Pub. No. 2007/0259410 and U.S. Appl. Pub. No. 2009/0155870.

In one embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

- 10        – a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;

– b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;

- 15        – c) acetoin to 3-amino-2-butanol, which may be catalyzed, for example, acetoin aminase;

– d) 3-amino-2-butanol to 3-amino-2-butanol phosphate, which may be catalyzed, for example, by aminobutanol kinase;

– e) 3-amino-2-butanol phosphate to 2-butanone, which may be catalyzed, for example, by aminobutanol phosphate phosphorylase; and,

- 20        – f) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

In another embodiment, the 2-butanol biosynthetic pathway comprises the following substrate to product conversions:

- 25        – a) pyruvate to alpha-acetolactate, which may be catalyzed, for example, by acetolactate synthase;

– b) alpha-acetolactate to acetoin, which may be catalyzed, for example, by acetolactate decarboxylase;

– c) acetoin to 2,3-butanediol, which may be catalyzed, for example, by butanediol dehydrogenase;

- 30        – d) 2,3-butanediol to 2-butanone, which may be catalyzed, for example, by dial dehydratase; and,

– e) 2-butanone to 2-butanol, which may be catalyzed, for example, by butanol dehydrogenase.

In some embodiments of the invention, a recombinant host cell comprises a biosynthetic pathway. The biosynthetic pathway can comprise reduced or eliminated aldehyde dehydrogenase activity and an isobutanol or 1-butanol biosynthetic pathway wherein the pathway

5 comprises the substrate to product conversion pyruvate to acetolactate. In some embodiments, a host cell comprising a biosynthetic pathway capable of converting pyruvate to acetolactate comprises a polynucleotide encoding a polypeptide having acetolactate synthase activity. For example, the biosynthetic pathway can be a butanol

10 producing pathway or a butanediol producing pathway. The biosynthetic pathway can also be a branched-chain amino acid (e.g., leucine, isoleucine, valine) producing pathway.

In other embodiments, the recombinant host cell can comprise an isobutanol, 1-butanol, or a 2-butanol biosynthetic pathway as described

15 herein. In some embodiments, the butanol biosynthetic pathway is an isobutanol biosynthetic pathway. Production of isobutanol or 2-butanol in a recombinant host cell disclosed herein may benefit from a reduction, substantial elimination or elimination of an acetolactate reductase activity.

20 Modifications

Functional deletion of the pyruvate decarboxylase gene has been used to increase the availability of pyruvate for utilization in biosynthetic product pathways. For example, U.S. Application Publication No. US 2007/0031950 A1,

25 discloses a yeast strain with a disruption of one or more pyruvate decarboxylase genes and expression of a D-lactate dehydrogenase gene, which is used for production of D-lactic acid. U.S. Application Publication No. US 2005/0059136 A1,

discloses glucose tolerant two carbon source independent (GCSI)

30 yeast strains with no pyruvate decarboxylase activity, which can have an exogenous lactate dehydrogenase gene. Nevoigt and Stahl (*Yeast* 12:1331-1337 (1996)) describe the impact of reduced pyruvate decarboxylase and increased NAD-dependent glycerol-3-phosphate

dehydrogenase in *Saccharomyces cerevisiae* on glycerol yield. U.S. Appl. Pub. No. 2009/0305363,

discloses increased conversion of pyruvate to acetolactate by engineering yeast for expression of a cytosol-localized acetolactate  
5 synthase and substantial elimination of pyruvate decarboxylase activity.

In embodiments of the invention, a recombinant host cell disclosed herein can comprise a modification in an endogenous polynucleotide encoding a polypeptide having pyruvate decarboxylase (PDC) activity or a modification in an endogenous polypeptide having PDC activity. In  
10 embodiments, a recombinant host cell disclosed herein can have a modification or disruption of a polynucleotide, gene and/or polypeptide encoding PDC. In embodiments, a recombinant host cell comprises a deletion, mutation, and/or substitution in an endogenous polynucleotide or gene encoding a polypeptide having PDC activity, or in an endogenous  
15 polypeptides having PDC activity. Such modifications, disruptions, deletions, mutations, and/or substitutions can result in PDC activity that is reduced or eliminated, resulting, for example, in a PDC knock-out (PDC-KO) phenotype.

In embodiments of the invention, an endogenous pyruvate  
20 decarboxylase activity of a recombinant host cell disclosed herein converts pyruvate to acetaldehyde, which can then be converted to ethanol or to acetyl-CoA via acetate. In other embodiments, a recombinant host cell is *Kluyveromyces lactis* containing one gene encoding pyruvate decarboxylase, *Candida glabrata* containing one gene encoding pyruvate  
25 decarboxylase, or *Schizosaccharomyces pombe* containing one gene encoding pyruvate decarboxylase.

In other embodiments, a recombinant host cell is *Saccharomyces cerevisiae* containing three isozymes of pyruvate decarboxylase encoded by the PDC1, PDC5, and PDC6 genes, as well as a pyruvate  
30 decarboxylase regulatory gene, PDC2. In a non-limiting example in *S. cerevisiae*, the PDC1 and PDC5 genes, or the PDC1, PDC5, and PDC6 genes, are disrupted. In another non-limiting example in *S. cerevisiae*, pyruvate decarboxylase activity can be reduced by disrupting the PDC2

regulatory gene. In another non-limiting example in *S. cerevisiae*, polynucleotides or genes encoding pyruvate decarboxylase proteins such as those having about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%,  
 5 about 96%, about 97%, about 98%, or about 99% sequence identity to PDC1, PDC2, PDC5 and/or PDC6 can be disrupted.

In embodiments, a polypeptide having PDC activity or a polynucleotide or gene encoding a polypeptide having PDC activity corresponds to Enzyme Commission Number EC 4.1.1.1. In other  
 10 embodiments, a PDC gene of a recombinant host cell disclosed herein is not active under the fermentation conditions used, and therefore such a gene would not need to be modified or inactivated.

Examples of recombinant host cells with reduced pyruvate decarboxylase activity due to disruption of pyruvate decarboxylase  
 15 encoding genes have been reported, such as for *Saccharomyces* in Flikweert *et al.* (*Yeast* (1996) 12:247-257), for *Kluyveromyces* in Bianchi *et al.* (*Mol. Microbiol.* (1996) 19(1):27-36), and disruption of the regulatory gene in Hohmann (*Mol. Gen. Genet.* (1993) 241:657-666).

*Saccharomyces* strains having no pyruvate decarboxylase activity are  
 20 available from the ATCC with Accession #200027 and #200028.

Examples of PDC polynucleotides, genes and/or polypeptides that can be targeted for modification or inactivation in the recombinant host cells disclosed herein include, but are not limited to, those of the following Table  
 8.

25 Table 8. Pyruvate decarboxylase target gene coding regions and proteins.

| Description                                                             | SEQ ID NO:<br>Nucleic acid | SEQ ID NO:<br>Amino acid |
|-------------------------------------------------------------------------|----------------------------|--------------------------|
| <i>PDC1</i> pyruvate decarboxylase from <i>Saccharomyces cerevisiae</i> | 648                        | 649                      |
| <i>PDC5</i> pyruvate decarboxylase from <i>Saccharomyces cerevisiae</i> | 650                        | 651                      |
| <i>PDC6</i> pyruvate decarboxylase from <i>Saccharomyces cerevisiae</i> | 652                        | 653                      |
| pyruvate decarboxylase from <i>Candida glabrata</i>                     | 654                        | 655                      |

|                                                                |     |     |
|----------------------------------------------------------------|-----|-----|
| <i>PDC1</i> pyruvate decarboxylase from <i>Pichia stipitis</i> | 656 | 657 |
| <i>PDC2</i> pyruvate decarboxylase from <i>Pichia stipitis</i> | 658 | 659 |
| pyruvate decarboxylase from <i>Kluyveromyces lactis</i>        | 660 | 661 |
| pyruvate decarboxylase from <i>Yarrowia lipolytica</i>         | 662 | 663 |
| pyruvate decarboxylase from <i>Schizosaccharomyces pombe</i>   | 664 | 665 |
| pyruvate decarboxylase from <i>Zygosaccharomyces rouxii</i>    | 666 | 667 |

Other examples of PDC polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to, PDC polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 8, wherein such a polynucleotide or gene encodes, or such a polypeptide has, PDC activity. Still other examples of PDC polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 8, wherein such a polynucleotide or gene encodes, or such a polypeptide has, PDC activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding a PDC sequence disclosed herein or known in the art can be modified, as disclosed above for aldehyde dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding PDC can be used to identify another PDC polynucleotide, gene and/or polypeptide sequence or to identify a PDC homolog in other cells, as disclosed above for acetolactate dehydrogenase. Such a PDC encoding sequence can be identified, for example, in the literature and/or in bioinformatics databases well known to the skilled person. For example, the identification of a PDC encoding sequence in other cell types using bioinformatics can be accomplished through BLAST (as described above) searching of publicly available databases with a known PDC encoding

DNA and polypeptide sequence, such as those provided herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

5           The modification of PDC in a recombinant host cell disclosed herein to reduce or eliminate PDC activity can be confirmed using methods known in the art. For example, disruption of a particular pyruvate decarboxylase could be confirmed with PCR screening using primers external to the gene sequence, or by Southern blot using a probe  
10       designed to the pyruvate decarboxylase gene sequence. Alternatively, one could utilize analytical methods such as gas chromatography or HPLC to screen strains for decreased or eliminated production of acetaldehyde and/or ethanol.

          Functional deletion of the hexokinase 2 gene has been used to  
15       reduce glucose repression and to increase the availability of pyruvate for utilization in biosynthetic pathways. For example, International Publication No. WO 2000/061722 A1,

          discloses the production of yeast biomass by aerobically growing yeast having one or more functionally deleted hexokinase 2 genes or  
20       analogs. In addition, Rossell et al. (Yeast Research 8:155-164 (2008)) found that *Saccharomyces cerevisiae* with a deletion of the hexokinase 2 gene showed 75% reduction in fermentative capacity, defined as the specific rate of carbon dioxide production under sugar-excess and anaerobic conditions. After starvation, the fermentation capacity was  
25       similar to that of a strain without the hexokinase 2 gene deletion. Diderich et al. (Applied and Environmental Microbiology 67:1587-1593 (2001)) found that *S. cerevisiae* with a deletion of the hexokinase 2 gene had lower pyruvate decarboxylase activity.

          In embodiments, a recombinant host cell disclosed herein can  
30       comprise a modification in an endogenous polynucleotide encoding a polypeptide having hexokinase 2 activity and/or a modification in a polypeptide having hexokinase 2 activity. In embodiments, a recombinant host cell disclosed herein can have a modification or disruption of a

polynucleotide, gene or polypeptide encoding hexokinase 2. In  
embodiments, a recombinant host cell comprises a deletion, mutation,  
and/or substitution in an endogenous polynucleotide or gene encoding a  
polypeptide having hexokinase 2 activity, or an endogenous polypeptide  
5 having hexokinase 2 activity. Such modifications, disruptions, deletions,  
mutations, and/or substitutions can result in hexokinase 2 activity that is  
reduced or eliminated, resulting, for example, in a hexokinase 2 knockout  
(H XK2-KO) phenotype. In embodiments, the host cell comprises a  
modification as described in U.S. Appn. Serial. Nos. 2011/0124060 A1 or  
10 2012/0015416 A1.

In embodiments, a polypeptide having hexokinase 2 activity can  
catalyze the conversion of hexose to hexose-6-phosphate, and/or can  
catalyze the conversion of D-glucose to D-glucose 6-phosphate, D-  
15 fructose to D-fructose 6-phosphate, and/or D-mannose to D-mannose 6-  
phosphate. In other embodiments, a polynucleotide, gene or polypeptide  
having hexokinase 2 activity can correspond to Enzyme Commission  
Number EC 2.7.1.1.

In embodiments of the invention, a recombinant host cell can be *S.*  
20 *cerevisiae* and a polynucleotide, gene or polypeptide having hexokinase 2  
activity can be H XK2. In other embodiments, a recombinant host cell can  
be *K. lactis* and a polynucleotide, gene or polypeptide having hexokinase  
2 activity can be RAG5. In other embodiments, a recombinant host cell  
can be *H. polymorpha* and a polynucleotide, gene or polypeptide having  
25 hexokinase 2 activity can be HPGLK1. In other embodiments, a  
recombinant host cell can be *S. pombe* and a polynucleotide, gene or  
polypeptide having hexokinase 2 activity can be H XK2.

Examples of hexokinase 2 polynucleotides, genes and polypeptides  
that can be targeted for modification or inactivation in a recombinant host  
30 cell disclosed herein include, but are not limited to, those of the following  
Table 9.

Table 9. Hexokinase 2 target gene coding regions and proteins.



|                                                      |                                      |
|------------------------------------------------------|--------------------------------------|
| <i>HXK2</i> from<br><i>S. cerevisiae</i>             | <u>Nucleic acid (SEQ ID NO: 668)</u> |
|                                                      | <u>Amino acid (SEQ ID NO: 669)</u>   |
| <i>RAG5</i> from<br><i>K. lactis</i>                 | <u>Nucleic acid (SEQ ID NO: 670)</u> |
|                                                      | <u>Amino acid (SEQ ID NO: 671)</u>   |
| <i>HPGLK1</i><br>from <i>H.</i><br><i>polymorpha</i> | <u>Nucleic acid (SEQ ID NO: 672)</u> |
|                                                      | <u>Amino acid (SEQ ID NO: 673)</u>   |
| <i>HXK2</i> from<br><i>S. pombe</i>                  | <u>Nucleic acid (SEQ ID NO: 674)</u> |
|                                                      | <u>Amino acid (SEQ ID NO: 675)</u>   |

Other examples of hexokinase 2 polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to,

5 hexokinase 2 polynucleotides, genes and/or polypeptides having at least about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to any one of the sequences of Table 9, wherein such a polynucleotide or gene encodes, or

10 such a polypeptide has, hexokinase 2 activity. Still other examples of hexokinase 2 polynucleotides, genes and polypeptides that can be targeted for modification or inactivation in a recombinant host cell disclosed herein include, but are not limited to an active variant, fragment or derivative of any one of the sequences of Table 9, wherein such a

15 polynucleotide or gene encodes, or such a polypeptide has, hexokinase 2 activity.

In embodiments, a polynucleotide, gene and/or polypeptide encoding a hexokinase 2 sequence disclosed herein or known in the art can be modified or disrupted, as disclosed above for aldehyde

20 dehydrogenase. In other embodiments, a polynucleotide, gene and/or polypeptide encoding hexokinase 2 can be used to identify another

hexokinase 2 polynucleotide, gene and/or polypeptide sequence or to identify a hexokinase 2 homolog in other cells, as disclosed above for aldehyde dehydrogenase. Such a hexokinase 2 encoding sequence can be identified, for example, in the literature and/or in bioinformatics

5 databases well known to the skilled person. For example, the identification of a hexokinase 2 encoding sequence in other cell types using bioinformatics can be accomplished through BLAST (as described above) searching of publicly available databases with a known hexokinase 2 encoding DNA and polypeptide sequence, such as those provided  
10 herein. Identities are based on the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.1, and Gonnet 250 series of protein weight matrix.

The modification of hexokinase 2 in a recombinant host cell disclosed herein to reduce or eliminate hexokinase 2 activity can be  
15 confirmed using methods known in the art. For example, disruption of hexokinase 2 could be confirmed with PCR screening using primers external to the hexokinase 2 gene, or by Southern blot using a probe designed to the hexokinase 2 gene sequence. Alternatively, one could examine putative hexokinase 2 knockout strains for increased biomass  
20 yield on glucose-containing media.

Examples of additional modifications that can be useful in cells provided herein include modifications to reduce glycerol-3-phosphate dehydrogenase activity and/or disruption in at least one gene encoding a polypeptide having pyruvate decarboxylase activity or a disruption in at  
25 least one gene encoding a regulatory element controlling pyruvate decarboxylase gene expression as described in U.S. Patent Appl. Pub. No. 2009/0305363 , modifications to a host cell that provide for increased carbon flux through an Entner-Doudoroff Pathway or reducing equivalents balance as described in U.S.  
30 Patent Appl. Pub. No. 2010/0120105 .

Other modifications include integration of at least one polynucleotide encoding a polypeptide that catalyzes a step in a pyruvate-utilizing biosynthetic pathway described in PCT Appn. Pub. No. WO 2012/033832 .

A genetic

modification which has the effect of reducing glucose repression wherein the yeast production host cell is *pdc-* is described in U.S. Appl. Publ No. US 2011/0124060 .

5

U.S. Appl. Publ. No. 20120064561A1,

discloses recombinant host cells comprising (a) at least one heterologous polynucleotide encoding a polypeptide having dihydroxy-acid dehydratase activity; and (b)(i) at least one deletion, mutation, and/or substitution in an endogenous gene encoding a polypeptide affecting Fe-S cluster biosynthesis; and/or (ii) at least one heterologous polynucleotide encoding a polypeptide affecting Fe-S cluster biosynthesis. In 10 embodiments, the polypeptide affecting Fe-S cluster biosynthesis is encoded by *AFT1*, *AFT2*, *FRA2*, *GRX3*, or *CCC1*. In embodiments, the polypeptide affecting Fe-S cluster biosynthesis is constitutive mutant *AFT1* 15 L99A, *AFT1* L102A, *AFT1* C291F, or *AFT1* C293F.

Additionally, host cells can comprise heterologous polynucleotides encoding a polypeptides with phosphoketolase activity and/or a heterologous polynucleotide encoding a polypeptide with 20 phosphotransacetylase activity such as, for example, those encoded by SEQ ID NOs: 962 and 963, and as described in PCT Appn. Pub. No. WO 2011/159853 .

#### Isobutanol and Other Products

25 In embodiments of the invention, methods for the production of a product of a biosynthetic pathway are provided which comprise (a) providing a recombinant host cell disclosed herein; and (b) growing the host cell under conditions whereby the product of the biosynthetic pathway is produced. In other embodiments, the product is produced as a co- 30 product along with ethanol. In still other embodiments, the product of the biosynthetic pathway is isobutanol.

In other embodiments of the invention, the product of the biosynthetic pathway is produced at a greater yield or amount compared

to the production of the same product in a recombinant host cell that does not comprise reduced or eliminated aldehyde dehydrogenase and/or aldehyde oxidase activity and/or acetolactate reductase activity. In embodiments, yield is increased by at least about 2%, at least about 5% or at least about 10%. In embodiments, this greater yield includes production at a yield of greater than about 10% of theoretical, at a yield of greater than about 20% of theoretical, at a yield of greater than about 25% of theoretical, at a yield of greater than about 30% of theoretical, at a yield of greater than about 40% of theoretical, at a yield of greater than about 50% of theoretical, at a yield of greater than about 60% of theoretical, at a yield of greater than about 75% of theoretical, at a yield of greater than about 80% of theoretical at a yield of greater than about 85% of theoretical, at a yield of greater than about 90% of theoretical, at a yield of greater than about 95% of theoretical, at a yield of greater than about 96% of theoretical, at a yield of greater than about 97% of theoretical, at a yield of greater than about 98% of theoretical, at a yield of greater than about 99% of theoretical, or at a yield of about 100% of theoretical. In other embodiments, the product is produced as a co-product along with ethanol. In still other embodiments, the product of the biosynthetic pathway is isobutanol.

Any product of a biosynthetic pathway that has the conversion of isobutyraldehyde to isobutyric acid as a pathway by-product can be produced with greater effectiveness in a recombinant host cell disclosed herein having the described modification of aldehyde dehydrogenase and/or aldehyde oxidase activity. A list of such products includes, but is not limited to, isobutanol.

#### Microbial Hosts for Isobutanol Production

Microbial hosts for isobutanol production can be selected from bacteria, cyanobacteria, filamentous fungi and yeasts. The microbial host used for butanol production should be tolerant to isobutanol so that the yield is not limited by butanol toxicity. Although butanol-tolerant mutants have been isolated from solventogenic *Clostridia*, little information is

available concerning the butanol tolerance of other potentially useful bacterial strains. Most of the studies on the comparison of alcohol tolerance in bacteria suggest that butanol is more toxic than ethanol (de Cavalho, et al., Microsc. Res. Tech., 64: 215-22, 2004) and (Kabelitz, et al., FEMS Microbiol. Lett., 220: 223-227, 2003, Tomas, et al., J. Bacteriol., 186: 2006-2018, 2004) report that the yield of 1-butanol during fermentation in *Clostridium acetobutylicum* can be limited by 1-butanol toxicity. The primary effect of 1-butanol on *Clostridium acetobutylicum* is disruption of membrane functions (Hermann et al., Appl. Environ. Microbiol., 50: 1238-1243, 1985).

The microbial hosts selected for the production of isobutanol should be tolerant to isobutanol and should be able to convert carbohydrates to isobutanol. The criteria for selection of suitable microbial hosts include the following: intrinsic tolerance to isobutanol, high rate of glucose utilization, availability of genetic tools for gene manipulation, and the ability to generate stable chromosomal alterations.

Suitable host strains with a tolerance for isobutanol can be identified by screening based on the intrinsic tolerance of the strain. The intrinsic tolerance of microbes to isobutanol can be measured by determining the concentration of isobutanol that is responsible for 50% inhibition of the growth rate ( $IC_{50}$ ) when grown in a minimal medium. The  $IC_{50}$  values can be determined using methods known in the art. For example, the microbes of interest can be grown in the presence of various amounts of isobutanol and the growth rate monitored by measuring the optical density at 600 nanometers. The doubling time can be calculated from the logarithmic part of the growth curve and used as a measure of the growth rate. The concentration of isobutanol that produces 50% inhibition of growth can be determined from a graph of the percent inhibition of growth versus the isobutanol concentration. In one embodiment, the host strain has an  $IC_{50}$  for isobutanol of greater than about 0.5%.

The microbial host for isobutanol production should also utilize glucose at a high rate. Most microbes are capable of metabolizing

carbohydrates. However, certain environmental microbes cannot metabolize carbohydrates to high efficiency, and therefore would not be suitable hosts.

The ability to genetically modify the host is essential for the production of any recombinant microorganism. The mode of gene transfer technology can be by electroporation, conjugation, transduction or natural transformation. A broad range of host conjugative plasmids and drug resistance markers are available. The cloning vectors are tailored to the host microorganisms based on the nature of antibiotic resistance markers that can function in that host.

The microbial host also has to be manipulated in order to inactivate competing pathways for carbon flow by deleting various genes. This requires the availability of either transposons to direct inactivation or chromosomal integration vectors. Additionally, the production host should be amenable to chemical mutagenesis so that mutations to improve intrinsic isobutanol tolerance can be obtained.

Based on the criteria described above, suitable microbial hosts for the production of isobutanol include, but are not limited to, members of the genera *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Vibrio*, *Lactobacillus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Brevibacterium*, *Pichia*, *Candida*, *Issatchenkia*, *Hansenula*, *Kluyveromyces*, and *Saccharomyces*. Suitable hosts include: *Escherichia coli*, *Alcaligenes eutrophus*, *Bacillus licheniformis*, *Paenibacillus macerans*, *Rhodococcus erythropolis*, *Pseudomonas putida*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus faecalis*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. In some embodiments, the host cell is *Saccharomyces cerevisiae*. *S. cerevisiae* yeast are known in the art and are available from a variety of sources, including, but not limited to, American Type Culture Collection (Rockville, MD), Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, LeSaffre, Gert Strand AB, Ferm Solutions, North American Bioproducts, Martrex, and Lallemand. *S. cerevisiae* include, but

are not limited to, BY4741, CEN.PK 113-7D, Ethanol Red® yeast, Ferm Pro™ yeast, Bio-Ferm® XR yeast, Gert Strand Prestige Batch Turbo alcohol yeast, Gert Strand Pot Distillers yeast, Gert Strand Distillers Turbo yeast, FerMax™ Green yeast, FerMax™ Gold yeast, Thermosacc® yeast,  
5 BG-1, PE-2, CAT-1, CBS7959, CBS7960, and CBS7961.

#### Construction of Production Host

Recombinant microorganisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a fermentable  
10 carbon substrate to butanol can be constructed using techniques well known in the art. In the present invention, genes encoding the enzymes of one of the isobutanol biosynthetic pathways of the invention, for example, acetolactate synthase, acetohydroxy acid isomeroreductase, acetohydroxy acid dehydratase, branched-chain  $\alpha$ -keto acid decarboxylase, and  
15 branched-chain alcohol dehydrogenase, can be isolated from various sources, as described above.

Methods of obtaining desired genes from a genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries can be created  
20 by restriction endonuclease digestion and can be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA can be amplified using standard primer-directed amplification methods such as polymerase chain reaction (U.S. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate  
25 vectors. Tools for codon optimization for expression in a heterologous host are readily available. Some tools for codon optimization are available based on the GC content of the host microorganism.

Once the relevant pathway genes are identified and isolated they can be transformed into suitable expression hosts by means well known in  
30 the art. Vectors or cassettes useful for the transformation of a variety of host cells are common and commercially available from companies such as EPICENTRE® (Madison, WI), Invitrogen Corp. (Carlsbad, CA), Stratagene (La Jolla, CA), and New England Biolabs, Inc. (Beverly, MA).

Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. Both control regions can be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions can also be derived from genes that are not native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements, including those used in the Examples, is suitable for the present invention including, but not limited to, *CYC1*, *HIS3*, *GAL1*, *GAL10*, *ADH1*, *PGK*, *PHO5*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO*, *TPI* (useful for expression in *Saccharomyces*); *AOX1* (useful for expression in *Pichia*); and *lac*, *ara*, *tet*, *trp*, *IP<sub>L</sub>*, *IP<sub>R</sub>*, *T7*, *tac*, and *trc* (useful for expression in *Escherichia coli*, *Alcaligenes*, and *Pseudomonas*) as well as the *amy*, *apr*, *npr* promoters and various phage promoters useful for expression in *Bacillus subtilis*, *Bacillus licheniformis*, and *Paenibacillus macerans*. For yeast recombinant host cells, a number of promoters can be used in constructing expression cassettes for genes, including, but not limited to, the following constitutive promoters suitable for use in yeast: *FBA1*, *TDH3* (*GPD*), *ADH1*, *ILV5*, and *GPM1*; and the following inducible promoters suitable for use in yeast: *GAL1*, *GAL10*, *OLE1*, and *CUP1*. Other yeast promoters include hybrid promoters *UAS(PGK1)-FBA1p* (SEQ ID NO: 406), *UAS(PGK1)-ENO2p* (SEQ ID NO: 538), *UAS(FBA1)-PDC1p* (SEQ ID NO: 539), *UAS(PGK1)-PDC1p* (SEQ ID NO: 540), and *UAS(PGK)-OLE1p* (SEQ ID NO: 541).

Promoters, transcriptional terminators, and coding regions can be cloned into a yeast 2 micron plasmid and transformed into yeast cells



(Ludwig, et al. *Gene*, 132: 33-40, 1993; US Appl. Pub. No. 20080261861A1).

Adjusting the amount of gene expression in a given host may be achieved by varying the level of transcription, such as through selection of  
5 native or artificial promoters. In addition, techniques such as the use of promoter libraries to achieve desired levels of gene transcription are well known in the art. Such libraries can be generated using techniques known in the art, for example, by cloning of random cDNA fragments in front of gene cassettes (Goh *et al.* (2002) *AEM* 99, 17025), by modulating  
10 regulatory sequences present within promoters (Ligr *et al.* (2006) *Genetics* 172, 2113), or by mutagenesis of known promoter sequences (Alper *et al.* (2005) *PNAS*, 12678; Nevoigt *et al.* (2006) *AEM* 72, 5266).

Termination control regions can also be derived from various genes native to the hosts. Optionally, a termination site can be unnecessary or  
15 can be included.

Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three related vectors-pRK437, pRK442, and pRK442(H) are available. These derivatives have proven to  
20 be valuable tools for genetic manipulation in Gram-negative bacteria (Scott et al., *Plasmid*, 50: 74-79, 2003). Several plasmid derivatives of broad-host-range Inc P4 plasmid RSF1010 are also available with promoters that can function in a range of Gram-negative bacteria. Plasmid pAYC36 and pAYC37, have active promoters along with multiple  
25 cloning sites to allow for the heterologous gene expression in Gram-negative bacteria.

Chromosomal gene replacement tools are also widely available. For example, a thermosensitive variant of the broad-host-range replicon pVW101 has been modified to construct a plasmid pVE6002 which can be  
30 used to effect gene replacement in a range of Gram-positive bacteria (Maguin et al., *J. Bacteriol.*, 174: 5633-5638, 1992). Additionally, in vitro transposomes are available to create random mutations in a variety of genomes from commercial sources such as EPICENTRE®.

The expression of a butanol biosynthetic pathway in various microbial hosts is described in more detail below.

Expression of a butanol biosynthetic pathway in *E. coli*

Vectors or cassettes useful for the transformation of *E. coli* are common and commercially available from the companies listed above. For example, the genes of an isobutanol biosynthetic pathway can be isolated from various sources, cloned into a modified pUC19 vector and transformed into *E. coli* NM522.

Expression of a butanol biosynthetic pathway in *Rhodococcus erythropolis*

A series of *E. coli*-*Rhodococcus* shuttle vectors are available for expression in *R. erythropolis*, including, but not limited to, pRhBR17 and pDA71 (Kostichka et al., Appl. Microbiol. Biotechnol., 62: 61-68, 2003). Additionally, a series of promoters are available for heterologous gene expression in *R. erythropolis* (Nakashima et al., Appl. Environ. Microbiol., 70: 5557-5568, 2004 and Tao et al., Appl. Microbiol. Biotechnol., 68: 346-354, 2005). Targeted gene disruption of chromosomal genes in *R. erythropolis* can be created using the method described by Tao et al., *supra*, and Brans et al. (Appl. Environ. Microbiol., 66: 2029-2036, 2000).

The heterologous genes required for the production of isobutanol, as described above, can be cloned initially in pDA71 or pRhBR71 and transformed into *E. coli*. The vectors can then be transformed into *R. erythropolis* by electroporation, as described by Kostichka et al., *supra*. The recombinants can be grown in synthetic medium containing glucose and the production of isobutanol can be followed using methods known in the art.

Expression of a butanol biosynthetic pathway in *B. subtilis*

Methods for gene expression and creation of mutations in *B. subtilis* are also well known in the art. For example, the genes of an isobutanol biosynthetic pathway can be isolated from various sources, cloned into a modified pUC19 vector and transformed into *Bacillus subtilis* BE1010. Additionally, the five genes of an isobutanol biosynthetic pathway can be split into two operons for expression. The three genes of the pathway (*bubB*, *ilvD*, and *kivD*) can be integrated into the chromosome of *Bacillus*

*subtilis* BE1010 (Payne, et al., J. Bacteriol., 173, 2278-2282, 1991). The remaining two genes (*ilvC* and *bdhB*) can be cloned into an expression vector and transformed into the *Bacillus* strain carrying the integrated isobutanol genes

5 Expression of a butanol biosynthetic pathway in *B. licheniformis*

Most of the plasmids and shuttle vectors that replicate in *B. subtilis* can be used to transform *B. licheniformis* by either protoplast transformation or electroporation. The genes required for the production of isobutanol can be cloned in plasmids pBE20 or pBE60 derivatives

10 (Nagarajan et al., Gene, 114: 121-126, 1992). Methods to transform *B. licheniformis* are known in the art (Fleming et al. Appl. Environ. Microbiol., 61: 3775-3780, 1995). The plasmids constructed for expression in *B. subtilis* can be transformed into *B. licheniformis* to produce a recombinant microbial host that produces isobutanol.

15 Expression of a butanol biosynthetic pathway in *Paenibacillus macerans*

Plasmids can be constructed as described above for expression in *B. subtilis* and used to transform *Paenibacillus macerans* by protoplast transformation to produce a recombinant microbial host that produces isobutanol.

20 Expression of the butanol biosynthetic pathway in *Alcaligenes (Ralstonia) eutrophus*

Methods for gene expression and creation of mutations in *Alcaligenes eutrophus* are known in the art (Taghavi et al., Appl. Environ. Microbiol., 60: 3585-3591, 1994). The genes for an isobutanol

25 biosynthetic pathway can be cloned in any of the broad host range vectors described above, and electroporated to generate recombinants that produce isobutanol. The poly(hydroxybutyrate) pathway in *Alcaligenes* has been described in detail, a variety of genetic techniques to modify the *Alcaligenes eutrophus* genome is known, and those tools can be applied  
30 for engineering an isobutanol biosynthetic pathway.

Expression of a butanol biosynthetic pathway in *Pseudomonas putida*

Methods for gene expression in *Pseudomonas putida* are known in the art (see for example Ben-Bassat et al., U.S. Patent No. 6,586,229).

The butanol pathway genes can be inserted into pPCU18 and this ligated DNA can be electroporated  
5 into electrocompetent *Pseudomonas putida* DOT-T1 C5aAR1 cells to generate recombinants that produce isobutanol.

Expression of a butanol biosynthetic pathway in *Saccharomyces cerevisiae*

10 Methods for gene expression in *Saccharomyces cerevisiae* are known in the art (e.g., Methods in Enzymology, Volume 194, Guide to Yeast Genetics and Molecular and Cell Biology, Part A, 2004, Christine Guthrie and Gerald R. Fink, eds., Elsevier Academic Press, San Diego, CA). Expression of genes in yeast typically requires a promoter, followed  
15 by the gene of interest, and a transcriptional terminator. A number of yeast promoters, including those used in the Examples herein, can be used in constructing expression cassettes for genes encoding an isobutanol biosynthetic pathway, including, but not limited to constitutive promoters FBA, GPD, ADH1, and GPM, and the inducible promoters  
20 GAL1, GAL10, and CUP1. Suitable transcriptional terminators include, but are not limited to FBAt, GPDt, GPMt, ERG10t, GAL1t, CYC1, and ADH1. For example, suitable promoters, transcriptional terminators, and the genes of an isobutanol biosynthetic pathway can be cloned into *E. coli*-yeast shuttle vectors and transformed into yeast cells as described in U.S.  
25 App. Pub. No. 20100129886. These vectors allow strain propagation in both *E. coli* and yeast strains. Typically the vector contains a selectable marker and sequences allowing autonomous replication or chromosomal integration in the desired host. Typically used plasmids in yeast are shuttle vectors pRS423, pRS424, pRS425, and pRS426 (American Type  
30 Culture Collection, Rockville, MD), which contain an *E. coli* replication origin (e.g., pMB1), a yeast 2 $\mu$  origin of replication, and a marker for nutritional selection. The selection markers for these four vectors are His3 (vector pRS423), Trp1 (vector pRS424), Leu2 (vector pRS425) and Ura3

(vector pRS426). Construction of expression vectors with genes encoding polypeptides of interest can be performed by either standard molecular cloning techniques in *E. coli* or by the gap repair recombination method in yeast.

5           The gap repair cloning approach takes advantage of the highly efficient homologous recombination in yeast. Typically, a yeast vector DNA is digested (e.g., in its multiple cloning site) to create a "gap" in its sequence. A number of insert DNAs of interest are generated that contain a  $\geq 21$  bp sequence at both the 5' and the 3' ends that

10 sequentially overlap with each other, and with the 5' and 3' terminus of the vector DNA. For example, to construct a yeast expression vector for "Gene X", a yeast promoter and a yeast terminator are selected for the expression cassette. The promoter and terminator are amplified from the yeast genomic DNA, and Gene X is either PCR amplified from

15 its source organism or obtained from a cloning vector comprising Gene X sequence. There is at least a 21 bp overlapping sequence between the 5' end of the linearized vector and the promoter sequence, between the promoter and Gene X, between Gene X and the terminator sequence, and between the terminator and the 3' end of the

20 linearized vector. The "gapped" vector and the insert DNAs are then co-transformed into a yeast strain and plated on the medium containing the appropriate compound mixtures that allow complementation of the nutritional selection markers on the plasmids. The presence of correct insert combinations can be confirmed by PCR

25 mapping using plasmid DNA prepared from the selected cells. The plasmid DNA isolated from yeast (usually low in concentration) can then be transformed into an *E. coli* strain, e.g. *TOP10*, followed by mini preps and restriction mapping to further verify the plasmid construct. Finally the construct can be verified by sequence analysis.

30           Like the gap repair technique, integration into the yeast genome also takes advantage of the homologous recombination system in yeast. Typically, a cassette containing a coding region plus control elements (promoter and terminator) and auxotrophic marker is PCR-

amplified with a high-fidelity DNA polymerase using primers that hybridize to the cassette and contain 40-70 base pairs of sequence homology to the regions 5' and 3' of the genomic area where insertion is desired. The PCR product is then transformed into yeast and plated  
5 on medium containing the appropriate compound mixtures that allow selection for the integrated auxotrophic marker. For example, to integrate "Gene X" into chromosomal location "Y", the promoter-coding regionX-terminator construct is PCR amplified from a plasmid DNA construct and joined to an autotrophic marker (such as *URA3*) by  
10 either SOE PCR or by common restriction digests and cloning. The full cassette, containing the promoter-coding regionX-terminator-*URA3* region, is PCR amplified with primer sequences that contain 40-70 bp of homology to the regions 5' and 3' of location "Y" on the yeast chromosome. The PCR product is transformed into yeast and  
15 selected on growth media lacking uracil. Transformants can be verified either by colony PCR or by direct sequencing of chromosomal DNA.

20 Expression of a butanol biosynthetic pathway in *Lactobacillus plantarum*

The *Lactobacillus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Bacillus subtilis* and *Streptococcus* can be used for *Lactobacillus*. Non-limiting examples  
25 of suitable vectors include pAM $\beta$ 1 and derivatives thereof (Renault et al., Gene 183:175-182, 1996); and (O'Sullivan et al., Gene, 137: 227-231, 1993); pMBB1 and pHV800, a derivative of pMBB1 (Vyckoff et al., Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520  
30 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267, 2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Several plasmids from *Lactobacillus plantarum* have also been

reported (van Kranenburg R, et al. Appl. Environ. Microbiol., 71: 1223-1230, 2005).

Expression of a butanol biosynthetic pathway in various *Enterococcus* species (*E. faecium*, *E. gallinarum*, and *E. faecalis*)

5           The *Enterococcus* genus belongs to the *Lactobacillales* family and many plasmids and vectors used in the transformation of *Lactobacilli*, *Bacilli* and *Streptococci* species can be used for *Enterococcus* species. Non-limiting examples of suitable vectors include pAM $\beta$ 1 and derivatives thereof (Renault et al., Gene, 183: 175-182, 1996); and (O'Sullivan et al.,  
10   Gene, 137: 227-231, 1993); pMBB1 and pHW800, a derivative of pMBB1 (Wyckoff et al. Appl. Environ. Microbiol., 62: 1481-1486, 1996); pMG1, a conjugative plasmid (Tanimoto et al., J. Bacteriol., 184: 5800-5804, 2002); pNZ9520 (Kleerebezem et al., Appl. Environ. Microbiol., 63: 4581-4584, 1997); pAM401 (Fujimoto et al., Appl. Environ. Microbiol., 67: 1262-1267,  
15   2001); and pAT392 (Arthur et al., Antimicrob. Agents Chemother., 38: 1899-1903, 1994). Expression vectors for *E. faecalis* using the *nisA* gene from *Lactococcus* can also be used (Eichenbaum et al., Appl. Environ. Microbiol., 64: 2763-2769, 1998). Additionally, vectors for gene replacement in the *E. faecium* chromosome can be used (Nallaapareddy  
20   et al., Appl. Environ. Microbiol., 72: 334-345, 2006).

Fermentation Media

          Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates can include but are not limited to  
25   monosaccharides such as glucose and fructose, oligosaccharides such as lactose, maltose, galactose, sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally the carbon substrate can also be  
30   one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. In addition to one and two carbon substrates methylotrophic microorganisms are also known to utilize a number of other

carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., Microb. Growth C1 Compd., [Int. Symp.], 7th  
5 (1993), 415-32. (eds): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., Arch. Microbiol., 153: 485-489, 1990). Hence it is contemplated that the source of carbon utilized in the present invention can encompass a wide variety of carbon  
10 containing substrates and will only be limited by the choice of microorganism.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, in some embodiments, the carbon substrates are glucose, fructose, and  
15 sucrose, or mixtures of these with C5 sugars such as xylose and/or arabinose for yeasts cells modified to use C5 sugars. Sucrose can be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and dextrose can be derived from renewable grain sources through saccharification of  
20 starch based feedstocks including grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars can be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in U.S. Patent App. Pub. No. 2007/0031918 A1.

25 Biomass refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass can also comprise additional components, such as protein and/or lipid. Biomass can be derived from a single source,  
30 or biomass can comprise a mixture derived from more than one source; for example, biomass can comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste,



industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for growth of the cultures and promotion of the enzymatic pathway necessary for butanol production described herein.

#### 15 Culture Conditions

Typically cells are grown at a temperature in the range of about 20 °C to about 40 °C in an appropriate medium. Suitable growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Medium (YM) broth or broth that includes yeast nitrogen base, ammonium sulfate, and dextrose (as the carbon/energy source) or YPD Medium, a blend of peptone, yeast extract, and dextrose in optimal proportions for growing most *Saccharomyces cerevisiae* strains. Other defined or synthetic growth media can also be used, and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2',3'-monophosphate (cAMP), can also be incorporated into the fermentation medium.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred for the initial condition. Suitable pH ranges for the fermentation of yeast are typically between about pH 3.0 to about pH 9.0. In one embodiment, about pH 5.0 to about

pH 8.0 is used for the initial condition. Suitable pH ranges for the fermentation of other microorganisms are between about pH 3.0 to about pH 7.5. In one embodiment, about pH 4.5 to about pH 6.5 is used for the initial condition.

- 5            Fermentations can be performed under aerobic or anaerobic conditions. In one embodiment, anaerobic or microaerobic conditions are used for fermentation.

#### Industrial Batch and Continuous Fermentations

- 10           The present processes may employ a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the medium is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired
- 15           microorganism or microorganisms, and fermentation is permitted to occur without adding anything to the system. Typically, however, a "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions
- 20           of the system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of
- 25           production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch fermentation processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the fermentation progresses.

- 30           Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated

on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO<sub>2</sub>. Batch and Fed-Batch fermentations are common and well known in the art and examples can be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, or Deshpande, Mukund (Appl. Biochem. Biotechnol., 36: 227, 1992).

Although the present invention is performed in batch mode it is contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to the medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

It is contemplated that the present invention can be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells can be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isobutanol production.

### Methods for Butanol Isolation from the Fermentation Medium

Bioproducted butanol can be isolated from the fermentation medium using methods known in the art for ABE fermentations (see, e.g., Durre, *Appl. Microbiol. Biotechnol.* 49:639-648 (1998), Groot *et al.*, *Process. Biochem.* 27:61-75 (1992), and references therein). For example, solids can be removed from the fermentation medium by centrifugation, filtration, decantation, or the like. Then, the butanol can be isolated from the fermentation medium using methods such as distillation, azeotropic distillation, liquid-liquid extraction, adsorption, gas stripping, membrane evaporation, or pervaporation.

Because butanol forms a low boiling point, azeotropic mixture with water, distillation can be used to separate the mixture up to its azeotropic composition. Distillation can be used in combination with another separation method to obtain separation around the azeotrope. Methods that can be used in combination with distillation to isolate and purify butanol include, but are not limited to, decantation, liquid-liquid extraction, adsorption, and membrane-based techniques. Additionally, butanol can be isolated using azeotropic distillation using an entrainer (see, e.g., Doherty and Malone, *Conceptual Design of Distillation Systems*, McGraw Hill, New York, 2001).

The butanol-water mixture forms a heterogeneous azeotrope so that distillation can be used in combination with decantation to isolate and purify the butanol. In this method, the butanol containing fermentation broth is distilled to near the azeotropic composition. Then, the azeotropic mixture is condensed, and the butanol is separated from the fermentation medium by decantation. The decanted aqueous phase can be returned to the first distillation column as reflux. The butanol-rich decanted organic phase can be further purified by distillation in a second distillation column.

The butanol can also be isolated from the fermentation medium using liquid-liquid extraction in combination with distillation. In this method, the butanol is extracted from the fermentation broth using liquid-liquid extraction with a suitable solvent. The butanol-containing organic phase is then distilled to separate the butanol from the solvent.

Distillation in combination with adsorption can also be used to isolate butanol from the fermentation medium. In this method, the fermentation broth containing the butanol is distilled to near the azeotropic composition and then the remaining water is removed by use of an adsorbent, such as molecular sieves (Aden *et al.*, *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, Report NREL/TP-510-32438, National Renewable Energy Laboratory, June 2002).

Additionally, distillation in combination with pervaporation can be used to isolate and purify the butanol from the fermentation medium. In this method, the fermentation broth containing the butanol is distilled to near the azeotropic composition, and then the remaining water is removed by pervaporation through a hydrophilic membrane (Guo *et al.*, *J. Membr. Sci.* 245, 199-210 (2004)).

*In situ* product removal (ISPR) (also referred to as extractive fermentation) can be used to remove butanol (or other fermentative alcohol) from the fermentation vessel as it is produced, thereby allowing the microorganism to produce butanol at high yields. One method for ISPR for removing fermentative alcohol that has been described in the art is liquid-liquid extraction. In general, with regard to butanol fermentation, for example, the fermentation medium, which includes the microorganism, is contacted with an organic extractant at a time before the butanol concentration reaches a toxic level. The organic extractant and the fermentation medium form a biphasic mixture. The butanol partitions into the organic extractant phase, decreasing the concentration in the aqueous phase containing the microorganism, thereby limiting the exposure of the microorganism to the inhibitory butanol.

Liquid-liquid extraction can be performed, for example, according to the processes described in U.S. Patent Appl. Pub. No. 2009/0305370 .

U.S. Patent Appl.

Pub. No. 2009/0305370 describes methods for producing and recovering butanol from a fermentation broth using liquid-liquid extraction, the

methods comprising the step of contacting the fermentation broth with a water immiscible extractant to form a two-phase mixture comprising an aqueous phase and an organic phase. Typically, the extractant can be an organic extractant selected from the group consisting of saturated, mono-  
5 unsaturated, poly-unsaturated (and mixtures thereof) C<sub>12</sub> to C<sub>22</sub> fatty alcohols, C<sub>12</sub> to C<sub>22</sub> fatty acids, esters of C<sub>12</sub> to C<sub>22</sub> fatty acids, C<sub>12</sub> to C<sub>22</sub> fatty aldehydes, and mixtures thereof. The extractant(s) for ISPR can be non-alcohol extractants. The ISPR extractant can be an exogenous organic extractant such as oleyl alcohol, behenyl alcohol, cetyl alcohol,  
10 lauryl alcohol, myristyl alcohol, stearyl alcohol, 1-undecanol, oleic acid, lauric acid, myristic acid, stearic acid, methyl myristate, methyl oleate, undecanal, lauric aldehyde, 20-methylundecanal, and mixtures thereof.

In some embodiments, the ester can be formed by contacting the alcohol in a fermentation medium with a carboxylic acid (e.g., fatty acids)  
15 and a catalyst capable of esterifying the alcohol with the carboxylic acid, as described in PCT Appn. Pub. No. WO/2011/159998.

In such embodiments, the carboxylic acid can serve as an ISPR extractant into which the alcohol esters partition. The carboxylic acid can be supplied to the fermentation  
20 vessel and/or derived from the biomass supplying fermentable carbon fed to the fermentation vessel. Lipids present in the feedstock can be catalytically hydrolyzed to carboxylic acid, and the same catalyst (e.g., enzymes) can esterify the carboxylic acid with the alcohol. The catalyst can be supplied to the feedstock prior to fermentation, or can be supplied  
25 to the fermentation vessel before or contemporaneously with the supplying of the feedstock. When the catalyst is supplied to the fermentation vessel, alcohol esters can be obtained by hydrolysis of the lipids into carboxylic acid and substantially simultaneous esterification of the carboxylic acid with butanol present in the fermentation vessel. Carboxylic acid and/or  
30 native oil not derived from the feedstock can also be fed to the fermentation vessel, with the native oil being hydrolyzed into carboxylic acid. Any carboxylic acid not esterified with the alcohol can serve as part of the ISPR extractant. The extractant containing alcohol esters can be

separated from the fermentation medium, and the alcohol can be recovered from the extractant. The extractant can be recycled to the fermentation vessel. Thus, in the case of butanol production, for example, the conversion of the butanol to an ester may reduce the free butanol concentration in the fermentation medium, shielding the microorganism from the toxic effect of increasing butanol concentration. In addition, unfractionated grain can be used as feedstock without separation of lipids therein, since the lipids can be catalytically hydrolyzed to carboxylic acid, thereby decreasing the rate of build-up of lipids in the ISPR extractant.

5        *In situ* product removal can be carried out in a batch mode or a continuous mode. In a continuous mode of *in situ* product removal, product is continually removed from the reactor. In a batchwise mode of *in situ* product removal, a volume of organic extractant is added to the fermentation vessel and the extractant is not removed during the process.

10        For *in situ* product removal, the organic extractant can contact the fermentation medium at the start of the fermentation forming a biphasic fermentation medium. Alternatively, the organic extractant can contact the fermentation medium after the microorganism has achieved a desired amount of growth, which can be determined by measuring the optical

15        density of the culture. Further, the organic extractant can contact the fermentation medium at a time at which the product alcohol level in the fermentation medium reaches a preselected level. In the case of butanol production according to some embodiments of the present invention, the carboxylic acid extractant can contact the fermentation medium at a time

20        before the butanol concentration reaches a toxic level, so as to esterify the butanol with the carboxylic acid to produce butanol esters and consequently reduce the concentration of butanol in the fermentation vessel. The ester-containing organic phase can then be removed from the fermentation vessel (and separated from the fermentation broth which

25        constitutes the aqueous phase) after a desired effective titer of the butanol esters is achieved. In some embodiments, the ester-containing organic phase is separated from the aqueous phase after fermentation of the

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available fermentable sugar in the fermentation vessel is substantially complete.

5 EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can  
10 ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

15 GENERAL METHODS:

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.,  
20 1989, by T. J. Silhavy, M. L. Bannan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987.

Materials and methods suitable for the maintenance and growth of  
25 bacterial cultures are also well known in the art. Techniques suitable for use in the following Examples can be found in Manual of Methods for General Bacteriology, Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, DC., 1994,  
30 or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA, 1989. All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals



(Milwaukee, WI), BD Diagnostic Systems (Sparks, MD), Life Technologies (Rockville, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified.

The meaning of abbreviations used is as follows: "Å" means

- 5    Angstrom, "min" means minute(s), "h" means hour(s), "μl" means microliter(s), "ng/μl" means nano gram per microliter, "pmol/μl" means pico mole per microliter, "ml" means milliliter(s), "L" means liter(s), "g/L" means gram per liter, "ng" means nano gram, "sec" means second(s), "ml/min" means milliliter per minute(s), "w/v" means weight per volume,
- 10    "v/v" means volume per volume, "nm" means nanometer(s), "mm" means millimeter(s), "cm" means centimeter(s), "mM" means millimolar, "M" means molar, "g" means gram(s), "μg" means microgram(s), "mg" means milligram(s), "g" means the gravitation constant, "rpm" means revolutions per minute, "HPLC" means high performance liquid chromatography, "MS"
- 15    means mass spectrometry, "HPLC/MS" means high performance liquid chromatography/mass spectrometry, "EDTA" means ethylenediamine-tetraacetic acid, "dNTP" means deoxynucleotide triphosphate, "°C" means degrees Celsius, and "V" means voltage.

#### High throughput screening assay of gene libraries

- 20       High throughput screening of the gene libraries of mutant KARI enzymes was performed as described herein (with the exception of Examples 16 and 21): 10× freezing medium containing 554.4 g/L glycerol, 68 mM of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 mM MgSO<sub>4</sub>, 17 mM sodium citrate, 132 mM KH<sub>2</sub>PO<sub>4</sub>, 36 mM K<sub>2</sub>HPO<sub>4</sub> was prepared with molecular pure water and
- 25    filter-sterilized. Freezing medium was prepared by diluting the 10× freezing medium with the LB medium. An aliquot (200 μl) of the freezing medium was used for each well of the 96-well archive plates (cat #3370, Corning Inc. Corning, NY).

- 30       Clones from the LB agar plates were selected and inoculated into the 96-well archive plates containing the freezing medium and grown overnight at 37 °C without shaking. The archive plates were then stored at -80°C. *E. coli* strain Bw25113 transformed with *pBAD-HisB* (Invitrogen) was always used as the negative control. The positive controls for the

libraries in Examples 3, 4, and 5 are the wild type K9-KARI, AB1D3, AB1D3 respectively.

Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0  $\mu$ l of cells from thawed archive plates, 200  $\mu$ l of the LB medium containing 100  $\mu$ g/ml ampicillin and 0.02%(w/v) arabinose as the inducer. Cells were grown overnight at 37 °C with 80% humidity while shaking (900rpm), harvested by centrifugation (4000 rpm, 5 min at 25 °C). (Eppendorf centrifuge, Brinkmann Instruments, Inc. Westbury, NY) and the cell pellet was stored at -20 °C for later analysis.

The assay substrate, (*R,S*)-acetolactate, was synthesized as described by Aulabaugh and Schloss (Aulabaugh and Schloss, Biochemistry, 29: 2824-2830, 1990). All other chemicals used in the assay were purchased from Sigma.

The enzymatic conversion of acetolactate to  $\alpha,\beta$ -dihydroxyisovalerate by KARI was followed by measuring the disappearance of the cofactor, NADPH or NADH, from the reaction at 340 nm using a plate reader (Molecular Device, Sunnyvale, CA). The activity was calculated using the molar extinction coefficient of 6220 M<sup>-1</sup>cm<sup>-1</sup> for either NADPH or NADH. The stock solutions used were: K<sub>2</sub>HPO<sub>4</sub> (0.2 M); KH<sub>2</sub>PO<sub>4</sub> (0.2 M); EDTA (0.5 M); MgCl<sub>2</sub> (1.0 M); NADPH (2.0 mM); NADH (2.0 mM) and acetolactate (45 mM). The 100 ml reaction buffer (pH 6.8) containing: 2.0 ml K<sub>2</sub>HPO<sub>4</sub>, 3.0 ml KH<sub>2</sub>PO<sub>4</sub>, 4.0 ml MgCl<sub>2</sub>, 0.1 ml EDTA and 90.9 ml water was prepared.

Frozen cell pellet in deep-well plates and BugBuster were warmed up at room temperature for 30 min at the same time. Each well of 96-well assay plates was filled with 120  $\mu$ l of the reaction buffer and 20  $\mu$ l of NADH (2.0 mM). 75  $\mu$ l of 50% BugBuster (v/v in water) was added to each well after 30 min warm-up and cells were suspended using plate shaker. The plates were incubated at room temperature for 20 min. An aliquot (15 to 25  $\mu$ l depending the expected activity) of cell lysate was transferred into each well of 96-well assay plates. Absorbance at 340 nm was recorded as background, 16  $\mu$ l of acetolactate (4.5 mM, diluted with the reaction buffer) was added to each well and mixed with shaking by the plate reader.

Absorbance at 340 nm was recorded at 0, and 10 to 30 minutes depending the expected activity after substrate addition. The difference in absorbance (before and after substrate addition) was used to determine the activity of the mutants. Mutants with higher KARI activity compared to the positive control were selected for re-screening.

The number of clones screened for the libraries in Example 1, 2 and 3 are about 12,000, 12,000 and 92 respectively. The top performers from each library were re-screened described below as secondary assay.

#### Secondary assay of active mutants

Cells containing selected mutants identified by high throughput screening (above) were grown overnight, at 37°C, in 3.0 ml of the LB medium containing 100 µg/ml ampicillin and 0.025% (w/v) arabinose as the inducer while shaking at 250 rpm. The cells were then aliquoted into 96 deepwell plates (200 µl per well) and harvested by centrifugation at 4,000 xg for 5 min at room temperature. 75 µl of 50% BugBuster (v/v in water) was added to each well and cells were suspended using plate shaker. The plates were incubated at room temperature for 20 min. An aliquot (15 to 25 µl depending the expected activity) of cell lysate was transferred into each well of 96-well assay plates, which contain 120 µl of the reaction buffer and 20 µl of NADH (2.0 mM) per well. Absorbance at 340 nm was recorded as background, 16 µl of acetolactate (4.5 mM, diluted with the reaction buffer) was added to each well and mixed with shaking by the plate reader. Absorbance at 340 nm was recorded at 0, and 5 to 10 minutes depending the expected activity after substrate addition. The difference in absorbance (before and after substrate addition) was used to determine the activity of the mutants. Mutants with higher KARI activity compared to the positive control were selected for further characterization.

#### Measurement of NADH and NADPH Michaelis Constants

KARI enzyme activity can be routinely measured by NADH or NADPH oxidation as described above, however to measure the Michaelis constant ( $K_M$ ) for these pyridine nucleotides formation of the 2,3-dihydroxyisovalerate product was measured directly using HPLC/MS.

Protein concentration of crude cell extract from Bugbuster lysed cells (as described above) was measured using the BioRad protein assay reagent (BioRad Laboratories, Inc., Hercules, CA 94547). Between 0.2 and 1.0 micrograms of crude extract protein was added to a reaction buffer consisting of 100 mM MOPS KOH, pH 6.8, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM glucose-6-phosphate (Sigma-Aldrich), 0.2 Units of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma-Aldrich), and various concentrations of NADH or NADPH, to a volume of 90  $\mu$ L. The reaction was initiated by the addition of 10  $\mu$ L of [S]-acetolactate to a final concentration of 2.5 mM and a final volume of 100  $\mu$ L. After 10 min incubations at 30°C, the reaction was quenched by withdrawing 50  $\mu$ L of the reaction mixture and adding it to 150  $\mu$ L of 0.1 % formic acid. To measure the K<sub>M</sub> of NADH and NADPH, the concentrations used were 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mM.

To analyze for 2,3-dihydroxyisovalerate, 2  $\mu$ L of the formic acid quenched reaction mixture was injected into a Waters Acquity HPLC equipped with Waters SQD mass spectrometer (Waters Corporation, Milford, MA). The chromatography conditions were: flow rate (0.5 ml/min), on a Waters Acquity HSS T3 column (2.1 mm diameter, 100 mm length). Buffer A consisted of 0.1% (v/v) in water, Buffer B was 0.1% formic acid in acetonitrile. The sample was analyzed using 1% buffer B (in buffer A) for 1 min, followed by a linear gradient from 1% buffer B at 1 min to 75% buffer B at 1.5 min. The reaction product, 2,3-dihydroxyisovalerate, was detected by ionization at m/z=133, using electrospray ionization -30 V cone voltage. The amount of product 2,3-dihydroxyisovalerate was calculated by comparison to an authentic standard.

To calculate the K<sub>M</sub> for NADH and NADPH, the rate data for DHIV formation measured in assays at a fixed concentration of S-acetolactate (2.5 mM) was fitted to the single substrate Michaelis-Menten equation, using a least-squares regression in Microsoft Excel, assuming saturating acetolactate concentration.

Construction of plasmids pYZ058, pLH550, pLH556, and pLH702

pYZ058 (pHR81-P<sub>CUP1</sub>-AlsS-P<sub>ILV5</sub>-yeast KARI; SEQ ID NO: 176) was derived from pYZ090 (pHR81-P<sub>CUP1</sub>-AlsS-P<sub>ILV5</sub>-lactis KARI; SEQ ID NO: 195). pYZ090 was cut with PmeI and SfiI enzymes, and ligated with a

5 PCR product of yeast KARI. The PCR product was amplified from genomic DNA of *Saccharomyces cerevisiae* BY4741 (Research Genetics Inc.) strain using upper primer 5'-

catcatcacagtttaaacagtatgtgaagcaaatcaactcgggtgg-3' (SEQ ID NO: 272) and lower primer 5'- ggacggggccctgcaggcctattggtttctggtctcaactttctgac-3'

10 (SEQ ID NO: 273), and digested with PmeI and SfiI enzymes. pYZ058 was confirmed by sequencing.

pLH550 (pHR81-PCUP1-AlsS-P<sub>ILV5</sub>-Pf5.KARI, SEQ ID NO: 175) was derived from pYZ058 (SEQ ID NO: 176). The wild type Pf5.KARI gene was PCR amplified with OT1349 (5'-

15 catcatcacagtttaaacagtatgaaagtttctacgataaagactgcgacc-3'; SEQ ID NO: 177) and OT1318 (5'-gcactlgaagggcctgcagggccttagttcttggttgcgacgatttg-3'; SEQ ID NO: 178), digested with PmeI and SfiI enzymes and ligated with pYZ058 vector cut with PmeI and SfiI. The vector generated, pLH550, was confirmed by sequencing.

20 pLH556 (SEQ ID NO: 138; FIGURE 4) was derived from pLH550 by digesting the vector with SpeI and NotI enzymes, and ligating with a linker annealed from OT1383 (5'-ctagtcaccggtggc-3', SEQ ID NO: 179) and OT1384 (5'-ggccgccaccggtga-3', SEQ ID NO: 180) which contains overhang sequences for SpeI and NotI sites. This cloning step eliminates

25 the AlsS gene and a large fragment of the PCUP1 promoter, with 160 bp residual upstream sequence that is not functional. pLH556 was confirmed by sequencing.

pHR81::ILV5p-K9D3 (pLH702, SEQ ID NO: 181) was derived from pLH556. The K9D3 mutant KARI gene was excised from vector pBAD-K9D3 using PmeI and SfiI enzymes, and ligated with pLH556 at PmeI and SfiI sites, replacing the Pf5.KARI gene with the K9D3 gene. The

30 constructed vector was confirmed by sequencing.

Example 1

### Construction of yeast isobutanol pathway strains containing various KARI genes

To identify polypeptides having KARI activity and performance in yeast isobutanol production, biodiversity screening of KARI-encoding genes from various bacterial and fungal species was carried out. The KARI genes were codon optimized based on codon preferences of *Saccharomyces cerevisiae* genes where indicated in Table 10. For each KARI gene, a PmeI restriction site and additional 3 bp (AGT) was added to the 5' end with the sequence 5'-GTTTAAACAGT-3' (SEQ ID NO: 136) before the ATG start codon, and a SfiI restriction site was added to the 3' end with the sequence 5'-GGCCCTGCAGGCC-3' (SEQ ID NO: 137). All of the KARI genes were synthesized by GenScript USA Inc. (Piscataway, NJ). Each KARI gene was subcloned into pHR81-PCUP1-AlsS-PIIv5-Pf5.Ilv5 vector (SEQ ID NO: 175) via the PmeI and SfiI sites (Ilv5 encodes for yeast ketol-acid reductoisomerase). This vector contains two expression cassettes: *Bacillus subtilis* acetolactate synthase (AlsS) gene under the yeast CUP1 promoter, and yeast Ilv5 gene controlled by the Ilv5 promoter. Sequence analysis was performed to confirm the KARI gene sequences.

The pHR81-PCUP1-AlsS-PIIv5-KARI vectors carrying the KARI genes were co-transformed with pLH468 (pRS423-P<sub>FBA1</sub>-DHAD- P<sub>TDH3</sub>-kivD-P<sub>GPM1</sub>-hADH1; SEQ ID NO: 139) into host strain BP1135 (PNY1505; Example 8) (CEN.pk 113-7D delta ura3::loxP delta his3 delta pdc6 delta pdc1::ilvD.Sm delta pdc5::sadB delta gpd2::loxP delta fra2). The yeast transformants were selected on minimum drop-out media plates (SE-Ura-His, 2% ethanol) after 5-7 days at 30°C, and restreaked on SE-Ura-His to obtain cell patches after additional 3 day incubation. The cell patches were used for shake flask inoculation.

### 30 Example 2

#### Screening the KARI diversity collection for isobutanol production

The various KARI genes were evaluated based on their "effective productivities" in yeast. The effective productivity was determined after a

certain period of growth under progressively oxygen-limited conditions (e.g. 48 h). The yeast biomass was calculated with the assumption that 1 OD<sub>600</sub> of yeast cells is equivalent to 0.3 g/L.

5 The yeast isobutanol pathway strains carrying various KARI genes were inoculated into 10 mL SEG-Ura,His media with 0.2% glucose and 0.2% ethanol, and grown aerobically overnight at 30°C, to about 2 OD. The cultures were centrifuged and a portion of the cells were resuspended in SEG-Ura,His (2% glucose, 1% ethanol) to an initial OD<sub>600</sub> of 0.4 in 25 mL total volume in a 125 mL shake flask. The shake flasks were closed  
10 with a screw-on solid plastid cap, and the cultures were grown under progressively oxygen-limited conditions in the flask under minimal air and oxygen exchange with the outside environment. After 48 h incubation at 30°C, 250 RPM, the cultures were removed for OD<sub>600</sub> measurement and HPLC analysis to measure isobutanol production.

15 From the KARI genes screened, as shown below, multiple had comparable or better isobutanol titers than *Lactococcus lactis* KARI. In particular, the K9 (*Anaerostipes caccae* DSM 14662) KARI clone showed a high isobutanol titer and effective isobutanol productivity, as measured after 48 h of growth under progressively oxygen-limited conditions (Table  
20 10).

Table 10. Isobutanol titers and effective productivities from yeast isobutanol production strains carrying various KARI genes measured after  
25 48 h of growth under progressively oxygen-limited conditions in shake flasks at 30°C.

| KARI clone       | SEQ ID NO:<br>(nucleic acid,<br>amino acid)<br><br>*All nucleic<br>acid seqs<br>except LTS<br>and S2 are<br>codon-<br>optimized | Isobutanol titer<br>(g/L) | Effective<br>Isobutanol<br>Productivity<br>(g/g) | Source Organism                                        |
|------------------|---------------------------------------------------------------------------------------------------------------------------------|---------------------------|--------------------------------------------------|--------------------------------------------------------|
| B3K01<br>("K1")  | 140, 141                                                                                                                        | 2.6                       | 4.1                                              | <i>Bifidobacterium<br/>angulatum</i> DSM<br>20098      |
| B3K02<br>("K2")  | 142, 143                                                                                                                        | 3.5                       | 3.7                                              | <i>Bifidobacterium<br/>dentium</i> ATCC 27678          |
| B3K09<br>("K9")  | 26, 27                                                                                                                          | 4.3                       | 5.2                                              | <i>Anaerostipes caccae</i><br>DSM 14662                |
| B3K25<br>("K25") | 375, 376                                                                                                                        | 3.6                       | 4.4                                              | <i>Enterococcus<br/>gallinarum</i> EG2                 |
| B3K26<br>("K26") | 381, 382                                                                                                                        | 4.4                       | 3.2                                              | <i>Streptococcus<br/>thermophilus</i> LMD-9            |
| B3K29<br>("K29") | 377, 378                                                                                                                        | 4.1                       | 3.3                                              | <i>Lactococcus lactis</i><br>subsp. cremoris<br>MG1363 |
| LTS              | 379, 380                                                                                                                        | 2.7                       | 3.1                                              | <i>Lactococcus lactis</i>                              |
| B3K07<br>("K7")  | 274, 275                                                                                                                        | 3.7                       | 2.8                                              | <i>Clostridium<br/>beijerinckii</i> NCIMB<br>8052      |
| S2               | 276, 277                                                                                                                        | 3.6                       | 1.5                                              | <i>Zymomonas mobilis</i>                               |

Example 3KARI enzyme analysis of the yeast isobutanol pathway strains



IpOHA (N-isopropyl oxalylhydroxamic acid) is a mimic of a reaction intermediate for the reaction catalyzed by the KARI enzyme. It is a tight binding inhibitor that binds to the active site of the KARI enzyme. The synthesis of IpOHA and its tight binding to KARI from *E. coli* is described in literature (A. Aulabaugh and J. V. Schloss, Biochemistry, 1990, 29, 2824-2830). Its use for active site titration has not been reported before. IpOHA was synthesized from [ $^{14}\text{C}$ ]-oxalate according to literature.

The yeast cultures from Example 2 were harvested and analyzed for KARI enzyme activities. 25 mL of the cultures was pelleted and resuspended in 10 mL of 50 mM Tris-HCl, pH 7.5. The cells were centrifuged again to remove the buffer and the cell pellets are stored at -70°C. The cell pellets were resuspended in 1 mL of 50 mM Tris-HCl pH 7.5 and sonicated. Soluble crude cell extracts were used to perform the enzyme assays. A portion of enzyme was incubated with a molar excess of [ $^{14}\text{C}$ ]-IpOHA, and saturating concentrations of NAD(P)H and  $\text{Mg}^{2+}$ . Because a reversible, dilution-sensitive complex forms first, extract concentrations were kept high, to favor complexation and thus reduce the time taken for tight complex formation. Because it was not known *a priori* how long it would take each KARI to form the tight complex, two time points were taken for each sample to verify that the results agree. At the end of the incubation time, small molecules were separated from protein molecules by ultrafiltration using Microcon® (Millipore Inc., Billerica, MA), and the high molecular weight fraction was counted. The concentration of KARI in the sample in either  $\mu\text{M}$  or  $\text{mg/ml}$  was back-calculated from the  $^{14}\text{C}$  dpm, the volumes, and the KARI subunit molecular weight. A fixed-time enzyme assay was run concurrently, and the data were used to calculate U/ml. The specific activity was calculated by dividing U/ml by  $\text{mg/ml}$  for a given sample. The assumption made was that full activity and the ability to bind IpOHA are strictly correlated. The specific activities of the KARI enzymes thus measured are listed in Table 11. The KARI activity in "Units per mg" represents the activity per milligram of KARI enzyme as quantitated using the IpOHA assay. Total protein concentration was determined by the Bradford method, and the

expression level of KARI is calculated by dividing KARI enzyme amount by the amount of total soluble cellular proteins.

Table 11. KARI enzyme activities measured by the IPOHA assay

5

| KARI clone | KARI activity U/mg | KARI % total protein | Organism                                                |
|------------|--------------------|----------------------|---------------------------------------------------------|
| B3K01      | 0.56               | 21                   | <i>Bifidobacterium angulatum</i> DSM 20098              |
| B3K02      | 0.44               | 28                   | <i>Bifidobacterium dentium</i> ATCC 27678               |
| B3K09      | 2.4                | 15                   | <i>Anaerostipes caccae</i> DSM 14662                    |
| B3K25      | 1.3                | 21                   | <i>Enterococcus gallinarum</i> EG2                      |
| B3K26      | 1.5                | 17                   | <i>Streptococcus thermophilus</i> LMD-9                 |
| B3K29      | 1.6                | 14                   | <i>Lactococcus lactis</i> subsp. <i>cremonis</i> MG1363 |
| LTS        | 0.8                | 23.0                 | <i>Lactococcus lactis</i>                               |

#### Example 4

##### Construction of a site-saturation gene library to identify variants utilizing NADH with $K_M$ lower than wild type

10 To construct the pBAD-based bacterial expression vector for K9 KARI, the K9 KARI gene (synthesized by Genscript, Piscataway, NJ) was subcloned into pBAD-ps-JEA1 vector (SEQ ID NO: 905) via the PmeI and SfiI sites. The ketol-acid reductoisomerase (KARI) from *Anaerostipes* *caccae* (called K9-KARI) was used for the library construction. One gene  
 15 library was constructed using the commercially available kits, T4 polynucleotide kinase (PNK) (USB Corporation, Cleveland, Ohio, #70031Z) and Chang\_IT Multiple Mutation Site Directed Mutagenesis Kit (USB Corporation, Cleveland, Ohio, #78480).

The oligonucleotides (K9\_56\_58\_060210f:

20 GAAGGANNKAAANNKTGGAAGAGAGC, SEQ ID NO: 144; and K9\_56\_58\_060210r: GCTCTCTTCCAMNNTTTMNNTCCTTC, SEQ ID NO: 145) were synthesized by Integrated DNA Technologies, Inc (Coralville IA). They were first phosphorylated by T4 PNK. In brief, A 30  $\mu$ l reaction mixture contained: 3.0  $\mu$ l of 10x T4 PNK buffer supplied with the

kit, 4.0  $\mu$ l of primer (about 35  $\mu$ M) , 0.8  $\mu$ l of 100 mM ATP mix, 0.6  $\mu$ l T4 PNK and 22  $\mu$ l of water. The reaction mixture was incubated at 37 °C for 1.0 hr and T4 PNK was then deactivated at 65 °C for 20 min.

- The phosphorylated primers were then directly used for the
- 5 subsequent PCR reaction to introduce the mutations at two sites into K9 KARI wild type using the kit. In brief, a 30  $\mu$ l reaction mixture contained: 3.0  $\mu$ l of 10x reaction buffer supplied with the kit, 3.0  $\mu$ l of phosphorylated forward primer and reverse primer, 2.0  $\mu$ l of K9 KARI wild type (50 ng/ $\mu$ l), 1.2  $\mu$ l Chang\_IT enzyme and 17.8  $\mu$ l of water. This reaction mixture was
- 10 placed into thin well 200  $\mu$ l-capacity PCR tubes and the following PCR reaction program were used for the PCR: The starting temperature was 95°C for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 95°C for 30 sec, 55°C for 30 sec, and 68°C for 20 min. At the completion of the temperature cycling, the samples were kept at 68° C for
- 15 25 min more, and then held at 4°C for later processing. The PCR reaction was cleaned up using the Zymo DNA clean-up kit (Zymo Research Corporation, Orange CA, #D4004). DNA was eluted out of membrane using 84  $\mu$ l of water. The DNA template was removed with Dpn I (Promega, Madison WI, #R6231) at 37 °C for 3 hr (reaction mixture: 10  $\mu$ l
- 20 of 10x reaction buffer, 1.0  $\mu$ l BSA, 6.0  $\mu$ l of Dpn I and 83  $\mu$ l cleaned PCR DNA). The Dpn I digested DNA was cleaned up again with Zymo DNA clean-up kit and digested again with Dpn I to completely remove the DNA template (reaction mixture: 1.5  $\mu$ l of 10x reaction buffer, 0.15  $\mu$ l BSA, 0.85  $\mu$ l of Dpn I and 83  $\mu$ l cleaned PCR DNA). The reaction mixture was
- 25 directly used to transform an electro-competent strain of *E. coli* Bw25113( $\Delta ilvC$ ) (described in U.S. Patent No. 8,129,162 )
- using a BioRad Gene Pulser II
- (Bio-Rad Laboratories Inc., Hercules, CA). The transformed clones were streaked on agar plates containing the LB medium and 100  $\mu$ g/ml
- 30 ampicillin (Cat#L1004, Teknova Inc. Hollister, CA) and incubated at 37 °C overnight. Clones were screened for activity using NADH.  $K_M$  for the variants was measured (Table 12).

**Table 12**

Kinetic Values for K9 KARI Variants in *E. coli* Extracts, as Determined via  
DHIV Formation Assays

| Mutant                                   | SEQ ID NO:<br>(nucleic<br>acid, amino<br>acid) | Mutations | K <sub>M</sub> (μM)<br>(NADH) | K <sub>M</sub> (μM)<br>(NADPH) |
|------------------------------------------|------------------------------------------------|-----------|-------------------------------|--------------------------------|
| K9 Wt                                    | 26, 27                                         |           | 326                           | 0.2                            |
| AB1D1                                    | 28, 29                                         | S56A      | 164                           | 1                              |
| 495B5                                    | 30, 31                                         | S56A/S58H | 44                            | 4                              |
| AB1D3 (also<br>referred to as<br>"K9D3") | 32, 33                                         | S56A/S58D | 38                            | 9                              |
| AB1G9 (also<br>referred to as<br>"K9G9") | 34, 35                                         | S56A/S58E | 47                            | 23                             |

5

**EXAMPLE 5****Construction of site saturation gene libraries to lower K<sub>M</sub> for NADH**

Based on work with *Pseudomonas fluorescens* KARI (PF5-KARI) positions 24, 33, 61, 80, 156 and 170 were targeted as mutagenesis targets for K9 KARI. Through multiple sequence alignment (MSA) between  
10 PF5-KARI and K9 KARI (Fig. 2), the corresponding positions are 30, 39, 67, 86, 162, and 176.

To identify more mutagenesis targets, MSA of existing KARI enzymes (K1, K2, K7, K9, K25, K26, *L. Lactis* and S2), determined to produce isobutanol in a butanologen strain (see other examples) was used  
15 to identify more mutagenesis targets. Positions 41, 87, 131, 191, 227, and 246 were selected as mutagenesis targets.

The oligonucleotides targeting positions 30, 39, 41, 67, 86, 87, 131, 162, 176, 191, 227, and 246 were commercially synthesized by Integrated DNA Technologies, Inc (Coralville IA) (Table 13). Eight pairs of  
20 oligonucleotides targeting positions 30, 67, 131, 162, 176, 191, 227, and 246 were used to generate Megaprimers using Supermix from Invitrogen (Cat#10572-014, Invitrogen, Carlsbad, CA). For each PCR reaction, a pair

of primers, any combination of one forward primer and one reverse primer encoding different positions from those eight pairs of oligonucleotides (e.g. K9\_30\_101110f and K9\_67\_101110r), were used. There are total  $P_i^2$  or 56 combinations. A 25  $\mu$ l reaction mixture contained: 22.5  $\mu$ l of Supermix solution, 1.0  $\mu$ l of forward primer and 1.0  $\mu$ l of reverse primer, 0.5  $\mu$ l of AB1D3 DNA template (50 ng/ $\mu$ l). The mixture was placed in a thin well 200  $\mu$ l tube for the PCR reaction in a Mastercycler gradient equipment (Brinkmann Instruments, Inc. Westbury, NY). The following conditions were used for the PCR reaction: The starting temperature was 95°C for 1.0 min followed by 35 heating/cooling cycles. Each cycle consisted of 95°C for 20 sec, 55°C for 20 sec, and 72°C for 1.0 min. At the completion of the temperature cycling, the samples were kept at 72° C for 2.0 min more, and then held awaiting sample recovery at 4°C. The PCR product was cleaned up using a DNA cleaning kit (Cat#D4003, Zymo Research, Orange, CA) as recommended by the manufacturer.

The Megaprimers were then used to generate gene libraries using the QuickChange II XL site directed mutagenesis kit (Catalog # 200524, Stratagene, La Jolla CA). A 25  $\mu$ l reaction mixture contained: 2.5  $\mu$ l of 10x reaction buffer, 1.0  $\mu$ l of 50 ng/ $\mu$ l template, 20.5  $\mu$ l of Megaprimer, 0.5  $\mu$ l of 40 mM dNTP mix, 0.5  $\mu$ l pfu-ultra DNA polymerase. Except for the Megaprimer and the templates, all reagents used here were supplied with the kit indicated above. This reaction mixture was placed in a thin well 200  $\mu$ l-capacity PCR tube and the following reactions were used for the PCR: The starting temperature was 95°C for 30 sec followed by 25 heating/cooling cycles. Each cycle consisted of 95°C for 30 sec, 55°C for 1 min, and 68°C for 6 min. At the completion of the temperature cycling, the samples were kept at 68° C for 8 min more, and then held at 4°C for later processing. The PCR reaction mixture was processed with Dpn I restriction enzyme same as that used in Example 4.

The oligonucleotides K9\_37&39\_101110f, K9\_37&39\_101110r and K9\_86&87\_101110f, K9\_86&87\_101110r were directly then used to generate gene libraries using the QuickChange II XL site directed

mutagenesis kit. Two 25 µl reaction mixtures for the two oligonucleotide sets. each 25 µl reaction mixture contained: 2.5 µl of 10x reaction buffer, 1.0 µl of 50 ng/µl template, 1.0 µl of forward primer, 1.0 µl reverse primer, 0.5 µl of 40 mM dNTP mix, 0.5 µl pfu-ultra DNA polymerase and 18.5 µl of water. The PCR program and the subsequent Dpn I processing are the same.

The Dpn I processed DNA mixture was cleaned up using Zymo DNA clean-up kit following the manufacturer's protocol. The cleaned-up DNA was used to transform an electro-competent strain of *E. coli* Bw25113 ( $\Delta ilvC$ ) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, CA). The transformed clones were streaked on agar plates containing the LB medium and 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, CA) and incubated at 37 °C overnight. Clones were screened for improved activity using NADH.  $K_M$  for the improved mutants was measured (Table 14).

Table 13: Primers

| Targeted position(s) of K9-KARI | Primers                                                                                                                                      |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|
| 30                              | K9_30_101110f: gactatcgccgttatcggtNNKgggtclcaagggtcac SEQ ID NO: 146<br>K9_30_101110r: GTGACCTTGAGAACCMNNACCGATAACGGCGATAGTC SEQ ID NO: 147  |
| 67                              | K9_67_101110f: gagctgaagaacaaggNNKgaagctacaccgtgc SEQ ID NO: 148<br>K9_67_101110r: GCAGCGGTGTAGACTTCMNNACCTTGTTCTTCAGCTC SEQ ID NO: 149      |
| 131                             | K9_131_101110f: caaaggacgttgatgctNNKatgatcgctccaaag SEQ ID NO: 150<br>K9_131_101110r: CTTTGGAGCGATCATMNNGACATCAACGTCCTTT SEQ ID NO: 151      |
| 162                             | K9_162_101110f: gctgtcgaacaagacNNKactggcaaggcttg SEQ ID NO: 152<br>K9_162_101110r: CAAAGCCTTGCCAGTMNNGTCTTGTTGACAGC SEQ ID NO: 153           |
| 176                             | K9_176_101110f: gcttggcctacgcttaNNKatcggtggtgctagagc SEQ ID NO: 154<br>K9_176_101110r: GCTCTAGCACCACCGATMNNATAAGCGTAGGCCAAAGC SEQ ID NO: 155 |
| 191                             | K9_191_101110f: gaaactacctcagaNNKgaactgaaaccgac SEQ ID NO: 156<br>K9_191_101110r: GTCGGTTTCAGTTTCMNNCTGAAGGTAGTTTC SEQ ID NO: 157            |
| 227                             | K9_227_101110f: gccggttacgaccaNNKaacgctacttcgaatg SEQ ID NO: 158<br>K9_227_101110r: CATTCGAAGTAAGCGTTMNNNTGGGTCGTAACCGGC SEQ ID NO: 159      |
| 246                             | K9_246_101110f: gttgactgatctacNNKtctggttctccggtatgc SEQ ID NO: 160<br>K9_246_101110r: GCATACCGGAGAAACCAGAMNNGTAGATCAAGTCAAC SEQ ID NO: 161   |

|        |                                                                                                                                                          |
|--------|----------------------------------------------------------------------------------------------------------------------------------------------------------|
| 39, 41 | K9_37&39_101110f: gttctcaaggcacgctNNKgccNNKaatgctaagggaatcc SEQ ID NO: 162<br>K9_37&39_101110r: GGATTCCTTAGCATTMNNGGCMNNAGCGTGACCTTGAGAAC SEQ ID NO: 163 |
| 86, 87 | K9_86&87_101110f: gacatcattatgactctgNNKNNKgatgaaaagcaggc SEQ ID NO: 164<br>K9_86&87_101110r: GCCTGCTTTTCATCMNNMNNCAAGATCATAATGATGTC SEQ ID NO: 165       |

**Table 14**List of some mutants with their measured  $K_M$  values

| Mutant | SEQ ID NO:<br>(nucleic acid,<br>amino acid) | Mutations             | $K_M$ ( $\mu$ M)<br>(NADH) | $K_M$ ( $\mu$ M)<br>(NADPH) |
|--------|---------------------------------------------|-----------------------|----------------------------|-----------------------------|
| AO7A9  | 38, 39                                      | S56A/S58D/I86T/N87P   | 15                         | 7                           |
| AO7B5  | 36, 37                                      | S56A/S58D/I86V/N87P   | 8                          | 4                           |
| AO7H8  | 40, 41                                      | S56A/S58D/N87P        | 8                          | 6                           |
| AO7D8  | 42, 43                                      | S56A/S58D/T131C/T191S | 26                         | 6                           |
| AO7F7  | 44, 45                                      | S56A/S58D/T131V/T191A | 28                         | 7                           |
| AO7H7  | 46, 47                                      | S56A/S58D/T191S       | 29                         | 8                           |

5

**EXAMPLE 6****Construction of a combinatorial library to lower  $K_M$  for NADH**

Based on the mutagenesis results (Example 4), T131L, T131A,  
 10 T131V, T131M, T131C, T191D, T191C, T191S, and T191G are  
 considered as beneficial mutations to improve  $K_M$  for NADH. A  
 combinatorial library to introduce these beneficial mutations into AO7B5  
 was made.

All oligonucleotides were synthesized by the Integrated DNA  
 15 Technologies, Inc (Coralville IA). They were first phosphorylated by T4  
 PNK. In brief, a 20  $\mu$ l reaction mixture contained: 2.0  $\mu$ l of 10x T4 PNK  
 buffer supplied with the kit, 2.85  $\mu$ l of primer (about 35  $\mu$ M), 0.6  $\mu$ l of 100  
 mM ATP mix, 0.4  $\mu$ l T4 PNK and 14.15  $\mu$ l of water. The reaction mixture  
 was incubated at 37 °C for 1.0 hr and T4 PNK was then deactivated at 65  
 20 °C for 20 min.

The phosphorylated primers were then directly used for the subsequent PCR reaction to introduce the mutations at two sites into A07B5 using the kit. In brief, a 50  $\mu$ l reaction mixture contained: 5.0  $\mu$ l of 10x reaction buffer supplied with the kit, 2.5  $\mu$ l of phosphorylated forward primer (0.5  $\mu$ l of each forward primer shown in Table 15), 2.5  $\mu$ l reverse primer (0.625  $\mu$ l of each forward primer shown at Table 15), 2.5  $\mu$ l of A07B5 (50 ng/ $\mu$ l), 2.5  $\mu$ l Chang\_IT enzyme and 35  $\mu$ l of water. This reaction mixture was placed into thin well 200  $\mu$ l-capacity PCR tubes and the following PCR reaction program were used for the PCR: The starting temperature was 95°C for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 95°C for 30 sec, 55°C for 30 sec, and 68°C for 20 min. At the completion of the temperature cycling, the samples were kept at 68°C for 25 min more, and then held at 4°C for later processing. The PCR reaction was cleaned up using the Zymo DNA clean-up kit (Zymo Research Corporation, Orange CA, #D4004). DNA was eluted out of membrane using 84  $\mu$ l of water. The DNA template was removed with Dpn I (Promega, Madison WI, #R6231) at 37 °C for 3 hr (reaction mixture: 10  $\mu$ l of 10x reaction buffer, 1.0  $\mu$ l BSA, 6.0  $\mu$ l of Dpn I and 83  $\mu$ l cleaned PCR DNA). The Dpn I digested DNA was cleaned up again with Zymo DNA clean-up kit and digested again with Dpn I to completely remove the DNA template (reaction mixture: 1.5  $\mu$ l of 10x reaction buffer, 0.15  $\mu$ l BSA, 0.85  $\mu$ l of Dpn I and 83  $\mu$ l cleaned PCR DNA).

The Dpn I processed DNA mixture was cleaned up using Zymo DNA clean-up kit following the manufacturer's protocol. The cleaned-up DNA was used to transform an electro-competent strain of *E. coli* Bw25113 ( $\Delta ilvC$ ) using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, CA). The transformed clones were streaked on agar plates containing the LB medium and 100  $\mu$ g/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, CA) and incubated at 37 °C overnight. Clones were screened for improved activity using NADH.  $K_M$  for the improved mutants was measured (Table 16).

Table 15: Primers for example 6



| Targeted position(s) of K9-KARI | Primers                                                                                                                                                                                                                                                                                                                 |
|---------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 131                             | K9_131L_112210f: ggacgttgatgtcTTGatgatcgctcc SEQ ID NO: 166<br>K9_131A_112210f: ggacgttgatgtcGCAatgatcgctcc SEQ ID NO: 167<br>K9_131V_112210f: ggacgttgatgtcGTTatgatcgctcc SEQ ID NO: 168<br>K9_131M_112210f: ggacgttgatgtcATGatgatcgctcc SEQ ID NO: 169<br>K9_131C_112210f: ggacgttgatgtcTGAatgatcgctcc SEQ ID NO: 170 |
| 191                             | K9_191D_112210r: GGTTCAGTTTCGTCTCTGAAGGTAGTTTC SEQ ID NO: 171<br>K9_191C_112210r: GGTTCAGTTTCGCATCTGAAGGTAGTTTC SEQ ID NO: 172<br>K9_191S_112210r: GGTTCAGTTTCCGATCTGAAGGTAGTTTC SEQ ID NO: 173<br>K9_191G_112210r: GGTTCAGTTTCGCCTCTGAAGGTAGTTTC SEQ ID NO: 174                                                        |

Table 16

List of some mutants with their measured  $K_M$  values

| Mutant | SEQ ID NO:<br>(nucleic acid,<br>amino acid) | Mutations                       | $K_M$ ( $\mu$ M)<br>(NADH) | $K_M$ ( $\mu$ M)<br>(NADPH) |
|--------|---------------------------------------------|---------------------------------|----------------------------|-----------------------------|
| AWB9   | 52, 53                                      | S56A/S58D/I86V/N87P/T131A       | 10                         | 4                           |
| AWC1   | 54, 55                                      | S56A/S58D/I86V/N87P/T131V       | 9                          | 3                           |
| AWD6   | 62, 63                                      | S56A/S58D/I86V/N87P/T131V/T191S | 5                          | 2                           |
| AWD10  | 64, 65                                      | S56A/S58D/I86V/N87P/T131A/T191C | 8                          | 3                           |
| AWF4   | 56, 57                                      | S56A/S58D/I86V/N87P/N107S/T131V | 7                          | 3                           |
| AWF6   | 58, 59                                      | S56A/S58D/I86V/N87P/T131V/T191D | 7                          | 2                           |
| AWG4   | 50, 51                                      | S56A/S58D/I86V/N87P/T131M       | 7                          | 3                           |
| AWH3   | 60, 61                                      | S56A/S58D/I86V/N87P/T131V/T191G | 6                          | 2                           |
| AS6F1  | 48, 49                                      | S56A/S58D/I86V/N87P/T131M/T191G | 4                          | 1                           |

5

Example 7Isobutanol production from K9 KARI variants

The following variants of K9 KARI were generated as described above.

10

Table 17.

KARI variants and the corresponding yeast expression vectors

| Clone Names | Yeast Vector Name      | Nucleotide Point Mutation Locations |     |     |     |     | Amino Acid Mutation Positions |
|-------------|------------------------|-------------------------------------|-----|-----|-----|-----|-------------------------------|
|             |                        | 166                                 | 168 | 172 | 173 | 174 |                               |
| WT K9 KARI  | pHR81-Pilv5-KARI-K9    | T                                   | T   | T   | C   | C   | S56, S58                      |
| AB1G9       | pHR81-Pilv5-KARI-K9.G9 | G                                   | G   | G   | A   | G   | S56A, S58E                    |
| 495B5       | pHR81-Pilv5-KARI-K9.B5 | G                                   | T   | C   | A   | T   | S56A, S58H                    |
| AB1D3       | pHR81-Pilv5-KARI-K9.D3 | G                                   | T   | G   | A   | T   | S56A, S58D                    |
| AB1D1       | pHR81-Pilv5-KARI-K9.D1 | G                                   | T   | T   | C   | C   | S56A                          |

5

The yeast expression plasmids were made by subcloning of the variant KARI genes from *E.coli* vectors (pBAD.KARI) into pHR81-Pilv5-Pf5.KARI vector pLH556 (Figure 4, SEQ ID NO: 138) at PmeI and SfiI sites. Yeast pathway strains were made in PNY2204 host (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-pUC19-loxP-kanMX-loxP-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD\_LI(y)-ADH1t; Example 13) by co-transforming the KARI vectors as pathway plasmid #1, and pBP915 (pRS423-P<sub>FBA1</sub>-DHAD-P<sub>GPM1</sub>-hADH1; SEQ ID NO: 182) as pathway plasmid #2. Transformants were patched to the same medium containing 2% glucose and 0.1% ethanol as carbon sources. Three patches were tested for isobutanol production under microaerobic conditions in serum vials. A clone that was transformed with pBP915 and the pLH702 plasmid which expresses K9D3 was designated PNY1910.

20

Yeast colonies from the transformation on SE-Ura-His plates appeared after 5-7 days. The colonies were patched onto fresh SE-Ura-His plates, incubate at 30°C for 3 days. The patched cells were inoculated into 25 mL SEG-Ura,HIS media with 0.2% glucose and 0.2% ethanol and grown aerobically for 1-2 days at 30°C, to 2-3 OD. The cells were centrifuged and re-suspended in 1 mL of SEG-Ura,HIS media (2% glucose, 0.1% ethanol, 10 mg/L ergosterol, 50 mM MES, pH 5.5, thiamine 30 mg/L, nicotinic acid 30 mg/L). A calculated amount of cells were transferred to 45 mL total volume of the same media for a starting OD=0.2 in a 60 mL serum vial, with the top closed tightly by a crimper. This step was done in the regular bio-hood in air. The serum vials were incubated at 30C, 200 rpm for 2 days. At 48 h, the samples were removed for OD and HPLC analysis of glucose, isobutanol and pathway intermediates. 24 h samples were taken in an anaerobic chamber to maintain the anaerobic condition in the serum vials. In the initial phase of the 48h incubation, the air present in the head space (~15 mL) and the liquid media is consumed by the growing yeast cells. After the oxygen in the head space is consumed, the culture becomes anaerobic. Therefore this experiment includes switching condition from aerobic to oxygen limiting and anaerobic conditions.

Of the four K9 variants, AB1G9 and AB1D3 produced relatively high isobutanol titers, while 495B5 and AB1D1 have lower titer. Wild type K9 KARI strain produced the lowest titer. While not wishing to be bound by theory, it is believed that the lower titer is due to the shifted balance of NADH and NADPH when cells are switched from aerobic to anaerobic conditions. By this rationale, under anaerobic conditions, NADH concentration and availability increased significantly, favoring the variant KARI enzymes that use NADH. Based on the kinetic analysis, AB1G9 ("K9G9") and AB1D3 ("K9D3") mutants have relatively high  $K_M$  for NADPH (23 & 9.2  $\mu\text{M}$ ), in addition to their relative low  $K_M$  for NADH (47 & 38  $\mu\text{M}$ ). As comparison, 495B5 and AB1D1's  $K_M$ 's are 2.5 and 1.1  $\mu\text{M}$  respectively for NADPH, and wt K9's  $K_M$  is 0.10  $\mu\text{M}$ . The low NADH  $K_M$  of AB1G9 and AB1D3, together with the high NADPH  $K_M$  of AB1G9 and AB1D3 may

have led to reduced NADPH utilization under anaerobic conditions, and relatively high NADH utilization. As evidence, AB1G9 and AB1D3 have lower glycerol accumulation (isobutanol:glycerol = 3.3) compared to 495B5 and AB1D1 (2-3). The isobutanol:glycerol ratio is for the wild type K9 is 1:1 under the same switched aerobic to anaerobic condition.

Table 18.

Kinetic properties of wild type and variant K9 KARI enzymes, and isobutanol titer and productivity measured from aerobic to anaerobic switch experiment in serum vials.

| Clone Names | K <sub>M</sub> (NADPH) | NADPH V <sub>max</sub> (U/mg) | K <sub>M</sub> (NADH) | NADH V <sub>max</sub> (U/mg) | Isobutanol g/L | Effective Isobutanol Productivity (g/g cells) |
|-------------|------------------------|-------------------------------|-----------------------|------------------------------|----------------|-----------------------------------------------|
| WT K9 KARI  | 0.19                   | 2.0                           | 326                   | 1.5                          | 0.9            | 3.1                                           |
| AB1G9       | 23                     | 2.4                           | 47                    | 2.0                          | 3.4            | 10.5                                          |
| 495B5       | 3.5                    | 2.5                           | 44                    | 1.9                          | 2.2            | 9.2                                           |
| AB1D3       | 9.2                    | 2.1                           | 38                    | 1.9                          | 3.3            | 10.8                                          |
| AB1D1       | 1.1                    | 2.8                           | 164                   | 2.1                          | 2.3            | 9.5                                           |

### Example 8

#### Construction of *Saccharomyces cerevisiae* strains BP1135 (PNY1505) and PNY1507 and Isobutanol-Producing Derivatives

This example describes construction of *Saccharomyces cerevisiae* strains BP1135 and PNY1507. These strains were derived from PNY1503 (BP1064). PNY1503 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands). BP1135 contains an additional deletion of the *FRA2* gene.

PNY1507 was derived from BP1135 with additional deletion of the *ADH1* gene, with integration of the *kivD* gene from *Lactococcus lactis*, codon optimized for expression in *Saccharomyces cerevisiae*, into the *ADH1* locus.

5           Deletions/integrations were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and the *URA3* gene for selection of transformants. The *URA3* gene was removed by homologous recombination to create a scarless deletion/integration.

10           The scarless deletion/integration procedure was adapted from Akada *et al.*, *Yeast*, 23:399, 2006. In general, the PCR cassette for each deletion/integration was made by combining four fragments, A-B-U-C, and the gene to be integrated by cloning the individual fragments into a plasmid prior to the entire cassette being amplified by PCR for the  
15           deletion/integration procedure. The gene to be integrated was included in the cassette between fragments A and B. The PCR cassette contained a selectable/counter-selectable marker, *URA3* (Fragment U), consisting of the native CEN.PK 113-7D *URA3* gene, along with the promoter (250 bp upstream of the *URA3* gene) and terminator (150 bp downstream of the  
20           *URA3* gene) regions. Fragments A and C (each approximately 100 to 500 bp long) corresponded to the sequence immediately upstream of the target region (Fragment A) and the 3' sequence of the target region (Fragment C). Fragments A and C were used for integration of the cassette into the chromosome by homologous recombination. Fragment B  
25           (500 bp long) corresponded to the 500 bp immediately downstream of the target region and was used for excision of the *URA3* marker and Fragment C from the chromosome by homologous recombination, as a direct repeat of the sequence corresponding to Fragment B was created upon integration of the cassette into the chromosome.

30

#### *FRA2* Deletion

The *FRA2* deletion was designed to delete 250 nucleotides from the 3' end of the coding sequence, leaving the first 113 nucleotides of the

*FRA2* coding sequence intact. An in-frame stop codon was present 7 nucleotides downstream of the deletion. The four fragments for the PCR cassette for the scarless *FRA2* deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and

5 CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). *FRA2* Fragment A was amplified with primer oBP594 (SEQ ID NO: 183) and primer oBP595 (SEQ ID NO: 184), containing a 5' tail with homology to the 5' end of *FRA2* Fragment B. *FRA2* Fragment B was amplified with primer oBP596 (SEQ

10 ID NO: 185), containing a 5' tail with homology to the 3' end of *FRA2* Fragment A, and primer oBP597 (SEQ ID NO:186), containing a 5' tail with homology to the 5' end of *FRA2* Fragment U. *FRA2* Fragment U was amplified with primer oBP598 (SEQ ID NO: 187), containing a 5' tail with homology to the 3' end of *FRA2* Fragment B, and primer oBP599 (SEQ ID

15 NO: 188), containing a 5' tail with homology to the 5' end of *FRA2* Fragment C. *FRA2* Fragment C was amplified with primer oBP600 (SEQ ID NO:189), containing a 5' tail with homology to the 3' end of *FRA2* Fragment U, and primer oBP601 (SEQ ID NO:190). PCR products were purified with a PCR Purification kit (Qiagen). *FRA2* Fragment AB was

20 created by overlapping PCR by mixing *FRA2* Fragment A and *FRA2* Fragment B and amplifying with primers oBP594 (SEQ ID NO:183) and oBP597 (SEQ ID NO:186). *FRA2* Fragment UC was created by overlapping PCR by mixing *FRA2* Fragment U and *FRA2* Fragment C and amplifying with primers oBP598 (SEQ ID NO:187) and oBP601 (SEQ ID

25 NO:190). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The *FRA2* ABUC cassette was created by overlapping PCR by mixing *FRA2* Fragment AB and *FRA2* Fragment UC and amplifying with primers oBP594 (SEQ ID NO:183 and oBP601 (SEQ ID NO:190). The PCR product was purified with a PCR

30 Purification kit (Qiagen).

Competent cells of PNY1503 were made and transformed with the *FRA2* ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, CA). Transformation mixtures were plated on

synthetic complete media lacking uracil supplemented with 1% ethanol at 30 °C. Transformants with a *fra2* knockout were screened for by PCR with primers oBP602 (SEQ ID NO:191) and oBP603 (SEQ ID NO:192) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen).

- 5 A correct transformant was grown in YPE (yeast extract, peptone, 1% ethanol) and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30 °C to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR with primers oBP602 (SEQ ID NO:191) and oBP603 (SEQ ID NO:192) using genomic
- 10 DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the *FRA2* gene from the isolate was demonstrated by a negative PCR result using primers specific for the deleted coding sequence of *FRA2*, oBP605 (SEQ ID NO:193) and oBP606 (SEQ ID NO:194). The correct isolate was selected as strain CEN.PK 113-7D
- 15 MATa *ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t pdc5Δ..P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ* and designated as PNY1505 (BP1135).

- This strain was transformed with isobutanol pathway plasmids (pYZ090, SEQ ID NO: 195) and pLH468 (SEQ ID NO: 139), and one clone
- 20 was designated BP1168 (PNY1506).

- pYZ090 (SEQ ID NO: 195) was constructed to contain a chimeric gene having the coding region of the *alsS* gene from *Bacillus subtilis* (nt position 457-2172) expressed from the yeast CUP1 promoter (nt 2-449) and followed by the CYC1 terminator (nt 2181-2430) for expression of
- 25 ALS, and a chimeric gene having the coding region of the *ilvC* gene from *Lactococcus lactis* (nt 3634-4656) expressed from the yeast ILV5 promoter (2433-3626) and followed by the ILV5 terminator (nt 4682-5304) for expression of KARI.

### 30 *ADH1* Deletion and *kivD* LI(y) Integration

The *ADH1* gene was deleted and replaced with the *kivD* coding region from *Lactococcus lactis* codon optimized for expression in *Saccharomyces cerevisiae*. The scarless cassette for the *ADH1* deletion-

*kivD\_LI(y)* integration was first cloned into plasmid pUC19-URA3MCS, as described in U.S. Appln. No. 61/356379, filed June 18, 2010.

The vector is pUC19 based and contains the sequence of the *URA3* gene from *Saccharomyces cerevisiae* CEN.PK 113-7D situated within a multiple cloning site (MCS). pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for *URA3*, the sequences from upstream (250 bp) and downstream (150 bp) of this gene are present for expression of the *URA3* gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.

The *kivD* coding region from *Lactococcus lactis* codon optimized for expression in *Saccharomyces cerevisiae* was amplified using pLH468 (SEQ ID NO:139) as template with primer oBP562 (SEQ ID NO:197), containing a PmeI restriction site, and primer oBP563 (SEQ ID NO:198), containing a 5' tail with homology to the 5' end of ADH1 Fragment B. ADH1 Fragment B was amplified from genomic DNA prepared as above with primer oBP564 (SEQ ID NO:199), containing a 5' tail with homology to the 3' end of *kivD\_LI(y)*, and primer oBP565 (SEQ ID NO:200), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). *kivD\_LI(y)*-ADH1 Fragment B was created by overlapping PCR by mixing the *kivD\_LI(y)* and ADH1 Fragment B PCR products and amplifying with primers oBP562 (SEQ ID NO:197) and oBP565 (SEQ ID NO:200). The resulting PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. ADH1 Fragment A was amplified from genomic DNA with primer oBP505 (SEQ ID NO:201), containing a SacI restriction site, and primer oBP506 (SEQ ID NO:202), containing an AscI restriction site. The ADH1 Fragment A PCR product was digested with SacI and AscI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing *kivD\_LI(y)*-ADH1 Fragment B. ADH1 Fragment C was amplified from genomic DNA with primer oBP507 (SEQ ID NO:203), containing a PacI restriction site, and



primer oBP508 (SEQ ID NO:204), containing a Sall restriction site. The ADH1 Fragment C PCR product was digested with PaeI and Sall and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing ADH1 Fragment A-kivD\_LI(y)-ADH1 Fragment B. The hybrid promoter UAS(PGK1)-P<sub>FBA1</sub> was amplified from vector pRS316-  
 5 UAS(PGK1)-P<sub>FBA1</sub>-GUS (SEQ ID NO:209) with primer oBP674 (SEQ ID NO:205), containing an Ascl restriction site, and primer oBP675 (SEQ ID NO:206), containing a PmeI restriction site. The UAS(PGK1)-P<sub>FBA1</sub> PCR product was digested with Ascl and PmeI and ligated with T4 DNA ligase  
 10 into the corresponding sites of the plasmid containing kivD\_LI(y)-ADH1 Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP505 (SEQ ID NO:201) and oBP508 (SEQ ID NO:204) and purified with a PCR Purification kit (Qiagen).

Competent cells of PNY1505 were made and transformed with the  
 15 ADH1-kivD\_LI(y) PCR cassette constructed above using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30 °C. Transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium containing 5-fluoro-orotic acid  
 20 (0.1%) at 30 °C to select for isolates that lost the URA3 marker. The deletion of *ADH1* and integration of *kivD\_LI(y)* were confirmed by PCR with external primers oBP495 (SEQ ID NO:207) and oBP496 (SEQ ID NO:208) and with *kivD\_LI(y)* specific primer oBP562 (SEQ ID NO:197) and external primer oBP496 (SEQ ID NO:208) using genomic DNA  
 25 prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D MATa *ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1tpdc5Δ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ adh1Δ::UAS(PGK1)P[FBA1]-kivD\_LI(y)-ADH1t* and designated as PNY1507 (BP1201). PNY1507 was  
 30 transformed with isobutanol pathway plasmids pYZ090 (SEQ ID NO:195) and pBP915 (SEQ ID NO: 182) and the resultant strain was named PNY1513.

#### Construction of the pRS316-UAS(PGK1)-FBA1p-GUS vector

To clone a cassette UAS(PGK1)-FBA1p (SEQ ID NO:766, first a 602bp FBA1 promoter (FBA1p) was PCR-amplified from genomic DNA of CEN.PK with primers T-FBA1(Sall) (SEQ ID NO:767) and B-FBA1(SpeI) (SEQ ID NO:768), and cloned into Sall and SpeI sites on the plasmid

5 pWS358-PGK1p-GUS (SEQ ID NO:769) after the PGK1p promoter was removed with a Sall/SpeI digest of the plasmid, yielding pWS358-FBA1p-GUS. The pWS358-PGK1p-GUS plasmid was generated by inserting a PGK1p and beta-glucuronidase gene (GUS) DNA fragments into multiple cloning site of pWS358, which was derived from pRS423 vector

10 (Christianson et al., Gene, 110:119-122, 1992). Secondly, the resulting pWS358-FBA1p-GUS plasmid was digested with Sall and SacI, a DNA fragment containing a FBA1p promoter, GUS gene, and FBA1 terminator gel-purified, and cloned into Sall/SacI sites on pRS316 to create pRS316-FBA1p-GUS. Thirdly, a 118bp DNA fragment containing an upstream

15 activation sequence (UAS) located between positions -519 and -402 upstream of the 3-phosphoglycerate kinase (PGK1) open reading frame, namely UAS(PGK1), was PCR-amplified from genomic DNA of CEN.PK with primers T-U/PKG1(KpnI) (SEQ ID NO:770) and B-U/PKG1(Sall) (SEQ ID NO:771). The PCR product was digested with KpnI and Sall and

20 cloned into KpnI/Sall sites on pRS316-FBA1p-GUS to create pRS316-UAS(PGK1)-FBA1p-GUS.

#### Example 9

##### 25 Improved recombinant host cells comprising elimination of ALD6

The purpose of this example is to describe methods to modify a yeast host strain for improved production of isobutanol. These modifications include integration of genes encoding isobutyraldehyde reductase activity and elimination of the native genes ALD6 and

30 YMR226c, encoding NADP<sup>+</sup>-dependent acetaldehyde dehydrogenase and a NADPH-dependent dehydrogenase, respectively.

##### Construction of *S. cerevisiae* Strain PNY2211

PNY2211 was constructed in several steps from *S. cerevisiae* strain PNY1507 (Example 8) as described in the following paragraphs. First PNY1507 was modified to contain a phosphoketolase gene. Next, an acetolactate synthase gene (*alsS*) was added to the strain, using an

5 integration vector targeted to sequences adjacent to the phosphoketolase gene. Finally, homologous recombination was used to remove the phosphoketolase gene and integration vector sequences, resulting in a scarless insertion of *alsS* in the intergenic region between *pdv1Δ::ilvD* (described in Example 12) and the native *TRX1* gene of chromosome XII.

10 The resulting genotype of PNY2211 is MATa *ura3Δ::loxP* *his3Δ* *pdv6Δ* *pdv1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-CYC1t* *pdv5Δ::P[PDC5]-ADH| sadB\_Ax-PDC5t* *gpd2Δ::loxP* *fra2Δ* *adh1Δ::UAS(PGK1)P[FBA1]-kivD\_LI(y)-ADH1t*.

A phosphoketolase gene cassette was introduced into PNY1507 by

15 homologous recombination. The integration construct was generated as follows. The plasmid pRS423::CUP1-*alsS*+FBA-*budA* (previously described in US2009/0305363)

was digested with *NotI* and *XmaI* to remove the 1.8 kb FBA-*budA* sequence, and the vector was religated after treatment with Klenow

20 fragment. Next, the CUP1 promoter was replaced with a TEF1 promoter variant (M4 variant previously described by Nevoigt *et al. Appl. Environ. Microbiol.* 72: 5266-5273 (2006)),

via DNA synthesis and vector construction service from DNA2.0 (Menlo Park, CA). The resulting plasmid, pRS423::TEF(M4)-*alsS*

25 was cut with *StuI* and *MluI* (removes 1.6 kb portion containing part of the *alsS* gene and *CYC1* terminator), combined with the 4 kb PCR product generated from pRS426::GPD-*xpk1*+ADH-*eutD* (SEQ ID NO:383) with primers N1176 (SEQ ID NO:282) and N1177 (SEQ ID NO:283) and an 0.8 kb PCR product DNA (SEQ ID NO: 284) generated from yeast genomic

30 DNA (*ENO1* promoter region) with primers N822 (SEQ ID NO:285) and N1178 (SEQ ID NO:286) and transformed into *S. cerevisiae* strain BY4741 (ATCC #201388); gap repair cloning methodology, see Ma *et al. Gene* 58:201-216 (1987). Transformants were obtained by plating cells on

synthetic complete medium without histidine. Proper assembly of the expected plasmid (pRS423::TEF(M4)-xpk1+ENO1-eutD, SEQ ID NO:293) was confirmed by PCR (primers N821 (SEQ ID NO:287) and N1115 (SEQ ID NO:288)) and by restriction digest (BglI). Two clones were

5 subsequently sequenced. The 3.1 kb TEF(M4)-xpk1 gene was isolated by digestion with *SacI* and *NotI* and cloned into the pUC19-URA3::ilvD-TRX1 vector (Clone A, cut with *AflI*). Cloning fragments were treated with Klenow fragment to generate blunt ends for ligation. Ligation reactions were transformed into *E. coli* Stbl3 cells, selecting for ampicillin resistance.

10 Insertion of TEF(M4)-xpk1 was confirmed by PCR (primers N1110 (SEQ ID NO:367) and N1114 (SEQ ID NO:290)). The vector was linearized with *AflI* and treated with Klenow fragment. The 1.8 kb *KpnI*-*HincII* geneticin resistance cassette (SEQ ID NO: 384) was cloned by ligation after Klenow fragment treatment. Ligation reactions were transformed into *E. coli* Stbl3

15 cells, selecting for ampicillin resistance. Insertion of the geneticin cassette was confirmed by PCR (primers N160SeqF5 (SEQ ID NO:210) and BK468 (SEQ ID NO:368)). The plasmid sequence is provided as SEQ ID NO: 291 (pUC19-URA3::pdc1::TEF(M4)-xpk1::kan).

The resulting integration cassette (pdc1::TEF(M4)-

20 xpk1::KanMX::TRX1) was isolated (*AscI* and *NaeI* digestion generated a 5.3 kb band that was gel purified) and transformed into PNY1507 using the Zymo Research Frozen-EZ Yeast Transformation Kit (Cat. No. T2001). Transformants were selected by plating on YPE plus 50 µg/ml G418. Integration at the expected locus was confirmed by PCR (primers N886

25 (SEQ ID NO:211) and N1214 (SEQ ID NO:281)). Next, plasmid pRS423::GAL1p-Cre (SEQ ID NO:271), encoding Cre recombinase, was used to remove the loxP-flanked KanMX cassette. Proper removal of the cassette was confirmed by PCR (primers oBP512 (SEQ ID NO: 337) and N160SeqF5 (SEQ ID NO:210)). Finally, the *alsS* integration plasmid

30 described in Example 13, pUC19-kan::pdc1::FBA-*alsS*::TRX1, clone A) was transformed into this strain using the included geneticin selection marker. Two integrants were tested for acetolactate synthase activity by transformation with plasmids pYZ090Δ*alsS* (SEQ ID NO:371) and pBP915

(SEQ ID NO:182) (transformed using Protocol #2 in Amberg, Burke and Strathern "Methods in Yeast Genetics" (2005)), and evaluation of growth and isobutanol production in glucose-containing media (methods for growth and isobutanol measurement are as follows: All strains were grown  
5 in synthetic complete medium, minus histidine and uracil containing 0.3 % glucose and 0.3 % ethanol as carbon sources (10 mL medium in 125 mL vented Erlenmeyer flasks (VWR Cat. No. 89095-260). After overnight incubation (30 °C, 250 rpm in an Innova@40 New Brunswick Scientific Shaker), cultures were diluted back to 0.2 OD (Eppendorf BioPhotometer  
10 measurement) in synthetic complete medium containing 2% glucose and 0.05% ethanol (20 ml medium in 125 mL tightly-capped Erlenmeyer flasks (VWR Cat. No. 89095-260)). After 48 hours incubation (30 °C, 250 rpm in an Innova@40 New Brunswick Scientific Shaker), culture supernatants (collected using Spin-X centrifuge tube filter units, Costar Cat. No. 8169)  
15 were analyzed by HPLC per methods described in U.S. Appl. Pub. No. 2007/0092957)

One of the two clones was positive and was named PNY2218.

PNY2218 was treated with Cre recombinase and the resulting clones were screened for loss of the xpk1 gene and pUC19 integration  
20 vector sequences by PCR (primers N886 (SEQ ID NO: 211) and N160SeqR5 (SEQ ID NO: 388)). This left only the alsS gene integrated in the pdc1-TRX1 intergenic region after recombination the DNA upstream of xpk1 and the homologous DNA introduced during insertion of the integration vector (a "scarless" insertion since vector, marker gene and  
25 loxP sequences are lost). Although this recombination could have occurred at any point, the vector integration appeared to be stable even without geneticin selection and the recombination event was only observed after introduction of the Cre recombinase. One clone was designated PNY2211.

30 An isolate of PNY2218 containing the plasmids pYZ090ΔalsS and pBP915 was designated PNY2209.

PNY1528 (hADH integrations in PNY2211)

Deletions/integrations were created by homologous recombination with PCR products containing regions of homology upstream and downstream of the target region and the *URA3* gene for selection of transformants. The *URA3* gene was removed by homologous

5 recombination to create a scarless deletion/integration.

YPRCΔ15 deletion and horse liver *adh* integration

The YPRCΔ15 locus was deleted and replaced with the horse liver *adh* gene, codon optimized for expression in *Saccharomyces cerevisiae*, along with the PDC5 promoter region (538 bp) from *Saccharomyces*  
10 *cerevisiae* and the ADH1 terminator region (316 bp) from *Saccharomyces cerevisiae*. The scarless cassette for the YPRCΔ15 deletion- P[PDC5]-*adh*\_HL(y)-ADH1t integration was first cloned into plasmid pUC19-URA3MCS (described in Example 8).

Fragments A-B-U-C were amplified using Phusion High Fidelity  
15 PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). YPRCΔ15 Fragment A was amplified from genomic DNA with primer oBP622 (SEQ ID NO: 212), containing a KpnI restriction site, and primer oBP623 (SEQ ID NO: 213),  
20 containing a 5' tail with homology to the 5' end of YPRCΔ15 Fragment B. YPRCΔ15 Fragment B was amplified from genomic DNA with primer oBP624 (SEQ ID NO: 214), containing a 5' tail with homology to the 3' end of YPRCΔ15 Fragment A, and primer oBP625 (SEQ ID NO: 215), containing a FseI restriction site. PCR products were purified with a PCR  
25 Purification kit (Qiagen). YPRCΔ15 Fragment A - YPRCΔ15 Fragment B was created by overlapping PCR by mixing the YPRCΔ15 Fragment A and YPRCΔ15 Fragment B PCR products and amplifying with primers oBP622 (SEQ ID NO: 212) and oBP625 (SEQ ID NO: 215). The resulting PCR product was digested with KpnI and FseI and ligated with T4 DNA ligase  
30 into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. YPRCΔ15 Fragment C was amplified from genomic DNA with primer oBP626 (SEQ ID NO: 216), containing a NotI restriction site, and primer oBP627 (SEQ ID NO: 217), containing a PacI restriction

site. The YPRCΔ15 Fragment C PCR product was digested with NotI and  
 PacI and ligated with T4 DNA ligase into the corresponding sites of the  
 plasmid containing YPRCΔ15 Fragments AB. The PDC5 promoter region  
 was amplified from CEN.PK 113-7D genomic DNA with primer HY21 (SEQ  
 5 ID NO: 218), containing an Ascl restriction site, and primer HY24 (SEQ ID  
 NO: 219), containing a 5' tail with homology to the 5' end of adh\_HL(y).  
 adh\_HL(y)-ADH1t was amplified from pBP915 (SEQ ID NO: 182) with  
 primers HY25 (SEQ ID NO: 220), containing a 5' tail with homology to the  
 3' end of P[PDC5], and HY4 (SEQ ID NO: 221), containing a PmeI  
 10 restriction site. PCR products were purified with a PCR Purification kit  
 (Qiagen). P[PDC5]-adh\_HL(y)-ADH1t was created by overlapping PCR by  
 mixing the P[PDC5] and adh\_HL(y)-ADH1t PCR products and amplifying  
 with primers HY21 (SEQ ID NO: 218) and HY4 (SEQ ID NO: 221). The  
 resulting PCR product was digested with Ascl and PmeI and ligated with  
 15 T4 DNA ligase into the corresponding sites of the plasmid containing  
 YPRCΔ15 Fragments ABC. The entire integration cassette was amplified  
 from the resulting plasmid with primers oBP622 (SEQ ID NO: 212) and  
 oBP627 (SEQ ID NO: 217).

Competent cells of PNY2211 were made and transformed with the  
 20 YPRCΔ15 deletion- P[PDC5]-adh\_HL(y)-ADH1t integration cassette PCR  
 product using a Frozen-EZ Yeast Transformation II kit (Zymo Research;  
 Orange, CA). Transformation mixtures were plated on synthetic complete  
 media lacking uracil supplemented with 1% ethanol at 30 °C.  
 Transformants were screened for by PCR with primers URA3-end F (SEQ  
 25 ID NO: 222) and oBP637 (SEQ ID NO: 224). Correct transformants were  
 grown in YPE (1% ethanol) and plated on synthetic complete medium  
 supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at  
 30 C to select for isolates that lost the URA3 marker. The deletion of  
 YPRCΔ15 and integration of P[PDC5]-adh\_HL(y)-ADH1t were confirmed  
 30 by PCR with external primers oBP636 (SEQ ID NO: 223) and oBP637  
 (SEQ ID NO: 224) using genomic DNA prepared with a YeaStar Genomic  
 DNA kit (Zymo Research). A correct isolate of the following genotype was  
 selected for further modification: CEN.PK 113-7D MATa ura3Δ::loxP his3Δ

$pdc6\Delta$   $pdc1\Delta::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-$   
 $CYC1t$   $pdc5\Delta::P[PDC5]-ADH|sadB\_Ax-PDC5t$   $gpd2\Delta::loxP$   $fra2\Delta$   
 $adh1\Delta::UAS(PGK1)P[FBA1]-kivD\_LI(y)-ADH1t$   $yprc\Delta15\Delta::P[PDC5]-$   
 $ADH|adh\_HI-ADH1t$ .

5

#### Horse liver *adh* integration at *fra2* $\Delta$

The horse liver *adh* gene, codon optimized for expression in  
*Saccharomyces cerevisiae*, along with the *PDC1* promoter region (870 bp)  
 from *Saccharomyces cerevisiae* and the *ADH1* terminator region (316 bp)  
 10 from *Saccharomyces cerevisiae*, was integrated into the site of the *fra2*  
 deletion. The scarless cassette for the *fra2* $\Delta$ -  $P[PDC1]-adh\_HL(y)-ADH1t$   
 integration was first cloned into plasmid pUC19-URA3MCS.

Fragments A-B-U-C were amplified using Phusion High Fidelity  
 PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-  
 15 7D genomic DNA as template, prepared with a Gentra Puregene  
 Yeast/Bact kit (Qiagen; Valencia, CA). *fra2* $\Delta$  Fragment C was amplified  
 from genomic DNA with primer oBP695 (SEQ ID NO: 229), containing a  
 NotI restriction site, and primer oBP696 (SEQ ID NO: 230), containing a  
 PacI restriction site. The *fra2* $\Delta$  Fragment C PCR product was digested  
 20 with NotI and PacI and ligated with T4 DNA ligase into the corresponding  
 sites of pUC19-URA3MCS. *fra2* $\Delta$  Fragment B was amplified from genomic  
 DNA with primer oBP693 (SEQ ID NO: 227), containing a PmeI restriction  
 site, and primer oBP694 (SEQ ID NO: 228), containing a FseI restriction  
 site. The resulting PCR product was digested with PmeI and FseI and  
 25 ligated with T4 DNA ligase into the corresponding sites of the plasmid  
 containing *fra2* $\Delta$  fragment C after digestion with the appropriate enzymes.  
*fra2* $\Delta$  Fragment A was amplified from genomic DNA with primer oBP691  
 (SEQ ID NO: 225), containing BamHI and AsiSI restriction sites, and  
 primer oBP692 (SEQ ID NO: 226), containing AscI and SwaI restriction  
 30 sites. The *fra2* $\Delta$  fragment A PCR product was digested with BamHI and  
 AscI and ligated with T4 DNA ligase into the corresponding sites of the  
 plasmid containing *fra2* $\Delta$  fragments BC after digestion with the appropriate  
 enzymes. The *PDC1* promoter region was amplified from CEN.PK 113-7D



genomic DNA with primer HY16 (SEQ ID NO: 231), containing an *Ascl* restriction site, and primer HY19 (SEQ ID NO: 232), containing a 5' tail with homology to the 5' end of *adh\_Hl(y)*. *adh\_Hl(y)*-ADH1t was amplified from pBP915 with primers HY20 (SEQ ID NO: 233), containing a 5' tail with homology to the 3' end of P[PDC1], and HY4 (SEQ ID NO: 221), containing a *PmeI* restriction site. PCR products were purified with a PCR Purification kit (Qiagen). P[PDC1]-*adh\_Hl(y)*-ADH1t was created by overlapping PCR by mixing the P[PDC1] and *adh\_Hl(y)*-ADH1t PCR products and amplifying with primers HY16 (SEQ ID NO: 231) and HY4 (SEQ ID NO: 221). The resulting PCR product was digested with *Ascl* and *PmeI* and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing *fra2Δ* Fragments ABC. The entire integration cassette was amplified from the resulting plasmid with primers oBP691 (SEQ ID NO: 225) and oBP696 (SEQ ID NO: 230).

Competent cells of the PNY2211 variant with *adh\_Hl(y)* integrated at *YPRCΔ15* were made and transformed with the *fra2Δ*-P[PDC1]-*adh\_Hl(y)*-ADH1t integration cassette PCR product using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30 °C. Transformants were screened for by PCR with primers URA3-end F (SEQ ID NO: 222) and oBP731 (SEQ ID NO: 235). Correct transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoro-orotic acid (0.1%) at 30 °C to select for isolates that lost the *URA3* marker. The integration of P[PDC1]-*adh\_Hl(y)*-ADH1t was confirmed by colony PCR with internal primer HY31 (SEQ ID NO: 236) and external primer oBP731 (SEQ ID NO: 235) and PCR with external primers oBP730 (SEQ ID NO: 234) and oBP731 (SEQ ID NO: 235) using genomic DNA prepared with a YeaStar Genomic DNA kit (Zymo Research). A correct isolate of the following genotype was designated PNY1528: CEN.PK 113-7D MATa *ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-*

ADH|adh<sub>HI</sub>-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD<sub>LI(y)</sub>-ADH1t  
yprcΔ15Δ::P[PDC5]-ADH|adh<sub>HI</sub>-ADH1t.

PNY2237 (scarless YMR226c deletion)

5           The gene YMR226c was deleted from *S. cerevisiae* strain  
PNY1528 by homologous recombination using a PCR amplified 2.0 kb  
linear scarless deletion cassette. The cassette was constructed from  
spliced PCR amplified fragments comprised of the URA3 gene, along with  
10 downstream homology sequences flanking the YMR226c gene  
chromosomal locus to promote integration of the deletion cassette and  
removal of the native intervening sequence and a repeat sequence to  
promote recombination and removal of the URA3 marker. Forward and  
reverse PCR primers (N1251 and N1252, SEQ ID NOs: 247 and 248,  
15 respectively), amplified a 1,208 bp URA3 expression cassette originating  
from pLA33 (pUC19::loxP-URA3-loxP (SEQ ID NO: 268)). Forward and  
reverse primers (N1253 and N1254, SEQ ID NOs: 249 and 250,  
respectively), amplified a 250 bp downstream homology sequence with a  
3' URA3 overlap sequence tag from a genomic DNA preparation of *S.*  
20 *cerevisiae* strain PNY2211 (above). Forward and reverse PCR primers  
(N1255 and N1256, SEQ ID NOs: 251 and 252, respectively) amplified a  
250 bp repeat sequence with a 5' URA3 overlap sequence tag from a  
genomic DNA preparation of *S. cerevisiae* strain PNY2211. Forward and  
reverse PCR primers (N1257 and N1258, SEQ ID NOs: 253 and 254,  
25 respectively) amplified a 250 bp upstream homology sequence with a 5'  
repeat overlap sequence tag from a genomic DNA preparation of *S.*  
*cerevisiae* strain PNY2211.

Approximately 1.5 μg of the PCR amplified cassette was  
transformed into strain PNY1528 (above) made competent using the  
30 ZYMO Research Frozen Yeast Transformation Kit and the transformation  
mix plated on SE 1.0% -uracil and incubated at 30<sup>0</sup>C for selection of cells  
with an integrated ymr226cΔ::URA3 cassette. Transformants appearing  
after 72 to 96 hours are subsequently short-streaked on the same medium

and incubated at 30°C for 24 to 48 hours. The short-streaks are screened for ymr226cΔ::URA3 by PCR, with a 5' outward facing URA3 deletion cassette-specific internal primer (N1249, SEQ ID NO: 245) paired with a flanking inward facing chromosome-specific primer (N1239, SEQ ID NO: 243) and a 3' outward-facing URA3 deletion cassette-specific primer (N1250, SEQ ID NO: 246) paired with a flanking inward-facing chromosome-specific primer (N1242, SEQ ID NO: 244). A positive PNY1528 ymr226cΔ::URA3 PCR screen resulted in 5' and 3' PCR products of 598 and 726 bp, respectively.

Three positive PNY1528 ymr226cΔ::URA3 clones were picked and cultured overnight in a YPE 1% medium of which 100μL was plated on YPE 1% + 5-FOA for marker removal. Colonies appearing after 24 to 48 hours were PCR screened for marker loss with 5' and 3' chromosome-specific primers (N1239 and N1242). A positive PNY1528 ymr226cΔ markerless PCR screen resulted in a PCR product of 801 bp. Multiple clones were obtained and one was designated PNY2237.

#### PNY2238 and PNY2243 (ALD6 deletion strains)

A vector was designed to replace the *ALD6* coding sequence with a Cre-lox recyclable URA3 selection marker. Sequences 5' and 3' of *ALD6* were amplified by PCR (primer pairs N1179 and N1180 and N1181 and N1182, respectively; SEQ ID NOs: 237, 238, 239, and 240, respectively). After cloning these fragments into TOPO vectors (Invitrogen Cat. No. K2875-J10) and sequencing (M13 forward (SEQ ID NO:269) and reverse (SEQ ID NO:270) primers), the 5' and 3' flanks were cloned into pLA33 (pUC19::loxP::URA3::loxP) (SEQ ID NO:268) at the *EcoRI* and *SphI* sites, respectively. Each ligation reaction was transformed into *E. coli* Stbl3 cells, which were incubated on LB Amp plates to select for transformants. Proper insertion of sequences was confirmed by PCR (primers M13 forward (SEQ ID NO: 269) and N1180 (SEQ ID NO:238) and M13 reverse (SEQ ID NO:270) and N1181 (SEQ ID NO:239), respectively).

The vector described above (pUC19::ald6Δ::loxP-URA3-loxP) was linearized with *AhdI* and transformed into PNY1528 and PNY2237 using

the standard lithium acetate method (except that incubation of cells with DNA was extended to 2.5h). Transformants were obtained by plating on synthetic complete medium minus uracil that provided 1% ethanol as the carbon source. Patched transformants were screened by PCR to confirm

5 the deletion/integration, using primers N1212 (SEQ ID NO: 241) and N1180 (5' end) (SEQ ID NO: 238) and N1181 (SEQ ID NO: 239) and N1213 (SEQ ID NO: 242) (3' end). A plasmid carrying Cre recombinase (pRS423::GAL1p-Cre = SEQ ID No. 271) was transformed into the strain using histidine marker selection. Transformants were passaged on YPE

10 supplemented with 0.5% galactose. Colonies were screened for resistance to 5-FOA (loss of URA3 marker) and for histidine auxotrophy (loss of the Cre plasmid). Proper removal of the URA3 gene via the flanking loxP sites was confirmed by PCR (primers N1262 and N1263, SEQ ID NOs: 255 and 256, respectively). Additionally, primers internal to

15 the ALD6 gene (N1230 and N1231; SEQ ID NOs: 261 and 262, respectively) were used to insure that no merodiploids were present. Finally, *ald6Δ::loxP* clones were screened by PCR to confirm that a translocation between *ura3Δ::loxP* (N1228 and N1229, SEQ ID NOs: 259 and 260) and *gpd2Δ::loxP* (N1223 and N1225, SEQ ID NOs: 257 and 258)

20 had not occurred. Two positive clones were identified from screening of transformants of PNY1528. Clone B has been designated PNY2243. Three positive clones were identified from screening transformants of PNY2237. Clones E and K were both assessed for isobutanol production at small scale (below). Although statistically identical in most parameters,

25 Clone E was selected (PNY2238) for further development.

### Example 10

#### Isobutanol pathway plasmids

The purpose of this example is to describe construction or

30 modification of isobutanol pathway plasmids for production of isobutanol in host strains.

pYZ067 (SEQ ID NO:374) was constructed to contain the following chimeric genes: 1) the coding region of the *ilvD* gene from *S. mutans*

UA159 with a C-terminal Lumio tag expressed from the yeast FBA1 promoter followed by the FBA1 terminator for expression of dihydroxy acid dehydratase, 2) the coding region for horse liver ADH expressed from the yeast GPM1 promoter followed by the ADH1 terminator for expression of  
5 alcohol dehydrogenase, and 3) the coding region of the KivD gene from *Lactococcus lactis* expressed from the yeast TDH3 promoter followed by the TDH3 terminator for expression of ketoisovalerate decarboxylase.

pYZ067 $\Delta$ kivD $\Delta$ hADH (SEQ ID NO: 385) was constructed from pYZ067 (SEQ ID NO: 374) by deleting the promoter-gene-terminator  
10 cassettes for both *kivD* and *adh*. pYZ067 was digested with BamHI and SacI (New England BioLabs; Ipswich, MA), and the 7934 bp fragment was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, CA). The isolated fragment of DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich,  
15 MA) and then self-ligated with T4 DNA ligase and used to transform competent TOP10 *Escherichia coli* (Invitrogen; Carlsbad, CA). Plasmids from transformants were isolated and checked for the proper deletion by sequence analysis. A correct plasmid isolate was designated pYZ067 $\Delta$ kivD $\Delta$ hADH.

20 pYZ067 $\Delta$ kivD $\Delta$ ilvD (SEQ ID NO: 772) was constructed to contain a chimeric gene having the coding region of the *adh* gene from horse liver (nt position 3148-2021), codon optimized for expression in *Saccharomyces cerevisiae*, expressed from the yeast GPM promoter (nt 3916-3160) and followed by the ADH1 terminator (nt 2012-1697) for  
25 expression of ADH. pYZ067 $\Delta$ kivD $\Delta$ ilvD was constructed from pYZ067 by deleting the promoter-gene-terminator cassettes for both *kivD* and *ilvD*. pYZ067 was digested with AatII and SacI (New England BioLabs; Ipswich, MA) and the 10196 bp fragment was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, CA). The isolated fragment of  
30 DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich, MA) and then self-ligated with T4 DNA ligase. The resulting plasmid was then digested with NgoMIV and BamHI (New England BioLabs; Ipswich, MA) and the 7533 bp fragment was purified on

an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, CA).

The isolated fragment of DNA was treated with DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs; Ipswich, MA) and then self-ligated with T4 DNA ligase. Plasmids were isolated and checked for the proper deletions by sequence analysis. A correct plasmid isolate was designated pYZ067DkivDDilvD.

pK9G9.OLE1p.ilvD (SEQ ID NO: 773), derived from pYZ090 (SEQ ID NO: 195), was constructed to contain a chimeric gene having the coding region of the ilvD gene from *Streptococcus mutans* (nt position 5377-3641) expressed from the yeast OLE1 promoter (nt 5986-5387) and followed by the FBA1 terminator (nt 3632-3320) for expression of DHAD, and a chimeric gene having the coding region of the variant K9G9 of the ilvC gene from *Anaerostipes caccae* (nucleic acid and amino acid SEQ ID NOs: 774 and 647) (nt 1628-2659) expressed from the yeast ILV5 promoter (nt 427-1620) and followed by the ILV5 terminator (nt 2685-3307) for expression of KARI. Construction of the plasmid was as follows. The chimeric gene from plasmid pYZ067 having the coding region of the ilvD gene from *Streptococcus mutans* expressed from the yeast FBA1 promoter and followed by the FBA1 terminator was ligated into pYZ090 after digestion with restriction enzymes NgoMIV and BamHI. The alsS coding region and 280 bp from the 3' end of the CUP1 promoter was deleted from the resulting plasmid by digesting with the restriction enzymes SpeI and PacI and self-ligating the resulting large DNA fragment. The yeast FBA1 promoter upstream of ilvD was removed from the resulting plasmid by digesting with the restriction enzymes NgoMIV and PmlI and was replaced with the yeast OLE1 promoter amplified with primers pOLE1-NgoMI (SEQ ID NO: 775) and pOLE1-PmlI (SEQ ID NO: 776). The coding region of the ilvC gene from *Lactococcus lactis* was deleted from the resulting plasmid by digestion with restriction enzymes PmeI and SfiI followed by gel purification of the large DNA fragment. The coding region of the variant K9G9 ilvC gene (SEQ ID NO: 777) from *Anaerostipes caccae* was digested out of pLH701 (SEQ ID NO: 778) with

PmeI and SfiI and gel purified. The two DNA fragments were ligated to generate pK9G9.OLE1p.ilvD.

#### EXAMPLE 11

##### 5                    Construction of PNY2240 and PNY2242

Strain PNY2240 was derived from PNY2211 after transformation with plasmids pLH702 (SEQ ID NO: 181) and pBP915 (SEQ ID NO: 182). Transformants were plated on synthetic complete medium without  
10 histidine or uracil (1% ethanol as carbon source). Transformants were patched to the same medium containing, instead, 2% glucose and 0.05% ethanol as carbon sources. Three patches were used to inoculate liquid medium (synthetic complete minus uracil with 0.3% glucose and 0.3% ethanol as carbon sources). To test isobutanol production, liquid cultures  
15 were sub-cultured into synthetic complete medium minus uracil containing 2% glucose and 0.05% ethanol as carbon sources that also contained BME vitamin mix (Sigma Cat. No. B6891). Cultures were incubated in sealed serum vials (10 ml medium in 15 ml vials) at 30°C with shaking (250 rpm in an Infors Multitron shaker). After 48 hours, culture medium  
20 was filtered (Spin-X column) and analyzed by HPLC (as described in US App. Pub. No. 2007/0092957).

One clone was designated PNY2240.

Strain PNY2242 was derived from PNY2238 after transformation with plasmids pLH702 (SEQ ID NO: 181) and pYZ067 $\Delta$ kivD $\Delta$ hADH  
25 (described herein above). Transformants were plated on synthetic complete medium without histidine or uracil (1% ethanol as carbon source). Transformants were patched to the same medium containing, instead, 2% glucose and 0.05% ethanol as carbon sources. Three patches were tested for isobutanol production, as described above. All  
30 three performed similarly in terms of glucose consumption and isobutanol production. One clone was designated PNY2242 and was further characterized under fermentation conditions, as described herein below.

### Example 12

#### Construction of *Saccharomyces cerevisiae* strain BP1064

##### (PNY1503)

5           The strain BP1064 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands) and contains deletions of the following genes: URA3, HIS3, PDC1, PDC5, PDC6, and GPD2. BP1064 was transformed with plasmids pYZ090 (SEQ ID NO:195) and pLH468 (SEQ ID NO:139) to create strain  
10   NGCI-070 (BP1083; PNY1504).

          Deletions, which completely removed the entire coding sequence, were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and either a G418 resistance marker or URA3 gene for selection of  
15   transformants. The G418 resistance marker, flanked by loxP sites, was removed using Cre recombinase (pRS423::PGAL1-cre; SEQ ID NO: 271). The URA3 gene was removed by homologous recombination to create a scarless deletion, or if flanked by loxP sites was removed using Cre recombinase.

##### 20   URA3 Deletion

          To delete the endogenous URA3 coding region, a *ura3::loxP-kanMX-loxP* cassette was PCR-amplified from pLA54 template DNA (SEQ ID NO:386). pLA54 contains the *K. lactis* TEF1 promoter and kanMX marker, and is flanked by loxP sites to allow recombination with Cre  
25   recombinase and removal of the marker. PCR was done using Phusion DNA polymerase and primers BK505 and BK506 (SEQ ID NOs:294 and 295). The URA3 portion of each primer was derived from the 5' region upstream of the URA3 promoter and 3' region downstream of the coding region such that integration of the loxP-kanMX-loxP marker resulted in  
30   replacement of the URA3 coding region. The PCR product was transformed into CEN.PK 113-7D using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on



YPD containing G418 (100 µg/ml) at 30 °C. Transformants were screened to verify correct integration by PCR using primers LA468 and LA492 (SEQ ID NOs:296 and 297) and designated CEN.PK 113-7D Δura3::kanMX.

#### HIS3 Deletion

- 5           The four fragments for the PCR cassette for the scarless HIS3 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs; Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen; Valencia, CA). HIS3 Fragment A was amplified with primer oBP452 (SEQ
- 10 ID NO:298) and primer oBP453 (SEQ ID NO:299), containing a 5' tail with homology to the 5' end of HIS3 Fragment B. HIS3 Fragment B was amplified with primer oBP454 (SEQ ID NO:300), containing a 5' tail with homology to the 3' end of HIS3 Fragment A, and primer oBP455 (SEQ ID
- 15 U. HIS3 Fragment U was amplified with primer oBP456 (SEQ ID NO:302), containing a 5' tail with homology to the 3' end of HIS3 Fragment B, and primer oBP457 (SEQ ID NO:303), containing a 5' tail with homology to the 5' end of HIS3 Fragment C. HIS3 Fragment C was amplified with primer
- 20 oBP458 (SEQ ID NO:304), containing a 5' tail with homology to the 3' end of HIS3 Fragment U, and primer oBP459 (SEQ ID NO:305). PCR products were purified with a PCR Purification kit (Qiagen). HIS3 Fragment AB was created by overlapping PCR by mixing HIS3 Fragment A and HIS3
- 25 Fragment B and amplifying with primers oBP452 (SEQ ID NO:298) and oBP455 (SEQ ID NO:301). HIS3 Fragment UC was created by overlapping PCR by mixing HIS3 Fragment U and HIS3 Fragment C and amplifying with primers oBP456 (SEQ ID NO:302) and oBP459 (SEQ ID
- 30 NO:305). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The HIS3 ABUC cassette was created by overlapping PCR by mixing HIS3 Fragment AB and HIS3
- Fragment UC and amplifying with primers oBP452 (SEQ ID NO:298) and oBP459 (SEQ ID NO:305). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-7D  $\Delta$ ura3::kanMX were made and transformed with the HIS3 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research; Orange, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30 °C. Transformants with a his3 knockout were screened for by PCR with primers oBP460 (SEQ ID NO:306) and oBP461 (SEQ ID NO:307) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain CEN.PK 113-7D  $\Delta$ ura3::kanMX  $\Delta$ his3::URA3.

10 KanMX Marker Removal from the  $\Delta$ ura3 Site and URA3 Marker Removal from the  $\Delta$ his3 Site

The KanMX marker was removed by transforming CEN.PK 113-7D  $\Delta$ ura3::kanMX  $\Delta$ his3::URA3 with pRS423::PGAL1-cre (SEQ ID NO: 271,) using a Frozen-EZ Yeast Transformation II kit (Zymo Research) and plating on synthetic complete medium lacking histidine and uracil supplemented with 2% glucose at 30 °C. Transformants were grown in YP supplemented with 1% galactose at 30 °C for ~6 hours to induce the Cre recombinase and KanMX marker excision and plated onto YPD (2% glucose) plates at 30 °C for recovery. An isolate was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30 °C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in and plated on YPD for removal of the pRS423::PGAL1-cre plasmid. Isolates were checked for loss of the KanMX marker, URA3 marker, and pRS423::PGAL1-cre plasmid by assaying growth on YPD+G418 plates, synthetic complete medium lacking uracil plates, and synthetic complete medium lacking histidine plates. A correct isolate that was sensitive to G418 and auxotrophic for uracil and histidine was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3 and designated as BP857. The deletions and marker removal were confirmed by PCR and sequencing with primers oBP450 (SEQ ID NO:308) and oBP451 (SEQ ID NO:309) for  $\Delta$ ura3 and primers oBP460 (SEQ ID NO:306) and oBP461 (SEQ ID NO:307) for  $\Delta$ his3 using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen).

PDC6 Deletion

- The four fragments for the PCR cassette for the scarless PDC6 deletion were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template,
- 5 prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). PDC6 Fragment A was amplified with primer oBP440 (SEQ ID NO:310) and primer oBP441 (SEQ ID NO:311), containing a 5' tail with homology to the 5' end of PDC6 Fragment B. PDC6 Fragment B was amplified with primer oBP442 (SEQ ID NO:312), containing a 5' tail with homology to the 3' end
- 10 of PDC6 Fragment A, and primer oBP443 (SEQ ID NO:313), containing a 5' tail with homology to the 5' end of PDC6 Fragment U. PDC6 Fragment U was amplified with primer oBP444 (SEQ ID NO:314), containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP445 (SEQ ID NO:315), containing a 5' tail with homology to the 5' end of PDC6
- 15 Fragment C. PDC6 Fragment C was amplified with primer oBP446 (SEQ ID NO:316), containing a 5' tail with homology to the 3' end of PDC6 Fragment U, and primer oBP447 (SEQ ID NO:317). PCR products were purified with a PCR Purification kit (Qiagen). PDC6 Fragment AB was created by overlapping PCR by mixing PDC6 Fragment A and PDC6
- 20 Fragment B and amplifying with primers oBP440 (SEQ ID NO:310) and oBP443 (SEQ ID NO:313). PDC6 Fragment UC was created by overlapping PCR by mixing PDC6 Fragment U and PDC6 Fragment C and amplifying with primers oBP444 (SEQ ID NO:314) and oBP447 (SEQ ID NO:317). The resulting PCR products were purified on an agarose gel
- 25 followed by a Gel Extraction kit (Qiagen). The PDC6 ABUC cassette was created by overlapping PCR by mixing PDC6 Fragment AB and PDC6 Fragment UC and amplifying with primers oBP440 (SEQ ID NO:310) and oBP447 (SEQ ID NO:317). The PCR product was purified with a PCR Purification kit (Qiagen).
- 30 Competent cells of CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3 were made and transformed with the PDC6 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with

2% glucose at 30 °C. Transformants with a *pdc6* knockout were screened for by PCR with primers oBP448 (SEQ ID NO:318) and oBP449 (SEQ ID NO:319) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). A correct transformant was selected as strain

5 CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6::URA3.

CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6::URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30 °C to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR and  
 10 sequencing with primers oBP448 (SEQ ID NO:318) and oBP449 (SEQ ID NO:319) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC6 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC6, oBP554 (SEQ ID NO:320) and oBP555 (SEQ  
 15 ID NO:321). The correct isolate was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6 and designated as BP891.

#### PDC1 Deletion-*ilvDSm* Integration

The PDC1 gene was deleted and replaced with the *ilvD* coding region from *Streptococcus mutans* ATCC #700610. The A fragment  
 20 followed by the *ilvD* coding region from *Streptococcus mutans* for the PCR cassette for the PDC1 deletion-*ilvDSm* integration was amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and NYLA83 genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). NYLA83 is a strain which carries the PDC1  
 25 deletion-*ilvDSm* integration described in U.S. Patent Application Publication No. 2009/0305363 .

PDC1 Fragment A-*ilvDSm* (SEQ ID NO:322) was amplified with primer oBP513 (SEQ ID NO:326) and primer oBP515 (SEQ ID NO:327), containing a 5' tail with homology to the 5' end of PDC1  
 30 Fragment B. The B, U, and C fragments for the PCR cassette for the PDC1 deletion-*ilvDSm* integration were amplified using Phusion High Fidelity PCR Master Mix (New England BioLabs) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra Puregene Yeast/Bact

kit (Qiagen). PDC1 Fragment B was amplified with primer oBP516 (SEQ ID NO:328) containing a 5' tail with homology to the 3' end of PDC1 Fragment A-ilvDSm, and primer oBP517 (SEQ ID NO:329), containing a 5' tail with homology to the 5' end of PDC1 Fragment U. PDC1 Fragment U was amplified with primer oBP518 (SEQ ID NO:330), containing a 5' tail with homology to the 3' end of PDC1 Fragment B, and primer oBP519 (SEQ ID NO:331), containing a 5' tail with homology to the 5' end of PDC1 Fragment C. PDC1 Fragment C was amplified with primer oBP520 (SEQ ID NO:332), containing a 5' tail with homology to the 3' end of PDC1 Fragment U, and primer oBP521 (SEQ ID NO:333). PCR products were purified with a PCR Purification kit (Qiagen). PDC1 Fragment A-ilvDSm-B was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm and PDC1 Fragment B and amplifying with primers oBP513 (SEQ ID NO:326) and oBP517 (SEQ ID NO:329). PDC1 Fragment UC was created by overlapping PCR by mixing PDC1 Fragment U and PDC1 Fragment C and amplifying with primers oBP518 (SEQ ID NO:330) and oBP521 (SEQ ID NO:333). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC1 A-ilvDSm-BUC cassette (SEQ ID NO:323) was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm-B and PDC1 Fragment UC and amplifying with primers oBP513 (SEQ ID NO:326) and oBP521 (SEQ ID NO:333). The PCR product was purified with a PCR Purification kit (Qiagen).

Competent cells of CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdcc6 were made and transformed with the PDC1 A-ilvDSm-BUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30 °C. Transformants with a pdc1 knockout ilvDSm integration were screened for by PCR with primers oBP511 (SEQ ID NO:336) and oBP512 (SEQ ID NO:337) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC1 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC1, oBP550 (SEQ ID NO:338) and oBP551 (SEQ ID NO:339). A

correct transformant was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6  $\Delta$ pdc1::ilvDSm-URA3.

CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6  $\Delta$ pdc1::ilvDSm-URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30 °C to select for isolates that lost the URA3 marker. The deletion of PDC1, integration of ilvDSm, and marker removal were confirmed by PCR and sequencing with primers oBP511 (SEQ ID NO:336) and oBP512 (SEQ ID NO:337) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6  $\Delta$ pdc1::ilvDSm and designated as BP907.

#### PDC5 Deletion sadB Integration

The PDC5 gene was deleted and replaced with the sadB coding region from *Achromobacter xylosoxidans* (the sadB gene is described in U.S. Patent Appl. No. 2009/0269823 ).

A segment of the PCR cassette for the PDC5 deletion-sadB integration was first cloned into plasmid pUC19-URA3MCS.

pUC19-URA3MCS is pUC19 based and contains the sequence of the URA3 gene from *Saccharomyces cerevisiae* situated within a multiple cloning site (MCS). pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream and downstream of this gene were included for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.

The DNA encompassing the URA3 coding region along with 250 bp upstream and 150 bp downstream of the URA3 coding region from *Saccharomyces cerevisiae* CEN.PK 113-7D genomic DNA was amplified with primers oBP438 (SEQ ID NO:334), containing BamHI, Ascl, PmeI, and FseI restriction sites, and oBP439 (SEQ ID NO:335), containing XbaI, PaeI, and NotI restriction sites, using Phusion High-Fidelity PCR Master Mix (New England BioLabs). Genomic DNA was prepared using a Gentra Puregene Yeast/Bact kit (Qiagen). The PCR product and pUC19 (SEQ ID

NO:325) were ligated with T4 DNA ligase after digestion with BamHI and XbaI to create vector pUC19-URA3MCS. The vector was confirmed by PCR and sequencing with primers oBP264 (SEQ ID NO:342) and oBP265 (SEQ ID NO:343).

- 5           The coding sequence of sadB and PDC5 Fragment B were cloned into pUC19-URA3MCS to create the sadB-BU portion of the PDC5 A-sadB-BUC PCR cassette. The coding sequence of sadB was amplified using pLH468-sadB (SEQ ID NO:359) as template with primer oBP530 (SEQ ID NO:344), containing an Ascl restriction site, and primer oBP531 (SEQ ID NO:345), containing a 5' tail with homology to the 5' end of PDC5
- 10           Fragment B. PDC5 Fragment B was amplified with primer oBP532 (SEQ ID NO:346), containing a 5' tail with homology to the 3' end of sadB, and primer oBP533 (SEQ ID NO:347), containing a PmeI restriction site. PCR products were purified with a PCR Purification kit (Qiagen). sadB-PDC5
- 15           Fragment B was created by overlapping PCR by mixing the sadB and PDC5 Fragment B PCR products and amplifying with primers oBP530 (SEQ ID NO:344) and oBP533 (SEQ ID NO:347). The resulting PCR product was digested with Ascl and PmeI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the
- 20           appropriate enzymes. The resulting plasmid was used as a template for amplification of sadB-Fragment B-Fragment U using primers oBP536 (SEQ ID NO:348) and oBP546 (SEQ ID NO:349), containing a 5' tail with homology to the 5' end of PDC5 Fragment C. PDC5 Fragment C was
- 25           amplified with primer oBP547 (SEQ ID NO:350) containing a 5' tail with homology to the 3' end of PDC5 sadB-Fragment B-Fragment U, and primer oBP539 (SEQ ID NO:351). PCR products were purified with a PCR Purification kit (Qiagen). PDC5 sadB-Fragment B-Fragment U-Fragment
- 30           C was created by overlapping PCR by mixing PDC5 sadB-Fragment B-Fragment U and PDC5 Fragment C and amplifying with primers oBP536 (SEQ ID NO:348) and oBP539 (SEQ ID NO:351). The resulting PCR product was purified on an agarose gel followed by a Gel Extraction kit (Qiagen). The PDC5 A-sadB-BUC cassette (SEQ ID NO:324) was created by amplifying PDC5 sadB-Fragment B-Fragment U-Fragment C with

primers oBP542 (SEQ ID NO:352), containing a 5' tail with homology to the 50 nucleotides immediately upstream of the native PDC5 coding sequence, and oBP539 (SEQ ID NO:351). The PCR product was purified with a PCR Purification kit (Qiagen).

- 5           Competent cells of CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdcc6  $\Delta$ pdcc1::ilvDSm were made and transformed with the PDC5 A-sadB-BUC PCR cassette using a Frozen-EZ Yeast Transformation II kit (Zymo Research). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose) at 30C.
- 10       Transformants with a pdcc5 knockout sadB integration were screened for by PCR with primers oBP540 (SEQ ID NO:353) and oBP541 (SEQ ID NO:354) using genomic DNA prepared with a Gentra Puregene Yeast/Bact kit (Qiagen). The absence of the PDC5 gene from the isolate
- 15       was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC5, oBP552 (SEQ ID NO:355) and oBP553 (SEQ ID NO:356). A correct transformant was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdcc6  $\Delta$ pdcc1::ilvDSm  $\Delta$ pdcc5::sadB-URA3.

- 20       CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdcc6  $\Delta$ pdcc1::ilvDSm  $\Delta$ pdcc5::sadB-URA3 was grown overnight in YPE (1% ethanol) and plated on synthetic complete medium supplemented with ethanol (no glucose) and containing 5-fluoro-orotic acid (0.1%) at 30 C to select for isolates that lost the URA3 marker. The deletion of PDC5, integration of sadB, and marker removal were confirmed by PCR with primers oBP540 (SEQ ID NO:353) and oBP541 (SEQ ID NO:354) using genomic DNA prepared
- 25       with a Gentra Puregene Yeast/Bact kit (Qiagen). The correct isolate was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdcc6  $\Delta$ pdcc1::ilvDSm  $\Delta$ pdcc5::sadB and designated as BP913.

#### GPD2 Deletion

- 30       To delete the endogenous GPD2 coding region, a gpd2::loxP-URA3-loxP cassette (SEQ ID NO:361) was PCR-amplified using loxP-URA3-loxP PCR (SEQ ID NO:360) as template DNA. loxP-URA3-loxP contains the URA3 marker from (ATCC # 77107) flanked by loxP recombinase sites. PCR was done using Phusion DNA polymerase and



primers LA512 and LA513 (SEQ ID NOs:340 and 341). The GPD2 portion of each primer was derived from the 5' region upstream of the GPD2 coding region and 3' region downstream of the coding region such that integration of the loxP-URA3-loxP marker resulted in replacement of the GPD2 coding region. The PCR product was transformed into BP913 and transformants were selected on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose). Transformants were screened to verify correct integration by PCR using primers oBP582 and AA270 (SEQ ID NOs:357 and 358).

The URA3 marker was recycled by transformation with pRS423::PGAL1-cre (SEQ ID NO:271) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30 C. Transformants were streaked on synthetic complete medium supplemented with 1% ethanol and containing 5-fluoro-uracil (0.1%) and incubated at 30 C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in YPE (1% ethanol) for removal of the pRS423::PGAL1-cre plasmid. The deletion and marker removal were confirmed by PCR with primers oBP582 (SEQ ID NO:357) and oBP591 (SEQ ID NO:362). The correct isolate was selected as strain CEN.PK 113-7D  $\Delta$ ura3::loxP  $\Delta$ his3  $\Delta$ pdc6  $\Delta$ pdc1::ilvDSm  $\Delta$ pdc5::sadB  $\Delta$ gpd2::loxP and designated as BP1064 (PNY1503).

### EXAMPLE 13

#### Construction of PNY2204 and Isobutanol Pathway Plasmids

The purpose of this example is to describe construction of a vector to enable integration of a gene encoding acetolactate synthase into the naturally occurring intergenic region between the PDC1 and TRX1 coding sequences in Chromosome XII. Strains resulting from the use of this vector are also described.

#### Construction of integration vector pUC19-kan::pdc1::FBA-alsS::TRX1

The FBA-alsS-CYCt cassette was constructed by moving the 1.7kb BbvCI/PacI fragment from pRS426::GPD::alsS::CYC (described in U.S.

Patent No. 7,851,188),

to pRS426::FBA::ILV5::CYC (described in U.S. Patent No. 7,851,188), which had been previously digested with BbvCI/PacI to release the ILV5 gene.

- 5 Ligation reactions were transformed into *E. coli* TOP10 cells and transformants were screened by PCR using primers N98SeqF1 (SEQ ID NO:363) and N99SeqR2 (SEQ ID NO:365). The FBA-alsS-CYCt cassette was isolated from the vector using BglII and NotI for cloning into pUC19-URA3::ilvD-TRX1 (clone "B") at the AflII site (Klenow fragment was used
- 10 to make ends compatible for ligation). Transformants containing the alsS cassette in both orientations in the vector were obtained and confirmed by PCR using primers N98SeqF4 (SEQ ID NO:364) and N1111 (SEQ ID NO:366) for configuration "A" and N98SeqF4 (SEQ ID NO:364) and N1110 (SEQ ID NO:367) for configuration "B". A geneticin selectable
- 15 version of the "A" configuration vector was then made by removing the URA3 gene (1.2 kb *NotI*/*NaeI* fragment) and adding a geneticin cassette. Klenow fragment was used to make all ends compatible for ligation, and transformants were screened by PCR to select a clone with the geneticin
- 20 resistance gene in the same orientation as the previous URA3 marker using primers BK468 (SEQ ID NO:368) and N160SeqF5 (SEQ ID NO:210). The resulting clone was called pUC19-kan::pdc1::FBA-alsS::TRX1 (clone A)(SEQ ID NO:387).

#### Construction of alsS integrant strains and isobutanol-producing derivatives

- 25 The pUC19-kan::pdc1::FBA-alsS integration vector described above was linearized with *PmeI* and transformed into PNY1507 (Example 8). *PmeI* cuts the vector within the cloned pdc1-TRX1 intergenic region and thus leads to targeted integration at that location (Rodney Rothstein, Methods in Enzymology, 1991, volume 194, pp. 281-301). Transformants
- 30 were selected on YPE plus 50 µg/ml G418. Patched transformants were screened by PCR for the integration event using primers N160SeqF5 (SEQ ID NO:210) and oBP512 (SEQ ID NO:337). Two transformants were tested indirectly for acetolactate synthase function by evaluating the strains ability to make isobutanol. To do this, additional isobutanol

pathway genes were supplied on *E. coli*-yeast shuttle vectors (pYZ090 $\Delta$ alsS and pBP915, described below). One clone was designated as PNY2205. The plasmid-free parent strain was designated PNY2204 (MATa ura3 $\Delta$ ::loxP his3 $\Delta$  pdc6 $\Delta$  pdc1 $\Delta$ ::P[PDC1]-DHAD|kivD\_Sm-PDC1t-  
 5 pUC19-loxP-kanMX-loxP-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5 $\Delta$ ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2 $\Delta$ ::loxP fra2 $\Delta$  adh1 $\Delta$ ::UAS(PGK1)P[FBA1]-kivD\_LI(y)-ADH1t).

Isobutanol pathway plasmids (pYZ090 $\Delta$ alsS and pBP915)

10 pYZ090 (SEQ ID NO:195) was digested with *SpeI* and *NotI* to remove most of the CUP1 promoter and all of the alsS coding sequence and CYC terminator. The vector was then self-ligated after treatment with Klenow fragment and transformed into *E. coli* Stb13 cells, selecting for ampicillin resistance. Removal of the DNA region was confirmed for two  
 15 independent clones by DNA sequencing across the ligation junction by PCR using primer N191 (SEQ ID NO:370). The resulting plasmid was named pYZ090 $\Delta$ alsS (SEQ ID NO:371). The pLH468 plasmid was constructed for expression of DHAD, KivD and HADH in yeast. pBP915 (SEQ ID NO: 182) was constructed from pLH468 (SEQ ID NO:139) by  
 20 deleting the *kivD* gene and 957 base pairs of the *TDH3* promoter upstream of *kivD*. pLH468 was digested with *SwaI* and the large fragment (12896 bp) was purified on an agarose gel followed by a Gel Extraction kit (Qiagen; Valencia, CA). The isolated fragment of DNA was self-ligated with T4 DNA ligase and used to transform electrocompetent TOP10  
 25 *Escherichia coli* (Invitrogen; Carlsbad, CA). Plasmids from transformants were isolated and checked for the proper deletion by restriction analysis with the *SwaI* restriction enzyme. Isolates were also sequenced across the deletion site with primers oBP556 (SEQ ID NO:372) and oBP561 (SEQ ID NO:373). A clone with the proper deletion was designated pBP915  
 30 (pLH468 $\Delta$ kivD)(SEQ ID NO:182).

pYZ090 is based on the pHR81 (ATCC #87541, Manassas, VA) backbone. pYZ090 was constructed to contain a chimeric gene having the coding region of the alsS gene from *Bacillus subtilis* (nt position 457-2172)

expressed from the yeast CUP1 promoter (nt 2-449) and followed by the  
CYC1 terminator (nt 2181-2430) for expression of ALS, and a chimeric  
gene having the coding region of the *ilvC* gene from *Lactococcus lactis* (nt  
3634-4656) expressed from the yeast ILV5 promoter (2433-3626) and  
5 followed by the ILV5 terminator (nt 4682-5304) for expression of KARI.

#### Example 14.

##### Isobutanol production-PNY1910 and PNY2242

Methods:

##### 10 Preparation of inoculum medium

1 L of inoculum medium contained: 6.7 g, Yeast Nitrogen Base w/o  
amino acids (Difco 0919-15-3); 2.8 g, Yeast Synthetic Drop-out Medium  
Supplement Without Histidine, Leucine, Tryptophan and Uracil (Sigma  
Y2001); 20 mL of 1% (w/v) L-Leucine; 4 mL of 1% (w/v) L-Tryptophan; 3 g  
15 of ethanol; 10 g of glucose.

##### Preparation of defined fermentation medium

The volume of broth after inoculation was 800 mL, with the  
following final composition, per liter: 5 g ammonium sulfate, 2.8 g  
20 potassium phosphate monobasic, 1.9 g magnesium sulfate septahydrate,  
0.2 mL antifoam (Sigma DF204), Yeast Synthetic Drop-out Medium  
Supplement without Histidine, Leucine, Tryptophan, and Uracil (Sigma  
Y2001), 16 mg L-leucine, 4 mg L-tryptophan, 6 mL of a vitamin mixture (in  
1 L water, 50 mg biotin, 1 g Ca-pantothenate, 1 g nicotinic acid, 25 g myo-  
25 inositol, 1 g thiamine chloride hydrochloride, 1 g pyridoxol hydrochloride,  
0.2 g p-aminobenzoic acid) 6 mL of a trace mineral solution (in 1 L water,  
15 g EDTA, 4.5 g zinc sulfate heptahydrate, 0.8 g manganese chloride  
dehydrate, 0.3 g cobalt chloride hexahydrate, 0.3 g copper sulfate  
pentahydrate, 0.4 g disodium molybdenum dehydrate, 4.5 g calcium  
30 chloride dihydrate, 3 g iron sulfate heptahydrate, 1 g boric acid, 0.1 g  
potassium iodide), 30 mg thiamine HCl, 30 mg nicotinic acid. The pH was  
adjusted to 5.2 with 2N KOH and glucose added to 10 g/L.

### Preparation of inoculum

A 125 mL shake flask was inoculated directly from a frozen vial by pipetting the whole vial culture (approx. 1 ml) into 10 mL of the inoculum medium. The flask was incubated at 260 rpm and 30 °C. The strain was  
5 grown overnight until OD about 1.0. OD at  $\lambda = 600$  nm was determined in a Beckman spectrophotometer (Beckman, USA).

### Bioreactor experimental design

Fermentations were carried out in 1L Biostat B DCU3 fermenters  
10 (Sartorius, USA) with a working volume on 0.8 L. Off-gas composition was monitored by a Prima DB mass spectrometer (Thermo Electron Corp., USA). The temperature was maintained at 30C and pH controlled at 5.2 with 2N KOH throughout the entire fermentation. Directly after inoculation with 80 mL of the inoculum, dO was controlled by agitation at 30%, pH  
15 was controlled at 5.25, aeration was controlled at 0.2 L/min. Once OD of approximately 3 was reached, the gas was switched to N2 for anaerobic cultivation. Throughout the fermentation, glucose was maintained in excess (5-20 g/L) by manual additions of a 50% (w/w) solution.

### Methods for analyzing cultivation experiments

OD at  $\lambda = 600$  nm was determined in a spectrophotometer by pipetting a well mixed broth sample into a cuvette (CS500 VWR International, Germany). If biomass concentration of the sample exceeded the linear absorption range of the spectrophotometer (typically  
25 OD values from 0.000 to 0.300), the sample was diluted with 0.9% NaCl solution to yield values in the linear range.

Measurements of glucose, isobutanol, and other fermentation by-products in the culture supernatant were carried out by HPLC, using a Bio-Rad Aminex HPX-87H column (Bio-Rad, USA), with refractive index (RI)  
30 and a diode array (210 nm) detectors. Chromatographic separation was achieved using 0.01 N H<sub>2</sub>SO<sub>4</sub> as the mobile phase with a flow rate of 0.6 mL/min and a column temperature of 40 °C. Isobutanol retention time is

32.2 minutes under these conditions. Isobutanol concentration in off-gas samples was determined by mass-spectrometer.

### Results

5           Maximal biomass concentration measured as optical density (OD), volumetric rate of isobutanol production, final isobutanol titer, and isobutanol yield on glucose are presented in the table below. The strain PNY2242 had higher titers and faster rates than the strain PNY1910 and produced isobutanol with higher specific rate and titer. The specific rates  
10 are shown in Figure 5. Accumulation of the DHIV + DHMB in the culture supernatant was three times higher with PNY1910 compared to the PNY2242 strain (Figure 6). Yield of glycerol, pyruvic acid, BDO, DHIV+DHMB\*,  $\alpha$ KIV, and isobutyric acid on glucose is shown in Figure 7.

15           \*DHIV analyzed by HPLC method includes both DHIV and DHMB.

Table 19

| Strain  | Max.<br>OD600 | Rate<br>(g/L/h) | Titer<br>(g/L) | Yield<br>(g/g) |
|---------|---------------|-----------------|----------------|----------------|
| PNY1910 | 5.0           | 0.16            | 10.9           | 0.25           |
| PNY2242 | 5.0           | 0.23            | 16.1           | 0.27           |

### Example 15.

#### Construction of K9G9 error prone PCR library

20           Error prone PCR of K9G9 was performed to generate a library that can be screened for variants with increases in the Km values for NADPH relative to NADH. Mutagenic PCR of K9G9 was performed with the  
25   GeneMorph® II EZClone Domain Mutagenesis Kit (Catalog #200552; Agilent Technologies, Stratagene Products Division, La Jolla, CA). Primers K9G9\_EZ\_F1 (AAA CAT GGA AGA ATG TAA GAT GGC; SEQ ID NO: 390) and K9G9\_EZ\_R1 (TCA GTT GTT AAT CAA CTT GTC TTC G; SEQ ID NO: 391) were commercially synthesized by Integrated DNA  
30   Technologies, Inc (Coralville IA). Other than the primers, template, and ddH<sub>2</sub>O, reagents used here were supplied with the kit indicated above.

- The mutagenic PCR mixture consisted of 4 µl of pHR81-PIIv5-KARI-K9.G9 (SEQ ID NO: 392) (770 ng/µg), 1.25 µl of each primer (100 ng/ µl stocks), 5 µl of 10× Mutazyme II reaction buffer, 1 µl of 40 mM dNTP mix, 1.5 µl of Mutazyme II DNA polymerase, and 36 µl of ddH<sub>2</sub>O. The following
- 5 conditions were used for the PCR reaction: The starting temperature was 95°C for 2.0 min followed by 30 heating/cooling cycles. Each cycle consisted of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 2.0 min. At the completion of the temperature cycling, the sample was kept at 72° C for 10.0 min more, and then held awaiting sample recovery at 4°C. The
- 10 reaction product was separated from the template via agarose gel electrophoresis (1% agarose, 1X TBE buffer) and recovered using the StrataPrep® DNA Gel Extraction Kit (Cat# 400766, Agilent Technologies, Stratagene Products Division, La Jolla, CA) as recommended by the manufacturer.
- 15 The isolated reaction product was employed as a megaprimer to generate gene libraries in the "EZClone reaction" of the kit indicated above. Other than the megaprimer, template, and ddH<sub>2</sub>O, reagents used here were supplied with the kit indicated above. The reaction consisted of 25 µl of the 2× EZClone enzyme mix, 4 µl of megaprimer (125 ng/ µl), 2 µl
- 20 of K9G9 in a pBAD.KARI vector (25 ng/ µl), 3 µl of EZClone solution, and 16µl of ddH<sub>2</sub>O. The following conditions were used for the reaction: The starting temperature was 95°C for 1.0 min followed by 30 heating/cooling cycles. Each cycle consisted of 95°C for 50 sec, 60°C for 50 sec, and 68°C for 10.0 min. At the completion of the temperature cycling, the
- 25 samples were kept at 72° C for 10.0 min more, and then held awaiting sample recovery at 4°C. 1 µl of the Dpn I (10 U/µl) was added and the mixture was incubated for 4 hours at 37°C.
- 30 4 µl of the Dpn I digested "EZClone reaction" product was then transformed into 50 µl XL10-Gold® Ultracompetent *E. coli* cells (provided in the GeneMorph® II EZClone Domain Mutagenesis Kit) as recommended by the manufacturer. The transformants were spread on agar plates containing the LB medium and 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, CA), incubated at 37°C overnight, and

store at 4°C. These steps were repeated with 4 µl Dpn I digested "EZClone reaction" product and 50 µl cells per transformation for a total of 10 transformations. The resultant library in XL-Gold was scraped off the agar plates with a solution containing M9 salts, combined, diluted into media  
5 containing the LB medium and 100 µg/ml ampicillin, and incubated at 37°C overnight. The library DNA was isolated from the cells with the QIAprep Spin Miniprep Kit (Catalog #2706; Qiagen, Valencia, CA) according to the protocol provided by the manufacturer. The amplified library was then used to transform an electro-competent strain of *E. coli* Bw25113 ( $\Delta$ ilvC)  
10 using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, CA). The transformed clones were spread on agar plates containing the LB medium and 100 µg/ml ampicillin (#101320-154, Teknova Inc. Hollister, CA) and incubated at 37°C overnight. Clones were employed for high throughput screening as described in Example 16.

15

#### Example 16.

##### Identification of K9G9 variants with increased $K_M$ for NADPH via screening for diminished NADP<sup>+</sup> inhibition of NADH activity

The K9G9 library described in Example 15 was screened for  
20 variants with reduced NADP<sup>+</sup> inhibition of NADH-dependent KARI activity. A K9G9 variant with reduced NADP<sup>+</sup> inhibition of activity with NADH can potentially exhibit an increase in the ratio of the  $K_M$  for NADPH to the  $K_M$  for NADH. With a specific objective to increase  $K_M$  for NADPH relative to  $K_M$  for NADH, the hits from the screen were partially purified and kinetic  
25 analyses were performed to determine  $V_{max}$  and  $K_M$  parameters with NADH and with NADPH.

##### High throughput screening assay of K9G9 gene library

High throughput screening of the gene libraries of mutant KARI  
30 enzymes was performed as described herein: 10× freezing medium containing 554.4 g/L glycerol, 68 mM of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 mM MgSO<sub>4</sub>, 17 mM sodium citrate, 132 mM KH<sub>2</sub>PO<sub>4</sub>, 36 mM K<sub>2</sub>HPO<sub>4</sub> was prepared with molecular pure water and filter-sterilized. Freezing medium was prepared by diluting the 10x freezing medium with the LB medium. An aliquot (200



μL) of the 1x freezing medium was used for each well of the 96-well archive plates (cat #3370, Corning Inc. Corning, NY).

Clones from the LB agar plates were selected and inoculated into the 96-well archive plates containing the freezing medium and grown overnight at 37 °C without shaking. The archive plates were then stored at -80°C. *E. coli* strain Bw25113(ΔilvC), as described in U.S. Patent 8,129,162, transformed with *pBAD-HisB* (Invitrogen) was always used as the negative control. The positive control for the library was K9G9-KARI in *E. coli* strain Bw25113 (ΔilvC), as described in U.S. Patent 8,129,162.

Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0 μl of cells from thawed archive plates, 200 μl of the LB medium containing 100 μg/ml ampicillin and 0.02%(w/v) arabinose as the inducer. Cells were grown overnight at 37°C with 80% humidity while shaking (900 rpm), harvested by centrifugation (3750 rpm, 5 min at 25°C). (Eppendorf centrifuge, Brinkmann Instruments, Inc. Westbury, NY) and the cell pellet was stored at -20°C for later analysis.

The assay substrate, (*R,S*)-acetolactate, was synthesized as described by Aulabaugh and Schloss (Aulabaugh and Schloss, Biochemistry, 29: 2824-2830, 1990). All other chemicals used in the assay were purchased from Sigma. The enzymatic conversion of acetolactate to α,β-dihydroxyisovalerate by KARI was followed by measuring the oxidation of the cofactor, NADH, from the reaction at 340 nm using a plate reader (Sapphire 2, Tecan, Mannedorf, Switzerland). The activity was calculated using the molar extinction coefficient of 6220 M<sup>-1</sup>cm<sup>-1</sup> NADH.

Frozen cell pellet in deep-well plates and BugBuster (Novagen 71456, Darmstadt, Germany) were warmed up at room temperature for 30 min at the same time. 75 μl of 50% BugBuster (v/v in water) was added to each well after 30 min warm-up and cells were suspended using plate shaker. The plates with cell pellet/50% Bug Buster suspension were incubated at room temperature for 30 min. Cell lysate diluted with 75 μL d.d water, resulting in 0.5X lysate. Assays of the diluted cell free extracts were performed at 30 °C in buffer containing 2.4 mM (*R/S*)-acetolactate,

100 mM HEPES pH 6.8, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 150  $\mu$ M NADH, 12.5  $\mu$ L 0.5 X cell lysate with or without 2.5 mM NADP<sup>+</sup>.

Identification of K9G9 variants with reduced NADP<sup>+</sup> inhibition of NADH

5 KARI activity

The ratio for the measured rate of NADH oxidation in the presence of NADP<sup>+</sup> to the measured rate of NADH oxidation in the absence of NADP<sup>+</sup> was calculated for each variant and positive control well (2 per plate). The mean and standard deviation of ratios for all of the positive control wells (104 total) were calculated.

10 A variant well was considered to contain an initial hit if the rate in the absence of NADP<sup>+</sup> was greater than 0.1 OD/hr and the rate ratio was both greater than 0.45 (three standard deviations higher than the positive control mean) and less than 1. A total of 521 hits were identified from a pool of 4607 potential variants. These initial hits were consolidated, forming a smaller library for further analysis.

Secondary screening of initial library hit

20 The consolidated hit library was grown in biological triplicate and cell free extracts were prepared and assayed as described above. Rate ratios were then calculated for the variants and positive controls as above. Final hits that were selected for detailed kinetic analysis met the following criteria: the rate in the absence of NADP<sup>+</sup> was greater than 0.6 OD/hr, rate ratio was greater than 0.51 and less than 1, and at least two out of three biological replicates passed the criteria. Seventeen hits were identified for kinetic analysis and streaked out on to LB plates with 100  $\mu$ g/mL ampicillin added.

Sequence analysis of K9G9 variants

30 DNA sequencing of the seventeen variants identified from the secondary HTS screening was accomplished by using TempliPhi™ (GE Healthcare) with the primers pBAD-For (ATGCCATAGCATTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394).

**Table 20. Amino Acid Substitutions for K9G9 Variants**

| Variant | Seq | Amino Acid Substitutions        |
|---------|-----|---------------------------------|
| 878 C1  | 873 | None identified                 |
| 879 A7  | 874 | K90M                            |
| 879 C2  | 875 | H37Q                            |
| 880 A11 | 876 | A182T, P320Q                    |
| 880 B4  | 877 | K57E                            |
| 880 D11 | 878 | K90M, A174V                     |
| 881 A2  | 879 | K90M, I133V, K282T              |
| 881 G3  | 880 | Y53F, E74G                      |
| 881 G9  | 881 | K90E                            |
| 882 B12 | 882 | H118R                           |
| 882 C10 | 883 | G31S, R61S, C121Y, D129N, G183D |
| 882 C7  | 933 | E54G                            |
| 882 F9  | 934 | K90E, Q160H                     |
| 882 G6  | 935 | G55A                            |
| 882 G12 | 936 | V142L, S285Y                    |
| 883 C4  | 937 | A170V                           |
| 883 G9  | 938 | L197M, K310M                    |

5 Kinetic analysis of partially purified variant protein

*E. coli* strain Bw25113 ( $\Delta$ ilvC), as described in U.S. Patent

8,129,162, was used to express the seventeen variants and positive control K9G9. Strains were grown for 8 hours in 10 mL of LB broth (#46-060-CM, Mediatech, Manassas, VA) containing 100  $\mu$ g/mL ampicillin at

- 10 37°C with shaking in 125 mL baffled, vented filtered lid flasks. 200  $\mu$ L of this culture was used to inoculate 100 mL LB broth with 100  $\mu$ g/mL ampicillin and 0.2% (w/v) arabinose added. These cultures were grown for 16 to 18 hours at 37°C with shaking in 500 mL baffled, vented filtered lid flasks. Cells were harvested in a 20 mL and two 40 mL aliquots,
- 15 supernatants were decanted and the pellets were frozen at -80°C.

To partially purify the protein, the cell pellet corresponding with the 20 mL cell culture harvest was thawed and resuspended in 1 mL Bug Buster Master Mix (Novagen 71456, Darmstadt, Germany). The cell suspension was incubated at room temperature for 15 minutes followed by

20 15 minute incubation at 60°C to denature the heat liable proteins. Cell debris and denatured proteins were pelleted by centrifugation for 30

minutes at 4°C. Supernatant containing the heat stable cytosolic protein, including K9G9 and variants, was recovered and stored at 4°C.

The total protein of the heat stable cytosolic protein fraction was measured by the Bradford Assay using Coomassie Plus (Thermo

- 5 Scientific #23238, Rockford, Ill.). BSA was employed as the standard. The concentration of protein was measured by determining the absorbance at 595 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, DE).

- To determine  $V_{\max}$  and  $K_M$  values for NADH and NADPH, the  
 10 partially purified proteins were assayed at various concentrations of NADH (0, 16.4, 32.8, 65.7, 98.5, 164.3 and 246.5  $\mu\text{M}$ ) and NADPH (0, 12.8, 25.6, 51.2, 76.8 and 128  $\mu\text{M}$ ). Assays were conducted at 30°C in 100 mM HEPES (pH 6.8), 10 mM  $\text{MgCl}_2$ , 100 mM KCl and 4.8 mM R/S-acetolactate. Between 0.1 to 0.35 mg/mL total protein was added to the  
 15 assay. The rate of conversion of S-acetolactate to DHIV was measured via monitoring the oxidation of NAD(P)H at 340 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, DE). The activity was calculated using the molar extinction coefficient of  $6220 \text{ M}^{-1}\text{cm}^{-1}$ .  $V_{\max}$  and  $K_m$  values were calculated by plotting specific activity (U/mg) vs.  
 20 cofactor concentration and the data were fit to the Michaelis-Menten equation using Kaleidagraph software (Synergy, Reading, PA).

**Table 21. Kinetic Values for Partially Purified K9G9 Variants as Determined via NAD(P)H Consumption Assays**

| Variant | $V_{\max}$<br>NADPH,<br>U/mg | $K_m$<br>NADPH,<br>$\mu\text{M}$ | $V_{\max}/K_m$<br>NADPH,<br>L/min*mg | $V_{\max}$<br>NADH,<br>U/mg | $K_m$<br>NADH,<br>$\mu\text{M}$ | $V_{\max}/K_m$<br>NADH,<br>L/min*mg |
|---------|------------------------------|----------------------------------|--------------------------------------|-----------------------------|---------------------------------|-------------------------------------|
| K9G9    | 1.53                         | 45.5                             | 0.034                                | 1.09                        | 67.4                            | 0.016                               |
| 878 C1  | 0.75                         | 42.2                             | 0.018                                | 0.62                        | 107.8                           | 0.006                               |
| 879 A7  | 2.51                         | 546                              | 0.005                                | 1.44                        | 263                             | 0.006                               |
| 879 C2  | 1.27                         | 103                              | 0.012                                | 1.23                        | 187                             | 0.007                               |
| 880 A11 | 0.72                         | 86.9                             | 0.008                                | 0.51                        | 117                             | 0.004                               |
| 880 B4  | 1.23                         | 233                              | 0.005                                | 1.14                        | 133                             | 0.009                               |
| 880 D11 | 1.38                         | 130                              | 0.011                                | 1.50                        | 232                             | 0.006                               |
| 881 A2  | 0.88                         | 93.5                             | 0.009                                | 1.13                        | 166.8                           | 0.007                               |
| 881 G3  | 0.69                         | 99.2                             | 0.007                                | 0.69                        | 61.8                            | 0.011                               |
| 881 G9  | 1.03                         | 158                              | 0.007                                | 0.96                        | 310                             | 0.003                               |

|         |      |      |       |      |      |       |
|---------|------|------|-------|------|------|-------|
| 882 B12 | 0.87 | 30.3 | 0.029 | 0.49 | 78.9 | 0.006 |
| 882 C10 | 0.71 | 34.1 | 0.021 | 0.56 | 97.9 | 0.006 |
| 882 C7  | 1.62 | 45.3 | 0.036 | 0.96 | 75.6 | 0.013 |
| 882 F9  | 1.39 | 256  | 0.005 | 1.19 | 335  | 0.004 |
| 882 G6  | 0.95 | 47.4 | 0.020 | 0.74 | 98.7 | 0.007 |
| 882 G12 | 1.06 | 63.5 | 0.017 | 0.75 | 81.2 | 0.009 |
| 883 C4  | 1.26 | 46.8 | 0.027 | 0.67 | 83.9 | 0.008 |
| 883 G9  | 1.26 | 38   | 0.033 | 1.01 | 71.9 | 0.014 |

Example 17.Manual recombination of K9 KARI variants via site directed mutagenesis

- 5           Site directed mutagenesis of the K9G9 derivatives K9JB4 and K9JG3 (identified in Example 16 as 880 B4 and 881 G3, respectively) was performed to incorporate other amino acid changes described in the examples. The initial step was to add to the N87P substitution, which is described in Example 5. Mutations were introduced into the KARI genes
- 10   with primers N87PC1  
(CTGACATCATTATGATCTTGATCCCAGATGAAAAGCAGGCTACCATG  
TAC; SEQ ID NO: 395) and N87PC1r  
(GTACATGGTAGCCTGCTTTTCATCTGGGATCAAGATCATAATGATGT  
CAG; SEQ ID NO: 396), employing the QulkChange® II Site-Directed
- 15   Mutagenesis Kit (Catalog #200523; Agilent Technologies, Stratagene Products Division, La Jolla, CA). Except for the primers, templates, and ddH<sub>2</sub>O, all reagents used here were supplied with the kit indicated above. Primers were commercially synthesized by Integrated DNA Technologies, Inc (Coralville IA). Templates were K9 KARI variants in *E. coli* vectors
- 20   (pBAD.KARI). For mutagenesis of K9JB4, the reaction mixture contained 1 µl K9JB4 (50 ng/µl), 1 µl of each primer (150 ng/ul), 5 µl of 10× reaction buffer, 1 µl of dNTP mix, 1 µl of Pfu Ultra HF DNA polymerase, and 40 µl of ddH<sub>2</sub>O. For, the K9JG3 reaction mixture, 1 µl K9JB4 (50 ng/ul) was substituted with 1 µl K9JG3 (50 ng/µl). The following conditions were used
- 25   for both reactions: The starting temperature was 95°C for 30 sec followed by 16 heating/cooling cycles. Each cycle consisted of 95°C for 30 sec, 55°C for 30 sec, and 68°C for 5.0 min. At the completion of the

temperature cycling, the samples held awaiting sample recovery at 4°C. 1 µl of the Dpn I (10 U/µl) was added to each reaction and the mixtures were incubated for 1 hour at 37°C.

- 2 µl of each mutagenic reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, Catalog #C404003) according to the manufacturer's instructions. The transformants were spread on agar plates containing the LB medium and 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, CA) and incubated at 37°C overnight. Multiple transformants were then selected for TempliPhi™ (GE Healthcare) based DNA sequencing employing primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394). Transformants with confirmed KARI sequences were inoculated into LB medium containing 100 µg/ml ampicillin and incubated at 33°C with shaking at 225 rpm.
- Plasmid DNA was isolated from the cells with the QIAprep Spin Miniprep Kit (Catalog #2706; Qiagen, Valencia, CA) according to the protocol provided by the manufacturer. The resultant clones K9JB4P and K9JG3P were derived from K9JB4 and K9JG3, respectively.
- Additional site directed mutagenesis was performed as described above with modifications.

- Variant K9JA1 was derived from K9JG3P employing primers oK57E1 (GGTTTATTCGAAGGTGCGGAGGAGTGGAAAAGAGCTG; SEQ ID NO: 397) and oK57E1r (CAGCTCTTTTCCACTCCTCCGCACCTTCGAATAAACC; SEQ ID NO: 398). The mutagenesis reaction contained 1 µl K9JG3P (50 ng/µl), 1 µl of each primer (150 ng/ul), 5 µl of 10× reaction buffer, 1 µl of dNTP mix, 1 µl of PfuUltra HF DNA polymerase, and 40 µl of ddH<sub>2</sub>O. Liquid cultures for *E. coli* transformants were incubated at 37°C instead of 33°C.
- Variant K9SB2 was derived from K9JB4P employing primers oY53F1 (GTAACGTTATCATTGGTTTATACGAAGGTGCGGAGGAG; SEQ ID NO: 399) and oY53F1r

(CTCCTCCGCACCTTCGAATAAACCAATGATAACGTTAC; SEQ ID NO: 400). The mutagenesis reaction contained 1  $\mu$ l K9JB4P (50 ng/ $\mu$ l), 1  $\mu$ l of each primer (150 ng/ $\mu$ l), 5  $\mu$ l of 10 $\times$  reaction buffer, 1  $\mu$ l of dNTP mix, 1  $\mu$ l of PfuUltra HF DNA polymerase, and 40  $\mu$ l of ddH<sub>2</sub>O. Liquid cultures for

5 *E. coli* transformants were incubated at 37°C instead of 33°C.

Variant K9SB2-K90L was derived from K9SB2 employing primers oK90L1(GATCTTGATCCCAGATGAATTGCAGGCTACCATGTACAAAAA C; SEQ ID NO: 401) and oK90L1r (GTT TTT GTA CAT GGT AGC CTG

10 CAA TTC ATC TGG GAT CAA GAT C; SEQ ID NO: 402). The mutagenesis reaction contained 2.5  $\mu$ l K9SB2 (50 ng/ $\mu$ l), 1  $\mu$ l of each primer (150 ng/ $\mu$ l), 5  $\mu$ l of 10 $\times$  reaction buffer, 1  $\mu$ l of dNTP mix, 1  $\mu$ l of PfuUltra HF DNA polymerase, and 38.5  $\mu$ l of ddH<sub>2</sub>O. For the heating/cooling cycles, the step of 55°C for 30 sec was increased to 1 min.

15 Liquid cultures for *E. coli* transformants were incubated at 37°C instead of 33°C.

Variant K9SB2-K90M was derived from K9SB2 employing primers oK90M1

20 (CTTGATCCCAGATGAAATGCAGGCTACCATGTACAAAAAC; SEQ ID NO: 403) and oK90M1r (GTT TTT GTA CAT GGT AGC CTG CAT TTC ATC TGG GAT CAA G; SEQ ID NO: 404). The mutagenesis reaction contained 2.5  $\mu$ l K9SB2 (50 ng/ $\mu$ l), 1  $\mu$ l of each primer (150 ng/ $\mu$ l), 5  $\mu$ l of 10 $\times$  reaction buffer, 1  $\mu$ l of dNTP mix, 1  $\mu$ l of PfuUltra HF DNA

25 polymerase, and 38.5  $\mu$ l of ddH<sub>2</sub>O. For the heating/cooling cycles, the step of 55°C for 30 sec was increased to 1 min. Liquid cultures for *E. coli* transformants were incubated at 37°C instead of 33°C.

30 **Table 22. Amino Acid Substitutions of K9G9 Variants and Combinations**

| Variant | Amino Acid Seq ID No: | Nucleic Acid SEQ ID NO: | Amino Acid Substitutions |
|---------|-----------------------|-------------------------|--------------------------|
| K9JB4   | 417                   | 418                     | S56A, K57E, S58E         |
| K9JB4P  | 419                   | 420                     | S56A, K57E, S58E, N87P   |
| K9JG3   | 421                   | 422                     | Y53F, S56A, S58E, E74G   |

|            |     |     |                                    |
|------------|-----|-----|------------------------------------|
| K9JG3P     | 423 | 424 | Y53F, S56A, S58E, E74G, N87P       |
| K9JA1      | 425 | 426 | Y53F, S56A, K57E, S58E, E74G, N87P |
| K9SB2      | 427 | 428 | Y53F, S56A, K57E, S58E, N87P       |
| K9SB2-K90L | 429 | 430 | Y53F, S56A, K57E, S58E, N87P, K90L |
| K9SB2-K90M | 431 | 432 | Y53F, S56A, K57E, S58E, N87P, K90M |

#### Example 18

##### Kinetic characterization of purified K9G9 derivatives with increased ratios of $K_M$ NADPH to $K_M$ NADH

- 5 K9G9 and variants were overexpressed in *E. coli* strain Bw25113 ( $\Delta ilvC$ ), as described in U.S. Patent 8,129,162, and purified in order to obtain a more accurate determination of cofactor affinity and maximum velocity.
- 10 For expression and characterization, *E. coli* plasmids (pBAD.KARI) were used to transform an electro-competent strain of *E. coli* Bw25113 ( $\Delta ilvC$ ) as described in U.S. Patent 8,129,162, using a BioRad Gene Pulser II (Bio-Rad Laboratories Inc., Hercules, CA). The transformed clones were spread on agar plates containing the LB medium and 100
- 15  $\mu\text{g/ml}$  ampicillin (#101320-154, Teknova Inc. Hollister, CA) and incubated at 37°C overnight. A single transformant for each strain was streaked out onto LB plates with 100  $\mu\text{g/mL}$  ampicillin. A single colony from each of these plates was used to inoculate 10 mL LB broth with 100  $\mu\text{g/mL}$  ampicillin. These cultures were grown for 8 hours at 37°C with shaking in
- 20 125 mL baffled flasks with vented, filtered lids. 200  $\mu\text{L}$  of this culture was used to inoculate two 500 mL baffled flasks with filtered vented lids containing LB broth with 100  $\mu\text{g/mL}$  ampicillin and 0.2% (w/v) arabinose. The expression cultures were grown for 16 – 18 hours at 37°C with shaking. Cells were harvested in 40 mL aliquots via centrifugation; the
- 25 supernatant was discarded and cell pellets were frozen at -80°C until purification.

- K9G9 and all variants were purified using the same process. Two cell pellets, representing 40 mL cell culture aliquots each, were resuspended in 4 mL Bug Buster Master Mix (Novagen 71456, Darmstadt,
- 30 Germany) and incubated for 15 minutes at room temperature followed by



15 minutes at 60°C. Denatured proteins and cell debris was pelleted by centrifugation at 7,000 rpm for 30 minutes and 4°C. The supernatant was decanted, save and filtered through a Acrodisc 0.2 µm syringe filter (PN4192, Pall, Ann Arbor, MI). K9G9 was purified from the filtered heat  
5 treated cell free extract using a GE Healthcare HiLoad 26/60 Superdex 200 gel filtration column (17-1071-01, Buckinghamshire, England). The column was pre-equilibrated with 0.2 CV equilibration with 50 mM HEPES (pH 7.5) 5 mM MgCl<sub>2</sub> buffer at a 2.0 mL/min flow rate prior to protein loading. K9G9 and variants were eluted over a 1.5 CV isocratic step  
10 consisting of 50 mM HEPES (pH 7.5) 5 mM MgCl<sub>2</sub> buffer at a 2.0 mL/min flow rate. Fractions 2.5 mL in volume were collected using a Frac-950 fraction collector (Buckinghamshire, England) in a serpentine pattern. K9G9 and variants all eluted between fractions D5 – E5 or D6 – E4. Fractions were pooled using a 15 mL Amicon Ultra YM-30 spin filter  
15 (UFC903008, Millipore, Billerica, MA) and washed with 10 mL 100 mM HEPES (pH 6.8) and 10 mM MgCl<sub>2</sub> buffer. Filtrate was discarded and the purified protein was eluted from the membrane using 1 mL buffer containing 100 mM HEPES (pH 6.8) and 10 mM MgCl<sub>2</sub>.

To determine  $V_{max}$  and  $K_M$  values for NADH and NADPH, the  
20 purified proteins were assayed at various concentrations of NAD(P)H (0 to 1000 µM) coupled with a NAD(P)H regeneration system. Assays were conducted at 30°C in a buffer containing 100mM MOPS, pH 6.8, 10mM MgCl<sub>2</sub>, 1mM EDTA, 5 mM (R/S)-acetolactate, 1mM glucose-6-phosphate, 3 mU/µL glucose-6-phosphate dehydrogenase. The reaction as quenched  
25 after ten minutes with three volumes 0.1% formic acid. DHIV concentration was measured using LC-MS. The rate of conversion of S-acetolactate to DHIV was determined by measuring the amount of DHIV produced at a fixed time point.  $V_{max}$  and  $K_m$  values were calculated by plotting specific activity (U/mg) vs. cofactor concentration and the data were fit to the  
30 Michaelis-Menten equation. Measurements of acetolactate  $K_m$  values (at a fixed concentration of NADH) indicated that the fixed acetolactate concentration employed for the cofactor  $K_m$  determinations was saturating.

**Table 23. Kinetic Values for Purified K9G9 Variants as Determined via DHIV Formation Assays**

| Variant    | $V_{max}$<br>NADPH,<br>U/mg | $K_m$<br>NADPH,<br>$\mu$ M | $V_{max}/K_m$<br>NADPH,<br>L/min*mg | $V_{max}$<br>NADH,<br>U/mg | $K_m$<br>NADH,<br>$\mu$ M | $V_{max}/K_m$<br>NADH,<br>L/min*mg |
|------------|-----------------------------|----------------------------|-------------------------------------|----------------------------|---------------------------|------------------------------------|
| K9G9       | 2.2                         | 24.1                       | 0.091                               | 1.9                        | 78.2                      | 0.024                              |
| K9JB4      | 2.7                         | 249                        | 0.011                               | 3.4                        | 115                       | 0.030                              |
| K9JB4P     | 2                           | 83.2                       | 0.024                               | 2.9                        | 34.1                      | 0.085                              |
| K9G3       | 3.1                         | 113                        | 0.027                               | 2.8                        | 106                       | 0.026                              |
| K9G3P      | 1.8                         | 33.6                       | 0.054                               | 2.1                        | 18.1                      | 0.116                              |
| K9JA1      | 2.6                         | 63.4                       | 0.041                               | 3.4                        | 14                        | 0.243                              |
| K9SB2      | 1.7                         | 44.8                       | 0.038                               | 1.8                        | 11.6                      | 0.155                              |
| K9SB2-K90L | 2.1                         | 173                        | 0.012                               | 2.4                        | 28.6                      | 0.084                              |
| K9SB2-K90M | 1.8                         | 245                        | 0.007                               | 2.2                        | 41.3                      | 0.053                              |

5

**Example 19.****Isobutanol production of K9G9 derivatives with increased ratios of  $K_m$  NADPH to  $K_m$  NADH**

The yeast expression plasmids for K9JB4, K9JB4P, K9JG3, K9JG3P, K9JA1, and K9SB2 were made by subcloning of the variant KARI genes from *E.coli* vectors (pBAD.KARI) into pHR81-Pilv5-KARI-K9.G9 at PmeI and SfiI sites. The resultant plasmids together with pHR81-Pilv5-KARI-K9.G9 and pHR81-Pilv5-KARI-K9.D3 (SEQ ID NO: 181) were analyzed for isobutanol production and by-product formation in yeast. Yeast pathway strains were made in PNY2259 (MATa ura3 $\Delta$ ::loxP his3 $\Delta$  pdc6 $\Delta$  pdc1 $\Delta$ ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5 $\Delta$ ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2 $\Delta$ ::loxP fra2 $\Delta$ ::P[PDC1]-ADH|adh\_HI-ADH1t adh1 $\Delta$ ::UAS(PGK1)P[FBA1]-kivD\_Lg(y)-ADH1t yprc15 $\Delta$ ::P[PDC5]-ADH|adh\_HI-ADH1t ymr226c $\Delta$  ald6 $\Delta$ ::loxP; Example 22) host by co-transforming the KARI vectors as pathway plasmid #1, and pBP915 (pRS423-P<sub>FBA1</sub>-DHAD-P<sub>GPM1</sub>-hADH1; SEQ ID NO: 182) as pathway plasmid #2. The transformed cells were plated on synthetic medium without histidine or uracil (1% ethanol as carbon source). Three transformants were transferred to fresh plates of the same media. The transformants were tested for isobutanol production under anaerobic conditions in serum vials.

Yeast colonies from the transformation on SE-Ura-His plates appeared after 3-5 days. The three colonies from each variant were patched onto fresh SE-Ura-His plates, incubate at 30°C for 3 days.

5 Growth media and procedure

Two types of media were used during the growth procedure of yeast strains: an aerobic pre-culture media and an anaerobic culture media. All chemicals were obtained from Sigma unless otherwise noted (St. Louis, MO)

10 Aerobic pre-culture media (SE-Ura-His): 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, MD), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.2% ethanol, 0.2% glucose, 0.01% w/v leucine and 0.002% w/v tryptophan.

15 Anaerobic culture media (SEG-Ura-His): 50 mM MES (pH 5.5, 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, MD), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.1% ethanol, 3% glucose, 0.01% leucine, 0.002% tryptophan, 30 mg/L nicotinic acid, 30 mg/L thiamine and 10 mg/L ergosterol made up in 50/50 v/v Tween /ethanol solution.

20 The patched cells were inoculated into 25 mL SEG-Ura,His media with 0.2% glucose and 0.2% ethanol, and grown under progressively oxygen-limited conditions with lid closed for approximately 48 hours at 30°C with shaking, until a target OD<sub>600</sub> value of approximately 1.5 to 2 was  
25 achieved. OD<sub>600</sub> values were recorded. Cells were pelleted via centrifugation and the supernatant was discarded. Cell pellets were transferred into a Coy Anaerobic Bag (Grass Lake, MI) where pellets were resuspended in 1.0 mL anaerobic growth media (SEG-Ura-His). The resuspended cell pellets were used to inoculate 30 mL SEG-Ura-His  
30 media in 50 mL serum bottles (Wheaton, 223748, Millville, NJ) to a target initial OD<sub>600</sub> value of 0.2. All anaerobic media, serum vials, stoppers and crimps were allowed to degas in the anaerobic bag for at least 24 hours prior to inoculation. Serum bottles were stoppered, crimped and transferred out of the anaerobic bag and grown at 30°C with shaking at

240 rpm. Anaerobic cultures were grown for 24 to 72 hours with a target OD<sub>600</sub> value of at least 1.2. Additional anaerobic growth steps used the cells from the previous anaerobic culture step as inoculant. Three transformants were evaluated for each variant.

5

HPLC analysis of yeast strains with K9G9 KARI variants

Samples were taken for HPLC analysis and to obtain OD<sub>600</sub> values at the end of the anaerobic growth period. HPLC analysis was performed using a Waters 2695 separations unit, 2996 photodiode array detector, and 2414 refractive index detector (Waters, Milford, MA) with a Shodex Sugar SH-G pre-column and Shodex Sugar SH1011 separations column (Shodex, JM Science, Grand Island, NY). Compounds were separated by isocratic elution at 0.01 N sulfuric acid with a flow rate of 0.5 mL/min. Chromatograms were analyzed using the Waters Empower Pro software.

15

Molar yields for glycerol, isobutanol and the glycerol/isobutanol ratio were determined. Mean and standard deviations were calculated from triplicate analyses for each variant. Student's t-test was then employed to determine if the difference in the values was statistically significant from the K9D3 control values. For the new variants, the increases in K<sub>M</sub> values for NADPH relative to K<sub>M</sub> for NADH are expected result reduced NADPH utilization. Results reported in the Table below and in Figure 9 indicate that the new variants with increased ratios of K<sub>M</sub> NADPH to K<sub>M</sub> NADH exhibit higher isobutanol to glycerol ratios relative to K9D3 and K9G9. K9SB2 demonstrated a 35% increase in isobutanol titer compared to K9D3.

25

**Table 24. K9G9 Variants Kinetic and Isobutanol Data**

|                 | Variant | K <sub>m</sub><br>(NADPH)/<br>K <sub>m</sub> (NADH) | Isobutanol/<br>Glycerol<br>Ratio | Isobutanol<br>Molar Yield | Isobutanol<br>Titer, mM |
|-----------------|---------|-----------------------------------------------------|----------------------------------|---------------------------|-------------------------|
| Experiment<br>1 | K9D3    | 0.24                                                | 1.67 ± 0.02                      | 0.581 ±<br>0.007          | 33.9 ± 1.8              |
|                 | K9JB4   | 2.2                                                 | 2.10 ± 0.06                      | 0.603 ±<br>0.006          | 35.3 ± 1.3              |
|                 | K9JG3   | 1.1                                                 | 2.07 ± 0.06                      | 0.598 ±<br>0.004          | 39.1 ± 1.6              |
| Experiment<br>2 | K9D3    | 0.24                                                | 1.75 ± 0.06                      | 0.586 ±<br>.0100          | 63.7 ± 0.6              |
|                 | K9JA1   | 4.5                                                 | 2.24 ± 0.04                      | 0.611 ±                   | 78.2 ± 5.4              |

|              |        |      |             |               |            |
|--------------|--------|------|-------------|---------------|------------|
|              |        |      |             | 0.002         |            |
|              | K9SB2  | 3.9  | 2.21 ± 0.10 | 0.608 ± 0.007 | 77.2 ± 1.4 |
| Experiment 3 | K9G9   | 0.31 | 2.10 ± 0.03 | 0.624 ± 0.011 | 54.2 ± 1.5 |
|              | K9JG3P | 1.9  | 2.56 ± 0.08 | 0.652 ± 0.009 | 66.3 ± 4.0 |
|              | K9JB4P | 2.4  | 2.54 ± 0.07 | 0.654 ± 0.006 | 61.5 ± 3.4 |

Example 20.5      Construction of K9SB2 error prone PCR library

The K9SB2 error prone PCR library was constructed in a similar manner as the K9G9 library with the following modifications. The mutagenic PCR mixture consisted of 9.5 µl K9SB2 in a pBAD.KARI vector (190 ng/ µl), 1.25 µl of primer K9G9\_EZ\_F1 (100 ng/ µl), 1.25 µl of primer K9G9\_EZ\_R1 (100 ng/ µl), 5 µl of 10× Mutazyme II reaction buffer, 1 µl of 40 mM dNTP mix, 1.5 µl of Mutazyme II DNA polymerase, and 30.5 µl of ddH<sub>2</sub>O. The "EZclone reaction" contained 25 µl of the 2× EZClone enzyme mix, 3 µl of megaprimer (K9SB2 mutagenic PCR product, 190 ng/ µl), 2.6 µl of K9SB2 template DNA (19 ng/ µl), 3 µl of EZClone solution 1, and 16µl of ddH<sub>2</sub>O. For the Dpn I step, the mixture was incubated for 3 hr at 37°C. Clones were employed for high throughput screening as described in Example 21.

20      Example 21.

Screening for K9SB2 variants with further increased ratios of Km NADPH to Km NADPH based on increased NADH to NADPH activity ratios

The K9SB2 library described in Example 20 was screened for variants with reduced NADPH affinity. With the specific objective to increase Km for NADPH relative to Km for NADH, the hits from the screen were partially purified and kinetic analyses were performed to determine V<sub>max</sub> and K<sub>m</sub> parameters with NADH and with NADPH.

High throughput screening assay of K9SB2 gene library  
 30      Variants were screened using HTS as described in Example 16, with the following exceptions. Assays buffer consisted of 2.4 mM (R/S)-

acetolactate, 100 mM HEPES pH 6.8, 10 mM MgCl<sub>2</sub>, 150 μM NADH or 100 μM NADPH and 12.5 μL 0.5 X cell lysate.

The ratio for the measured rate for oxidation of 100 μM NADPH to the measured rate for oxidation of 150 μM NADH was calculated for each variant and positive control well (2 per plate). A variant well was considered to contain an initial hit if the NADH rate was greater than 0.6 OD/hr and the rate ratio (NADPH/NADH) was less than 0.37 (three standard deviations lower than the positive control mean). A total of 218 hits were identified from a pool of 4947 potential variants. These initial hits were consolidated, forming a smaller library for further analysis.

The consolidated initial hit library was grown in biological triplicate and cell free extracts were prepared and assayed as described above. Rate ratios were then calculated for the variants and positive controls as above. Final hits that were selected for detailed kinetic analysis met the following criteria: the NADPH/NADH rate ratio was less than 0.45, the NADH rate was greater than 0.6 OD/hr and at least two out of three biological replicates passed the criteria. 107 variants were identified.

Data were also analyzed to identify variants that had a higher rate of conversion for S-acetolactate to DHIV with the NADH cofactor. The average rate and standard deviation of NADH oxidation was calculated for all the positive controls. A variant was considered a potential hit if the rate of NADH oxidation was at least 3 standard deviations higher than the rate of the positive control (2.524 OD/hr). 68 variants were identified and sequence analysis determined that 17 had at least one amino acid substitution. The substitutions T93A and T93I each appeared twice and variants 2017 B12 and D6 have been selected for further analysis.

DNA sequencing of the 107 variants identified from the secondary HTS screening was accomplished by using TempliPhi™ (GE Healthcare) with the primers pBAD-For (ATGCCATAGCATTTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394). 105 sequences were different from the parent and the amino acid substitutions are listed in the first of the following two tables.

DNA sequencing of the 68 variants identified from the NADH rate screening was accomplished by using TempliPhi™ (GE Healthcare) with primers pBAD-For (ATGCCATAGCATTTTATCC; SEQ ID NO: 393) and pBAD-Rev (CTGATTTAATCTGTATCAGGCT; SEQ ID NO: 394). 17

- 5 sequences were different from wild type and the amino acid substitutions of the 2 substitutions that appeared repeatedly are listed in the second table below.

**Table 25. K9SB2 Variants' Amino Acid Substitutions**

| Variant  | Seq | Amino Acid Substitutions                                |
|----------|-----|---------------------------------------------------------|
| K9SB2    | 427 | Y53F, S56A, K57E, S58E, N87P                            |
| 2011 A2  | 433 | Y53F, G55D, S56A, K57E, S58E, N87P                      |
| 2011 A3  | 434 | Y53F, S56A, K57E, S58E, N87P, M94I                      |
| 2011 A5  | 435 | Y53F, S56A, K57E, S58E, M83I, N87P, L185M, E217D        |
| 2011 A7  | 436 | Y53F, S56A, K57E, S58E, N87P, D98V                      |
| 2011 A9  | 437 | Y53F, S56A, K57E, S58E, F67I, N87P                      |
| 2011 A11 | 438 | Y53F, S56A, K57E, S58E, N87P, M94T, K126E, T273A        |
| 2011 B1  | 439 | Y53F, S56A, K57E, S58E, N87P, M94I, A279T               |
| 2011 B2  | 440 | Y53F, S56A, K57E, S58E, N87P, Q91L                      |
| 2011 B3  | 441 | Y53L, S56A, K57E, S58E, N87P                            |
| 2011 B4  | 442 | Y53F, S56A, K57E, S58E, N87P, P135T                     |
| 2011 B7  | 443 | Y53F, S56A, K57E, S58E, N87P, L185M                     |
| 2011 B8  | 444 | Y53F, S56A, K57E, S58E, N87P, C233S, F296Y              |
| 2011 B10 | 445 | Y53F, S56A, K57E, S58E, N87P, A303D                     |
| 2011 C1  | 446 | Y53F, S56A, K57E, S58E, E63K, N87P, G251D, K294R        |
| 2011 C3  | 448 | Y53F, S56A, K57E, S58E, A72V, N87P, N102Y, F189I, Y245H |
| 2011 C6  | 449 | Y53F, S56A, K57E, S58E, I84F, N87P                      |
| 2011 C7  | 450 | E13V, Y53F, S56A, K57E, S58E, M94I, N87P, T141I         |
| 2011 C8  | 451 | Y53F, S56A, K57E, S58E, A72V, N87P, N102Y, F189I, Y245H |
| 2011 C9  | 447 | Y53F, S56A, K57E, S58E, N87P, E194D                     |
| 2011 C10 | 452 | Y53F, A56G, K57E, S58E, K60N, N87P                      |
| 2011 C12 | 453 | Y53F, S56A, K57E, S58E, I84L, N87P, N97T                |
| 2011 D1  | 454 | L39M, Y53F, S56A, K57E, S58E, E68G, N87P                |
| 2011 D2  | 455 | Y53F, S56A, K57E, S58E, N87P, M94I, V307I               |
| 2011 D3  | 456 | Y53F, S56A, K57E, S58E, F67I, N87P                      |
| 2011 D4  | 457 | Y53F, S56A, K57E, S58E, N87P, P135L, A202V              |
| 2011 D5  | 458 | Y53F, S56A, K57E, S58E, N87P, G164S, G199A              |
| 2011 D6  | 500 | Y53F, S56A, K57E, S58E, N87P, S247C                     |
| 2011 D8  | 459 | Y53F, S56A, K57E, S58E, N87P, N116I                     |
| 2011 D9  | 460 | Y53F, S56A, K57E, S58E, N87P, K90M                      |
| 2011 D11 | 461 | Y53F, S56A, K57E, S58E, N87P, M94L, T259I               |
| 2011 D12 | 462 | Y53F, S56A, K57E, S58E, M83K, N87P                      |

|          |     |                                                                 |
|----------|-----|-----------------------------------------------------------------|
| 2011 E3  | 463 | Y53F, S56A, K57E, S58E, N87P, I122V, L297W                      |
| 2011 E4  | 464 | Y53F, S56A, K57E, S58E, N87P, A112S, Q160R                      |
| 2011 E8  | 465 | Y53F, S56A, K57E, S58E, N87P, V142I, P320L                      |
| 2011 E11 | 466 | Y53F, S56A, K57E, S58E, N87P, Q91L                              |
| 2011 F2  | 467 | Y53F, S56V, K57E, S58E, N87P, A210T                             |
| 2011 F4  | 468 | Y53F, S56A, K57E, S58E, N87P, F189I                             |
| 2011 F6  | 469 | Y53F, S56G, K57E, S58E, K60N, N87P                              |
| 2011 F9  | 470 | A41V, Y53F, S56A, K57E, S58E, N87P, S305P                       |
| 2011 F10 | 471 | Y53F, S56A, K57E, S58E, L85M, N87P                              |
| 2011 G1  | 472 | E13V, Y53F, S56A, K57E, S58E, M94I, N87P, T141I                 |
| 2011 G3  | 473 | H35Q, Y53F, S56A, K57E, S58E, N87P                              |
| 2011 G4  | 474 | Y53F, S56A, K57E, S58E, I84F, N87P                              |
| 2011 G8  | 475 | A26T, Y53F, S56A, K57E, S58E, N87P, K90M                        |
| 2011 G9  | 476 | Y53F, S56T, K57E, S58E, N87P                                    |
| 2011 G10 | 477 | Y53F, S56A, K57E, S58E, I84F, N87P                              |
| 2011 H1  | 478 | Y53F, S56A, K57E, S58E, N87P, M94I, V156L                       |
| 2011 H5  | 479 | Y53F, S56A, K57E, S58E, I84L, N87P                              |
| 2011 H7  | 480 | Y53F, S56T, K57E, S58E, N87P                                    |
| 2011 H9  | 481 | Y53F, G55C, S56A, K57E, S58E, N87P                              |
| 2012 A2  | 482 | Y53F, S56A, K57E, S58E, R61G, I86V, N87P                        |
| 2012 A7  | 483 | Y53F, S56A, K57E, S58E, T71S, A76V, N87P                        |
| 2012 A8  | 484 | Y53F, S56A, K57E, S58E, N87P, M212T                             |
| 2012 A9  | 485 | H35Q, Y53F, S56A, K57E, S58E, A72T, N87P                        |
| 2012 A10 | 486 | A36T, Y53F, S56A, K57E, S58E, N87P                              |
| 2012 A11 | 487 | Y53F, S56A, K57E, S58E, N87P, S247T                             |
| 2012 B3  | 488 | Y53F, S56A, K57E, S58E, N87P, P135S                             |
| 2012 B4  | 489 | Y53F, S56A, K57E, S58E, W59R, N87P, K278E                       |
| 2012 B5  | 490 | Y53F, S56V, K57E, S58E, N87P, I234V                             |
| 2012 B6  | 491 | Y30H, Y53F, S56A, K57E, S58E, N87P                              |
| 2012 B9  | 492 | I50M, Y53F, S56A, K57E, S58E, N87P                              |
| 2012 C2  | 493 | Y53L, S56A, K57E, S58E, N87P                                    |
| 2012 C3  | 494 | Y53F, S56A, K57E, S58E, N87P, F115V, T191S, V208I, C209W, F292I |
| 2012 C5  | 495 | Y53F, S56A, K57E, S58E, N87P, M94I                              |
| 2012 C6  | 496 | Y53F, S56A, K57E, S58E, F67L, N87P                              |
| 2012 C8  | 497 | Y53F, S56A, K57E, S58E, N87P, M94I, M169T                       |
| 2012 C10 | 498 | Y53F, S56A, K57E, S58E, F67I, N87P, T276I                       |
| 2012 D1  | 499 | Y53F, S56A, K57E, S58E, I84F, N87P, M132T                       |
| 2012 D8  | 501 | Y53F, S56A, K57E, S58E, N87P, P135S                             |
| 2012 D11 | 502 | K8N, Y53F, S56A, K57E, S58E, N87P, K90M, T141I                  |
| 2012 E5  | 503 | Y53F, S56A, K57E, S58E, I84L, N87P                              |
| 2012 E9  | 504 | Y53F, S56A, K57E, S58E, N87P, V142I, T191S, C233S               |
| 2012 F1  | 505 | Y53F, S56A, K57E, S58E, N87P, H235Y                             |
| 2012 F2  | 506 | Y53F, S56V, K57E, S58E, N87P, V232D                             |
| 2012 F3  | 507 | Y53F, S56A, K57E, S58E, N87P, K90M, V142I, T187S                |
| 2012 F4  | 508 | Y53F, S56A, K57E, S58E, N87P, M94I, G149D                       |
| 2012 F7  | 509 | E13V, Y53F, S56A, K57E, S58E, N87P, M94I, T141I                 |
| 2012 F10 | 510 | Y53F, S56A, K57E, S58E, Q65H, N87P, F189I                       |



|          |     |                                                        |
|----------|-----|--------------------------------------------------------|
| 2012 F12 | 511 | Y53F, S56V, K57E, S58E, N87P                           |
| 2012 G3  | 512 | Y53F, S56T, K57E, S58E, N87P, R190S                    |
| 2012 G4  | 513 | Y53F, S56A, K57E, S58E, N87P, N102S, V142I             |
| 2012 G5  | 514 | Y53F, S56A, K57E, S58E, I84L, N87P                     |
| 2012 G8  | 515 | Y53F, S56A, K57E, S58E, K77N, N87P, A92V               |
| 2012 G9  | 516 | Y53F, S56A, K57E, S58E, N87P, M94I, V307I              |
| 2012 G10 | 517 | Y53F, S56A, K57E, S58E, N87P, T195I                    |
| 2012 G12 | 518 | Y53F, S56A, K57E, S58E, N87P, F309I                    |
| 2012 H1  | 519 | Y53F, S56A, K57E, S58E, N87P, K90T, A180S              |
| 2012 H3  | 520 | Y53F, S56A, K57E, S58E, W59C, N87P                     |
| 2012 H7  | 521 | Y53F, S56A, K57E, S58E, N87P, M94I, A202T              |
| 2012 H9  | 522 | H35N, Y53F, S56A, K57E, S58E, N87P                     |
| 2012 H11 | 523 | Y53F, S56A, K57E, S58E, A72V, N87P, L211M, I240M       |
| 2013 A2  | 524 | Y53F, S56T, K57E, S58E, N87P, Q288H                    |
| 2013 A4  | 525 | Y53F, S56A, K57E, S58E, L85M, N87P                     |
| 2013 A5  | 526 | L52S, Y53F, S56A, K57E, S58E, N87P                     |
| 2013 B2  | 527 | A36T, Y53F, S56A, K57E, S58E, N87P, V203I              |
| 2013 B5  | 528 | Y53F, S56A, K57E, S58E, N87P, P135T                    |
| 2013 B7  | 529 | I9M, Y53F, S56A, K57E, S58E, N87P, K90E                |
| 2013 B8  | 530 | Y53F, G55C, S56A, K57E, S58E, N87P                     |
| 2013 B9  | 531 | Y53F, S56V, K57E, S58E, N87P                           |
| 2013 B11 | 532 | A38V, Y53F, S56A, K57E, S58E, N87P                     |
| 2013 C1  | 533 | Y53L, S56A, K57E, S58E, N87P, M237I                    |
| 2013 C6  | 534 | K23M, Y53F, S56A, K57E, S58E, N87P, E194D              |
| 2013 C8  | 535 | Y53F, S56A, K57E, S58E, N87P, P135T                    |
| 2013 C12 | 536 | Y53F, S56A, K57E, S58E, A72V, N87P, T93S, A176V, H235Y |
| 2013 D1  | 537 | Y53F, S56A, K57E, S58E, N87P, M94R, K310M              |

**Table 26. K9SB2 Variants' Amino Acid Substitutions**

| Variant  | AA<br>Seq ID<br>NO;<br>Nucleic<br>acid<br>SEQ<br>ID NO | Amino Acid Substitutions           |
|----------|--------------------------------------------------------|------------------------------------|
| K9SB2    | 427                                                    | Y53F, S56A, K57E, S58E, N87P       |
| 2017 B12 | 639;<br>640                                            | Y53F, S56A, K57E, S58E, N87P, T93I |
| 2017 D6  | 641;<br>642                                            | Y53F, S56A, K57E, S58E, N87P, T93A |

- 5 Kinetic analysis of partially purified K9SB2 variant proteins  
*E. coli* strain Bw25113 ( $\Delta ilvC$ ), as described in U.S. Patent  
8,129,162, was used to express the 107 variants from the secondary HTS

screening and positive control K9SB2. Clones from archive plates were inoculated into the 96-deep well plates. Each well contained 3.0  $\mu$ l of cells from thawed archive plates, 200  $\mu$ l of the LB medium containing 100  $\mu$ g/ml ampicillin and 0.02%(w/v) arabinose as the inducer. Cells were grown  
5 overnight at 37°C with 80% humidity while shaking (900 rpm), harvested by centrifugation (4000 rpm, 7 min at 4°C) (75004251, Thermo Scientific, Rockford, IL) and the cell pellet was stored at -80°C for later analysis.

Frozen cell pellets in deep-well plates were thawed at room temperature for 30 minutes at the same time. 75  $\mu$ l of 50% BugBuster  
10 (Novagen 71456, Darmstadt, Germany) (v/v in water) was added to each and cells were suspended using a plate shaker. The cells suspension in 50% Bug Buster was incubated for 30 minutes at room temperature which was then followed by a 15 minute incubation at 60°C. Cell debris and denatured heat labile proteins were pelleted by centrifugation (4000  
15 rpm, 15 min at 4°C) (75004251, Thermo Scientific, Rockford, IL) and 75  $\mu$ L of the supernatant was transferred to a flat bottomed 96-well plate (Corning, 3370, Corning, NY) and diluted two-fold with 75  $\mu$ L 100 mM HEPES (pH 6.8), 100 mM KCl, 10 mM  $MgCl_2$ .

Total protein was determined by using the Bradford Assay with  
20 Coomassie Plus (Thermo Scientific, #23238, Rockford, IL). BSA was employed as the standard. The concentration of protein was measured by determining the absorbance at 595 nm using a Cary 300 spectrophotometer (Agilent Technologies, Wilmington, DE).

To determine  $V_{max}$  and  $K_M$  values for NADH and NADPH, the  
25 partially purified proteins were assayed at various concentrations of NADH (20, 30, 40, 60, 80, 120, 200 and 300  $\mu$ M) and NADPH (60, 80, 120, 200, 300 and 400  $\mu$ M). Assays were conducted at 30°C in 100 mM HEPES (pH 6.8), 10 mM  $MgCl_2$ , 100 mM KCl and 4.8 mM *R/S*-acetolactate. Between 0.005 to 0.015 mg/mL total protein was added to the assay. The rate of  
30 conversion of *S*-acetolactate to DHIV was measured via monitoring the oxidation of NAD(P)H at 340 nm using a Spectramax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA). The activity was calculated using the molar extinction coefficient of 6220  $M^{-1}cm^{-1}$ .  $V_{max}$  and  $K_m$  values

were calculated by plotting specific activity (U/mg) vs. cofactor concentration and the data were fit to the Michaelis-Menten equation using Kaleidagraph software (Synergy, Reading, PA).

**Table 27. Kinetic Values for Partially Purified K9SB2 Variants as Determined via NAD(P)H Consumption Assays**

| Variant  | $V_{max}$<br>NADPH,<br>U/mg | $K_m$<br>NADPH,<br>$\mu$ M | $V_{max}/K_m$<br>NADPH,<br>L/min*mg | $V_{max}$<br>NADH,<br>U/mg | $K_m$<br>NADH,<br>$\mu$ M | $V_{max}/K_m$<br>NADH,<br>L/min*mg |
|----------|-----------------------------|----------------------------|-------------------------------------|----------------------------|---------------------------|------------------------------------|
| K9SB2    | 1.79                        | 153                        | 0.012                               | 2.10                       | 87.4                      | 0.024                              |
| 2011 A2  | 1.48                        | 897                        | 0.002                               | 1.94                       | 71.2                      | 0.027                              |
| 2011 A3  | 1.43                        | 371                        | 0.004                               | 1.33                       | 44.1                      | 0.030                              |
| 2011 A5  | 0.93                        | 109                        | 0.009                               | 0.23                       | 17.4                      | 0.017                              |
| 2011 A7  | 2.27                        | 334                        | 0.007                               | 1.70                       | 69.2                      | 0.025                              |
| 2011 A9  | 2.09                        | 266                        | 0.008                               | 0.40                       | n/d                       | n/a                                |
| 2011 A11 | 2.21                        | 294                        | 0.008                               | 1.25                       | 25.8                      | 0.048                              |
| 2011 B1  | 1.79                        | 421                        | 0.004                               | 64.5                       | 55171                     | 0.001                              |
| 2011 B2  | 0.33                        | 254                        | 0.001                               | 0.02                       | 264                       | 0.007                              |
| 2011 B3  | 1.87                        | 505                        | 0.004                               | 0.68                       | 225                       | 0.003                              |
| 2011 B4  | 0.36                        | 294                        | 0.001                               | 0.26                       | 171                       | 0.002                              |
| 2011 B7  | n/d                         | 1.03                       | n/a                                 | 0.48                       | 25.5                      | 0.019                              |
| 2011 B8  | 0.83                        | 109.5                      | 0.008                               | 0.61                       | 28.7                      | 0.021                              |
| 2011 B10 | 0.88                        | 171                        | 0.005                               | 0.46                       | 7.53                      | 0.061                              |
| 2011 C1  | 1.06                        | 404                        | 0.003                               | 1.51                       | 191                       | 0.008                              |
| 2011 C3  | 1.08                        | 844                        | 0.001                               | 2.53                       | 500                       | 0.005                              |
| 2011 C6  | 1.19                        | 388                        | 0.003                               | 1.90                       | 189                       | 0.010                              |
| 2011 C7  | 0.71                        | 946                        | 0.001                               | 1.95                       | 457                       | 0.004                              |
| 2011 C8  | 1.73                        | 1546                       | 0.001                               | 3.73                       | 750                       | 0.005                              |
| 2011 C9  | 1.02                        | 123                        | 0.008                               | 2.29                       | 177                       | 0.013                              |
| 2011 C10 | 1.02                        | 656                        | 0.002                               | 3.84                       | 899                       | 0.004                              |
| 2011 C12 | 2.74                        | 244                        | 0.011                               | 3.08                       | 99                        | 0.031                              |
| 2011 D1  | 4.68                        | 501                        | 0.009                               | 3.54                       | 80.4                      | 0.044                              |
| 2011 D2  | 2.34                        | 547                        | 0.004                               | 2.18                       | 77.3                      | 0.028                              |
| 2011 D3  | 0.05                        | 306                        | 0.0002                              | 0.05                       | 44.3                      | 0.001                              |
| 2011 D4  | 0.47                        | 857                        | 0.001                               | 0.44                       | 91.6                      | 0.005                              |
| 2011 D5  | 0.75                        | 550                        | 0.001                               | 0.42                       | 57.1                      | 0.007                              |
| 2011 D6  | 0.70                        | 200                        | 0.004                               | 0.52                       | 25.11                     | 0.021                              |
| 2011 D8  | 0.04                        | 214                        | 0.0002                              | 0.04                       | 38.9                      | 0.001                              |
| 2011 D9  | 0.18                        | 407                        | 0.0004                              | 0.16                       | 50.2                      | 0.003                              |
| 2011 D11 | 0.78                        | 185                        | 0.004                               | 0.61                       | 15.0                      | 0.041                              |
| 2011 D12 | 0.74                        | 190                        | 0.004                               | 0.80                       | 39.0                      | 0.021                              |
| 2011 E3  | 0.77                        | 163                        | 0.005                               | 1.54                       | 128                       | 0.012                              |
| 2011 E4  | 1.59                        | 270                        | 0.006                               | 3.78                       | 234                       | 0.016                              |
| 2011 E8  | 0.91                        | 435                        | 0.002                               | 2.16                       | 252                       | 0.009                              |
| 2011 E11 | 5.56                        | 6466                       | 0.001                               | 3.06                       | 511                       | 0.006                              |
| 2011 F2  | 0.39                        | 692                        | 0.001                               | 1.79                       | 136                       | 0.013                              |
| 2011 F4  | 2.07                        | 242                        | 0.009                               | 2.00                       | 68.3                      | 0.029                              |

|          |      |      |        |      |      |       |
|----------|------|------|--------|------|------|-------|
| 2011 F6  | 1.19 | 946  | 0.001  | 1.35 | 231  | 0.006 |
| 2011 F9  | 0.57 | 269  | 0.002  | 0.45 | 27.1 | 0.017 |
| 2011 F10 | 1.43 | 390  | 0.004  | 1.55 | 79.8 | 0.019 |
| 2011 G1  | 1.31 | 1533 | 0.001  | 0.50 | 40.1 | 0.013 |
| 2011 G3  | 0.61 | 1003 | 0.001  | 1.00 | 283  | 0.004 |
| 2011 G4  | 1.00 | 316  | 0.003  | 2.13 | 174  | 0.012 |
| 2011 G8  | 0.90 | 482  | 0.002  | 1.22 | 106  | 0.012 |
| 2011 G9  | 0.30 | 530  | 0.001  | 3.48 | 549  | 0.006 |
| 2011 G10 | 0.93 | 358  | 0.003  | 2.13 | 238  | 0.009 |
| 2011 H1  | 0.96 | 218  | 0.004  | 1.57 | 114  | 0.014 |
| 2011 H5  | 1.17 | 81.2 | 0.014  | 1.85 | 86.5 | 0.022 |
| 2011 H7  | 0.20 | 435  | 0.001  | 1.25 | 173  | 0.007 |
| 2011 H9  | 1.30 | 741  | 0.002  | 1.55 | 177  | 0.009 |
| 2012 A2  | 1.71 | 264  | 0.007  | 1.58 | 55.4 | 0.029 |
| 2012 A7  | 1.98 | 215  | 0.009  | 1.87 | 67.3 | 0.028 |
| 2012 A8  | 1.19 | 91.8 | 0.013  | 1.24 | 22.7 | 0.055 |
| 2012 A9  | 0.44 | 481  | 0.001  | 0.34 | 38.1 | 0.009 |
| 2012 A10 | 1.21 | 340  | 0.004  | 1.31 | 66.7 | 0.020 |
| 2012 A11 | 1.99 | 342  | 0.006  | 1.37 | 35.9 | 0.038 |
| 2012 B3  | 0.88 | 1214 | 0.001  | 0.42 | 63.8 | 0.007 |
| 2012 B4  | 4.34 | 1593 | 0.003  | 1.66 | 95.1 | 0.018 |
| 2012 B5  | 4.88 | 7389 | 0.001  | 1.19 | 85.2 | 0.014 |
| 2012 B6  | 3.72 | 428  | 0.009  | 2.23 | 51.3 | 0.044 |
| 2012 B9  | 1.17 | 523  | 0.002  | 0.87 | 63.7 | 0.014 |
| 2012 C2  | 4.43 | 923  | 0.005  | 3.16 | 189  | 0.017 |
| 2012 C3  | 1.40 | 203  | 0.007  | 1.80 | 68.9 | 0.026 |
| 2012 C5  | 1.73 | 348  | 0.005  | 3.66 | 268  | 0.014 |
| 2012 C6  | 2.18 | 234  | 0.009  | 2.90 | 103  | 0.028 |
| 2012 C8  | 1.53 | 394  | 0.004  | 2.63 | 194  | 0.014 |
| 2012 C10 | 1.12 | 286  | 0.004  | 1.41 | 78.9 | 0.018 |
| 2012 D1  | 0.72 | 599  | 0.001  | 0.58 | 64.5 | 0.009 |
| 2012 D8  | 1.17 | 1528 | 0.001  | 0.47 | 73.6 | 0.006 |
| 2012 D11 | 0.43 | 334  | 0.001  | 0.52 | 27.7 | 0.019 |
| 2012 E5  | 2.13 | 257  | 0.008  | 4.77 | 313  | 0.015 |
| 2012 E9  | 1.07 | 1326 | 0.001  | n/d  | n/d  | n/a   |
| 2012 F1  | 1.70 | 272  | 0.006  | 1.48 | 62.5 | 0.024 |
| 2012 F2  | 0.39 | 925  | 0.0004 | 0.79 | 97.8 | 0.008 |
| 2012 F3  | 1.40 | 2213 | 0.0006 | 0.94 | 142  | 0.007 |
| 2012 F4  | 2.70 | 719  | 0.004  | 1.50 | 72.9 | 0.021 |
| 2012 F7  | 0.86 | 840  | 0.001  | 0.77 | 117  | 0.007 |
| 2012 F10 | 1.92 | 170  | 0.011  | 1.75 | 27.4 | 0.064 |
| 2012 F12 | 0.90 | 1582 | 0.0006 | 1.75 | 117  | 0.015 |
| 2012 G3  | 1.47 | 1003 | 0.002  | 1.07 | 127  | 0.008 |
| 2012 G4  | 0.73 | 615  | 0.001  | 0.63 | 81.6 | 0.007 |
| 2012 G5  | 1.92 | 240  | 0.008  | 2.00 | 83.5 | 0.024 |
| 2012 G8  | 1.17 | 315  | 0.004  | 0.99 | 58.7 | 0.017 |
| 2012 G9  | 2.41 | 717  | 0.003  | 1.37 | 91.5 | 0.015 |

|          |       |       |        |       |      |        |
|----------|-------|-------|--------|-------|------|--------|
| 2012 G10 | 1.06  | 400   | 0.003  | 0.71  | 39.8 | 0.018  |
| 2012 G12 | 1.58  | 147   | 0.011  | 2.00  | 70.0 | 0.029  |
| 2012 H1  | 1.49  | 195   | 0.008  | 1.74  | 68.9 | 0.025  |
| 2012 H3  | 14.98 | 7389  | 0.002  | 1.45  | 99.0 | 0.015  |
| 2012 H7  | 1.14  | 246   | 0.005  | 1.30  | 76.2 | 0.017  |
| 2012 H9  | 0.37  | 210   | 0.002  | n/d   | n/d  | n/a    |
| 2012 H11 | 0.65  | 162   | 0.004  | 0.62  | 32.0 | 0.019  |
| 2013 A2  | 0.58  | 285   | 0.002  | 0.64  | 71.0 | 0.009  |
| 2013 A4  | 0.63  | 188   | 0.003  | 0.86  | 81.5 | 0.011  |
| 2013 A5  | 0.61  | 886   | 0.001  | 0.88  | 210  | 0.004  |
| 2013 B2  | 0.62  | 282   | 0.002  | 0.71  | 70.4 | 0.010  |
| 2013 B5  | 6.68  | 7389  | 0.001  | 0.083 | 150  | 0.006  |
| 2013 B7  | 1.22  | 433   | 0.003  | 1.12  | 79.0 | 0.014  |
| 2013 B8  | 0.42  | 90.7  | 0.005  | 1.27  | 191  | 0.007  |
| 2013 B9  | 5.31  | 13970 | 0.0004 | 1.38  | 217  | 0.006  |
| 2013 B11 | 0.48  | 212   | 0.002  | 0.60  | 63.2 | 0.010  |
| 2013 C1  | 0.49  | 149   | 0.003  | 0.68  | 64.8 | 0.0105 |
| 2013 C6  | 0.54  | 163   | 0.003  | 0.36  | 24.4 | 0.015  |
| 2013 C8  | 2.87  | 3752  | 0.001  | 0.70  | 188  | 0.004  |
| 2013 C12 | 0.75  | 495   | 0.002  | 0.79  | 115  | 0.007  |
| 2013 D1  | 1.31  | 1608  | 0.001  | 0.87  | 188  | 0.005  |

Example 22:Construction of strain PNY2259

The purpose of this example is to describe the assembly of the  
5 constructs used to replace the chromosomal copy of *kivD\_LI(y)* in  
PNY2238 at the *adh1Δ* locus with *kivD\_Lg(y)*.

The deletion/integration was created by homologous recombination  
with PCR products containing regions of homology upstream and  
downstream of the target region and the *URA3* gene for selection of  
10 transformants. The *URA3* gene was removed by homologous  
recombination to create a scarless deletion/integration. The plasmid to  
integrate *kivD\_Lg(y)* was derived from a plasmid constructed to integrate  
UAS(PGK1)P[FBA1]-*kivD\_LI(y)* into the *ADH1* locus of *Saccharomyces*  
*cerevisiae*. Construction of the plasmid used to integrate  
15 UAS(PGK1)P[FBA1]-*kivD\_LI(y)* into the *ADH1* locus is described below.  
The plasmids were constructed in pUC19-URA3MCS.

Construction of the *ADH1* deletion/UAS(PGK1)P[FBA1]-*kivD LI(y)*  
integration plasmid

The *kivD* coding region from *Lactococcus lactis* codon optimized for expression in *Saccharomyces cerevisiae*, *kivD\_LI(y)*, was amplified using pLH468 (SEQ ID NO: 139) as template with primer oBP562 (SEQ ID NO: 197), containing a PmeI restriction site, and primer oBP563 (SEQ ID NO: 198), containing a 5' tail with homology to the 5' end of ADH1 Fragment B. ADH1 Fragment B was amplified from *Saccharomyces cerevisiae* CEN.PK 113-7D genomic DNA with primer oBP564 (SEQ ID NO: 199), containing a 5' tail with homology to the 3' end of *kivD\_LI(y)*, and primer oBP565 (SEQ ID NO: 200), containing a FseI restriction site. PCR products were purified with a PCR Purification kit (Qiagen; Valencia, CA). *kivD\_LI(y)*-ADH1 Fragment B was created by overlapping PCR by mixing the *kivD\_LI(y)* and ADH1 Fragment B PCR products and amplifying with primers oBP562 (SEQ ID NO: 197) and oBP565 (SEQ ID NO: 200). The resulting PCR product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. ADH1 Fragment A was amplified from genomic DNA with primer oBP505 (SEQ ID NO: 201), containing a SacI restriction site, and primer oBP506 (SEQ ID NO: 202), containing an AsclI restriction site. The ADH1 Fragment A PCR product was digested with SacI and AsclI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing *kivD\_LI(y)*-ADH1 Fragment B. ADH1 Fragment C was amplified from genomic DNA with primer oBP507 (SEQ ID NO: 203), containing a PacI restriction site, and primer oBP508 (SEQ ID NO: 204), containing a Sall restriction site. The ADH1 Fragment C PCR product was digested with PacI and Sall and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing ADH1 Fragment A-*kivD\_LI(y)*-ADH1 Fragment B. The hybrid promoter UAS(PGK1)-P<sub>FBA1</sub> (SEQ ID NO: 406) was amplified from vector pRS316- UAS(PGK1)-P<sub>FBA1</sub>-GUS with primer oBP674 (SEQ ID NO: 205), containing an AsclI restriction site, and primer oBP675 (SEQ ID NO: 206), containing a PmeI restriction site. The UAS(PGK1)-P<sub>FBA1</sub> PCR product was digested with AsclI and PmeI and ligated with T4 DNA ligase into the corresponding sites of the plasmid containing *kivD\_LI(y)*-ADH1 Fragments ABC to generate pBP1181.

Construction of pBP1716 and pBP1719

kivD\_LI(y) was removed from the ADH1 deletion/UAS(PGK1)P[FBA1]-kivD\_LI(y) integration plasmid pBP1181. The  
5 plasmid was digested with PmeI and FseI and the large DNA fragment was purified on an agarose gel followed by a gel extraction kit (Qiagen). ADH1 fragment B was amplified from pBP1181 with primer oBP821 (SEQ ID NO: 407), containing a PmeI restriction site, and primer oBP484 (SEQ ID NO: 408), containing a FseI restriction site. The ADH1 fragment B PCR  
10 product was digested with PmeI and FseI and ligated with T4 DNA ligase into the corresponding sites of the gel purified large DNA fragment. A PCR fragment corresponding to the 3' 500bp of kivD\_LI(y) was cloned into the resulting vector for the targeted deletion of kivD\_LI(y) in PNY1528. The fragment was amplified from pBP1181 with primers oBP822 (SEQ ID NO:  
15 409), containing a NotI restriction site, and oBP823 (SEQ ID NO: 410), containing a PaeI restriction site. The fragment was digested with NotI and PaeI and ligated with T4 DNA ligase into the corresponding sites downstream of URA3 in the above plasmid with the kivD\_LI(y) deletion after digestion with the appropriate restriction enzymes. The resulting  
20 plasmid was designated pBP1716.

The *kivD* coding region from *Listeria grayi* codon optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NO: 411), kivD\_Lg(y), was synthesized by DNA2.0 (Menlo Park, CA). kivD\_Lg(y) was amplified with primers oBP828 (SEQ ID NO: 412), containing a PmeI restriction site,  
25 and oBP829 (SEQ ID NO: 413) containing a PmeI restriction site. The resulting PCR product was digested with PmeI and ligated with T4 DNA ligase into the corresponding site in pBP1716 after digestion with the appropriate enzyme. The orientation of the cloned gene was checked by PCR with primers FBAp-F (SEQ ID NO: 414) and oBP829 (SEQ ID NO:  
30 413). An isolate with kivD\_Lg(y) in the correct orientation was designated pBP1719.

Construction of strain PNY2259

The *kivD\_LI(y)* deletion/*kivD\_Lg(y)* integration cassette was amplified from pBP1719 with primers oBP505 (SEQ ID NO: 201) and oBP823 (SEQ ID NO: 410). Competent cells of the PNY2238 were made and transformed with the PCR product using a Frozen-EZ Yeast  
 5 Transformation II kit (Zymo Research; Orange, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol at 30C. Transformant strains were screened by PCR (JumpStart(TM) REDTaq (c) ReadyMix(TM)) using primers Ura3-end F (SEQ ID NO: 222) and HY-50 (SEQ ID NO: 415).  
 10 Transformants were grown in YPE (1% ethanol) and plated on synthetic complete medium supplemented with 1% EtOH and containing 5-fluoroorotic acid (0.1%) at 30 C to select for isolates that lost the *URA3* marker. The deletion of *kivD\_LI(y)* and integration of *kivD\_Lg(y)* was confirmed by PCR with primers HY-50 and oBP834 (SEQ ID NO: 416). One correct  
 15 isolate contained *kivD\_Lg(y)* at the same locus and expressed from the same promoter as *kivD\_LI(y)* in PNY2238 was designated PNY2259.

### Example 23

#### 20 Construction of two site-saturation gene libraries to identify variants with cofactor preference to NADH

In Example 4, primers having the degeneracy codon NNK were used (N represents all 4 nucleotides A, C G and T while K stands for G and T). In this Example, primer mixtures containing primers encoding each  
 25 individual amino acid change of A, C, D, E, F, G, H, I, L, M, N, P, Q, V, W, or Y for positions 53, 56 and 58 of K9 KARI were employed and substitutions to S, T, K, and R were excluded as non-preferred for these positions. The size of the saturation library targeting the three NADPH phosphate binding sites (53, 56 and 58) of K9 KARI is 4,096 (as compared  
 30 to 32\*32\*32 or 32,768 variants using NNK degeneracy code primers as in Example 4).

One library construction method started from position 58. Primer mixtures were first made by mixing all the primers targeting the same



positions (for example, the primer mixture, K9\_53f, was made by mixing equal mole of all 16 forward primers targeting position 53 (listed in the table below). Similarly, K9\_56f and K9\_58f were prepared. The shared reverse primer is K9\_191G\_112210r (SEQ ID NO: 174):

- 5 GGTTCAGTTTCGCCTCTGAAGGTAGTTTC (called SR in this example). The mutation at position 58 was first introduced into AS6F1 through PCR. The mutagenesis procedure is similar to the one described in Example 4. In brief, K9\_58f and SR were phosphorylated. The phosphorylated primers were then directly used to introduce mutation at position 58 into AS6F1
- 10 using USB Change\_It kit (USB Corporation, Cleveland, OH, #78480). The template was removed with Dpn I. The cleaned up PCR product (Zymo DNA Clean & Concentrator-5; Zymo Research Corporation, Irvine, CA, Cat #D4003) was transformed into KOBW-3a cells. After overnight growth on LB agar plates at a 37 °C incubator, all cells were collected and DNA
- 15 was extracted using the Qiaprep Spin miniprep kit (Qiagen Inc. Valencia, CA, Cat #27106)

The extracted DNA was then used as templates to introduce mutation at position 56 using K9\_56f and SR same as the mutagenesis for position 58. At last the mutation at position 53 was similarly introduced.

- 20 After mutations at all three positions (53, 56 and 58) were introduced into AS6F1, the new library was screened same as the one described in example 4 and some selected mutants are listed in the table below.

The other method began with the position 53. The primer mixtures K9\_56r and K9\_58r were similarly prepared using primers listed in the

- 25 table below). The shared forward primer is pBAD\_266f: CTCTCTACTGTTTCTCCATACCCG (SEQ ID NO: 634; called SF in this example). The mutation at position 53 was first introduced into AS6F1 through PCR. The mutagenesis procedure is similar to the one described above using AS6F1 as the template and K9\_53f and SR as the two PCR
- 30 primers. The resulted mutated DNA (at position 53) was used as templates and K9\_56r and SF were used as the mutagenesis primers to introduced mutation at position 56. At last the mutation at position 58 was similarly introduced using K9\_58r and SF. After mutations at all three

positions (53, 56 and 58) were introduced into AS6F1, the new library was screened same as above and some selected mutants were listed in the table below.

Table 28. forward mutational primers.

| Targeted position(s) of K9-KARI | Primers        |                                     |                  |
|---------------------------------|----------------|-------------------------------------|------------------|
| 53                              | K9_53F_031411f | GTTATCATCGGATTATTCGAAGGA            | (SEQ ID NO: 544) |
|                                 | K9_53L_031411f | GTTATCATCGGATTATTGGAAGGA            | (SEQ ID NO: 545) |
|                                 | K9_53Y_031411f | GTTATCATCGGATTATATGAAGGA            | (SEQ ID NO: 546) |
|                                 | K9_53C_031411f | GTTATCATCGGATTATGTGAAGGA            | (SEQ ID NO: 547) |
|                                 | K9_53W_031411f | GTTATCATCGGATTATGGGAAGGA            | (SEQ ID NO: 548) |
|                                 | K9_53P_031411f | GTTATCATCGGATTACCAGAAGGA            | (SEQ ID NO: 549) |
|                                 | K9_53H_031411f | GTTATCATCGGATTACATGAAGGA            | (SEQ ID NO: 550) |
|                                 | K9_53Q_031411f | GTTATCATCGGATTACAAGAAGGA            | (SEQ ID NO: 551) |
|                                 | K9_53I_031411f | GTTATCATCGGATTAATTGAAGGA            | (SEQ ID NO: 552) |
|                                 | K9_53M_031411f | GTTATCATCGGATTAATGGAAGGA            | (SEQ ID NO: 553) |
|                                 | K9_53N_031411f | GTTATCATCGGATTAAATGAAGGA            | (SEQ ID NO: 554) |
|                                 | K9_53V_031411f | GTTATCATCGGATTAGTTGAAGGA            | (SEQ ID NO: 555) |
|                                 | K9_53A_031411f | GTTATCATCGGATTAGCTGAAGGA            | (SEQ ID NO: 556) |
|                                 | K9_53D_031411f | GTTATCATCGGATTAGATGAAGGA            | (SEQ ID NO: 557) |
|                                 | K9_53E_031411f | GTTATCATCGGATTAGAAGAAGGA            | (SEQ ID NO: 558) |
|                                 | K9_53G_031411f | GTTATCATCGGATTAGGTGAAGGA            | (SEQ ID NO: 559) |
| 56                              | K9_56F_031411f | GGATTACCTGAAGGATTCAAA               | (SEQ ID NO: 560) |
|                                 | K9_56L_031411f | GGATTACCTGAAGGATTGAAA               | (SEQ ID NO: 561) |
|                                 | K9_56Y_031411f | GGATTACCTGAAGGATATAAA               | (SEQ ID NO: 562) |
|                                 | K9_56C_031411f | GGATTACCTGAAGGATGTAAA               | (SEQ ID NO: 563) |
|                                 | K9_56W_031411f | GGATTACCTGAAGGATGGAAA               | (SEQ ID NO: 564) |
|                                 | K9_56P_031411f | GGATTACCTGAAGGACCAAAA               | (SEQ ID NO: 565) |
|                                 | K9_56H_031411f | GGATTACCTGAAGGACATAAA               | (SEQ ID NO: 566) |
|                                 | K9_56Q_031411f | GGATTACCTGAAGGACAAAAA               | (SEQ ID NO: 567) |
|                                 | K9_56I_031411f | GGATTACCTGAAGGAATTAAT               | (SEQ ID NO: 568) |
|                                 | K9_56M_031411f | GGATTACCTGAAGGAATGAAA               | (SEQ ID NO: 569) |
|                                 | K9_56N_031411f | GGATTACCTGAAGGAAATAAA               | (SEQ ID NO: 570) |
|                                 | K9_56V_031411f | GGATTACCTGAAGGAGTTAAA               | (SEQ ID NO: 571) |
|                                 | K9_56A_031411f | GGATTACCTGAAGGAGCTAAA               | (SEQ ID NO: 572) |
|                                 | K9_56D_031411f | GGATTACCTGAAGGAGATAAA               | (SEQ ID NO: 573) |
|                                 | K9_56E_031411f | GGATTACCTGAAGGAGAAAAA               | (SEQ ID NO: 574) |
|                                 | K9_56G_031411f | GGATTACCTGAAGGAGGTAAA               | (SEQ ID NO: 575) |
| 58                              | K9_58F_051611f | GATTACCTGAAGGATTTAAATTTCTGGAAGAGAGC | (SEQ ID NO: 576) |
|                                 | K9_58L_051611f | GATTACCTGAAGGATTTAAATTTGTGGAAGAGAGC | (SEQ ID NO: 577) |
|                                 | K9_58Y_051611f | GATTACCTGAAGGATTTAAATATTGGAAGAGAGC  | (SEQ ID NO: 578) |
|                                 | K9_58C_051611f | GATTACCTGAAGGATTTAAATGTTGGAAGAGAGC  | (SEQ ID NO: 579) |
|                                 | K9_58W_051611f | GATTACCTGAAGGATTTAAATGGTGGGAAGAGAGC | (SEQ ID NO: 580) |
|                                 | K9_58P_051611f | GATTACCTGAAGGATTTAAACCATGGAAGAGAGC  | (SEQ ID NO: 581) |
|                                 | K9_58H_051611f | GATTACCTGAAGGATTTAAACATTGGAAGAGAGC  | (SEQ ID NO: 582) |
|                                 | K9_58Q_051611f | GATTACCTGAAGGATTTAAACAATGGAAGAGAGC  | (SEQ ID NO: 583) |
|                                 | K9_58I_051611f | GATTACCTGAAGGATTTAAATTTTGGGAAGAGAGC | (SEQ ID NO: 584) |
|                                 | K9_58M_051611f | GATTACCTGAAGGATTTAAATTTGTGGAAGAGAGC | (SEQ ID NO: 585) |
|                                 | K9_58N_051611f | GATTACCTGAAGGATTTAAAAATTTGGAAGAGAGC | (SEQ ID NO: 586) |
|                                 | K9_58V_051611f | GATTACCTGAAGGATTTAAAGTTTGGGAAGAGAGC | (SEQ ID NO: 587) |
|                                 | K9_58A_051611f | GATTACCTGAAGGATTTAAAGCTTGGGAAGAGAGC | (SEQ ID NO: 588) |
|                                 | K9_58D_051611f | GATTACCTGAAGGATTTAAAGATTGGGAAGAGAGC | (SEQ ID NO: 589) |
|                                 | K9_58E_051611f | GATTACCTGAAGGATTTAAAGAATGGGAAGAGAGC | (SEQ ID NO: 590) |

|                |                                                     |
|----------------|-----------------------------------------------------|
| K9_58G_051611f | GATTACCTGAAGGATTTAAAGGTTGGAAGAGAGC (SEQ ID NO: 591) |
|----------------|-----------------------------------------------------|

Table 29. forward mutational primers.

| Targeted position(s) of K9-KARI | Primers                                                    |
|---------------------------------|------------------------------------------------------------|
| 56                              | K9_56F_071211r GCTCTCTTCCATGGTTTGAATCCTTC (SEQ ID NO: 592) |
|                                 | K9_56L_071211r GCTCTCTTCCATGGTTTCAATCCTTC (SEQ ID NO: 593) |
|                                 | K9_56Y_071211r GCTCTCTTCCATGGTTTATATCCTTC (SEQ ID NO: 594) |
|                                 | K9_56C_071211r GCTCTCTTCCATGGTTTACATCCTTC (SEQ ID NO: 595) |
|                                 | K9_56W_071211r GCTCTCTTCCATGGTTTCCATCCTTC (SEQ ID NO: 596) |
|                                 | K9_56P_071211r GCTCTCTTCCATGGTTTTGTCCTTC (SEQ ID NO: 597)  |
|                                 | K9_56H_071211r GCTCTCTTCCATGGTTTATGTCCTTC (SEQ ID NO: 598) |
|                                 | K9_56Q_071211r GCTCTCTTCCATGGTTTTGTCCTTC (SEQ ID NO: 599)  |
|                                 | K9_56I_071211r GCTCTCTTCCATGGTTTAATTCCTTC (SEQ ID NO: 600) |
|                                 | K9_56M_071211r GCTCTCTTCCATGGTTTCATTCCTTC (SEQ ID NO: 601) |
|                                 | K9_56N_071211r GCTCTCTTCCATGGTTTATTCCTTC (SEQ ID NO: 602)  |
|                                 | K9_56V_071211r GCTCTCTTCCATGGTTTAACTCCTTC (SEQ ID NO: 603) |
|                                 | K9_56A_071211r GCTCTCTTCCATGGTTTAGCTCCTTC (SEQ ID NO: 604) |
|                                 | K9_56D_071211r GCTCTCTTCCATGGTTTATCTCCTTC (SEQ ID NO: 605) |
|                                 | K9_56E_071211r GCTCTCTTCCATGGTTTTCTCCTTC (SEQ ID NO: 606)  |
|                                 | K9_56G_071211r GCTCTCTTCCATGGTTTACCTCCTTC (SEQ ID NO: 607) |
| 58                              | K9_58F_071211r GTTCTTCTGCTCTCTTCCAGAATTT (SEQ ID NO: 608)  |
|                                 | K9_58L_071211r GTTCTTCTGCTCTCTTCCACAATTT (SEQ ID NO: 609)  |
|                                 | K9_58Y_071211r GTTCTTCTGCTCTCTTCCAATATTT (SEQ ID NO: 610)  |
|                                 | K9_58C_071211r GTTCTTCTGCTCTCTTCCACATTT (SEQ ID NO: 611)   |
|                                 | K9_58W_071211r GTTCTTCTGCTCTCTTCCACCATTT (SEQ ID NO: 612)  |
|                                 | K9_58P_071211r GTTCTTCTGCTCTCTTCCATGGTTT (SEQ ID NO: 613)  |
|                                 | K9_58H_071211r GTTCTTCTGCTCTCTTCCAATGTTT (SEQ ID NO: 614)  |
|                                 | K9_58Q_071211r GTTCTTCTGCTCTCTTCCATGTTT (SEQ ID NO: 615)   |
|                                 | K9_58I_071211r GTTCTTCTGCTCTCTTCCAAATTTT (SEQ ID NO: 616)  |
|                                 | K9_58M_071211r GTTCTTCTGCTCTCTTCCACATTTT (SEQ ID NO: 617)  |
|                                 | K9_58N_071211r GTTCTTCTGCTCTCTTCCAATTTT (SEQ ID NO: 618)   |
|                                 | K9_58V_071211r GTTCTTCTGCTCTCTTCCAAACTTT (SEQ ID NO: 619)  |
|                                 | K9_58A_071211r GTTCTTCTGCTCTCTTCCAAGCTTT (SEQ ID NO: 620)  |
|                                 | K9_58D_071211r GTTCTTCTGCTCTCTTCCAATCTTT (SEQ ID NO: 621)  |
|                                 | K9_58E_071211r GTTCTTCTGCTCTCTTCCATCTTT (SEQ ID NO: 622)   |
|                                 | K9_58G_071211r GTTCTTCTGCTCTCTTCCAACCTTT (SEQ ID NO: 623)  |

Table 30. List of some mutants with their measured  $K_M$  values

| Mutant | SEQ ID NO:<br>(nucleic<br>acid, amino<br>acid) | Mutations                                     | $K_M$ ( $\mu$ M)<br>(NADH) | $K_M$ ( $\mu$ M)<br>(NADPH) |
|--------|------------------------------------------------|-----------------------------------------------|----------------------------|-----------------------------|
| K9 Wt  | 26, 27                                         | --                                            | 326                        | 0.2                         |
| BI7D12 | 625, 624                                       | Y53Q, S56V, S58D, I86V,<br>N87P, T131M, T191G | 39                         | 196                         |
| BI10F1 | 627, 626                                       | Y53E, S56V, S58D, I86V,<br>N87P, T131M, T191G | 74                         | 573                         |
| BJ6G6  | 629, 628                                       | Y53P, S56D, S58Q, I86V,<br>N87P, T131M, T191G | 298                        | 672                         |

|       |          |                                               |     |     |
|-------|----------|-----------------------------------------------|-----|-----|
| BJ7D6 | 631, 630 | Y53P, S56V, S58E, I86V,<br>N87P, T131M, T191G | 37  | 236 |
| BJ7F7 | 633, 632 | Y53A, S56D, S58Q, I86V,<br>N87P, T131M, T191G | 269 | 762 |

#### Example 24

##### Construction of an ald6 $\Delta$ strain and isobutanol-producing derivatives

- 5        A 5.3 kb (BglII/EcoRV) DNA fragment from pRS426::GPD-xpk1+ADH-eutD (SEQ ID NO:383) containing expression cassettes for xpk1 and eutD genes from *Lactobacillus plantarum* was added between the ALD6 flanking sequences, at the SnaBI site of the pUC19::ald6D::loxP-URA3-loxP vector described in Example 9, above.
- 10      The ligation reaction was transformed into *E. coli* Stbl3 cells, which were incubated on LB Amp plates to select for transformants. Insertion of the xpk1-eutD cassette was confirmed by PCR (primers). A positive clone (pUC19:: $\Delta$ ald6::URA3::xpkS) was obtained
- 15        The vector described above was linearized with AhdI and transformed into PNY1507 (described herein) cells prepared with the Zymo Research Frozen-EZ Yeast Transformation Kit (Cat. No. T2001) with a modification to manufacturer's protocol that included an additional outgrowth incubation of 2.5 hrs. in 2.0 mL YPE (yeast extract, peptone with 1% ethanol) medium. Transformants were obtained by plating on
- 20      synthetic complete medium minus uracil that provided 1% ethanol as the carbon source. Patched transformants were screened by PCR to confirm the deletion/integration, using primers N1090 and N1213 (SEQ ID NOs: 779 and 242). A plasmid carrying Cre recombinase (pRS423::GAL1p-Cre; SEQ ID No. 271) was transformed into the strain using histidine marker
- 25      selection. Transformants were passaged on YPE supplemented with 0.5% galactose. Colonies were screened for resistance to 5-FOA (loss of URA3 marker) and for histidine auxotrophy (loss of the Cre plasmid). Proper removal of the URA3 gene via the flanking loxP sites was confirmed by PCR with primers N1212 and N1214 (SEQ ID NOs: 241 and
- 30      281). Finally, the alsS integration plasmid (SEQ ID NO:780) was

transformed into this strain using the included geneticin selection marker. Integrants were confirmed using primers N160SeqF5 and oBP512 (SEQ ID NO: 388 and 337).

Plasmids pYZ090 $\Delta$ alsS and pBP915 (SEQ ID NOs: 371 and 182) were transformed into the strain by lithium acetate transformation (Protocol #2 in "Methods in Yeast Genetics" 2005. Amberg, Burke and Strathern). Transformants were selected by plating on synthetic complete minus histidine and uracil with ethanol as the carbon source. Transformants were patched and then repatched onto synthetic complete minus histidine and uracil with 2% glucose and 0.05% ethanol. Six clones were evaluated for growth and isobutanol production. One of these has been designated PNY2216.

#### Example 25

#### YMR226c Deletion from *S. cerevisiae* Strain PNY2211 (Construction of PNY2240)

The gene YMR226c was deleted from *S. cerevisiae* strain PNY2211 (described in Example 9) by homologous recombination using a PCR amplified linear KanMX4-based deletion cassette available in *S. cerevisiae* strain BY4743 ymr226c $\Delta$ ::KanMX4 (ATCC 4020812). Forward and reverse PCR primers N1237 (SEQ ID NO:784) and N1238 (SEQ ID NO:785), amplified a 2,051 bp ymr226c $\Delta$ ::KanMX4 deletion cassette from chromosome XIII. The PCR product contained upstream and downstream sequences of 253 and 217 bp, respectively, flanking the ymr226c $\Delta$ ::KanMX4 deletion cassette, that are 100% homologous to the sequences flanking the native YMR226c locus in strain PNY2211. Recombination and genetic exchange occur at the flanking homologous sequences effectively deleting the YMR226c gene and integrating the ymr226c $\Delta$ ::KanMX4 deletion cassette.

Approximately 2.0  $\mu$ g of the PCR amplified product was transformed into strain PNY2211 made competent using the lithium-acetate method previously described in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202

(2005)), and the transformation mix was plated on YPE plus geneticin (50 µg/mL) and incubated at 30 °C for selection of cells with an integrated ymr226cΔ::KanMX4 cassette. Transformants were screened for ymr226cΔ::KanMX4 by PCR, with a 5' outward facing KanMX4 deletion cassette-specific internal primer N1240 (SEQ ID NO:786) paired with a flanking inward facing chromosome-specific primer N1239 (SEQ ID NO:243) and a 3' outward-facing KanMX4 deletion cassette-specific primer N1241 (SEQ ID NO:787) paired with a flanking inward-facing chromosome-specific primer N1242 (SEQ ID NO:244). Positive PNY2211 ymr226cΔ::KanMX4 clones were obtained, one of which was designated PNY2248.

#### Example 26

##### Production of Isobutanol with Decreased DHMB Yield in YMR226c Knock-

15

#### Out

PNY2211 ymr226cΔ::KanMX4 transformants and a non-deletion control (PNY2211 with native YMR226c) were tested for butanol production in glucose medium by first introducing the isobutanol pathway-containing plasmids pYZ090ΔalsS (SEQ ID NO:371) and pBP915 (SEQ ID NO:182) simultaneously by the Quick and Dirty lithium acetate transformation method described in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2005)). Plasmid selection was based on histidine and uracil auxotrophy on selection plates containing ethanol (synthetic complete medium with 1.0% ethanol -his -ura). After three to five days, several transformants showing the most robust growth were adapted to glucose medium by patching onto SD 2.0% glucose + 0.05% ethanol -his -ura and incubated 48 to 72 hours at 300 °C. Three streaks showing the most robust growth were used to inoculate a 10 mL seed culture in SD 0.2% glucose + 0.2% ethanol -his -ura in 125 mL vented flasks and grown at 30 °C, 250 rpm for approximately 24 hours. Cells were then subcultured into synthetic complete medium with 2% glucose + 0.05% ethanol -his -ura in 125 ml tightly-capped flasks and incubated 48 hours at 30 °C. Culture

supernatants collected after inoculation and after 48 hours incubation were analyzed by HPLC to determine production of isobutanol and by LC/MS to quantify DHMB. Controls strains were observed to produce DHMB at a molar yield of 0.03 to 0.07 mole per mole glucose. A peak corresponding to DHMB was not observed in culture supernatants of the ymr226cΔ strains, one of which was designated PNY2249.

#### Example 27

##### Identification of Genes that Encode Acetolactate Reductase (ALR) Activity Enzymes Using Yeast Knockout Library

From a knockout ("KO") collection of >6000 yeast strains derived from the strain BY4743, available from Open Biosystems® (a division of Thermo Fisher Scientific, Waltham, MA), 95 candidate dehydrogenase gene knockout strains were chosen. Starter cultures of knockout strains were grown in 96-well deepwell plates (Costar 3960, Corning Inc., Corning NY, or similar) on rich medium YPD, and subcultured at a starting OD<sub>600nm</sub> of ~0.3 in medium containing 0.67% Yeast Nitrogen Base, 0.1% casamino acids, 2% glucose, and 0.1 M K<sup>+</sup>-MES, pH 5.5. Samples were taken over a 5-day period for DHMB and DHIV measurements. DHIV and the two isomers of DHMB were separated and quantified by liquid chromatography-mass spectrometry ("LC/MS") on a Waters (Milford, MA) AcquityTQD system, using an Atlantis T3 (part #186003539) column. The column was maintained at 30 °C, and the flow rate was 0.5 ml/min. The A mobile phase was 0.1% formic acid in water, and the B mobile phase was 0.1% formic acid in acetonitrile. Each run consisted of 1 min at 99% A, a linear gradient over 1 min to 25% B, followed by 1 min at 99% A. The column effluent was monitored for peaks at m/z = 133 (negative ESI), with cone voltage 32.5V, by Waters ACQ\_TQD (s/n QBA688) mass spec detector. The so-called "fast DHMB" typically emerged at 1.10 min, followed by DHIV at 1.2 min, and "slow" DHMB emerged at 1.75 min. Baseline separation was obtained and peak areas for DHIV were converted to μM DHIV concentrations by reference to analyses of standards solutions made from a 1M aqueous stock. These measurements showed that most of the changes in DHMB levels occurred

in the first 48-60 hours, so a single sample was collected at about that time in subsequent experiments. In this experiment, fast DHMB was found at much higher levels than slow DHMB, which was not always detectable. The ratio of DHIV to fast DHMB in most cultures was ~ 3, but  
5 a strain lacking the YMR226C gene consistently showed very low levels of fast DHMB, and normal DHIV, so that the DHIV/fast DHMB ratio was about 100. This suggested that YMR226Cp is the major ALR in this background.

To confirm that YMR226Cp is the major ALR in this background,  
10 the *in vitro* levels of ALR and KARI were tested in the *ymr226c* deletion strain (American Type Culture Collection (ATCC), Manassas VA, ATCC #4020812) and its parent, BY4743 (ATCC #201390; American Type Culture Collection, Manassas VA). Fifty ml tubes containing 6 ml YPD were inoculated from YPD agar plates and allowed to grow overnight (30  
15 °C, 250 rpm). The cells were pelleted, washed once in water, and resuspended in 1 ml yeast cytoplasm buffer (Van Eunen *et al.* *FEBS Journal* 277: 749-760 (2010)) containing a yeast protease inhibitor cocktail (Roche, Basel, Switzerland, Cat # 11836170001, used as directed by the vendor, 1 tablet per 10 mls of buffer). Toluene (0.02 ml, Fisher  
20 Scientific, Fair Lawn NJ) was added, and the tubes were shaken at top speed for 10 min on a Vortex Genie 2 shaker (Scientific Industries, Bohemia NY, Model G-560) for permeabilization. The tubes were placed in a water bath at 30 °C, and substrates were added to the following final concentrations: (S)-acetolactate (made enzymatically as described below in Example 29) to 9.4 mM, NADPH (Sigma-Aldrich, St. Louis MO) 0.2 mM  
25 plus a NAD(P)H-regeneration system consisting of ~ 10 mM glucose-6-phosphate and 2.5 U/ml *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO, Cat # G8404). At timed intervals, aliquots (0.15 ml) were added to 0.15 ml aliquots of 2% formic acid to stop  
30 the reaction. The samples were then analyzed for DHIV and both isomers of DHMB by LC/MS as described above; only fast DHMB and DHIV were observed. The specific activities of the two enzymes in the two strains are shown in Table 31.



**Table 31. KARI and ALR Enzyme Activities**

| Strain                  | KARI              | ALR       |
|-------------------------|-------------------|-----------|
| BY4743                  | 1.7 mU/mg protein | 20 mU/mg  |
| YMR226C deletion strain | 2.2 mU/mg protein | 0.1 mU/mg |

The data suggests that the YMR226C gene product accounted for >99% of the ALR activity.

5

**Example 28**

**Identification of Genes that Encode Acetolactase Reductase (ALR) Activity  
Enzymes Using Yeast Overexpression Library**

From a "Yeast ORF" collection of >5000 transformants of Y258 each with a plasmid carrying a known yeast gene plus a C-terminal tag, under the control of an inducible promoter (Open Biosystems®, a division of Thermo Fisher Scientific, Waltham, MA), ninety-six strains with plasmids containing genes associated with dehydrogenase activity were grown in 96-well format by adaptation of the growth and induction protocol recommended by the vendor (Open Biosystems®). The cells were pelleted and permeabilized with toluene as described above, and a concentrated substrate mix was added to give final concentrations as in Example 27. Timed samples were taken and analyzed for DHIV and both isomers of DHMB. The ratios of the ALR/KARI were calculated and compared.

Strains with elevated ratios were candidates for overproduction of ALR activities. When the data for relative rates of fast DHMB and DHIV formation were displayed in a Minitab® (Microsoft Inc., Redmond, WA) boxplot, half the ratios fell between ~9-13, and most of the rest fell within 3 and 19. The exceptions identified as outliers included YER081W, YIL074C, YMR226C, YBR006W, and YOR375C, for which ratios of ALR/KARI fell between 22 and 40. In a similar analysis of relative rates of slow DHMB and DHIV formation, half the ratios fell between 9 and 11, but YMR226C, YPL275W, YER081W, AND YOL059W appeared as outliers, with ratios between 13 and 25. Thus, overexpression of YMR226C and YER081W, increased synthesis of both DHMBs. In addition, YIL074C, YBR006W, and YOR375C increased fast DHMB synthesis, and YPL275W and YOL059W increased slow DHMB synthesis. The genomic DNA

sequences (which may include introns) and ORF translation sequences of genes identified in overexpression are provided in Table 6.

5

#### Example 29

#### Inhibition of KARI by DHMB

#### Enzymatic Production of (S)-Acetolactate

(S)-acetolactate was used as a starting material for DHMB

10 synthesis. (S)-acetolactate was made enzymatically, as follows. An *E. coli* TOP10 strain (Invitrogen, Carlsbad, CA) modified to express *Klebsiella* BudB (previously described in US Patent No. 7,851,188 );

under IPTG control was used as a source of enzyme. It was grown in  
15 200-1000 ml culture volumes. For example, 200 ml was grown in Luria Broth (Mediatech, Manassas, VA) containing 0.1 mg/ml Ampicillin (Sigma, St. Louis, MO) in a 0.5 L conical flask, which was shaken at 250 rpm at 37 °C. At OD 600 ~0.4, isopropylthiogalactoside (Sigma, St. Louis, MO) was added to 0.4 mM, and growth was continued for 2 hours before the cells  
20 were collected by centrifugation, yielding ~ 1 g wet weight cells. Likewise, partial purifications were conducted at scales from ~0.5 to 5 g wet cells. For example, ~0.5 g cells were suspended in 2.5 ml buffer containing 25 mM Na-MES pH 6, broken by sonication at 0 °C, and clarified by centrifugation. Crude extract was supplemented with 0.1 mM thiamin  
25 pyrophosphate, 10 mM MgCl<sub>2</sub>, and 1 mM EDTA (all from Sigma, St. Louis, MO). Next, 0.07 ml of 10% w/v aqueous streptomycin sulfate (Sigma, St. Louis, MO) was added and the sample was heated in a 56 °C water bath for 20 min. It was clarified by centrifugation, and ammonium sulfate was added to 50% of saturation. The mixture was centrifuged, and  
30 the pellet was brought up in 0.5 ml 25 mM Na-MES, pH 6.2, and used without further characterization. Acetolactate syntheses were also conducted at various scales. A large preparation was conducted as follows: 5.5 g sodium pyruvate was dissolved in 25 mM Na-MES, pH 6.2, to ~ 45 ml and supplemented with 10 mM MgCl<sub>2</sub>, 1 mM thiamin

pyrophosphate, 1 mM EDTA (all from (Sigma, St. Louis, MO), 25 mM sodium acetate (Fisher Scientific, Fair Lawn NJ), and 0.25 ml of a BudB preparation. The mixture was stirred under a pH meter at room temperature. As the reaction proceeded, CO<sub>2</sub> was evolved, and the pH rose. Pyruvic acid (Alfa, Ward Hill, MA) was added slowly via peristaltic pump to keep the pH between 6 and 7. As the pH rises, the enzyme reaction slows, but if it is allowed to fall below 6, decarboxylation of acetolactic acid becomes a problem. When the reaction was complete, the mixture was stored at -80 °C.

#### 10 Synthesis of DHMB

DHMB was synthesized chemically from (S)-acetolactate. Three ml of a crude acetolactate preparation at ~0.8 M at pH ~8 was treated with 1.2 equiv NaBH<sub>4</sub> (Aldrich Chemical Co, Milwaukee, WI). The reaction was allowed to sit at room temperature overnight before being divided in two and desalted in two portions on a 60 cm x 1 cm diameter column of Biogel P-2 (Bio-Rad, Hercules, CA) using water as the mobile phase. The fractions containing mixed DHMBs were concentrated by rotary evaporation and adjusted to pH 2.2 with sulfuric acid.

The diastereomers of DHMB were separated using an HPLC system (consisting of an LKB 2249 pump and gradient controller (LKB, now a division of General Electric, Chalfont St Giles, UK) and a Hewlett-Packard (now Agilent, Santa Clara, CA) 1040A UV/vis detector ) with a Waters Atlantis T3 (5 µm, 4.6 x 150 mm) run at room temperature in 0.2% aqueous formic acid, pH 2.5, at a flow rate of 0.3 mL/min, with UV detection at 215 nm. "Fast" DHMB was eluted at 8.1 min and "slow" DHMB was eluted at 13.7 min. DHIV was not present. The pooled fractions were taken nearly to dryness, and coevaporated with toluene to remove residual formic acid. The residue was then dissolved in water and made basic with triethylamine (Fisher, Fair Lawn, NJ).

#### 30 Concentration Determination and Absolute Structure of DHMB

The concentration of purified DHMB solutions was determined as follows. The concentration was estimated based on the mmol acetolactate used in the NaBH<sub>4</sub> reduction. To portions of the DHMBs, a known quantity

of sodium benzoate (made by dissolving solid benzoic acid (ACS grade, Fisher Scientific, Fair Lawn, NJ) in aqueous NaOH)) was added to give two-component mixtures in (approximately) equimolar amounts. A similar sample of DHIV was also prepared from the solid sodium salt obtained via

5 custom synthesis (Albany Molecular Research, Albany NY). The samples were coevaporated several times with D<sub>2</sub>O (Aldrich, Milwaukee, WI) and redissolved in D<sub>2</sub>O. Integrated proton NMR spectra were obtained and used to determine the mole ratio of DHIV or DHMB to benzoate.

Comparison of the NMR spectra of the DHMBs with the literature spectra for the free acids in CDCl<sub>3</sub> (Kaneko *et al.*, *Phytochemistry* 39: 115-120 (1995)) showed that fast DHMB was the erythro isomer. Since enzymatically synthesized acetolactate has the (S) configuration at C-2, the fast DHMB has the 2S, 3S configuration. Slow DHMB has the threo 2S, 3R configuration.

10

15 Dilutions of the NMR samples were also analyzed by LC/MS using separately prepared benzoic acid solutions as standards. Benzoic acid, DHIV, and the two isomers of DHMB were separated and quantified by LC/MS on a Waters (Milford, MA) AcquityTQD system, using an Atlantis T3 (part #186003539) column, as described above. Benzoic acid was

20 detected at m/z=121 (negative ESI), and emerged at 2.05 min. The concentration of benzoate in the mixtures was within experimental uncertainty of the expected value. The experiment also showed that either isomer of DHMB had ~80% of the sensitivity of DHIV in LC/MS (*i.e.*, MS peak area observed /nmol injected) throughout the response range of the

25 instrument. Thus, if a DHIV standard is used to quantify DHMB found in cell extracts or in enzymatic reactions, the apparent DHMB concentrations need to be multiplied by 1.25.

#### Measuring Inhibition of KARI by DHMB

Purified KARI encoded by genes either from *Lactococcus lactis* (SEQ ID NO: 864), a derivative of *Pseudomonas fluorescens* KARI known as JEA1 (SEQ ID NO: 799; U.S. Appl. Pub No. 2010/0197519),

30 or a variant of

Anaerostipes caccae KARI known as K9D3 (SEQ ID NO:788), were tested

for their sensitivity to DHMB inhibition in spectrophotometric assays in a Shimadzu (Kyoto, Japan) UV160U instrument with a TCC240A temperature control unit, set at 30 °C. The buffer was 0.1 M K<sup>+</sup> Hepes, pH 6.8, containing 10 mM MgCl<sub>2</sub> and 1 mM EDTA. NADPH was present at 0.2 mM, and racemic acetolactate was present at either 3mM or 0.725 mM (S) isomer. The rate of NADPH oxidation in the presence and absence of either fast or slow DHMB was measured.  $V_{\max}$  for each sample was calculated from the observed rate and the known acetolactate  $K_M$  using the Michaelis-Menten equation. A volumetric  $K_i$  was estimated for each measurement in the presence of DHMB using the Michaelis-Menten equation as modified for competitive inhibition vs. acetolactate (the  $K_M$  term in the Michaelis-Menten equation is multiplied by  $(1+[I]/K_i)$ , and the equation is solved for  $K_i$ . The results were converted to mM upon completion of the NMR experiment and are shown in Table 32.

Table 32.  $K_i$  Values for KARI Inhibition by DHMB Isomers

| Strain           | Fast DHMB | Slow DHMB |
|------------------|-----------|-----------|
| JEA1             | 0.23 mM   | 0.23 mM   |
| K9D3             | 0.3 mM    | 0.2 mM    |
| <i>L. lactis</i> | 2.8 mM    | 2.3 mM    |

### Example 30

#### Inhibition of DHAD by DHMB

Purified dihydroxyacid dehydratase (DHAD) from *Staphylococcus mutans* was tested for inhibition of conversion of dihydroxyisovalerate (DHIV) to 2-ketoisovalerate (2-KIV) by DHMB by using a modification of a colorimetric assay as described by Szamosi *et al.*, *Plant Phys.* 101: 999-1004 (1993). The assay took place in a 2 mL Eppendorf tube placed in a heating block maintained at 30 °C. The assay mixture had a final volume of 0.8 mL containing 100 mM Hepes-KOH buffer, pH 6.8, 10 mM MgCl<sub>2</sub>, 0.5-10 mM DHIV, 0-40 mM DHMB, and 18 µg DHAD. The assay was initiated by adding a 10x concentrated stock of substrate. Samples were removed (0.35 mL) at times 0.1 and 30 minutes, and the reaction was stopped by mixing into 0.35 mL 0.1 N HCl with 0.05% 2,4-dinitrophenylhydrazine (Aldrich) in a second Eppendorf tube. After incubating 30 minutes at room temperature, 0.35 mL of 4N NaOH was

added to the mixture, mixed, and centrifuged at 15,000xG for 2 minutes in a centrifuge (Beckman-Coulter Microfuge 18). The absorbance of the solution at 540 nm was then measured in a 1 cm pathlength cuvette using a Cary 300 Bio UV-Vis spectrophotometer (Varian). Based on a standard

5 curve using authentic 2-KIV (Fluka), 1 OD absorbance at 540 nm is produced by 0.28 mM 2-KIV. The rate of 2-KIV formation was measured in the presence and absence of either fast or slow DHMB. Both forms of DHMB behaved like competitive inhibitors of DHIV. Their inhibition constants ( $K_i$ ) were calculated from the Michaelis-Menten equation for

10 simple competitive inhibition:  $v = S \cdot V_{max} / (S + K_M \cdot (1 + I/K_i))$ , where  $v$  is the measured rate of 2-KIV formation,  $S$  is the initial concentration of DHIV,  $V_{max}$  is the maximum rate calculated from the observed rate at 10 mM DHIV and no DHMB,  $K_M$  is a previously measured constant of 0.5 mM, and  $I$  is the concentration of DHMB. The fast and slow isomers of DHMB

15 had calculated inhibition constants of 7 mM and 5 mM, respectively.

#### Example 31

##### Identification of YMR226C Homologs

Homologs of the YMR226C gene of *Saccharomyces cerevisiae* were sought by BLAST searches of the GenBank non-redundant

20 nucleotide database ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)), the Fungal Genomes BLAST Search Tool at the Saccharomyces Genome Database ([www.yeastgenome.org/cgi-bin/blast-fungal.pl](http://www.yeastgenome.org/cgi-bin/blast-fungal.pl)), and the BLAST Tool of the Genolevures Project ([genolevures.org/blast.html#](http://genolevures.org/blast.html#)). Unique sequences from 18 yeast species showing high sequence identity to YMR226C were

25 identified, and the complete ORF for these genes was recovered from the accessioned record in the associated database. The polypeptide sequences encoded by these ORFs were determined by the Translation feature of Vector NTI (Invitrogen, Carlsbad CA). The polynucleotide and polypeptide sequences are shown below in Table 33. The yeast species,

30 nucleotide database accession number, and DNA and protein sequences are given in the Table. The *S. kluyveri* sequence is in the Genolevures database under the accession number given; the others are in GenBank. The percent identities between the sequences are shown in Table 34.

The 18 ORFs were aligned using AlignX (Vector NTI; the gene encoding a putative NADP<sup>+</sup>-dependent dehydrogenase from *Neurospora crassa* (XM\_957621, identified in the GenBank BLAST search using the YMR226C nucleotide sequence) was used as an outgroup. The resulting phylogenetic tree is shown in Figure 11, and a sequence alignment is shown in Figure 12.

The sequence identity of these homologs to YMR226C ranges from a minimum of 55% (*Yarrowia lipolytica* and *Schizosaccharomyces pombe*) to a maximum of 90% (*S. paradoxus*). A BLAST search also revealed a cDNA from *S. pastorianus* (accession number CJ997537) with 92% sequence identity over 484 base pairs, but since this species is a hybrid between *S. bayanus* (whose YMR226C homolog shows 82% identity to the *S. cerevisiae* sequence), and because only a partial ORF sequence was available, this sequence was not included in the comparison. When the YMR226C sequence from the canonical laboratory strain S288C was compared with the sequences from 12 other strains of *S. cerevisiae*, only 4 single-nucleotide polymorphisms are found (sequence identity 99.5%), indicating that this is a highly-conserved gene in that species.

Table 33: YMR226C Yeast Homologs

| Species                        | Accession #<br>(Date of database<br>accession) | Nucleic acid SEQ<br>ID NO: | Amino acid SEQ<br>ID NO: |
|--------------------------------|------------------------------------------------|----------------------------|--------------------------|
| <i>Saccharomyces paradoxus</i> | AABY01000127<br>(3/7/2011)                     | 698                        | 699                      |
| <i>Saccharomyces bayanus</i>   | AACA01000631<br>(3/7/2011)                     | 700                        | 701                      |
| <i>Saccharomyces castellii</i> | AACF01000116<br>(3/7/2011)                     | 702                        | 703                      |
| <i>Saccharomyces mikatae</i>   | AACH01000019<br>(3/7/2011)                     | 704                        | 705                      |
| <i>Ashbya gossypii</i>         | AE016819<br>(3/7/2011)                         | 706                        | 707                      |
| <i>Candida glabrata</i>        | CR380959<br>(3/7/2011)                         | 708                        | 709                      |
| <i>Debaryomyces hansenii</i>   | CR382139<br>(3/7/2011)                         | 710                        | 711                      |
| <i>Scheffersomyces</i>         | XM_001387479                                   | 712                        | 713                      |

| Species                                                                            | Accession #<br>(Date of database<br>accession) | Nucleic acid SEQ<br>ID NO: | Amino acid SEQ<br>ID NO: |
|------------------------------------------------------------------------------------|------------------------------------------------|----------------------------|--------------------------|
| <i>stipitis</i><br>(formerly <i>Pichia stipitis</i> )                              | (3/7/2011)                                     |                            |                          |
| <i>Meyerozyma guilliermondii</i><br>(formerly <i>Pichia guilliermondii</i> )       | XM_001482184<br>(3/7/2011)                     | 714                        | 715                      |
| <i>Vanderwaltozyma polyspora</i><br>(formerly <i>Kluyveromyces polysporus</i> )    | XM_001645671<br>(3/4/2011)                     | 716                        | 717                      |
| <i>Candida dubliniensis</i>                                                        | XM_002419771<br>(3/7/2011)                     | 718                        | 719                      |
| <i>Zygosaccharomyces rouxii</i>                                                    | XM_002494574<br>(3/7/2011)                     | 720                        | 721                      |
| <i>Lachancea thermotolerans</i><br>(formerly <i>Kluyveromyces thermotolerans</i> ) | XM_002553230<br>(3/4/2011)                     | 722                        | 723                      |
| <i>Kluyveromyces lactis</i>                                                        | XM_451902<br>(3/4/2011)                        | 724                        | 725                      |
| <i>Saccharomyces kluyveri</i>                                                      | SAKL0H04730<br>(3/7/2011)                      | 726                        | 727                      |
| <i>Yarrowia lipolytica</i>                                                         | XM_501554<br>(3/8/2011)                        | 728                        | 729                      |
| <i>Schizosaccharomyces pombe</i>                                                   | NM_001018495<br>(3/8/2011)                     | 730                        | 731                      |

Table 34: YMR226C Homolog Percent Identity

| Species | Sm | Sb | Sca | Ag | Dh | Ss | Mg | Cd | Cg | Vp | Sk | Kl | Lt | Zr | Sce | Sp | Yl | N |
|---------|----|----|-----|----|----|----|----|----|----|----|----|----|----|----|-----|----|----|---|
| Spa     | 88 | 82 | 70  | 64 | 62 | 62 | 58 | 57 | 67 | 68 | 68 | 69 | 68 | 68 | 90  | 55 | 55 | 5 |
| Sm      |    | 82 | 70  | 64 | 60 | 62 | 58 | 56 | 67 | 69 | 68 | 70 | 68 | 69 | 86  | 57 | 56 | 5 |
| Sb      |    |    | 71  | 63 | 59 | 62 | 58 | 53 | 67 | 66 | 68 | 70 | 69 | 67 | 82  | 56 | 56 | 5 |
| Sca     |    |    |     | 60 | 62 | 61 | 60 | 59 | 65 | 69 | 69 | 71 | 64 | 70 | 69  | 57 | 53 | 5 |
| Ag      |    |    |     |    | 56 | 60 | 57 | 54 | 59 | 61 | 62 | 62 | 62 | 62 | 63  | 54 | 55 | 5 |
| Dh      |    |    |     |    |    | 64 | 62 | 61 | 61 | 63 | 62 | 61 | 59 | 63 | 62  | 57 | 57 | 5 |
| Ss      |    |    |     |    |    |    | 68 | 64 | 61 | 62 | 62 | 64 | 62 | 63 | 62  | 56 | 58 | 5 |
| Mg      |    |    |     |    |    |    |    | 60 | 57 | 58 | 60 | 60 | 59 | 62 | 59  | 57 | 57 | 5 |
| Cd      |    |    |     |    |    |    |    |    | 57 | 62 | 59 | 60 | 54 | 60 | 58  | 57 | 53 | 4 |
| Cg      |    |    |     |    |    |    |    |    |    | 69 | 70 | 68 | 67 | 67 | 66  | 55 | 56 | 5 |
| Vp      |    |    |     |    |    |    |    |    |    |    | 71 | 72 | 67 | 70 | 71  | 58 | 52 | 5 |
| Sk      |    |    |     |    |    |    |    |    |    |    |    | 77 | 71 | 72 | 69  | 53 | 54 | 5 |
| Kl      |    |    |     |    |    |    |    |    |    |    |    |    | 71 | 72 | 71  | 56 | 52 | 5 |
| Lt      |    |    |     |    |    |    |    |    |    |    |    |    |    | 69 | 69  | 53 | 60 | 5 |
| Zr      |    |    |     |    |    |    |    |    |    |    |    |    |    |    | 69  | 58 | 55 | 5 |
| Sce     |    |    |     |    |    |    |    |    |    |    |    |    |    |    |     | 55 | 55 | 5 |
| Spo     |    |    |     |    |    |    |    |    |    |    |    |    |    |    |     |    | 58 | 6 |





13,000 rpm at 4 °C for 30 minutes. Typically, 0.06 to 0.1 mg of crude extract protein is used in a single assay. Protein in the crude extracts was determined by Bradford assay with Coomassie stain.

Aldehyde dehydrogenase activity is measured following a protocol given by Sigma-Aldrich and by Bostian and Betts (Bostian, K.A. and Betts, G.F. (1978) Biochemical Journal 173, 773-786). Crude extracts from the deletion strains above and commercially available aldehyde dehydrogenase are tested using this method.

An alternative assay is to add isobutyraldehyde at concentrations from 1 to 30 mM to approximately 0.1 mg of crude extract protein in a sealed glass GC vials which are incubated at 30 °C for 30 minutes. The extracts are then centrifuged through 0.22 µm spin filters (Corning, Cat #8169) at 3000 rpm for 3 minutes, and the filtrate is transferred to a GC vial for GC-MS analysis. Isobutyraldehyde and isobutyric acid are detected.

The GC method utilizes an Agilent 7890 GC equipped with a 5975 mass spectrometer for detection, and a DB-1701 column (30 m x 0.25 mm ID, 0.25 µm film) from Agilent (Santa Clara, CA). The carrier gas is helium at a constant flow rate of 1.0 ml/min; injector split is 1:10 at 250 °C; oven temperature is 40 °C for 1 min, 40 °C to 120 °C at 10 °C/min, and 120 °C to 240 °C at 30 °C/min. MS detection is used in full scan mode for identification and quantitation of isobutyraldehyde and isobutyric acid. Calibrated standard curves are generated for the following compounds: isobutyraldehyde, isobutyric acid, and isobutanol.

25

### Example 33

#### Construction of expression vectors for isobutanol pathway gene expression in *S. cerevisiae*

##### pLH475-JEA1 construction

30 The pLH475-JEA1 plasmid (SEQ ID NO: 419) was constructed for expression of ALS and KARI in yeast. pLH475-JEA1 is a pHR81 vector (ATCC #87541) containing the following chimeric genes: 1) the CUP1 promoter (SEQ ID NO: 789), acetolactate synthase coding region from

*Bacillus subtilis* (AlsS; SEQ ID NO: 790; protein SEQ ID NO: 791) and CYC1 terminator 2 (SEQ ID NO: 792); 2) an ILV5 promoter (SEQ ID NO: 793), Pf5.IlvC-JEA1 coding region and ILV5 terminator (SEQ ID NO: 794); and 3) the FBA1 promoter (SEQ ID NO: 795), *S. cerevisiae* KARI coding region (ILV5; SEQ ID NO: 796; protein SEQ ID NO: 797) and CYC1 terminator (SEQ ID NO: 798).

The Pf5.IlvC-JEA1 coding region is a sequence encoding KARI derived from *Pseudomonas fluorescens* but containing mutations, that was described in U.S. Patent Application Publication Nos. 2009/0163376 and 2010/0197519.

The Pf5.IlvC-JEA1 encoded KARI (nucleic acid and amino acid SEQ ID NOs: 799 and 800) has the amino acid changes as compared to the natural *Pseudomonas fluorescens* KARI.

#### 15 Expression Vector pLH468

The pLH468 plasmid (SEQ ID NO:139) was constructed for expression of DHAD, KivD, and HADH in yeast.

Coding regions for *L. lactis* ketoisovalerate decarboxylase (KivD) and Horse liver alcohol dehydrogenase (HADH) were synthesized by DNA2.0 based on codons that were optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NOs: 801 and 802, respectively) and provided in plasmids pKivDy-DNA2.0 and pHadhy-DNA2.0. The encoded proteins are SEQ ID NOs: 803 and 804, respectively. Individual expression vectors for KivD and HADH were constructed. To assemble pLH467 (pRS426::P<sub>TDH3</sub>-kivDy-TDH3t), vector pNY8; also named pRS426.GPD-ald-GPDt, described in US Patent App. Pub. US2008/0182308, Example 17), was digested with *AscI* and *SfiI* enzymes, thus excising the *GPD* promoter and the ald coding region. A *TDH3* promoter fragment (SEQ ID NO: 805) from pNY8 was PCR amplified to add an *AscI* site at the 5' end, and an *SpeI* site at the 3' end, using 5' primer OT1068 and 3' primer OT1067 (SEQ ID NOs: 806 and 807). The *AscI*/*SfiI* digested pNY8 vector fragment was ligated with the *TDH3* promoter PCR product digested with *AscI* and

SpeI, and the SpeI-SfiI fragment containing the codon optimized *kivD* coding region isolated from the vector pKivD-DNA2.0. The triple ligation generated vector pLH467 (pRS426::PTDH3-*kivDy*-TDH3t). pLH467 (SEQ ID NO: 808) was verified by restriction mapping and sequencing.

- 5 pLH435 (pRS425::PGPM1-Hadhy-ADH1t) was derived from vector pRS425::GPM-sadB (SEQ ID NO: 809) which is described in U.S. Appl. Pub. No. 2009/0305363, Example 3.

- 10 pRS425::GPM-sadB is the pRS425 vector (ATCC #77106) with a chimeric gene containing the GPM1 promoter (SEQ ID NO: 810), coding region from a butanol dehydrogenase of *Achromobacter xylosoxidans* (*sadB*; DNA SEQ ID NO: 811; protein SEQ ID NO: 812), and ADH1 terminator (SEQ ID NO: 444). pRS425::GPMp-sadB contains *BbvI* and *PacI* sites at the 5' and 3' ends of the *sadB* coding region, respectively. A *NheI* site was added at the 5' end of the *sadB* coding region by site-  
 15 directed mutagenesis using primers OT1074 and OT1075 (SEQ ID NO: 813 and 814) to generate vector pRS425-GPMp-sadB-NheI, which was verified by sequencing. pRS425::PGPM1-sadB-NheI was digested with *NheI* and *PacI* to drop out the *sadB* coding region, and ligated with the *NheI*-*PacI* fragment containing the codon optimized HADH coding region  
 20 from vector pHadhy-DNA2.0 to create pLH435 (SEQ ID NO: 815). To combine *KivD* and HADH expression cassettes in a single vector, yeast vector pRS411 (ATCC # 87474) was digested with *SacI* and *NotI*, and ligated with the *SacI*-*Sall* fragment from pLH467 that contains the P<sub>TDH3</sub>-*kivDy*-TDH3t cassette together with the *Sall*-*NotI* fragment from pLH435  
 25 that contains the P<sub>GPM1</sub>-Hadhy-ADH1t cassette in a triple ligation reaction. This yielded the vector pRS411::P<sub>TDH3</sub>-*kivDy*-P<sub>GPM1</sub>-Hadhy (pLH441, SEQ ID NO: 816), which was verified by restriction mapping.

- 30 In order to generate a co-expression vector for all three genes in the lower isobutanol pathway: *ilvD*, *kivDy* and Hadhy, we used pRS423 FBA *ilvD*(Strep) (SEQ ID NO: 817), as the source of the *ilvD* gene. This shuttle vector contains an F1 origin of replication (nt 1423 to 1879) for maintenance in *E. coli* and a 2 micron origin (nt 8082 to 9426) for replication in yeast. The vector has an FBA1 promoter (nt 2111 to 3108;

SEQ ID NO: 795) and FBA1 terminator (nt 4861 to 5860; SEQ ID NO: 818). In addition, it carries the His marker (nt 504 to 1163) for selection in yeast and ampicillin resistance marker (nt 7092 to 7949) for selection in *E. coli*. The *ilvD* coding region (nt 3116 to 4828; SEQ ID NO: 819; protein

5 SEQ ID NO: 820) from *Streptococcus mutans* UA159 (ATCC #700610) is between the FBA1 promoter and FBA1 terminator forming a chimeric gene for expression. In addition there is a lumio tag fused to the *ilvD* coding region (nt 4829-4849).

The first step was to linearize pRS423 FBA *ilvD*(Strep) (also called

10 pRS423-FBA(SpeI)-*ilvD*(*Streptococcus mutans*)-Lumio) with *SacI* and *SacII* (with *SacII* site blunt ended using T4 DNA polymerase), to give a vector with total length of 9,482 bp. The second step was to isolate the *kivDy*-*hADHy* cassette from pLH441 with *SacI* and *KpnI* (with *KpnI* site blunt ended using T4 DNA polymerase), which gives a 6,063 bp fragment.

15 This fragment was ligated with the 9,482 bp vector fragment from pRS423-FBA(SpeI)-*ilvD*(*Streptococcus mutans*)-Lumio. This generated vector pLH468 (pRS423::P<sub>FBA1</sub>-*ilvD*(Strep)Lumio-FBA1t-P<sub>TDH3</sub>-*kivDy*-*TDH3t*-P<sub>GPM1</sub>-*hadhy*-*ADH1t*), which was confirmed by restriction mapping and sequencing.

20

#### Example 34

##### Construction of *S. cerevisiae* host strain containing modifications in pyruvate decarboxylase and hexokinase 2

This example describes insertion-inactivation of endogenous PDC1,

25 PDC5, and PDC6 genes of *S. cerevisiae*. PDC1, PDC5, and PDC6 genes encode the three major isozymes of pyruvate decarboxylase. Hexokinase 2, which is responsible for phosphorylation of glucose and transcriptional repression, is also inactivated. The resulting PDC/HXK2 inactivation strain was used as a host for expression vectors pLH475-JEA1 and

30 pLH468.

##### Construction of *pdv6*:: P<sub>GPM1</sub>-*sadB* integration cassette and PDC6 deletion:

A *pd<sub>6</sub>::P<sub>GPM1</sub>-sadB-ADH1t-URA3r* integration cassette was made by joining the *GPM-sadB-ADH1t* segment (SEQ ID NO: 821) from pRS425::*GPM-sadB* (described in U.S. Patent App. Pub. No. 2009/0305363) to the *URA3r* gene from

- 5 pUC19-URA3r. pUC19-URA3r (SEQ ID NO: 822) contains the *URA3* marker from pRS426 (ATCC # 77107) flanked by 75 bp homologous repeat sequences to allow homologous recombination *in vivo* and removal of the *URA3* marker. The two DNA segments were joined by SOE PCR (as described by Horton *et al.* (1989) *Gene* 77:61-68) using as template
- 10 pRS425::*GPM-sadB* and pUC19-URA3r plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, MA; catalog no. F-540S) and primers 114117-11A through 114117-11D (SEQ ID NOs: 823, 824, 825, 826), and 114117-13A and 114117-13B (SEQ ID NOs: 827 and 828).

- The outer primers for the SOE PCR (114117-13A and 114117-13B)
- 15 contained 5' and 3' ~50 bp regions homologous to regions upstream and downstream of the *PDC6* promoter and terminator, respectively. The completed cassette PCR fragment was transformed into BY4700 (ATCC # 200866) and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30 °C using standard
  - 20 genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 112590-34G and 112590-34H (SEQ ID NOs: 829 and 830), and 112590-34F and 112590-49E (SEQ ID NOs: 831 and 832) to verify integration at the *PDC6* locus with deletion of the
  - 25 *PDC6* coding region. The *URA3r* marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD -URA media to verify the absence of growth. The resulting identified strain has the genotype:
  - 30 BY4700 *pd<sub>6</sub>::P<sub>GPM1</sub>-sadB-ADH1t*.

Construction of *pd<sub>1</sub>::P<sub>PDC1</sub>-ilvD* integration cassette and *PDC1* deletion:

A *pd<sub>1</sub>::P<sub>PDC1</sub>-ilvD-FBA1t-URA3r* integration cassette (SEQ ID NO: 833) was made by joining the *ilvD-FBA1t* segment from pLH468 to the

*URA3r* gene from pUC19-*URA3r* by SOE PCR (as described by Horton *et al.* (1989) *Gene* 77:61-68) using as template pLH468 and pUC19-*URA3r* plasmid DNAs, with Phusion DNA polymerase (New England Biolabs Inc., Beverly, MA; catalog no. F-540S) and primers 114117-27A through

5 114117-27D (SEQ ID NOs: 823, 824, 825, 826).

The outer primers for the SOE PCR (114117-27A and 114117-27D) contained 5' and 3' ~50 bp regions homologous to regions downstream of the *PDC1* promoter and downstream of the *PDC1* coding sequence. The completed cassette PCR fragment was transformed into BY4700

10 *pdv6::P<sub>GPM1</sub>-sadB-ADH1t* and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30 °C using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 114117-36D  
15 and 135 (SEQ ID NOs 834 and 835), and primers 112590-49E and 112590-30F (SEQ ID NOs 832 and 836) to verify integration at the *PDC1* locus with deletion of the *PDC1* coding sequence. The *URA3r* marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30 °C following standard protocols. Marker removal  
20 was confirmed by patching colonies from the 5-FOA plates onto SD -*URA* media to verify the absence of growth. The resulting identified strain "NYLA67" has the genotype: BY4700 *pdv6::P<sub>GPM1</sub>-sadB-ADH1t pdv1::P<sub>PDC1</sub>-ilvD-FBA1t*.

#### HIS3 deletion

25 To delete the endogenous *HIS3* coding region, a *his3::URA3r2* cassette was PCR-amplified from *URA3r2* template DNA (SEQ ID NO: 837). *URA3r2* contains the *URA3* marker from pRS426 (ATCC # 77107) flanked by 500 bp homologous repeat sequences to allow homologous recombination *in vivo* and removal of the *URA3* marker. PCR was done  
30 using Phusion DNA polymerase and primers 114117-45A and 114117-45B (SEQ ID NOs: 838 and 839) which generated a ~2.3 kb PCR product. The *HIS3* portion of each primer was derived from the 5' region upstream of the *HIS3* promoter and 3' region downstream of the coding region such

that integration of the *URA3r2* marker results in replacement of the *HIS3* coding region. The PCR product was transformed into NYLA67 using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30 °C. Transformants were screened to verify correct integration by replica plating of transformants onto synthetic complete media lacking histidine and supplemented with 2% glucose at 30 °C. The *URA3r* marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30 °C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain, called NYLA73, has the genotype: BY4700 *cdc6::P<sub>GPM1</sub>-sadB-ADH1t cdc1::P<sub>PDC1</sub>-ilvD-FBA1t* *Δhis3*.

#### Deletion of HXK2:

A *hxx2::URA3r* cassette was PCR-amplified from *URA3r2* template (described above) using Phusion DNA polymerase and primers 384 and 385 (SEQ ID NOs: 840 and 841) which generated a ~2.3 kb PCR product. The *HXK2* portion of each primer was derived from the 5' region upstream of the *HXK2* promoter and 3' region downstream of the coding region such that integration of the *URA3r2* marker results in replacement of the *HXK2* coding region. The PCR product was transformed into NYLA73 using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30 °C. Transformants were screened by PCR to verify correct integration at the *HXK2* locus with replacement of the *HXK2* coding region using primers N869 and N871 (SEQ ID NOs: 842 and 843). The *URA3r2* marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30 °C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD -URA media to verify the



absence of growth, and by PCR to verify correct marker removal using primers N946 and N947 (SEQ ID NOs: 844 and 845). The resulting identified strain named NYLA83 has the genotype: BY4700 *pdv6::P<sub>GPM1</sub>-sadB-ADH1t pdv1::P<sub>PDC1</sub>-ilvD-FBA1t Δhis3 Δhvk2*.

5 Construction of *pdv5::kanMX* integration cassette and *PDC5* deletion

A *pdv5::kanMX4* cassette was PCR-amplified from strain YLR134W chromosomal DNA (ATCC No. 4034091) using Phusion DNA polymerase and primers *PDC5::KanMXF* and *PDC5::KanMXR* (SEQ ID NOs: 846 and 847) which generated a ~2.2 kb PCR product. The *PDC5* portion of each  
 10 primer was derived from the 5' region upstream of the *PDC5* promoter and 3' region downstream of the coding region such that integration of the *kanMX4* marker results in replacement of the *PDC5* coding region. The PCR product was transformed into NYLA83, and transformants were selected and screened as described above. The identified correct  
 15 transformants named NYLA84 have the genotype: BY4700 *pdv6::P<sub>GPM1</sub>-sadB-ADH1t pdv1::P<sub>PDC1</sub>-ilvD-FBA1t Δhis3 Δhvk2 pdv5::kanMX4*. Plasmid vectors pLH468 and pLH475-JEA1 were simultaneously transformed into strain NYLA84 (BY4700 *pdv6::P<sub>GPM1</sub>-sadB-ADH1t pdv1::P<sub>PDC1</sub>-ilvD-FBA1t Δhis3 Δhvk2 pdv5::kanMX4*) using standard  
 20 genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the resulting strain was maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30 °C.

25 Example 35 (Prophetic)

Construction of *S. cerevisiae* host strain containing modifications in pyruvate decarboxylase, hexokinase 2, and aldehyde dehydrogenase.

This example describes inactivation of one or more aldehyde dehydrogenases that abolish or reduce formation of isobutyric acid.  
 30 Disruption of *ALD2* is used as an example, but the method could be used for disruption of any aldehyde dehydrogenase. The resulting NYLA84-derived strain is used as a host for expression vectors pLH475-JEA1 and pLH468.

Deletion of *ALD2*:

A *ald2::URA3r* cassette is PCR-amplified from *URA3r2* template (described above) using Phusion DNA polymerase and primers T001 and T002 (SEQ ID NOs: 848 and 849) which generates a ~2.3 kb PCR product. The *ALD2* portion of each primer is derived from the 5' sequence and 3' sequence of the *ALD2* gene such that integration of the *URA3r2* marker results in replacement of the *ALD2* coding region. The PCR product is transformed into NYLA84 using standard genetic techniques (*Methods in Yeast Genetics*, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants are selected on synthetic complete media lacking uracil and supplemented with 1% ethanol at 30 °C. Transformants are screened by PCR to verify correct integration at the *ALD2* locus with replacement of the *ALD2* coding region using primers T003 and T004 (SEQ ID NOs: 850 and 851). The *URA3r2* marker is recycled by plating on synthetic complete media supplemented with 1% ethanol and 5-FOA at 30 °C following standard protocols. Marker removal is confirmed by patching colonies from the 5-FOA plates onto SE-URA media to verify the absence of growth, and by PCR to verify correct marker removal using primers T004 and T005 (SEQ ID NOs: 851 and 852). The resulting identified strain is named NYLA84  $\Delta ald2$  and is of the genotype: BY4700 *pdh6::P<sub>GPM1</sub>-sadB-ADH1t pdh1::P<sub>PDC1</sub>-ilvD-FBA1t  $\Delta his3 \Delta hxx2 \Delta ald2$ .*

Example 3625 Production of isobutanol in recombinant *S. cerevisiae* in NYLA84HPLC Method

Analysis for glucose and fermentation by-product composition is well known to those skilled in the art. For example, one high performance liquid chromatography (HPLC) method utilizes a Shodex SH-1011 column with a Shodex SH-G guard column (both available from Waters Corporation, Milford, MA), with refractive index (RI) detection. Chromatographic separation is achieved using 0.01 M H<sub>2</sub>SO<sub>4</sub> as the

mobile phase with a flow rate of 0.5 mL/min and a column temperature of 50 °C. Isobutanol retention time is 47.6 minutes.

Production of isobutanol in recombinant *S. cerevisiae* in NYLA84

The purpose of this example is to describe the production of isobutanol in the yeast strain NYLA84. The yeast strain comprises deletions of PDC1, PDC5, and PDC6, genes encoding three isozymes of pyruvate decarboxylase, and deletion of HXK2 encoding hexokinase 2. The strain also contains constructs for heterologous expression of AlsS (acetolactate synthase), KARI (keto acid reductoisomerase), DHAD (dihydroxy acid dehydratase), KivD (ketoisovalerate decarboxylase), SadB (secondary alcohol dehydrogenase), and HADH (horse liver alcohol dehydrogenase).

Strain construction

Plasmids pLH468 and pLH475-JEA1 were introduced into NYLA84, by standard PEG/lithium acetate-mediated transformation methods. Transformants were selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) was used as the carbon source. After three days, transformants were patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. Freezer vials were made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

Production of isobutanol

Freezer vials were used to inoculate 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources.

Fermentation conditions:

A 1 liter fermenter was prepared and sterilized with 0.4L water. After cooling, filter sterilized medium was added to give the following final concentrations in 800 mL post-inoculation:

Medium (final concentration):

6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco)

2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without  
Histidine, Leucine, Tryptophan and Uracil (Sigma Y2001)

20 mL/L of 1% (w/v) L-Leucine

4 mL/L of 1% (w/v) L-Tryptophan

5 1 mL/L ergosterol/tween/ethanol solution

0.2 mL/L Sigma DF204

10 g/L glucose

The fermenter was set to control at pH 5.5 with KOH, 30% dO,  
temperature 30 °C, and airflow of 0.2 SLPM (or, 0.25 vvm). At  
10 inoculation, the airflow was set to 0.01 SLPM initially, then increased to  
0.2 SLPM once growth was established. Glucose was maintained at 5-15  
g/L throughout by manual addition.

Air was continuously supplied to the fermentor at 0.25 vvm.  
Continuous aeration led to significant stripping of isobutanol from the  
15 aqueous phase of the fermentor. To quantify the loss of isobutanol due to  
stripping, the off-gas from the fermentor was directly sent to a mass  
spectrometer (Prima dB mass spectrometer, Thermo Electron Corp.,  
Madison, WI) to quantify the amount of isobutanol in the gas stream. The  
isobutanol peaks at mass to charge ratios of 74 or 42 were monitored  
20 continuously to quantify the amount of isobutanol in the gas stream.  
Glucose and organic acids in the aqueous phase were monitored during  
the fermentation using HPLC. Glucose was also monitored quickly using  
a glucose analyzer (YSI, Inc., Yellow Springs, OH). Isobutanol in the  
aqueous phase was quantified by HPLC as described in HPLC Method  
25 herein above after the aqueous phase was removed periodically from the  
fermentor. The effective titer, corrected for the isobutanol lost due to  
stripping, was 7.5 g/L. The titer of isobutyric acid was 1.28 g/L (Figure  
14).

### 30 Example 37 (Prophetic)

#### Production of isobutanol in recombinant *S. cerevisiae* in NYLA84 $\Delta ald2$ .

The purpose of this example is to describe the production of  
isobutanol in the yeast strain NYLA84. Disruption of *ALD2* is used as an

example, but the example could be used for disruption of any aldehyde dehydrogenase. The NYLA84  $\Delta ald2$  yeast strain comprises deletions of *PDC1*, *PDC5*, and *PDC6*, genes encoding three isozymes of pyruvate decarboxylase, deletion of *HXK2* encoding hexokinase 2, and deletion of  
 5 *ALD2* encoding aldehyde dehydrogenase. The strain also contains constructs for heterologous expression of AlsS (acetolactate synthase), KARI (keto acid reductoisomerase), DHAD (dihydroxy acid dehydratase), KivD (ketoisovalerate decarboxylase), and SadB (secondary alcohol dehydrogenase).

#### 10 Strain construction

Plasmids pLH468 and pLH475-JEA1 are introduced into NYLA84  $\Delta ald2$ , by standard PEG/lithium acetate-mediated transformation methods. Transformants are selected on synthetic complete medium lacking glucose, histidine and uracil. Ethanol (1% v/v) is used as the carbon  
 15 source. After three days, transformants are patched to synthetic complete medium lacking histidine and uracil supplemented with both 2% glucose and 0.5% ethanol as carbon sources. Freezer vials are made by dilution of log-phase cultures with 45% glycerol to a final glycerol concentration of 15% (w/v).

#### 20 Production of isobutanol

Freezer vials are used to inoculate 80 ml of synthetic complete medium lacking histidine and uracil supplemented with both 0.25% glucose and 0.5% ethanol as carbon sources. A 1 liter fermenter is prepared and sterilized with 0.4L water. After cooling, filter sterilized  
 25 medium is added to give the following final concentrations in 800 mL post-inoculation:

Medium (final concentration):

6.7 g/L, Yeast Nitrogen Base w/o amino acids (Difco)

2.8 g/L, Yeast Synthetic Drop-out Medium Supplement Without Histidine,

30 Leucine, Tryptophan and Uracil (Sigma Y2001)

20 mL/L of 1% (w/v) L-Leucine

4 mL/L of 1% (w/v) L-Tryptophan

1 mL/L ergosterol/tween/ethanol solution

0.2 mL/L Sigma DF204

10 g/L glucose

The fermenter is set to control at pH 5.5 with KOH, 30% dO, temperature 30 °C, and airflow of 0.2 SLPM (or, 0.25 vvm). At

5 inoculation, the airflow is set to 0.01 SLPM initially, then increased to 0.2 SLPM once growth is established. Glucose is maintained at 5-15 g/L throughout by manual addition.

To quantify the loss of isobutanol due to stripping, the off-gas from the fermentor is directly sent to a mass spectrometer (Prima dB mass  
10 spectrometer, Thermo Electron Corp., Madison, WI) to quantify the amount of isobutanol in the gas stream. The isobutanol peaks at mass to charge ratios of 74 or 42 are monitored continuously to quantify the amount of isobutanol in the gas stream. Glucose and organic acids in the aqueous phase are monitored during the fermentation using HPLC.  
15 Glucose is also monitored quickly using a glucose analyzer (YSI, Inc., Yellow Springs, OH). Isobutanol and isobutyric acid in the aqueous phase are quantified by HPLC after the aqueous phase was removed periodically from the fermentor.

20

### Example 38

#### Analysis of isobutyric acid production

Strains were inoculated into synthetic complete medium, minus histidine and uracil, supplemented with 0.05% ethanol. Once cultures had reach stationary phase they were subcultured into fresh medium (starting  
25 OD = 0.2). For PNY2205 (Example 13), medium was supplemented with 0.05% ethanol to satisfy the C2 requirement observed in PDC KO yeast that do not have the phosphoketolase pathway. For PNY2209 (Example 13), cells were subcultured into media with and without ethanol. For the ald6Δ clones (PNY2216 and isogenic clones, Example 24), medium  
30 without ethanol was used. Cultures were grown in screw capped shake flasks (20 ml medium in 125 ml flasks). Culture supernatants were collected immediately after inoculation and again after 48 hours. These culture supernatants were analyzed by HPLC (described in

US20070092957 ) to determine glucose consumption and isobutyric acid concentration.

Table 35. Molar yield of isobutyric acid in ald6Δ strains.

| Strain                              | Isobutyric acid molar yield (mole/mole glucose consumed) |
|-------------------------------------|----------------------------------------------------------|
| PNY2205*                            | 0.09                                                     |
| PNY2209*                            | 0.07                                                     |
| PNY2209                             | 0.09                                                     |
| PNY2216 and 5 other isogenic clones | 0.03                                                     |

5

\*Indicates culture medium was supplemented with 0.05% ethanol

### Example 39

#### 10 Increased Production of Isobutanol and Decreased By-Product Production in New Strains

The purpose of this example is to describe small-scale culturing experiments with the newly constructed strains using plasmid pK9G9.OLE1p.ilvD to supply the remaining isobutanol pathway genes.

- 15 New host strains PNY1528, PNY2243, PNY2237 and PNY2238 were transformed with plasmid pK9G9.OLE1p.ilvD and tested for isobutanol production. Transformants were obtained by selection on synthetic complete medium minus uracil with 1% ethanol as the carbon source. Transformants were patched on synthetic complete medium minus uracil
- 20 with 2% glucose and 0.05% ethanol as carbon sources. Patches were used to inoculate liquid medium (synthetic complete minus uracil with 0.3% glucose and 0.3% ethanol as carbon sources). To test isobutanol production, liquid cultures were sub-cultured into synthetic complete medium minus uracil containing 2% glucose and 0.05% ethanol as carbon
- 25 sources that also contained BME vitamin mix (Sigma Cat. No. B6891). Cultures were incubated in sealed serum vials (10 ml medium in 15 ml vials) at 30°C with shaking (250 rpm in an Infors Multitron shaker). After 48 hours, culture medium was filtered (Spin-X column) and analyzed by HPLC (as described previously in US App. Pub. No. 20070092957). Molar

yield of the pathway by-product isobutyrate was decreased by 50% in strains carrying the *ald6Δ*. Strains based on PNY2238 were found to have higher glucose consumption and isobutanol titer (clone K results shown in the table below).

5 Table 36. Molar Yield of isobutyric acid in the *ald6Δ* strains

| Strain                              | Relevant phenotype    | Isobutyric acid molar yield (mole/mole glucose consumed) |
|-------------------------------------|-----------------------|----------------------------------------------------------|
| PNY2205*                            | ALD6+, C2-dependent   | 0.09                                                     |
| PNY2209*                            | ALD6+, C2-independent | 0.07                                                     |
| PNY2209                             | ALD6+, C2-independent | 0.09                                                     |
| PNY2216 and 5 other isogenic clones | ALD6-, C2-independent | 0.03                                                     |

\* indicates the culture medium was supplemented with 0.05% ethanol

All strains contained plasmid pK9G9.OLE1p.ilvD. For PNY2237- and PNY2238-derived strains, the data presented are an average of two biological replicates.

10 Example 40

Construction of a site-saturation gene library and screening the isobutanol production of the resultant variants in PNY2259

The reverse primer mixture (called K9\_309r in this example) containing primers encoding all 20 individual amino acid changes at position 309 (Table 37) and the forward primer K9\_131T\_080211f: GGACTTGATGTCACCTATGATC (called K9\_131Tf in this example) were employed to create a single-site saturation library targeting the position of 309 of K9 KARI. A plasmid containing variant K9SB2 (pHR81-ILV5p.K9SB2.TEF1(M4)p.ilvD (SEQ ID NO: 930, also called "pLH744").

20 In brief, a megaprimer was prepared through a regular PCR. The megaprimer PCR mixture consisted of 45 μl of SuperMix (Invitrogen, Carlsbad, CA, #10572063), 2.0 μl K9\_131Tf (20 μM), 2.0 μl K9\_309r (20 μM) and 1.0 μl template (50 ng/μl). The PCR program for making the megaprimer is: the starting temperature was 95°C for 1.0 min followed by 25 35 heating/cooling cycles. Each cycle consisted of 95°C for 20 sec, 55°C



for 20 sec, and 72°C for 1.0 min. The megaprimer was then used to introduce mutation into K9SB2 using the same procedure as shown in Example 5. Clones from the single-site saturation library were sequenced.

The resultant unique variants together with pLH744 were analyzed for isobutanol production and by-product formation in yeast (triple for each mutant). Yeast pathway strains were made in PNY2259 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh\_HI-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD\_Lg(y)-ADH1t yprcΔ15Δ::P[PDC5]-ADH|adh\_HI-ADH1t ymr226cΔ ald6Δ::loxP; Example 22) host by transforming the KARI vectors. The transformed cells were plated on synthetic medium without histidine or uracil (1% ethanol as carbon source). Three transformants were transferred to fresh plates of the same media. The transformants were tested for isobutanol production under anaerobic conditions in 48-well plates (Axygen, Union City, CA #391-05-061).

Yeast colonies from the transformation on SE-Ura plates appeared after 5-7 days. The three colonies from each variant were patched onto fresh SE-Ura plates, and incubated at 30°C for 3 days.

#### Growth media and procedure

Two types of media were used during the growth procedure of yeast strains: an aerobic pre-culture media and an anaerobic culture media. All chemicals were obtained from Sigma unless otherwise noted (St. Louis, MO)

Aerobic pre-culture media (SE-Ura): 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, MD), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil, 0.2% ethanol, 0.2% glucose, 0.01% w/v leucine, 0.002% w/v histidine, and 0.002% w/v tryptophan.

Anaerobic culture media (SEG-Ura-His): 50 mM MES (pH 5.5, 6.7 g/L yeast nitrogen base without amino acids (Difco, 291940, Sparks, MD), 1.4 g/L yeast synthetic drop-out medium supplement without histidine,

leucine, tryptophan and uracil, 0.1% ethanol, 3% glucose, 0.01% leucine, 0.002% w/v histidine, 0.002% tryptophan, 30 mg/L nicotinic acid, 30 mg/L thiamine and 10 mg/L ergosterol made up in 50/50 v/v Tween /ethanol solution.

5           The patched cells were inoculated into 48-well plates. Each well contains 1.5 ml aerobic media. The plates were covered with permeable foils and grown at 30°C with shaking overnight. The cell density ( $OD_{600}$ ) was then measured. The amount of cells to make 1.5 ml of cell suspension (in anaerobic media) with the final  $OD_{600}=0.2$  for each well  
10           were calculated and 1.5 ml cell suspension was prepared with anaerobic media and added into each well. Oxygen in 48-well plates was removed using an anaerobic chamber following the manufacturer's protocol (Coy Laboratory Products Inc. Grass Lake, MI). Cells were then grown at 30°C with shaking for two days. The cell density ( $OD_{600}$ ) was then measured.  
15           The best grown mutants underwent the same two-day growth in 48-well plates for isobutanol production measurement. After two days' anaerobic growth, the cell density ( $OD_{600}$ ) was then measured. Cells were centrifuged at 4,000 g for 5 min and the supernatant was collected for the isobutanol measurement using LC/MS.

20           LC/MS analysis of yeast strains with K9 KARI mutants

          Samples were taken for LCMS analysis at the end of the anaerobic growth period. LCMS analysis was performed using a Waters AcQuity UPLC separations unit and AcQuity TQD triple quad mass spectrometer (Waters, Milford, MA) with a Waters AcQuity UPLC HSS T3 separations  
25           column (Waters, Milford, MA). Compounds were separated using a reverse phase gradient of water (+ 0.1% formic acid) and acetonitrile (+ 0.1% formic acid) starting with 99% aqueous and ending with 99% organic, at a flow rate of 0.5 mL/min. Chromatograms were analyzed using Waters Masslynx 4.1 software (Waters, Milford, MA). Micro molar yields  
30           for isobutanol were calculated using Waters Quanlynx software (Waters, Milford, MA) using a calibration curve of triplicate analyses of standards.

Table 37. Reverse Primers

| Targeted position(s) of K9-KARI | Primers                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 309                             | K9_309I_111711r: CTTTCTCATAGCCTTAATGTGGAC (SEQ ID NO: 884)<br>K9_309L_111711r: CTTTCTCATAGCCTTTAAGTGGAC (SEQ ID NO: 885)<br>K9_309Y_111711r: CTTTCTCATAGCCTTATAGTGGAC (SEQ ID NO: 886)<br>K9_309C_111711r: CTTTCTCATAGCCTTACAGTGGAC (SEQ ID NO: 887)<br>K9_309W_111711r: CTTTCTCATAGCCTTCCAGTGGAC (SEQ ID NO: 888)<br>K9_309P_111711r: CTTTCTCATAGCCTTTGGGTGGAC (SEQ ID NO: 889)<br>K9_309H_111711r: CTTTCTCATAGCCTTATGGTGGAC (SEQ ID NO: 890)<br>K9_309Q_111711r: CTTTCTCATAGCCTTTTGGTGGAC (SEQ ID NO: 891)<br>K9_309M_111711r: CTTTCTCATAGCCTTCATGTGGAC (SEQ ID NO: 892)<br>K9_309N_111711r: CTTTCTCATAGCCTTATTGTGGAC (SEQ ID NO: 893)<br>K9_309V_111711r: CTTTCTCATAGCCTTAACGTGGAC (SEQ ID NO: 894)<br>K9_309A_111711r: CTTTCTCATAGCCTTAGCGTGGAC (SEQ ID NO: 895)<br>K9_309D_111711r: CTTTCTCATAGCCTTATCGTGGAC (SEQ ID NO: 896)<br>K9_309E_111711r: CTTTCTCATAGCCTTTTCGTGGAC (SEQ ID NO: 897)<br>K9_309G_111711r: CTTTCTCATAGCCTTACCGTGGAC (SEQ ID NO: 898)<br>K9_309S_111711r: CTTTCTCATAGCCTTAGAGTGGAC (SEQ ID NO: 899)<br>K9_309T_111711r: CTTTCTCATAGCCTTAGTGTGGAC (SEQ ID NO: 900)<br>K9_309R_111711r: CTTTCTCATAGCCTTCTGTGGAC (SEQ ID NO: 901)<br>K9_309K_111711r: CTTTCTCATAGCCTTCTGTGGAC (SEQ ID NO: 902) |

Table 38. List of some variants with OD<sub>600</sub> and isobutanol titer (mM) after two days anaerobic growth

| Variant | Amino Acid Seq ID No: | Repeat | OD <sub>600</sub> | Isobutanol titer (mM) |
|---------|-----------------------|--------|-------------------|-----------------------|
| K9C4    | 927                   | 1      | 0.5502            | 76.99                 |
|         |                       | 2      | 0.6578            | 84.05                 |
|         |                       | 3      | 0.7301            | 98.91                 |
| K9C8    | 928                   | 1      | 0.6887            | 116.57                |
|         |                       | 2      | 0.5309            | 78.77                 |
|         |                       | 3      | 0.6859            | 102.49                |
| K9SB2   | 427                   | 1      | 0.6314            | 88.39                 |
|         |                       | 2      | 0.5977            | 81.18                 |
|         |                       | 3      | 0.2325            | 44.60                 |

5

#### Example 41

##### Construction of K9SB2\_SH (K9SB2 short), a truncated version of K9SB2

A gene encoding a version of K9SB2 lacking the first five N-terminal amino acids (MEECK) was prepared by PCR with the Phusion® High-

10 Fidelity PCR Kit (Catalog #E0553L, New England Biolabs). Primers Kshort1

(AAACCGGTTTAAACAGTATGGCTAAGATTTACTACCAAGAAGACTG; SEQ ID NO: 903) and YGrev (TTCTGTTTTATCAGACCGCTTC; SEQ ID NO: 904) were synthesized by Integrated DNA Technologies, Inc (Coralville IA). Other than the primers, dNTP mix (Cat#18427-013, 5 Invitrogen, Carlsbad, CA), template, and ddH<sub>2</sub>O, reagents used here were supplied with the kit indicated above. The PCR mixture consisted of 1 µl of K9SB2 in a pBAD.KARI plasmid (20 ng/µl; plasmid preparation described in Example 17; SEQ ID NO: 428), 2 µl of each primer (20 µM stocks), 10 µl of 5× Phusion HF Buffer, 2 µl of 5 mM dNTP mix, 0.5 µl polymerase, 10 and 28 µl of ddH<sub>2</sub>O. The following conditions were used for the PCR reaction: The starting temperature was 98°C for 2 min followed by 30 heating/cooling cycles. Each cycle consisted of 98°C for 10 sec, 48°C for 30 sec, and 72°C for 1.5 min. At the completion of the temperature cycling, the sample was kept at 72° C for 10 min more, and then held awaiting 15 sample recovery at 4°C. The reaction product was separated from the template via agarose gel electrophoresis (1% agarose, 1X TBE buffer) and recovered using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (Cat# 28-9034-70, GE Healthcare Sciences, Piscataway, NJ) as recommended by the manufacturer. The purified PCR product was 20 digested with PmeI and SfiI and ligated into the corresponding sites of pBAD-ps-JEA1 (SEQ ID NO: 905) and the sequence of the resultant construct K9SB2\_SH in pBAD.KARI (SEQ ID NO: 929) was confirmed.

#### Example 42

##### Generation of yeast expression plasmids for studies of K9SB2 and other

##### 25 KARI enzymes

##### Construction of pHR81-ILV5p-K9SB2-TEF(M4)-IlvD (pLH744; SEQ ID NO: 930):

The K9SB2 gene was excised from pHR81-ILV5p-K9SB2 by PmeI and SfiI digestion, and ligated with pHR81-ILV5p-K9D3-TEF(M4)-IlvD 30 (pBP2090) at PmeI and SfiI sites. The ligated vector was named pHR81-ILV5p-K9SB2-TEF(M4)-IlvD (pLH744), and this vector was confirmed by sequencing.

##### Construction of K9SB2 SH DHAD (SEQ ID NO: 931):

The digested K9SB2\_SH PCR product (described in example 41) was ligated into PmeI and SfiI sites of pLH744 and confirmed by sequencing.

Construction of K9SB2\_SH 81 (SEQ ID NO: 932)

- 5 The digested K9SB2\_SH PCR product (described in example 41) was ligated into PmeI and SfiI sites of pHR81-Pilv5-KARI-K9.G9 and confirmed by sequencing.

Construction of plasmids for yeast expression of K9SB2 derivatives

- 10 The yeast expression plasmids for variants listed in Table 39 (described in examples 17 and 21) were generated by subcloning the corresponding KARI genes from pBAD.KARI plasmids into the PmeI and SfiI sites of plasmid pLH744 and plasmid pHR81-Pilv5-KARI-K9.G9. The constructs were confirmed by sequencing.

- 15 Table 39: K9SB2 derivatives subcloned into yeast expression plasmids

| <b>K9SB2 Derivative</b> | <b>Alternate name</b> | <b>Amino Acid Sequence ID</b> | <b>Nucleic Acid Sequence ID</b> |
|-------------------------|-----------------------|-------------------------------|---------------------------------|
| K9SB2-K90M              | K9_David              | 431                           | 432                             |
| K9SB2-G55D              | K9_Eliza              | 433                           | 946                             |
| K9SB2-Q91L              | K9_Frank              | 440                           | 947                             |
| K9SB2-A303D             | K9_Grace              | 445                           | 948                             |
| K9SB2-M94-V307I         | K9_Ingrid             | 455                           | 949                             |
| K9SB2-F67I              | K9_Jarvis             | 437                           | 950                             |
| K9SB2-A56G-K90N         | K9_Kelly              | 452                           | 951                             |
| K9SB2-G55C              | K9_Norman             | 481                           | 952                             |
| K9SB2-P135S             | K9_Ophelia            | 488                           | 953                             |
| K9SB2-F53L              | K9_Pat                | 441                           | 954                             |
| K9SB2-Q94I              | K9_Quentin            | 495                           | 955                             |
| K9SB2-F67L              | K9_Ralph              | 496                           | 956                             |
| K9SB2-K8N-K90M-T141I    | K9_Sophia             | 502                           | 957                             |
| K9SB2-E13V-M94I-T141I   | K9_Tiberius           | 509                           | 958                             |

|            |           |     |     |
|------------|-----------|-----|-----|
| K9SB2-A56V | K9_Ursala | 511 | 959 |
| K9SB2-I84L | K9_Victor | 514 | 960 |
| K9SB2-W59C | K9_Watson | 520 | 961 |
| K9SB2-T93A | K9_Xavier | 641 | 642 |

Construction of plasmids for yeast expression of truncated versions of K9SB2 derivatives

- N-terminally truncated versions of K9SB2 derivatives were prepared as described in example 41, with modifications. The primer Kshort1 was replaced with the 5'-phosphorylated primer KPSH1 (AAACAGTATG GCT AAG ATT TAC TAC CAA GAA GAC TG; SEQ ID NO: 906), which was synthesized by Integrated DNA Technologies, Inc (Coralville IA). K9SB2 in the PCR was replaced by pooled mixtures of the variants (pBAD.KARI plasmids) listed in Table 39. The purified PCR products were digested with SfiI and ligated into the PmeI and SfiI sites of pHR81-PIIv5-KARI-K9.G9. DNA sequencing with TempliPhi™ (GE Healthcare) and primers pHR81-F (ACACCCAGTATTTTCCCTTTCC) and pHR81- Rev (CTA GTG TAC AGA TGT ATG TCG G) (SEQ ID NOs: 924 and 925) was performed to identify each truncated derivative. The identified variants are indicated in Table 40. The coding sequences for the truncated versions were subsequently subcloned into the PmeI and SfiI sites of plasmid pLH744 and confirmed by sequencing.

Table 40. Truncated versions of K9SB2 derivatives

| Variant       | Amino Acid Sequence ID | Nucleic Acid Sequence ID |
|---------------|------------------------|--------------------------|
| K9_David_SH   | 196                    | 263                      |
| K9_Eliza_SH   | 266                    | 907                      |
| K9_Frank_SH   | 267                    | 908                      |
| K9_Grace_SH   | 389                    | 909                      |
| K9_Ingrid_SH  | 405                    | 910                      |
| K9_Jarvis_SH  | 781                    | 911                      |
| K9_Kelly_SH   | 782                    | 912                      |
| K9_Norman_SH  | 783                    | 913                      |
| K9_Ophelia_SH | 835                    | 914                      |
| K9_Pat_SH     | 853                    | 915                      |
| K9_Quentin_SH | 854                    | 916                      |
| K9_Ralph_SH   | 855                    | 917                      |
| K9_Ursala_SH  | 856                    | 918                      |

|              |     |     |
|--------------|-----|-----|
| K9_Watson_SH | 857 | 858 |
| K9_Xavier_SH | 859 | 919 |

Construction of K9\_Zeke\_SH (K9SB2-K90M-T93A) and K9\_Annabel\_SH (K9SB2-K90M-T93I)

K9\_Zeke\_SH (SEQ ID NO: 860, protein SEQ ID NO: 861) and  
5 K9\_Annabel\_SH (SEQ ID NO: 862, protein SEQ ID NO: 863) were derived from K9\_David\_SH via site directed mutagenesis employing the employing the QuikChange® Lightning Site-Directed Mutagenesis Kit (Catalog #210518; Agilent Technologies, Stratagene Products Division, La Jolla, CA). Except for the primers, templates, and ddH<sub>2</sub>O, all reagents  
10 used here were supplied with the kit indicated above. Primers were synthesized by Integrated DNA Technologies, Inc (Coralville IA).

For K9\_Zeke\_SH, primers employed were oMT93A (GATCCCAGATGAAATGCAGGCTGCCATGTACAAAAACGACATCG; SEQ ID NO: 920)) and oMT93Arev  
15 (CGATGTCGTTTTGTACATGGCAGCCTGCATTTTCATCTGGGATC; SEQ ID NO: 921). The reaction mixture contained 1 µl K9\_David\_SH in the pHR81-Pilv5-KARI-K9.G9-derived vector (20 ng/µl), 1 µl of each primer (150 ng/ul), 5 µl of 10× reaction buffer, 1 µl of dNTP mix, 1.5 µl QuikSolution, 1 ul QuikChange Lightning enzyme, and 38.5 µl ddH<sub>2</sub>O. For  
20 K9\_Annabel\_SH, the primers were replaced with oMT93I (GATCCCAGATGAAATGCAGGCTATCATGTACAAAAACGACATCG; SEQ ID NO: 922) and oMT93Irev (CGATGTCGTTTTGTACATGATAGCCTGCATTTTCATCTGGGATC; SEQ ID NO: 923).

25 The following conditions were used for both reactions: The starting temperature was 95°C for 2 min followed by 18 heating/cooling cycles. Each cycle consisted of 95°C for 20 sec, 60°C for 10 sec, and 68°C for 6 min. At the completion of the temperature cycling, the samples held awaiting sample recovery at 4°C. 2 µl of the Dpn I was added to each  
30 reaction and the mixtures were incubated for 1 hour at 37°C. 2 µl of each mutagenic reaction was transformed into One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, Catalog #C7373-03) according to the

manufacturer's instructions. The transformants were spread on agar plates containing the LB medium and 100 µg/ml ampicillin (Cat#L1004, Teknova Inc. Hollister, CA) and incubated at 37°C overnight. Multiple transformants were then selected for TempliPhi™ DNA sequencing™ (GE Healthcare), which employed primers pHR81-F (ACACCCAGTATTTTCCCTTTCC; SEQ ID NO: 924) and pHR81- Rev (CTA GTG TAC AGA TGT ATG TCG G; SEQ ID NO: 925). Variants with the confirmed sequences (K9\_Zeke\_SH and K9\_Annabel\_SH in pHR81-Pilv5-KARI-K9.G9 derived vectors) were subcloned into the PmeI and SfiI sites of pLH744 (SEQ ID NO: 930).

#### Example 43

##### Isobutanol production of K9SB2 and derivatives grown under anaerobic conditions in 48-well plates

The yeast expression plasmids for K9SB2, K9SB2\_SH, and K9SB2-T93A, prepared with vector derived from pLH744 as described in Example 42, were employed to evaluate isobutanol production in yeast grown under anaerobic conditions in a 48-well plate. Isobutanol production strains were made in host PNY2259 (MATa ura3Δ::loxP his3Δ pdc6Δ pdc1Δ::P[PDC1]-DHAD|ilvD\_Sm-PDC1t-P[FBA1]-ALS|alsS\_Bs-CYC1t pdc5Δ::P[PDC5]-ADH|sadB\_Ax-PDC5t gpd2Δ::loxP fra2Δ::P[PDC1]-ADH|adh\_HI-ADH1t adh1Δ::UAS(PGK1)P[FBA1]-kivD\_Lg(y)-ADH1t yprcΔ15Δ::P[PDC5]-ADH|adh\_HI-ADH1t ymr226cΔ ald6Δ::loxP) by transforming the plasmids containing the coding sequences for the KARI variants and plating on synthetic medium without uracil (1% ethanol as carbon source). Yeast colonies from the transformation on SE-Ura plates appeared after 3-5 days of incubation at 30°C. At least three colonies from each variant were patched onto fresh SE-Ura plates and incubated at 30°C.

##### Yeast Cultivation Conditions:

Aerobic cultivation medium: SE -Ura medium with 2 g/l ethanol.



Anaerobic cultivation medium: SEG –Ura with 30 g/l glucose and 1 g/l ethanol, supplemented with 10 mg/l ergosterol, 50 mM MES buffer (pH 5.5), 30 mg/l thiamine, and 30 mg/l nicotinic acid.

48-well plates: Axygen catalog # P-5ML-48-C-S, 5 ml/well total  
5 volume, culture volume of 1.5 ml/well.

Plates were covered with a permeable adhesive film (VWR ; catalog number 60941-086) for aerobic cultivation. Plates were shaken at 225 rpm at 30 °C. For anaerobic cultivation, freshly inoculated plates covered with permeable film were purged of oxygen by equilibration in an  
10 anaerobic chamber for 2 hours. The plate covers were then exchanged for adhesive aluminum covers (VWR ; catalog number 89049-034) and each plate was placed into an airtight plastic box (Mitsubishi Gas Chemical America, Inc; New York, NY; Catalog 50-25) along with a fresh oxygen scavenger pack (Mitsubishi Gas Chemical America, Inc; New York, NY;  
15 Catalog 10-01). The entire assembly (plate(s) and oxygen scavenger pack inside a sealed airtight plastic box) was removed from the anaerobic chamber and shaken at 225 rpm at 30 °C.

#### Experimental Protocol

Single yeast colonies on SE –Ura agar plates were streaked onto  
20 fresh SE –Ura agar plates and incubated at 30 °C until dense patches of cells had grown. Liquid precultures in 48-well plates were inoculated with loops of these cells for initial aerobic cultivation. After shaking overnight, the OD<sub>600</sub> of each culture well was measured by transferring 0.15 ml of each well into a flat-bottom 96-well plate and measuring the absorbance of  
25 each well at 600 nm with a Molecular Devices (Sunnyvale, CA) plate reader. A linear transformation based on an experimentally-determined calibration line was applied to these plate reader-measured optical densities to convert them into comparable absorbance values for a cuvette-based spectrophotometer.

30 A calculated portion of each aerobic preculture well was inoculated into the corresponding well of a fresh 48-well plate with 1.5 ml of the SEG –Ura medium, to achieve an initial OD<sub>600</sub> (in cuvette spectrophotometer absorbance units) of 0.2. In the process of inoculating the fresh plate, the

aerobic preculture plate was centrifuged, the supernatant was removed from each well, and the cells in each well were resuspended in fresh SEG –Ura medium. This anaerobic cultivation plate was shaken for 3 days. The isobutanol concentration in the culture supernatants was measured by

5 HPLC (Table 41).

Table 41. Isobutanol titers reached in the first anaerobic passaging cycle

| Variant    | # wells | Mean Isobutanol Titer (mM) | Standard Deviation of Isobutanol Titer (mM) |
|------------|---------|----------------------------|---------------------------------------------|
| K9SB2      | 16      | 35.2                       | 11.9                                        |
| K9SB2_SH   | 8       | 67.4                       | 12.2                                        |
| K9SB2-T93A | 8       | 40.8                       | 9.1                                         |

A follow-up anaerobic cultivation was initiated from the first  
 10 anaerobic cultivation as follows: A calculated portion of each anaerobic culture well was inoculated into the corresponding well of a fresh 48-well plate with 1.5 ml of the SEG –Ura medium, to achieve an initial OD<sub>600</sub> (in  
 cuvette spectrophotometer units) of 0.2. In the process of inoculating the  
 fresh plate, the growth plate was centrifuged, the supernatant was  
 15 removed from each well, and the cells in each well were resuspended in fresh SEG –Ura medium, in order to minimize carryover of metabolites  
 from one cultivation to the next. The follow-up (second-cycle) anaerobic  
 cultivation plate was shaken for 2 days. The isobutanol concentration in  
 the culture supernatants was measured by HPLC (Table 42).

20

Table 42. Isobutanol titers reached in the second anaerobic passaging cycle

| Variant    | # wells | Mean Isobutanol Titer (mM) | Standard Deviation of Isobutanol Titer (mM) |
|------------|---------|----------------------------|---------------------------------------------|
| K9SB2      | 16      | 67.6                       | 10.8                                        |
| K9SB2_SH   | 8       | 85.7                       | 9.2                                         |
| K9SB2-T93A | 8       | 76.3                       | 16.8                                        |

Example 44

Kinetic characterization of K9G9, K9SB2, and K9SB2\_SH

K9G9, K9SB2, K9SB2\_SH were overexpressed via pBAD.KARI plasmids in *E. coli* strain Bw25113 ( $\Delta ilvC$ ) and purified for detailed measurement of the kinetic parameters.

5 Expression, purification and cofactor kinetic analyses were performed as described in example 18, with the following modifications. Expression cultures were grown in 20 mL of LB with 100  $\mu$ g/mL ampicillin and 0.2% (w/v) arabinose in a 125 mL vented cap flask. The expression media was inoculated with 1/10 volume of 8 hour culture. Expression  
10 cultures were grown for 18 hours for K9 SB2 and 24 hours for K9SB2\_SH.

Table 43. Kinetic Parameters Comparing Reactions with NADH and NADPH

| Variant  | SEQ ID NO: | $V_{max}$ NADPH, U/mg | $K_m$ NADPH, $\mu$ M | $V_{max}/K_m$ NADPH, L/min*mg | $V_{max}$ NADH, U/mg | $K_m$ NADH, $\mu$ M | $V_{max}/K_m$ NADH, L/min*mg |
|----------|------------|-----------------------|----------------------|-------------------------------|----------------------|---------------------|------------------------------|
| K9G9     | 644        | 2.2                   | 24.1                 | 0.091                         | 1.9                  | 78.2                | 0.024                        |
| K9SB2    | 427        | 1.7                   | 44.8                 | 0.038                         | 1.8                  | 11.6                | 0.155                        |
| K9SB2 SH | 637        | 1.7                   | 109.9                | 0.015                         | 1.7                  | 13.3                | 0.128                        |

15

#### Example 45

#### Isobutanol production of K9SB2 and derivatives

Isobutanol production analysis in 48-well plates was performed, as described, in example 42, with the following modifications. Aerobic and  
20 anaerobic cultivation media are the same as those described in example 19, but with 0.01% w/v histidine added. OD<sub>600</sub> values of aerobic pre-cultures were measured using a Cary 300 spectrophotometer (Agilent Technology, Wilmington, DE). A Heraeus Multifug X1R with a M-20 rotor (Thermo Scientific, Waltham, MA) was used to pellet the aerobic pre-culture cells and the spent cultivation media was discarded. The plates  
25 with cell pellets were transferred into the Coy Anaerobic Bag (Grass Lake, MI) and 100  $\mu$ L of anaerobic cultivation media was added to each pellet. The anaerobic cultivation media was allowed to degas for at least 24 hours prior to the 48-well plate receiving 1.5 mL aliquots; this process was  
30 performed inside the anaerobic bag. The anaerobic culture plate was inoculated with the appropriate volume of cell resuspension and covered

- with an adhesive aluminum foil. Plates were placed into a MCG 2.5L AnaeroPack system (MCG, Japan). The box was sealed and removed from the anaerobic bag and placed in a 30°C incubator for 80 hours with shaking at 220 rpm. Three transformants were analyzed per variant, six transformations were analyzed for K9D3 and K92B2 (Table 44).

Table 44. Isobutanol Titers

| Variant     | SEQ ID NO: | Isobutanol Titer, mM |
|-------------|------------|----------------------|
| K9D3        | 645        | 76.4 ± 9.7           |
| K9SB2       | 427        | 93.8 ± 3.3           |
| K9 Frank    | 440        | 27.8 ± 0.5           |
| K9 Grace    | 445        | 89.4 ± 15.5          |
| K9 Ingrid   | 455        | 88.3 ± 5.4           |
| K9 Jarvis   | 437        | 91.8 ± 5.8           |
| K9 Kelly    | 452        | 40.9 ± 4.5           |
| K9 Norman   | 481        | 66.2 ± 10.6          |
| K9 Ophelia  | 488        | 28.3 ± 5.3           |
| K9 Pat      | 441        | 77.1 ± 25.6          |
| K9 Quentin  | 495        | 80.1 ± 10.8          |
| K9 Ralph    | 496        | 82.1 ± 21.3          |
| K9 Sophia   | 502        | 25.3 ± 13.4          |
| K9 Tiberius | 509        | 11.4 ± 10.4          |
| K9 Ursula   | 511        | 57.3 ± 26.2          |
| K9 Victor   | 514        | 93.0 ± 11.4          |

- Isobutanol production analysis in serum vials was performed for select variants as described in Example 19, with the following modifications. KARI variants were expressed in yeast from plasmids derived from pLH744, prepared as described in Example 42. Histidine was added to both the aerobic pre-culture and anaerobic growth media to a final concentration of 0.01% w/v. Three or four transformants were analyzed per variant (Table 45, 46, and 47).

Table 45. Isobutanol Titers Experiment 1

| Variant  | SEQ ID NO: | Passage | Hours | Isobutanol Titer, mM | Isobutanol/ Glycerol |  |
|----------|------------|---------|-------|----------------------|----------------------|--|
| K9SB2    | 427        | 1       | 75    | 28.2 ± 6.85          | 2.11 ± 0.16          |  |
| K9SB2_SH | 637        | 1       | 75    | 87.9 ± 1.04          | 2.49 ± 0.04          |  |
| K9SB2    | 427        | 2       | 48    | 85.0 ± 4.35          | 2.67 ± 0.06          |  |
| K9SB2_SH | 637        | 2       | 48    | 97.4 ± 4.79          | 2.49 ± 0.07          |  |

- Table 46. Isobutanol Titers Experiment 2

| Variant     | SEQ ID NO: | Passage | Hours | Isobutanol Titer, mM | Isobutanol/Glycerol |
|-------------|------------|---------|-------|----------------------|---------------------|
| K9SB2       | 427        | 1       | 44    | 30.2 ± 4.1           | 2.30 ± 0.15         |
| K9SB2_SH    | 637        | 1       | 44    | 40.3 ± 5.6           | 2.41 ± 0.07         |
| K9 David    | 431        | 1       | 44    | 36.2 ± 3.5           | 2.40 ± 0.10         |
| K9 David_SH | 196        | 1       | 44    | 41.9 ± 6.4           | 2.37 ± 0.04         |
| K9 Grace    | 445        | 1       | 44    | 40.2 ± 5.5           | 2.30 ± 0.05         |
| K9 Pat      | 441        | 1       | 44    | 29.0 ± 3.1           | 2.35 ± 0.10         |
| K9SB2       | 427        | 2       | 46    | 59.2 ± 0.6           | 2.61 ± 0.03         |
| K9SB2_SH    | 637        | 2       | 46    | 74.8 ± 3.0           | 2.58 ± 0.03         |
| K9 David    | 431        | 2       | 46    | 63.8 ± 0.6           | 2.65 ± 0.06         |
| K9 David_SH | 196        | 2       | 46    | 76.7 ± 1.5           | 2.53 ± 0.04         |
| K9 Grace    | 445        | 2       | 46    | 62.3 ± 3.1           | 2.52 ± 0.07         |
| K9 Pat      | 441        | 2       | 46    | 50.1 ± 2.1           | 2.48 ± 0.07         |

Table 47 Isobutanol Titers

| Variant    | SEQ ID NO: | Passage | Hours | Isobutanol Titer, mM |
|------------|------------|---------|-------|----------------------|
| K9SD2      | 427        | 2       | 50    | 66.2 ± 1.8           |
| Annabel_SH | 862        | 2       | 50    | 74.4 ± 2.4           |
| Zeke_SH    | 860        | 2       | 50    | 76.8 ± 8.3           |

Table 2

HMMER2.0 [220]  
NAME Functionally Verified KARIS  
LENG 354

ALPH Amino

MAP yes

COM hmmbuild -n Functionally Verified KARIS exp-KAR1hm exp-KAR1\_mod.aln

COM hmmscalibrate exp-KAR1hm

NSEQ 25

DATE Mon Dec 8 17:34:51 2008

XT -8455 -4 -1000 -1000 -8455 -4 -8455 -4

NULT -4 -8455

NULE 585 -1588 85 338 -284 453 -1168 187 249 902 -1085 -142 -21 -313 45 531 201 384 -1988 -544

EVD -333.712080.110102

| EVL -333 / 12/08 0.1 T102 |        |       |       |       |       |       |         |        |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
|---------------------------|--------|-------|-------|-------|-------|-------|---------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-----------------------|-------|
| HMM                       | A      | C     | D     | E     | F     | G     | H       | I      | K     | L     | M     | N     | P     | Q     | R     | S     | T     | V     | W     | Y     | Position in alignment |       |
|                           | m->m   | m->i  | m->d  | i->m  | i->i  | d->m  | d->d    | b->m   | m->e  |       |       |       |       |       |       |       |       |       |       |       |                       |       |
|                           | -650 * | -1463 |       |       |       |       |         |        |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 1(1Q)                     | -648   | -1356 | -136  | -44   | -1453 | -1166 | -219    | -1455  | 321   | -1417 | -911  | -227  | -1496 | 3263  | 122   | -643  | -694  | -1239 | -1542 | -1030 | 7100%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -38    | -5840 | -6882 | -894  | -1115 | -701  | -1318   | -650 * |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 2(2M)                     | -4231  | -3929 | -5215 | -5402 | -3438 | -4370 | -4526   | -3232  | -5113 | -2613 | -5320 | -5052 | -4790 | -4977 | -4823 | -4692 | -4459 | -3829 | -4103 | -4017 | 7200%                 |       |
| -                         | -147   | -501  | 232   | 42    | -382  | 397   | 104     | -625   | 209   | -467  | -722  | 276   | 395   | 44    | 95    | 361   | 121   | -368  | -296  | -251  |                       |       |
| -                         | -3303  | -3316 | -925  | -3473 | -136  | -701  | -1318 * | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 3(1+)                     | -1308  | -1104 | -2227 | -2120 | -3516 | -2093 | -244    | -196   | -1861 | 64    | 66    | -1526 | -2278 | -1503 | -1798 | -1617 | -1350 | -389  | 305   | 1335  | 8600%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -38    | -5840 | -6882 | -894  | -1115 | -701  | -1318 * | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 4(A)                      | 1615   | -1744 | 1125  | 33    | -2015 | -1540 | -262    | -1666  | 937   | -1765 | -911  | -252  | -1659 | 154   | -383  | 489   | 640   | 3     | -2038 | -1421 | 8700%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -901   | -7402 | -1125 | -894  | -1115 | -2352 | -314 *  | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 5(C)                      | -346   | 2578  | 1084  | -712  | 2092  | -1540 | -384    | -161   | -624  | -482  | -482  | 125   | -731  | -1705 | -451  | -883  | -631  | -338  | -50   | -774  | -133                  | 8800% |
| -                         | -149   | -500  | 235   | 43    | -381  | 398   | 106     | -626   | 210   | -466  | -721  | 275   | 394   | 45    | 96    | 359   | 118   | -369  | -295  | -249  |                       |       |
| -                         | -1009  | -1006 | -7567 | -131  | -3527 | -1916 | -444 *  | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 6(S)                      | 800    | -586  | -1937 | -1415 | -821  | -1740 | -964    | 1279   | -1204 | -584  | 19    | -1258 | -1964 | -1013 | -1358 | 1715  | -476  | 1117  | -1320 | -938  | 9000%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -17    | -5853 | -7995 | -894  | -1115 | -701  | -1318 * | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 7(K)                      | -955   | -2411 | -803  | 501   | -2743 | -1919 | -568    | -2483  | 2435  | -2420 | -1502 | 57    | -2010 | 1146  | 458   | 829   | 224   | -2040 | -2577 | -1913 | 9100%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -8     | -8139 | -9181 | -894  | -1115 | -701  | -1318 * | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |
| 8(V)                      | -2472  | -2010 | -5089 | -4702 | -2834 | -4789 | -4391   | 2241   | -4574 | -151  | -1318 | -4442 | -4600 | -4417 | -4628 | -4080 | -82   | 3023  | -3952 | -3510 | 9200%                 |       |
| -                         | -149   | -500  | 233   | 43    | -381  | 399   | 106     | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |                       |       |
| -                         | -8     | -8139 | -9181 | -894  | -1115 | -701  | -1318 * | *      |       |       |       |       |       |       |       |       |       |       |       |       |                       |       |

Table Z

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 9(Y)  | -4673 | -3685 | -5210 | -5505 | 2423  | -5059 | -1332 | -3424 | -5065 | -392  | -2838 | -3726 | -4920 | -3835 | -4458 | -4313 | -4533 | -581  | 4343  | 9300% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 10(Y) | -2170 | -2625 | -2489 | -2097 | -1555 | -2996 | -148  | -2628 | 908   | -2674 | -2098 | -2051 | -3206 | -1513 | -1078 | -2258 | 1039  | -2435 | -2009 | 4185  |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 11(D) | -2498 | -4412 | 3500  | 1042  | -4881 | -2437 | -1765 | -4500 | 733   | -4361 | -3682 | 515   | -2961 | -1429 | -2759 | -2158 | -2568 | -3974 | -4550 | -3541 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 12(K) | 11    | -2371 | 348   | 819   | -2692 | -535  | -527  | -2443 | 2294  | -2387 | -1461 | 550   | -1960 | -68   | 904   | -67   | -837  | -1993 | -2554 | -1871 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 13(D) | -2663 | -4633 | 3709  | 590   | -4189 | -2497 | -1972 | -4738 | 731   | -4578 | -3953 | -1072 | -3046 | -1551 | -2967 | -2292 | -2742 | -4201 | -4759 | -3703 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 14(C) | 2503  | 3193  | 4266  | -3618 | -2010 | -3276 | -2696 | 782   | -3517 | -1437 | -1031 | -3233 | -3509 | -3212 | -3411 | -2499 | -1792 | 1507  | -2796 | -2431 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 15(D) | -1363 | -2905 | 2748  | 542   | -3302 | -2072 | -920  | -2977 | 290   | -2912 | -2023 | 1270  | -2294 | -489  | -1766 | 53    | 1115  | -2519 | -3086 | -2349 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 16(L) | -1268 | -1113 | -3338 | -540  | -1057 | -2827 | -1716 | 559   | -2409 | 2299  | -236  | -2381 | -2862 | -2089 | -2316 | -232  | -1213 | 1305  | -1545 | -1304 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 17(S) | -1350 | -2877 | 588   | 1045  | -3189 | -495  | -920  | -2963 | -628  | -2901 | -2011 | -1860 | -2289 | -489  | -1784 | 2139  | 190   | -2503 | -3077 | -2343 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -2336 | -8139 | -325  | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 18(G) | -454  | -832  | -968  | -1110 | -2112 | 3143  | -1211 | -2091 | -1317 | -2764 | -1631 | -978  | -1499 | -1202 | -1421 | -846  | -774  | -1550 | -1916 | -1919 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -38   | -5840 | -6882 | -894  | -1115 | -3098 | -179  | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 19(H) | -898  | -1313 | -545  | -482  | -320  | -1336 | 4297  | -1552 | -160  | -1493 | -1036 | -579  | -1675 | -383  | -322  | -934  | -951  | -1354 | -725  | 107   |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -38   | -5840 | -6882 | -894  | -1115 | -3098 | -179  | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 20(I) | -872  | -1812 | 3234  | 432   | -2215 | -967  | -433  | -2172 | -569  | -2268 | -1704 | 99    | -1453 | -184  | -1741 | -728  | -973  | -1814 | -2146 | -1646 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -38   | -5840 | -6882 | -894  | -1115 | -3098 | -179  | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 21(E) | -766  | -1695 | 521   | 2831  | -2560 | -1029 | -269  | -1804 | -118  | -1919 | -1331 | 69    | -1441 | -4    | -527  | -653  | -814  | -1512 | -1988 | -1505 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -38   | -5840 | -6882 | -894  | -1115 | -3098 | -179  | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 22(Y) | -1337 | -1229 | -1661 | -1556 | 1268  | -1957 | 121   | -918  | -1294 | -769  | -535  | -1229 | -2163 | -1111 | -1301 | -1443 | -1369 | -932  | 592   | 3932  |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |
| -     | -38   | -5840 | -6882 | -894  | -1115 | -109  | -375  | *     |       |       |       |       |       |       |       |       |       |       |       |       |
| 23(I) | -2294 | -1931 | -4749 | -4227 | -1724 | -4227 | -3323 | 2306  | -3952 | 1990  | -634  | -3878 | 94    | -3538 | -3812 | -3411 | -2247 | 1515  | -2691 | -2629 |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |

CA 2831130 2018-08-03

[illegible]



Table Z

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 38(G) | 677   | 2128  | -3838 | -4171 | -4647 | 3536  | -3816 | -4506 | -4340 | -4749 | -3857 | -3009 | -3149 | -3871 | -4137 | -1784 | -2005 | -3297 | -4725 | -4735 | 13700% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 39(H) | -667  | -3375 | -2682 | -2114 | -3744 | -3201 | 4738  | -3782 | -445  | -3553 | -2886 | -2112 | 866   | -1265 | 1506  | -2614 | -2557 | -3469 | -3282 | -2903 | 13800% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 40(A) | 3631  | -2768 | -4492 | -4815 | -4888 | -2992 | -4271 | -4781 | -4818 | -5025 | -4365 | -3727 | -3728 | -4477 | -4545 | -2367 | -2762 | -3862 | -4724 | -4942 | 13900% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 41(H) | -3103 | -3404 | -2950 | -2573 | -783  | -3679 | 4649  | -3407 | -1372 | -3071 | -2715 | -2454 | -3764 | 2546  | -1429 | -2390 | -2976 | -3308 | 2269  | -295  | 14000% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 42(A) | 3367  | -1795 | -4134 | -4277 | -4057 | -2118 | -3648 | -3648 | -4035 | -4024 | -3192 | -2817 | -2900 | -3608 | -3823 | 217   | -1869 | -278  | -4363 | -4211 | 14100% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 43(Q) | -1061 | 1950  | -2044 | -1475 | -1236 | -2372 | -1154 | -789  | -1218 | 1062  | 1123  | 743   | -2446 | 2895  | -1441 | -1392 | -1005 | -639  | -1578 | -1278 | 14200% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 44(N) | -4000 | -4117 | -3389 | -3749 | -5073 | -3911 | -4123 | -6022 | -4503 | -5797 | -5419 | 4397  | -4479 | -4255 | -4552 | -4115 | 4312  | -5371 | -4650 | -4731 | 14300% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 45(L) | -4414 | -3800 | -5638 | -5628 | -2280 | -4980 | -4628 | -1886 | -5423 | 3316  | -1236 | -5514 | -4997 | -4750 | -5002 | -5379 | -4399 | -2629 | -3665 | -3650 | 14400% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 46(R) | -1731 | -3015 | 275   | 931   | -3467 | -2518 | -973  | -3116 | 2321  | -2955 | -2123 | 224   | -2603 | 256   | 2803  | -1596 | -1613 | -2730 | -2955 | -2515 | 14500% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 47(D) | -2866 | -4843 | 3855  | 944   | -5037 | -2600 | -2081 | -5052 | -2528 | -4903 | -4373 | -1209 | -3196 | -1786 | -3536 | -2501 | -3007 | -4517 | -5004 | -3956 | 14600% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 48(S) | -1536 | -2212 | -2363 | -2679 | -4233 | -2279 | -3081 | -4365 | -3331 | -4524 | -3676 | 288   | -3026 | -2967 | -3457 | 3508  | -1962 | -3269 | -4477 | -4066 | 14700% |
| -     | -149  | -500  | 232   | 44    | -381  | 398   | 106   | -627  | 211   | -465  | -721  | 275   | 393   | 45    | 95    | 360   | 118   | -370  | -295  | -250  |        |
| -     | -156  | -3318 | -9181 | -2405 | -302  | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 49(G) | -2521 | -3669 | 1232  | -911  | -4449 | 3373  | -2128 | -4854 | -2535 | -4752 | -4136 | -53   | -3115 | -1835 | -3440 | -2284 | -2715 | -4157 | -4880 | -3914 | 15400% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 50(V) | -2767 | -2324 | -5232 | -4770 | 396   | -4827 | -3784 | -96   | -4546 | 848   | -611  | -4472 | -4518 | -3960 | -4367 | -4081 | -2715 | 3323  | -3037 | -2660 | 15500% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 211   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -148  | -3381 | -9181 | -203  | -2328 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 51(D) | -1684 | -3285 | 2735  | 2014  | -3354 | -2196 | -1171 | -3350 | 92    | -3279 | -2427 | 692   | -2505 | -770  | -1595 | -1483 | -1666 | 332   | -3460 | -2678 | 15700% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 52(V) | -3122 | -2888 | -5092 | -5160 | -3322 | -4180 | -4681 | -905  | -5050 | -2626 | -2570 | -4662 | -4579 | -4940 | -4923 | -4013 | -3297 | 3796  | -4414 | -4190 | 15800% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

CA 2831130 2018-08-03

|       |       |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |        |
|-------|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 53(V) | 369   | 386   | -3075 | -2452 | -483  | -2557 | -1420 | 1415   | 378   | -757  | -117  | -2098 | -2610 | -1809 | -2037 | -1830 | 1186  | 2145  | -1385 | -343  | 15900% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 54(V) | -2624 | -2122 | -5302 | -4991 | -272  | -5108 | -5136 | 2623   | 4948  | -1533 | -1475 | -4794 | -4871 | -4894 | -5108 | -4488 | -2620 | 3088  | -4511 | -3996 | 16000% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 55(G) | 929   | -2107 | -3852 | -4182 | -4633 | 3492  | -3804 | -4466  | -4335 | -4732 | -3835 | -2997 | -3132 | -3963 | -4127 | -1761 | -1982 | -3275 | -4720 | -4725 | 16100% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 56(L) | -3427 | -2938 | -5791 | -5325 | -1449 | -5374 | -4410 | -543   | -5063 | 3041  | -255  | -5207 | -4820 | -4126 | -4691 | -4757 | -3351 | 883   | -3184 | -3234 | 16200% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -6    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 57(R) | -3040 | -3724 | -3266 | 82    | -4820 | -3470 | -1396 | -3905  | 804   | -3529 | -2874 | -2133 | -3439 | -978  | 3800  | -2894 | -2709 | -3682 | -3363 | -3267 | 16300% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 58(K) | 31    | -2412 | 803   | 1532  | -2243 | -1920 | -559  | -2483  | 1772  | -2421 | -1503 | -555  | 1229  | 727   | 1079  | -566  | -893  | -2041 | -2579 | -1915 | 16400% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 59(G) | -2671 | -4651 | 1614  | 587   | -4832 | 3103  | -1901 | -4803  | -2269 | -4648 | -4047 | 421   | -3049 | -1587 | -3230 | -2297 | -2766 | -4245 | -4850 | -3752 | 16500% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 60(S) | -1499 | -2308 | -1932 | -1859 | -4006 | 1604  | -2121 | -3754  | 1362  | -3793 | -2945 | -1833 | -2827 | -1794 | -1902 | 2786  | -1771 | -2970 | -3910 | -3479 | 16600% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -247  | -8139 | -2699 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 61(K) | 1362  | -2232 | -819  | -98   | -287  | -427  | -435  | -2309  | 1599  | -2265 | -1349 | 1101  | -1861 | 886   | -512  | 833   | -740  | -1868 | -2441 | -1767 | 16700% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -6    | -7900 | -8943 | -894  | -1115 | -344  | -2238* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 62(S) | -1288 | -1904 | -3742 | -4011 | -4394 | -2155 | -3593 | -4209  | -3996 | -4479 | -3573 | -2789 | -2948 | -3606 | -3832 | 3517  | 228   | -3028 | -4500 | -4451 | 16800% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 63(W) | 726   | -873  | -3261 | -2634 | 1926  | -2667 | -1425 | 660    | -2252 | -701  | -68   | -2174 | -2617 | -1898 | 18    | -1648 | -872  | 983   | 4091  | -988  | 16900% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 64(E) | 1527  | -2404 | 212   | 1635  | -2722 | -1878 | -555  | -2474  | 1241  | -2419 | -1497 | 350   | -1985 | -100  | -659  | 96    | 70    | -2025 | -2589 | -1903 | 17000% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 65(K) | 8     | -2242 | -885  | 770   | -2502 | -1953 | -609  | -2192  | 2589  | 22    | -1353 | -631  | -2052 | 692   | -617  | -389  | -905  | -361  | -2455 | -1806 | 17100% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 66(A) | 3531  | -2768 | -4492 | -4615 | -4888 | -2992 | -4271 | 4781   | -4818 | -5025 | 4365  | -3727 | -3728 | -4477 | -4545 | -2367 | -2762 | -3882 | -4724 | -4942 | 17200% |
| -     | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -     | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |

Table Z

|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 67(Q) | -1006 | -2441 | -869  | 1767  | -2780 | -1965 | -586  | -2510 | 1702  | -2445 | -1534 | -603  | -2052 | 1923  | 873   | 388   | 236   | -630  | -2596 | -1949 | 17300% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 68(A) | 1489  | -2333 | 167   | 1234  | -2711 | -1873 | -547  | -2462 | 895   | -2408 | -1485 | 1161  | -1977 | -90   | -648  | 666   | 141   | -2014 | -2577 | -1892 | 17400% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 69(D) | 2104  | -2898 | 2124  | 985   | -3163 | -2096 | 1397  | -2935 | -893  | -2897 | -2025 | -723  | -2329 | -543  | -1250 | -1245 | -1368 | -2501 | -3087 | -530  | 17500% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 70(G) | -2294 | -2898 | -2521 | -2885 | -4852 | 3641  | -3456 | -5042 | -3796 | -5094 | -4356 | 365   | -3545 | -3376 | -4005 | -2451 | -2700 | -3968 | -4706 | -4575 | 17600% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 71(F) | -2566 | -2256 | -4685 | -4188 | 3199  | -4138 | 1018  | 505   | -3812 | 1986  | -405  | -3595 | -3961 | -3157 | -3524 | -3277 | -2509 | -1337 | -1821 | -840  | 17700% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 72(K) | 47    | -2348 | 338   | 950   | -2668 | -1854 | -512  | -507  | 1721  | -2364 | -1438 | -490  | -1947 | 672   | 438   | 306   | 687   | -1970 | -2533 | -1851 | 17800% |
| -     | -149  | -500  | 233   | 46    | -381  | 399   | 106   | -627  | 210   | -466  | -721  | 277   | 393   | 45    | 95    | 359   | 119   | -370  | -295  | -250  |        |
| -     | -155  | -3318 | -9181 | -2159 | -556  | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 73(V) | -1810 | -1639 | -4149 | -3689 | -1859 | -3417 | -2822 | 29    | -3369 | 320   | -897  | -3230 | 112   | -3099 | -3251 | -2519 | -767  | 3269  | -2708 | -2354 | 18400% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 74(K) | 847   | -1093 | -2131 | -1554 | 304   | 127   | -1121 | -837  | 1445  | 645   | 1186  | -1534 | -2401 | -1174 | -1547 | -784  | -172  | -528  | -1519 | 1413  | 18500% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 75(T) | -1284 | -2794 | 1526  | 1290  | -3956 | -2041 | 1284  | -2963 | -548  | -2808 | -1914 | -668  | -2242 | -407  | -1055 | 1451  | 1827  | -2411 | -2986 | -2264 | 18600% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 76(V) | -1085 | -957  | -3143 | -2535 | -543  | -2618 | -1496 | 1052  | -2198 | -792  | 1859  | -146  | 686   | -1884 | -2111 | -1695 | 945   | 2346  | -1458 | -1106 | 18700% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 77(W) | 1606  | -2321 | -752  | 612   | -2828 | -323  | -527  | -2366 | 1480  | -2331 | -1416 | -510  | -789  | -73   | 421   | 23    | 629   | -1936 | 2212  | -1843 | 18800% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 78(E) | -1509 | -3540 | 1372  | 3127  | -3651 | -120  | -1391 | -3695 | -1319 | -3605 | -2737 | -900  | -2659 | -1005 | -1976 | -400  | -655  | -3134 | -3730 | -2657 | 18900% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 79(A) | 3390  | -1668 | -4092 | -4341 | -4332 | -2153 | -3680 | -3942 | -4157 | -4333 | -3471 | -2869 | -2948 | -3730 | -3919 | -1525 | 931   | -2894 | -4530 | -4483 | 19000% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 80(V) | 2003  | -1721 | -4449 | -3995 | -2160 | -3763 | -3240 | 1342  | -3745 | -1435 | -1124 | -3561 | -3855 | -3494 | -3700 | -2979 | -58   | 2574  | -3091 | -2698 | 19100% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 81(K) | 1714  | -2501 | -959  | 446   | -2858 | -2043 | -654  | -2574 | 1964  | -2506 | -1609 | -869  | -2135 | -203  | 1088  | 428   | -1032 | -2146 | -2652 | -2027 | 19200% |
| -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

Table Z

|        |       |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 382(W) | 265   | -2347 | 815   | 432   | -2663 | 634   | -519  | -2410  | 619   | -2361 | -1438 | -495  | -1952 | 1955  | -508  | -382  | 147   | -1958 | 2858  | -1853 | 19300% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 383(A) | 3391  | -1860 | -3998 | 4279  | 4411  | 2128  | -3684 | -4207  | -4197 | -4190 | -3665 | -2837 | -2929 | -3729 | -3959 | 706   | -1718 | -3001 | -4636 | -4534 | 19400% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 84(D)  | -2747 | -4755 | 3813  | 396   | -4912 | -2495 | -1935 | -4905  | -3234 | -4735 | -4166 | -1079 | -3082 | 603   | -3296 | -2553 | -2944 | -4347 | -4929 | -3805 | 19500% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 85(V)  | -2717 | -2220 | -5838 | -4951 | -2234 | -5099 | -4670 | 1963   | -4844 | 1553  | -1011 | -4759 | -4771 | -4509 | -4836 | -4427 | -2688 | 274   | -3899 | -3628 | 19600% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 86(V)  | -2635 | -2129 | -5906 | -4970 | -2652 | -5125 | -5011 | 2554   | -4915 | -354  | -1368 | -4781 | -4852 | -4798 | -5038 | -4487 | -2622 | 3019  | -4356 | -3902 | 19700% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 87(M)  | -1340 | -1208 | -3317 | -2708 | -988  | -2660 | -1708 | 577    | -2346 | 932   | 4131  | -2382 | -2878 | 250   | -2265 | -228  | -1278 | -506  | -1629 | -1313 | 19800% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 88(I)  | -2566 | -2177 | -5017 | -4470 | 659   | -4496 | -3487 | 2791   | -4191 | 1116  | 1394  | -4156 | -4228 | -3815 | -3972 | -3887 | -2499 | 1652  | -2860 | -2711 | 19900% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 89(L)  | -4414 | -3800 | -5638 | -5628 | -2230 | -4980 | -4628 | -1886  | -5423 | 3316  | -1236 | -5514 | -4997 | -4750 | -5002 | -5379 | -4399 | -2629 | -3665 | -3590 | 20000% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 90(I)  | 1212  | -1286 | -3946 | -3262 | -1380 | -3195 | -2166 | 1616   | -2918 | 1031  | -493  | -2824 | -3211 | -2583 | -2782 | -2108 | 1596  | 1259  | -2020 | -1668 | 20100% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 91(F)  | -1614 | -2214 | -3395 | -3710 | -4516 | -2407 | -3616 | -4516  | -3976 | -4705 | -3849 | -2890 | 3993  | -3625 | -3900 | 666   | -2068 | -3354 | -4610 | -4474 | 20200% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 92(D)  | -4680 | -4701 | 4174  | -3014 | -5700 | -3967 | -3905 | 6376   | -4478 | -6024 | -5744 | -3355 | -4501 | -3870 | -4926 | -4440 | -4750 | -5864 | -4622 | -5231 | 20300% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 93(E)  | -1123 | -2195 | -583  | 2715  | -2589 | -2045 | -942  | -2250  | -625  | -2356 | 1979  | -870  | -2250 | -554  | -1093 | 453   | 932   | -1902 | -2660 | -2064 | 20400% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 94(H)  | 399   | -1137 | -2012 | -14   | 1582  | -2305 | 1600  | 246    | -1252 | 190   | -325  | -1456 | -2374 | 1474  | -1479 | -84   | -905  | 896   | -1557 | -1158 | 20500% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 95(Q)  | -2742 | -3142 | -2766 | -2681 | -2790 | -3344 | -2460 | -160   | -1802 | -2456 | -2353 | -2682 | -3710 | 4317  | -1836 | -2694 | -2844 | -2558 | -3295 | -2711 | 20600% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |

Table Z

|        |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 96(A)  | 1981  | -2315 | 809   | -268  | -2645 | -531  | -576   | -2374 | 232   | -2350 | -1445 | -567  | 1217  | 711   | 445   | 447   | -874  | -1951 | -2540 | -1833 | 20700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 97(D)  | 491   | -2351 | 1394  | 1381  | -2611 | -1854 | 1082   | -2421 | 1010  | -2367 | -1440 | -489  | -1947 | 1017  | 362   | -760  | 250   | -623  | -2535 | -1832 | 20900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 98(V)  | -2039 | -1706 | -4456 | -3939 | -1846 | -3939 | -3049  | 1986  | -3656 | 1460  | -826  | 804   | -3870 | -3351 | -3565 | -3105 | -2000 | 2330  | -2796 | -2442 | 20900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 99(Y)  | 4840  | -3766 | -5230 | -5581 | 1698  | -5109 | -1300  | -3727 | -5135 | -3041 | -3132 | -3723 | -4964 | -3961 | -4501 | -4357 | -4690 | -3883 | 3325  | 4377  | 21000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 100(E) | -163  | -2363 | 734   | 1681  | -2674 | -1859 | 886    | -2422 | 1668  | -792  | -1443 | 777   | -1982 | 890   | 286   | -766  | 238   | -1975 | -2536 | -1856 | 21100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -257  | -8139 | -9649 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 101(E) | 1017  | -2763 | 862   | 2042  | -3060 | -1913 | -775   | -2836 | -495  | -2773 | -1866 | 1956  | -2143 | -136  | -1066 | 765   | -1185 | -2377 | -2948 | -2207 | 21200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -9    | -7891 | -8933 | -894  | -1115 | -338  | -2261* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 102(E) | -944  | -2422 | 863   | 2138  | -2740 | -436  | -567   | -2493 | 894   | -2437 | -1515 | -518  | -1994 | 1767  | -673  | 109   | -885  | -1023 | -2605 | -1917 | 21300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 103(I) | 2660  | -2166 | -6316 | -4965 | -2620 | -5119 | -4900  | 3165  | -4894 | 297   | -1251 | -4775 | -4828 | -4705 | -4975 | -470  | -2542 | 2240  | -4202 | -3814 | 21400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 104(E) | 1068  | -2341 | -760  | 2003  | 628   | -1887 | 876    | -2380 | 1240  | -2347 | -1436 | -529  | -1983 | 881   | -618  | -304  | -855  | -1954 | -2530 | -1852 | 21500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 105(P) | -343  | -3144 | 1561  | 442   | -3538 | -489  | -1216  | -3329 | -1038 | -3274 | -2420 | -848  | 2974  | -812  | -1635 | 469   | -1644 | -2849 | -3462 | -2693 | 21600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 106(N) | -1173 | -2375 | -814  | 827   | -2376 | -2071 | 1767   | -2279 | -479  | -2336 | -1509 | 3151  | -2218 | -415  | -957  | -1093 | -1120 | -198  | -2486 | 647   | 21700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 107(M) | -3415 | -2990 | -5826 | -5252 | -132  | -5488 | -4282  | 1361  | -5022 | 2621  | 2728  | -5181 | -4778 | 4005  | -4613 | -4776 | -3292 | 86    | -3071 | -3194 | 21800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 108(K) | -1941 | -3098 | -1997 | -1232 | -3650 | -2740 | -1025  | -3210 | 3059  | -3010 | -2208 | 499   | -2766 | 1457  | 1261  | -1817 | -90   | -2858 | -3002 | -2622 | 21900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 109(P) | 1129  | -2426 | -740  | 964   | -2747 | -1913 | -589   | -2491 | 1139  | -2440 | -1525 | -552  | 1941  | 1446  | -655  | -480  | -913  | -2050 | -2610 | -1935 | 22000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 110(G) | -2276 | -2907 | -2347 | -2709 | -4832 | 3654  | -3349  | -5005 | -3678 | -5083 | -4315 | 1193  | -3507 | -3243 | -3937 | -2418 | -2674 | -3974 | -4703 | -4521 | 22100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -486  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

CA 2831130 2018-08-03

|        |       |       |       |       |       |       |        |        |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
|--------|-------|-------|-------|-------|-------|-------|--------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|--|
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701   | -1376* | *     |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 111(A) | 1730  | -2349 | 958   | -198  | -2661 | -1868 | -535   | -2405  | 927   | -2362 | -1444 | 414   | -1866 | 788   | 45    | 96    | 359   | 117   | -369  | -294  | -1863  | 22200% |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 112(T) | 1365  | -1149 | -14   | -1461 | -1155 | -2314 | -1111  | 758    | -1275 | -1024 | 1167  | -1475 | -2388 | -1111 | -1501 | 334   | 1843  | 354   | -1581 | -1182 | 22300% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 113(L) | -3333 | -2796 | -8006 | -5293 | -1505 | -5335 | -4502  | 1096   | -5103 | 2935  | -282  | -5232 | -4657 | -4172 | -4762 | -4864 | -3236 | 506   | -3264 | -3351 | 22400% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 114(A) | 1769  | -1625 | 158   | -867  | -1603 | 148   | -891   | -1181  | -762  | 187   | -712  | -1040 | -2228 | 680   | -1135 | -1111 | -913  | 1306  | -1913 | -1442 | 22500% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 115(F) | 4110  | -3437 | -536  | -5431 | 4216  | -5143 | 2158   | -1742  | -5074 | 563   | -1124 | 4230  | 4871  | -3987 | 4561  | 4647  | 4016  | -2374 | -1366 | -292  | 22800% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 116(A) | 3091  | -1829 | -3998 | -4219 | -4413 | 119   | -363   | -4216  | -4134 | -4469 | -3623 | -2738 | -2896 | -3656 | -3927 | 1514  | -1679 | -2383 | -4632 | -4639 | 22700% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 117(H) | -5197 | -4539 | -4720 | -5009 | -4036 | -4506 | 5438   | -6314  | -4911 | -5786 | -5667 | -4954 | -4860 | -6011 | -4732 | -5391 | -5396 | -6022 | -4083 | -3641 | 22800% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 118(G) | 4436  | -4203 | -5052 | -5462 | -5893 | 3834  | -5028  | -6627  | -5766 | -6297 | -5970 | -5141 | -4804 | -6548 | -5385 | -4727 | -4815 | -5662 | -4924 | -5849 | 22900% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 119(F) | 4044  | -3387 | -5634 | -5444 | 4033  | -5246 | 2370   | -1514  | -5107 | 1089  | -868  | -4443 | -4880 | -3998 | -4592 | -4639 | -3834 | -2700 | -1536 | -523  | 23000% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 120(N) | 886   | -1899 | -2020 | -1781 | -2956 | -2135 | -1925  | -2602  | -1809 | 3     | -2052 | 3488  | -2633 | -1676 | -2141 | 413   | -1437 | -2139 | -3194 | -2737 | 23100% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 364   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 121(I) | -2673 | -2169 | -5324 | -4969 | -2477 | -5123 | -4876  | 3293   | -4893 | 358   | -1211 | -4780 | -4824 | -4681 | -4961 | 4472  | -2653 | 1969  | -4168 | -3791 | 23200% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 122(H) | -3381 | -3705 | -3197 | -3491 | -4166 | 638   | 5216   | -5496  | -3768 | -5304 | -4811 | -3481 | -4165 | -3770 | -3879 | -3608 | -3702 | -4763 | -4170 | -3759 | 23300% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 123(Y) | -4816 | -3767 | -6210 | -5649 | 3410  | -5097 | 2153   | -3719  | -5105 | -3041 | -3127 | -3715 | -4965 | -3851 | -4483 | -4344 | -4609 | -3870 | -547  | 3677  | 23400% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |
| 124(G) | -1065 | -2519 | 948   | -272  | -2820 | 1844  | 938    | -2666  | 972   | -284  | -1622 | 1553  | -2090 | -2279 | -802  | -936  | -1011 | -2133 | -2708 | -2021 | 23500% |        |  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626   | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |        |  |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1376* | *      |       |       |       |       |       |       |       |       |       |       |       |       |        |        |  |

Table Z

|        |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 125(O) | -412  | -2285 | -2468 | -2186 | -2088 | -2877 | -2018  | -1889 | -1538 | 1526  | -1121 | -2187 | -3153 | 3585  | -1718 | -2137 | -1954 | -1719 | -2789 | -2414 | 23500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 126(I) | -2254 | -1916 | -4813 | -4439 | -2456 | -4221 | -3933  | 3248  | -4248 | -1515 | -1324 | -4044 | -4259 | -4063 | -4255 | 230   | -2280 | 2003  | -3673 | -3237 | 23700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 127(K) | -888  | -2234 | 334   | 1172  | -2504 | -1881 | -546   | -93   | 1370  | -300  | -1337 | 465   | -1974 | 655   | -645  | -94   | -827  | 1245  | -2448 | -1798 | 23800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 128(P) | 715   | -1925 | -3618 | -3897 | -4494 | 653   | -3594  | -4274 | -4053 | -4520 | -3596 | -2770 | 3775  | -3593 | -3811 | -1550 | -1770 | -3067 | -4647 | -4548 | 23900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 129(P) | 479   | -2398 | -1173 | -637  | -2915 | -2106 | -648   | -2610 | -288  | -2586 | -1713 | -884  | 2238  | 1247  | 2196  | 51    | -1147 | -2184 | -2757 | -2174 | 24000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 130(A) | 1787  | -2663 | 1377  | 529   | -2976 | -1992 | -760   | -2756 | 1785  | -2680 | -1776 | -623  | -2161 | -319  | -936  | 297   | -1120 | -2285 | -2953 | -2145 | 24100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -2336 | -8139 | -325  | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 131(F) | -1308 | -1104 | -2227 | -2120 | 3516  | -2093 | -244   | -196  | -1891 | 64    | 66    | -1626 | -2276 | -1503 | -1796 | -1617 | -1350 | -389  | 305   | 1335  | 24200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -58   | -5840 | -6882 | -694  | -1115 | -3098 | -173*  | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 132(P) | -603  | -937  | -997  | -1058 | -1832 | -1041 | -109   | -1737 | -1074 | -1874 | -1416 | -392  | 3539  | -1055 | -1192 | -289  | -856  | -1383 | -1765 | -1661 | 24300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -38   | -5840 | -6882 | -694  | -1115 | -3098 | -173*  | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 133(K) | -804  | -1483 | -564  | -230  | -120  | -1335 | -101   | -1605 | 2889  | -1630 | -1021 | -349  | -1569 | 232   | 698   | -286  | -759  | -1358 | -1537 | -1317 | 24400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -38   | -5840 | -6882 | -694  | -1115 | -109  | -3773* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 134(D) | -2405 | -4159 | 3349  | -651  | -4360 | -261  | -1744  | -4307 | -1947 | -4207 | -3514 | 2151  | -2936 | -1416 | -2754 | -2102 | -2471 | -3802 | -4324 | 637   | 24500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 135(I) | -2047 | -1713 | -4504 | -3983 | -1821 | -3943 | -3061  | 2461  | -3697 | 1581  | -797  | -3593 | -3873 | -3371 | -3587 | -342  | -2008 | 1904  | -2784 | -2441 | 24600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 136(D) | -2024 | -3444 | 3495  | -680  | -3658 | -2331 | -1598  | -44   | -1632 | -3675 | -2915 | 685   | -2782 | -1248 | -2505 | 478   | -2068 | -3196 | -3911 | -3093 | 24700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 137(V) | -3122 | -2888 | -5092 | -5160 | -3022 | -4180 | -4687  | -905  | -5060 | -2626 | -2570 | -4662 | -4579 | -4940 | -4923 | -4073 | -3297 | 3796  | -4414 | -4190 | 24800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 138(I) | -63   | -875  | -3230 | -2609 | 1667  | -393  | -1422  | 2613  | -2236 | -723  | -81   | -2157 | -2606 | -1885 | -2086 | -1633 | 276   | -271  | -1325 | 844   | 24900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     |        |
| 139(M) | 315   | -2345 | -4754 | -4279 | -1396 | -4001 | -3301  | -697  | -3877 | 816   | 4676  | -3879 | -3994 | -3381 | -3676 | -3242 | -2531 | -1114 | -2746 | -2629 | 25000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

CA 2831130 2018-08-03

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 140(V) | -2623 | -2122 | -5301 | -4990 | -2770 | -5102 | -5132 | 2415  | -4945 | -1532 | -1474 | -4791 | -4869 | -4890 | -5102 | -4483 | -2619 | 3208  | -4506 | -3891 | 25100% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 141(A) | 3405  | 2528  | 4529  | 4796  | 4340  | 2257  | 385   | -3901 | 4447  | 4351  | -3532 | -3057 | -3052 | -3976 | -4112 | -1843 | -1844 | -2929 | -4572 | -4519 | 25200% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 142(P) | -4853 | 4332  | -5213 | -5573 | -5553 | -4406 | -507  | -6679 | -5780 | -6281 | -6067 | -5357 | 4310  | -5648 | -5396 | -5166 | -5194 | -6092 | -4900 | -5786 | 25300% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 143(K) | -4484 | -4357 | -4380 | -3992 | -5413 | -4236 | -3307 | -5555 | 3994  | -5171 | -4707 | -3921 | -4535 | -3079 | -2169 | -4529 | -4408 | -5264 | -4403 | -4729 | 25400% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 144(G) | 2167  | -1833 | -3963 | -4199 | -4430 | 2713  | -3642 | -4236 | -4146 | -4489 | -3540 | -2785 | -2898 | -3681 | -3639 | 910   | -1682 | -2964 | -4647 | -4558 | 25500% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 145(P) | -2604 | -2948 | -4094 | -4236 | -3644 | -3269 | -375  | -3253 | -3912 | -3066 | 2095  | -3659 | 4036  | -3912 | -3822 | -2883 | -2963 | -3249 | -4027 | -3787 | 25600% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 146(G) | -4435 | -4203 | -5092 | -5492 | -5893 | 3834  | -5028 | -6627 | -5765 | -6297 | -5970 | -5141 | -4804 | -5546 | -5385 | -4727 | -4815 | -5882 | -4924 | -5849 | 25700% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 147(H) | -2569 | -3440 | -1867 | -1702 | -3820 | -2996 | 4731  | -3830 | 634   | -3639 | -2963 | -1838 | 1551  | -1305 | -748  | -2470 | -2510 | -3482 | -3434 | -2990 | 25800% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 148(I) | 194   | -1498 | -3255 | -2899 | -2240 | -2226 | 229   | -1754 | -2652 | 1634  | -1430 | -2330 | -2747 | -2399 | -2684 | 567   | 2637  | -1484 | -2683 | -2351 | 25900% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 149(V) | -3122 | -2888 | -5092 | -5160 | -3322 | -4180 | -468  | -905  | -5060 | -2626 | -2570 | -4682 | -4579 | -4940 | -4923 | -4013 | -3297 | 3759  | -4414 | -4190 | 26000% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 150(R) | -4845 | -4446 | -5107 | -4682 | -5507 | -4412 | -379  | -5946 | -2789 | -5502 | -5118 | -4521 | -4754 | -3672 | 4219  | -4389 | -4832 | -5644 | -4538 | -4993 | 26100% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 151(R) | -982  | -2395 | -777  | 1012  | -2721 | 76    | 103   | -2459 | -142  | -2413 | -1501 | -580  | -2018 | -128  | 2308  | 1224  | -56   | -2023 | -2585 | -1919 | 26200% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 152(E) | -902  | -2032 | -899  | 2078  | -2728 | -1934 | -611  | -1897 | -259  | -221  | -1156 | 520   | -2024 | 816   | -736  | -358  | 1303  | -287  | -2295 | 537   | 26300% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 153(Y) | -4820 | -3765 | -5219 | -5565 | 3303  | -5093 | -131  | -3703 | -5127 | -3017 | -3111 | -3732 | -4959 | -3668 | -4500 | -4356 | -4679 | -3867 | -566  | 4052  | 26400% |
| -      | -     | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |        |



Table Z

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 154(V) | 129   | -1901 | -989  | 821   | -2650 | -1969 | -654  | -1704 | 498   | -52   | -1037 | -703  | -2057 | 665   | -796  | 443   | 1871  | -2192 | -1626 | 26500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 155(Q) | 576   | -2335 | 344   | 1156  | -2075 | -1856 | -515  | -508  | 1502  | -2370 | -1444 | 571   | -1949 | 1878  | 419   | -764  | -822  | -1916 | -2538 | -1856  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 156(G) | -3239 | -3889 | 516   | -2361 | -5325 | 3546  | -3331 | -5629 | -3818 | -5498 | -4951 | -2619 | -3905 | -3187 | -4377 | -3211 | -3532 | -4837 | -4695 | -4826  |
| -      | -149  | -500  | 233   | 44    | -381  | 399   | 106   | -627  | 211   | -466  | -721  | 277   | 393   | 45    | 95    | 359   | 117   | -369  | -295  | -250   |
| -      | -155  | -3318 | -9181 | -2159 | -366  | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 157(G) | 763   | -2516 | -789  | 488   | -2648 | 2300  | -672  | -2562 | 596   | -2529 | -1627 | 481   | -2112 | 224   | 471   | -362  | -1024 | -2149 | -2694 | -2033  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 158(G) | -62   | -2212 | -3792 | -4133 | -4698 | 3627  | -3843 | -4650 | -4356 | -4812 | -3937 | -3058 | -3216 | -3901 | -4170 | -1874 | -2095 | -3384 | -4734 | -4766  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 159(V) | -2485 | -2030 | -5123 | -4769 | -2667 | -4797 | -4593 | 2349  | -4661 | -1545 | -1424 | -4502 | -4648 | -4554 | -4752 | -4115 | 825   | 2985  | -4159 | -3678  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 160(P) | -2541 | -3139 | -2413 | -2753 | -4726 | -2991 | -3342 | -5055 | -3527 | -5058 | -4393 | 1199  | 4031  | -3244 | -3757 | -2365 | -2911 | -4149 | -4583 | -4362  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 161(C) | 1577  | 3078  | 1357  | -656  | -2664 | -219  | 891   | -2359 | -617  | -2434 | -1576 | -891  | -2199 | -545  | -1093 | 872   | -1043 | -1946 | -2701 | -2102  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 162(L) | -2140 | -2404 | -3955 | -3997 | -2553 | -3121 | -3283 | -1687 | -3689 | 3041  | -1200 | -3360 | -3626 | -3433 | -3567 | 480   | -2414 | -1913 | -3145 | -2755  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 163(I) | -2527 | -2092 | -5072 | -4613 | 2047  | -4674 | -3911 | 2668  | -4413 | 117   | -841  | -4323 | -4439 | -4023 | -4320 | -3916 | -2488 | 2115  | -3342 | -3040  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 164(A) | 3631  | -2768 | 4492  | -4815 | -4888 | -2992 | -4271 | -4781 | -4818 | -5025 | -4365 | -3727 | -3728 | -4477 | -4545 | -2567 | -2762 | -3852 | -4724 | -4942  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 165(V) | -2623 | -2122 | -5301 | -4990 | -2710 | -5102 | -5131 | 2426  | -4946 | -1532 | -1474 | -4791 | -4869 | -4891 | -5102 | -4493 | -2619 | 3200  | -4506 | -3991  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 166(H) | -496  | -2631 | 903   | -2051 | 722   | -3242 | 3753  | -2386 | -2056 | -2342 | -1863 | -2047 | -3330 | -1815 | -2362 | -2318 | -2233 | -2212 | -981  | 3315   |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 167(Q) | -4589 | -4392 | -3927 | -4146 | -5095 | -4221 | -4099 | -5973 | -3840 | -5564 | -5304 | -4230 | -4693 | 4575  | -3826 | -4704 | -4772 | -5612 | -4677 | -4751  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *     | *      |
| 168(D) | -2873 | -4605 | 3943  | -507  | -4448 | -2633 | -2151 | -5087 | -2604 | -4922 | -4387 | 428   | -3235 | -1872 | -3575 | -2522 | -3009 | -4491 | -4932 | -3946  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |

Table Z

|        |       |       |       |       |       |       |       |       |       |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| -      | -     | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |
| 169(A) | 1776  | -1612 | -1274 | 138   | -1698 | -2092 | -816  | -1295 | 150   |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 170(S) | 1545  | -2420 | 1001  | -1518 | -449  | -2206 | -2206 | -3839 | -2264 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 171(G) | -2999 | -3451 | -2978 | -3207 | -5151 | -3559 | -3451 | -5283 | 15    |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 172(N) | 887   | -2349 | -736  | 911   | -2668 | -1860 | -513  | -2415 | 711   |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 173(A) | 3631  | -2788 | 4492  | -4815 | -488  | -2892 | -4271 | -4781 | -4818 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 174(K) | -1368 | -2597 | -1358 | -720  | -2953 | -2798 | 1833  | -2603 | 1975  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 175(D) | -27   | -2613 | 2320  | 1049  | -2623 | -1962 | 1973  | -2684 | 544   |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 176(V) | -1096 | -938  | -3279 | -2858 | -89   | -2843 | -1513 | 1265  | 1006  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 177(A) | 3342  | -1826 | 4064  | -4295 | -438  | 111   | -3651 | -4129 | -4159 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 178(L) | -4414 | -3800 | -5638 | -5629 | -2290 | -4960 | -4623 | -1886 | -5423 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 179(S) | 2216  | -1831 | -3061 | -4157 | -4409 | 666   | -3608 | -4213 | -4076 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 180(Y) | -3634 | -3050 | -4918 | -4872 | 36    | -4597 | -1405 | 223   | -4437 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 181(A) | 3391  | -1860 | -3998 | -4279 | -4411 | -2128 | -3684 | -4207 | -4197 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 182(K) | 370   | 307   | 113   | -351  | -2880 | -1925 | 615   | 324   | 1888  |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |
| 183(M) | 1728  | -1728 | -2330 | -2330 | -2330 | -2330 | -2330 | -2330 | -2330 |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |

Table Z

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 183(G) | 2572  | -2028 | -3934 | -4246 | -4575 | 2752  | -3783 | -4406 | -4316 | -4661 | -3751 | -2958 | -3070 | -3837 | -4092 | -1679 | -1898 | -3191 | -4701 | -4686 | 29900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 184(I) | -2178 | -1808 | -4630 | -4153 | -2094 | -4190 | -5417 | 3121  | -3909 | 311   | -1023 | 698   | -4099 | 3956  | -3864 | -3388 | -2148 | 1742  | -3147 | -2761 | 30000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 185(G) | -4435 | -4203 | -5092 | -5462 | -5893 | 3834  | -5028 | -6827 | -5765 | -6297 | -5970 | -5141 | -4804 | -5546 | -5385 | -4727 | -4815 | -5862 | -4924 | -5849 | 30100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 186(G) | 1392  | 2751  | -4953 | -4536 | -4308 | 2864  | -3581 | -4084 | -4233 | -4354 | -3425 | -2859 | -2890 | -3744 | -3857 | 712   | -1858 | -2914 | -4553 | -4470 | 30200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 187(G) | 656   | -1822 | -3738 | -3768 | -4188 | 2507  | -3358 | -3950 | -3667 | -4196 | -3283 | -2667 | 966   | 3302  | -3621 | -1441 | 2334  | -2867 | -4408 | -4236 | 30300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 188(F) | -3706 | -3632 | -4490 | -3846 | 1391  | -4057 | -2273 | -3795 | -1906 | -3355 | -3181 | -3458 | -4263 | -2675 | 3948  | -3768 | -3671 | -3813 | -2293 | -1328 | 30400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -155  | -8139 | -3345 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 189(A) | 2844  | -1570 | -3814 | -3873 | -4048 | -1958 | -3316 | -3787 | -3686 | -4061 | -3156 | -2598 | -2734 | -3301 | -3572 | 1098  | 1907  | -2713 | -4286 | -4136 | 30500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -7992 | -9034 | -894  | -1115 | -1303 | -750  | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 190(G) | 4176  | -3995 | -4855 | -5222 | -5696 | 3828  | -4821 | -6386 | -5533 | -6087 | -5741 | -4896 | -4606 | -5312 | -5178 | -4661 | -4560 | -5613 | -4754 | -5635 | 30600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -7992 | -9034 | -894  | -1115 | -422  | -1998 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 191(V) | -2456 | -2036 | -5138 | -4788 | -2680 | -4825 | -4631 | 2622  | -4866 | -1549 | -1431 | -4527 | -4688 | -4594 | -4783 | -4148 | 629   | 2919  | -4191 | -3706 | 30700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 192(I) | -2760 | -2307 | -5270 | -4785 | 1172  | -4884 | -3997 | 3346  | -4576 | 662   | -572  | -4539 | -4535 | 4008  | -4400 | -4134 | -2703 | 757   | -3223 | -3041 | 30800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 193(E) | 454   | -3056 | -801  | 3279  | -4251 | -2346 | -1868 | -3919 | -1868 | -3932 | -3156 | -1291 | 1208  | -1533 | -2450 | -1842 | -2067 | -3305 | -4121 | -3395 | 30900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 194(T) | -2738 | -3109 | -4509 | -4810 | -4918 | -3305 | -4346 | -4865 | -4769 | -5072 | -4551 | -3987 | -3998 | -4590 | -4545 | -2999 | 4033  | -4113 | -4584 | -4915 | 31000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 196(T) | -1323 | -1975 | -2766 | -2978 | -4307 | -2152 | -3105 | -4004 | -3295 | -4229 | -3354 | 134   | -2901 | -2998 | -3387 | -113  | 3742  | -2969 | -4405 | -4119 | 31100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 196(F) | -4110 | -3437 | -5436 | -5431 | 4276  | -5143 | -2158 | -1742 | -5074 | 563   | -1124 | -4290 | -4871 | -3987 | -4661 | -4547 | -4016 | -2374 | -1366 | -292  | 31200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 197(K) | -1111 | -2844 | -1470 | 1220  | -3294 | -2415 | -860  | -2939 | 2448  | -2798 | -1945 | -1056 | -2475 | 767   | 2054  | -1421 | -1432 | -2844 | -2858 | -2358 | 31300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

CA 2831130 2018-08-03

[illegible]

Table Z

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 212(C) | -223  | 5044  | -4840 | -4445 | -1198 | -3905 | -3598 | -31   | -4138 | 449   | -930  | -3902 | -4040 | -3778 | -4010 | -3184 | -2306 | 1347  | -3209 | -2883 | 32800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 213(G) | -4435 | -4203 | -5092 | -5462 | -5893 | 3834  | -5023 | -6627 | -5765 | -6297 | -5970 | -5141 | -4804 | -5546 | -5385 | -4727 | -4815 | -5862 | -4924 | -5849 | 32900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 214(G) | 677   | -2128 | -3838 | -4171 | -4647 | 3536  | -3816 | -4506 | -4340 | -4749 | -3857 | -3009 | -3149 | -3871 | -4137 | -784  | -2005 | -3297 | -4725 | -4735 | 33000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 215(V) | 378   | 724   | -3707 | -3104 | -1180 | -2966 | -1919 | 1210  | -2734 | 1302  | -359  | -2627 | -3014 | -2382 | -2566 | -2089 | 1123  | 1949  | -1773 | -1423 | 33100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 216(M) | 948   | -1407 | -1515 | 158   | -1452 | -2164 | 1671  | -1030 | -821  | -1302 | 1976  | -1113 | -2246 | -718  | -1173 | 773   | 1715  | 1332  | -1794 | -1343 | 33200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 217(E) | 1397  | -2528 | -725  | 2285  | -2932 | 240   | -791  | -2681 | 328   | -2645 | -1744 | -674  | -2162 | -351  | -939  | 545   | -1095 | -2227 | -2828 | -2143 | 33300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 218(L) | -3705 | -3122 | -5060 | -5527 | -1359 | -5814 | -4569 | 1065  | -5292 | 3069  | -146  | -5564 | -4963 | -4103 | -4828 | -5215 | -3571 | -1219 | -3159 | -3298 | 33400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 219(V) | -2600 | -2108 | -5251 | -4894 | -2568 | -5025 | -4783 | 2479  | -4810 | -1354 | 1358  | -4583 | -4772 | -4554 | -4895 | -352  | -2584 | 3018  | -4181 | -3758 | 33500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 220(K) | -1633 | -2905 | -1573 | 708   | -3375 | -2487 | -501  | -3003 | 2825  | -2849 | -2008 | -1128 | -2541 | 1714  | 784   | -1509 | -105  | -2617 | -2894 | -2418 | 33600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 221(A) | 2352  | 2086  | -2593 | -2000 | -347  | -2434 | -1271 | -486  | -32   | -832  | 714   | -1817 | -2498 | -1493 | -1792 | -1483 | 274   | 433   | -1419 | 501   | 33700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 222(G) | 224   | -1905 | -3562 | -3696 | -3884 | 3361  | -3291 | -3220 | -3625 | 81    | -2896 | -2733 | -2877 | -3326 | -3545 | -1606 | -1763 | -2574 | -4088 | -3810 | 33800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 223(F) | -4781 | -3756 | -5207 | -5542 | -4341 | -5070 | -1342 | -3553 | -5111 | -2971 | -3085 | -3743 | -4949 | -3874 | -4496 | -4351 | -4650 | -3835 | -591  | 1725  | 33900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 224(E) | -2413 | -4114 | 221   | 3465  | -4392 | -2485 | -1688 | -4248 | -1608 | -4112 | -3336 | -1094 | -2951 | -1336 | 871   | -2119 | -2441 | -3763 | -4239 | -3395 | 34000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 225(T) | -1461 | -1864 | -3139 | -2645 | -2655 | -2483 | -2138 | -1734 | -1645 | -2359 | -1751 | -2258 | -2936 | -1995 | 920   | -1748 | 3354  | 967   | -2989 | -2654 | 34100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1373 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 226(L) | -3831 | -3266 | -5314 | -5148 | -673  | -5058 | -2478 | -1443 | -4706 | 3059  | -789  | -4359 | -4756 | -3864 | -4314 | -4452 | -3729 | -2115 | -1672 | 1736  | 34200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 108   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

Table-Z

[illegible]

Table 2

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |         |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|
| 241(L) | -3370 | -2647 | -5795 | -5233 | -1386 | -5465 | 4298  | 708   | -5010 | 2859  | 1349  | -5155 | -4777 | -4026 | -4822 | -4755 | -3254 | 814   | -3103 | -3213 | 357000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 242(H) | -2519 | -4224 | -445  | 946   | -4287 | -2505 | 4563  | -4377 | -1764 | -4237 | -3571 | 2007  | -3009 | -1439 | -2361 | -2209 | -2569 | -3893 | -4267 | -3353 | 358000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 243(E) | -3177 | 2571  | -2701 | 3711  | -4851 | -3438 | -3479 | -4765 | -3558 | -4932 | -4406 | -3081 | -4005 | -3370 | -3802 | -3269 | -3451 | -4280 | -4554 | -4524 | 359000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 244(L) | -85   | -1333 | -3893 | -3280 | -1111 | -3185 | -2063 | 1066  | -2910 | 2310  | 1961  | -2823 | -3170 | -2501 | -2721 | -2289 | -113  | 436   | -1859 | -1568 | 360000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 245(K) | -2513 | -3173 | -2941 | -2370 | -4002 | -3094 | -1824 | -9895 | 3666  | -3734 | -5063 | -2271 | -3377 | -1447 | -618  | -2537 | 751   | -3485 | -9625 | -3471 | 361000% |
| -      | -149  | -500  | 233   | 43    | -381  | 400   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -155  | -3318 | -9191 | -196  | -2374 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 246(L) | -3571 | -3023 | -5954 | -5375 | -1321 | -5648 | -4350 | -632  | -5138 | 2962  | 1671  | -5358 | -4852 | -4044 | -4689 | -4863 | -3436 | 742   | -3082 | -3239 | 363000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 247(I) | -2980 | -2484 | -5473 | -5109 | -1958 | -5196 | -4567 | 3728  | -4915 | 267   | -781  | -4933 | -4833 | -4427 | -4799 | -4598 | -2949 | -64   | -3627 | -3397 | 364000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 248(V) | -1685 | -1688 | -4095 | -3732 | -2281 | -3082 | -2863 | -227  | -3402 | -1488 | 1383  | -3123 | -3419 | -3145 | -3320 | 367   | -1807 | 332   | -2874 | -2504 | 365000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 249(D) | -2563 | -4569 | 3654  | -1039 | -4853 | -2751 | -2187 | -4998 | 767   | -4822 | -4260 | -1424 | -3314 | -1891 | -3072 | -2624 | -3060 | -4467 | -4770 | -3962 | 366000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 250(L) | -2768 | -2715 | -4842 | -4633 | -1875 | -3598 | -3750 | -1038 | -4207 | 3056  | -562  | -4150 | -4179 | -3740 | -3989 | -3399 | 699   | -1545 | -3154 | -3067 | 367000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 251(M) | -2822 | -2366 | 5342  | -4861 | -1759 | -4985 | -4151 | 2587  | -4663 | 173   | 4005  | -4649 | -4601 | -4076 | -4487 | -4251 | -2764 | 766   | -3321 | -3210 | 368000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 252(Y) | -4652 | -3630 | -5142 | -5401 | 1516  | -4592 | -1300 | -3544 | -4958 | -2563 | -2986 | -3671 | -4868 | -3786 | -4393 | 381   | -4432 | -3662 | 2413  | 4375  | 369000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 253(E) | -1959 | -3457 | -568  | 3135  | -3841 | -2347 | -1470 | -3622 | -1165 | -3547 | -2751 | -1000 | -2716 | 1879  | -1866 | -1757 | 692   | -3162 | -3709 | -2960 | 370000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 254(G) | -347  | -2818 | -1215 | 201   | -3253 | 2635  | -921  | -2921 | 1474  | -2822 | -1972 | -1002 | -2459 | -486  | 668   | -1397 | -1436 | -2521 | -2923 | -2378 | 371000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |         |
| 255(G) | -4435 | -4203 | -5092 | -5462 | -5893 | 3834  | -5028 | -6627 | -5765 | -6297 | -5670 | -5141 | -4804 | -5546 | -5385 | -4727 | -4815 | -5862 | -4924 | -5849 | 372000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |         |

CA 2831130 2018-08-03

|   |        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|---|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 256(I) | -2042 | -1769 | 4321  | -3740 | -1316 | -3753 | -2668 | 3134  | -3389 | 1017  | 1999  | 194   | -3853 | -2968 | -3221 | -884  | -1900 | -344  | -2325 | -2073 | 37300% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 257(A) | 1914  | -1640 | -1237 | 128   | -1748 | -661  | -763  | -1355 | -577  | -49   | -817  | -905  | -2155 | -498  | -993  | 1037  | 162   | -1149 | -2002 | 624   | 37400% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 258(N) | 365   | -3809 | 1001  | 557   | -4083 | 1196  | -1518 | -3930 | -1535 | -3938 | -3055 | 3219  | -2763 | -1148 | -2243 | -1845 | -2131 | -3433 | -4027 | -3144 | 37500% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 259(M) | -3656 | -3159 | 5815  | -5350 | -1349 | -5421 | -4248 | -822  | -4928 | 948   | 4520  | -5248 | -4638 | -4039 | -4539 | -4860 | -3558 | -1517 | -3044 | -3030 | 37600% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 260(R) | -1614 | -1949 | 2260  | -1863 | -886  | -2765 | -1089 | -1596 | -1089 | 360   | -1133 | 2239  | -2814 | -1215 | 2408  | -789  | -1535 | -1458 | 1995  | 1548  | 37700% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 261(Y) | -1548 | -2973 | 568   | -505  | -2846 | -2207 | 1172  | -2986 | 441   | -2941 | -2110 | 1645  | -2445 | -659  | -1264 | -389  | -1509 | -2552 | -2524 | 3695  | 37800% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 262(S) | 279   | -1844 | -3677 | -4131 | -4448 | 136   | -3634 | -4260 | -4132 | 4511  | -3561 | -2782 | -2903 | -3648 | -3936 | 3391  | -1692 | -3009 | -4661 | -4566 | 37900% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 263(I) | -2653 | -2149 | -5311 | -4961 | -2538 | -5114 | 4905  | 2957  | -4891 | 332   | -1267 | -4770 | -4827 | -4713 | -4978 | -4465 | -2636 | 2521  | -4218 | -3821 | 38000% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 264(S) | -212  | -2711 | -1019 | -4348 | -4897 | -2893 | -4045 | -4983 | -4527 | -5102 | -4364 | -3492 | -3638 | -4203 | -4355 | 3681  | -7664 | -3502 | -4615 | -4605 | 38100% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 265(N) | -2725 | -4778 | 2906  | 990   | -4866 | -2466 | -1922 | -4885 | -3320 | -4718 | -4140 | 3045  | -3069 | -1612 | -3311 | -334  | -2821 | -4325 | -4919 | -3794 | 38200% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 266(T) | -2061 | -3396 | -656  | 903   | -671  | -2367 | -1713 | -3874 | -1685 | -3859 | -3103 | 2125  | -2850 | -1369 | -2277 | -1897 | 3157  | -3351 | -4055 | -3271 | 38300% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 267(A) | 3410  | -2035 | -3979 | -4290 | -4573 | 659   | -3768 | 4400  | -4332 | -4650 | -3754 | -2978 | -3080 | -3558 | -4099 | -1690 | -1908 | -3194 | -4697 | -4687 | 38400% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 268(E) | -218  | -3486 | -1036 | 2964  | -9535 | -2588 | -1284 | -3595 | 1878  | -3417 | -2636 | -1209 | -2827 | 1323  | -773  | -1923 | -2032 | -3199 | -3455 | -2917 | 38500% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| - | 269(Y) | -4624 | -3618 | -5100 | -5310 | 1910  | -4972 | -1289 | -3522 | 634   | -2951 | -2965 | -3649 | -4847 | -3741 | -4239 | -4199 | -4391 | -3637 | 2997  | 4211  | 38600% |
| - | -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| - | -      | -155  | -8139 | -3345 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |



Table Z

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 270(G) | -4176 | -3995 | -4855 | -5222 | -5886 | 3828  | -4823 | -6386 | -5533 | -6087 | -5741 | -4896 | -4606 | -5312 | -5178 | -4481 | -4560 | -5613 | -4754 | -5635 | 38700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -7992 | -9034 | -894  | -1115 | -472  | -1961 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 271(D) | -2710 | -4705 | 3025  | 1828  | -4880 | 1758  | -1932 | -4863 | -2320 | -4703 | -4115 | -1084 | -3073 | -1621 | -3297 | -2330 | -2809 | -4301 | -4894 | -3793 | 38800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 272(Y) | -2497 | -2175 | 4651  | -4137 | 2447  | -4046 | -2215 | 255   | -3766 | 892   | 1558  | -3537 | -3886 | -3109 | -3468 | -3181 | -2410 | -1282 | -1615 | 3508  | 38800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 273(V) | -1425 | -1250 | -3480 | -2894 | -1283 | -3035 | -1955 | 591   | -2570 | -1    | -429  | -2568 | -3060 | 942   | -2514 | -2129 | 1701  | 2519  | -1907 | -1553 | 39000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 274(T) | 516   | -1643 | -1918 | -1401 | -2170 | -2112 | -1387 | -1759 | -1234 | -2016 | -1265 | -1442 | -2421 | -1149 | 887   | 1341  | 2345  | 822   | -2454 | -2008 | 39100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 275(G) | 677   | -2128 | -3838 | -4171 | -4647 | 3536  | 3815  | -4506 | -4340 | -4749 | -3857 | -3009 | -3149 | -3871 | -4137 | -1734 | -2005 | -3297 | -4725 | -4735 | 39200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 276(P) | -992  | -2210 | 343   | -359  | -2447 | -1960 | -675  | -2143 | 533   | -2204 | -1351 | -651  | 2813  | -260  | -802  | 465   | -939  | -873  | -2467 | 1093  | 39300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 277(R) | -1214 | -2548 | -1097 | 1072  | 175   | -2145 | -716  | -2587 | 848   | -2528 | -1653 | -795  | -2228 | -273  | 2862  | 417   | -1133 | -2151 | -2671 | -2084 | 39400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 278(V) | 289   | -2035 | -5133 | -4789 | -2922 | -4777 | -4639 | 2142  | -4699 | -1561 | -1443 | -4511 | -4649 | -4595 | -4764 | -4102 | -2487 | 3125  | -4202 | -3717 | 39500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 279(I) | -2265 | -1919 | -4828 | -4452 | -2473 | -4254 | -3954 | 3155  | -4255 | -1516 | -1326 | -4066 | -4279 | -4082 | -4274 | 276   | -2288 | 2132  | -3638 | -3250 | 39600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 280(D) | -1731 | -3162 | 2329  | -550  | -3318 | -2239 | -1273 | -3221 | -1145 | -3214 | -2403 | 2295  | -2573 | -899  | -1742 | -1561 | 1851  | -2804 | -3356 | 1327  | 39700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 281(E) | 1097  | -2699 | 1227  | 2368  | -2994 | -2011 | -756  | -2753 | 381   | -2704 | -1808 | -640  | -2190 | -358  | -992  | -1055 | -1162 | -2310 | -2885 | 1152  | 39800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 282(E) | 166   | -2372 | 859   | 1835  | -2922 | -1861 | 1182  | -2444 | 490   | -2388 | -1482 | 590   | 478   | 1355  | -620  | 116   | -837  | -1944 | -2555 | -1871 | 39900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 283(T) | 228   | -1688 | -3655 | -3444 | -3179 | -2145 | -2891 | -2611 | -3215 | -3076 | -2328 | -2557 | -2821 | -2916 | -3214 | 2251  | 2366  | 1397  | -3549 | -3270 | 40000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 284(K) | -2991 | -3623 | -3848 | -2331 | -4472 | -3512 | -1358 | -3783 | 2942  | -3413 | 1860  | -2788 | -3430 | -937  | 2705  | -2874 | -2650 | -3555 | -3257 | -3189 | 40100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 105   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -365  | -294  | -249  |        |

CA 2831130 2018-08-03

[illegible]

Table Z

|        |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 299(F) | -1347 | -3577 | -4619 | 543   | 3858  | -4820 | -1320 | -3438 | -4609 | -2900 | -2894 | -3532 | -4750 | -3628 | -4209 | -4078 | -4237 | -3546 | -501  | 2917  | 41600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 300(A) | 2827  | -1603 | -4068 | -3628 | -2047 | -3165 | -2821 | 1205  | -3349 | -1486 | -1089 | -3103 | -3432 | -3073 | -3299 | -2387 | 197   | 1100  | -2812 | -2447 | 41700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 301(K) | -2364 | -3353 | -2464 | 153   | -4038 | -3041 | -1188 | -3501 | 2928  | -3234 | -2487 | -1693 | -3047 | -758  | 2488  | 458   | -2152 | -3194 | -3159 | -2895 | 41800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 302(M) | -893  | -2351 | 740   | 1780  | -2680 | -537  | -524  | -2429 | 930   | -2378 | 1896  | -498  | -1968 | -66   | 722   | 775   | -833  | -1982 | -2545 | -1864 | 41900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 303(W) | -2965 | -2553 | -4795 | -4482 | 3045  | -4315 | -1773 | -1438 | -4098 | 92    | -987  | -3564 | -4179 | -3330 | -3743 | -3476 | -2878 | 604   | 4754  | -64   | 42000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 304(I) | -2621 | -2136 | -5246 | -4859 | -2454 | -4987 | -4571 | 3052  | -4747 | -250  | 1043  | -4641 | -4713 | -4488 | -4769 | -4301 | -2597 | 2384  | -3934 | -3595 | 42100% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 305(L) | 684   | -1319 | -1741 | 761   | -1375 | -2223 | -1037 | -927  | -1042 | 1693  | -533  | -1287 | -2325 | -915  | -1349 | 767   | -152  | 84    | -1756 | -1329 | 42200% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 306(E) | 364   | -4165 | 621   | 3314  | -4393 | -2398 | -1688 | -4282 | -1836 | -4169 | -3446 | 728   | -2893 | -1339 | -2628 | -2045 | -2402 | -3767 | -4362 | -3401 | 42300% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 307(N) | 939   | 979   | -1235 | -681  | -1738 | 1352  | 933   | -1357 | -549  | -1572 | -816  | 2186  | -2155 | -482  | 165   | -1022 | -880  | 30    | -1950 | 1446  | 42400% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 308(Q) | 667   | -2393 | -773  | 511   | -2721 | -316  | -544  | -2455 | 1211  | -2405 | -1434 | 584   | -1989 | 2135  | 1528  | 812   | -868  | -2019 | -2566 | -1895 | 42500% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 309(A) | 2050  | -1857 | -1081 | -526  | -2073 | -2012 | 188   | -1645 | -385  | -1213 | -1011 | 486   | -2103 | -346  | 1857  | -953  | 306   | -261  | -2169 | -1624 | 42600% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 310(G) | -1846 | -2469 | -2089 | -2292 | 182   | 2844  | -2358 | -3563 | -2904 | -3628 | -3017 | 2347  | -3167 | -2550 | -3185 | -986  | -2162 | -3065 | -2814 | -1874 | 42700% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 311(Y) | 475   | 1019  | -1606 | -1042 | 225   | -2192 | -935  | -946  | -891  | -1226 | 1222  | 357   | -2267 | 1002  | 1677  | -1172 | -888  | -800  | -1730 | 2446  | 42800% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 312(P) | -87   | -2372 | -1362 | 756   | -378  | -2205 | -2007 | -3445 | -1924 | -3575 | -2761 | -1555 | 3596  | -1697 | -2362 | -1666 | 286   | -2803 | -3832 | -3277 | 42900% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |
| -      | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378 | *     | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 313(K) | -804  | -1483 | -564  | -230  | -1920 | -1335 | -101  | -1606 | 2869  | -1630 | -1071 | -349  | -1569 | 232   | 698   | -796  | -759  | -1355 | -1637 | -1317 | 43000% |
| -      | -149  | -500  | 233   | 43    | -381  | 399   | 106   | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -389  | -294  | -249  |        |

Table Z

|          |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |        |
|----------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| -        | -38   | -5840 | -6882 | -894  | -1115 | -3098 | -179*  | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 31314(E) | -766  | -1695 | 521   | 2831  | -2050 | -1029 | -293   | -1804 | -118  | -1919 | -1331 | 69    | -1441 | -4    | -527  | -853  | -814  | -1512 | -1988 | 43100% |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -38   | -5840 | -6882 | -894  | -1115 | -109  | -377*  | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 315(T)   | -942  | -2382 | -739  | 1086  | -2714 | 151   | -581   | -2459 | -171  | -2415 | -1499 | 414   | -2004 | -128  | 839   | 1365  | 1730  | -2017 | -2592 | -1915  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 316(M)   | -2196 | -1920 | -4459 | -3691 | 1726  | -3822 | -2504  | -645  | -3523 | 1973  | 3030  | -3442 | -3673 | -2938 | -3257 | -2944 | -2114 | 325   | -2014 | 1662   |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 317(H)   | -883  | -2314 | -747  | 517   | -2618 | -1863 | 1714   | -647  | 1272  | -2322 | -1408 | 1011  | 472   | -59   | 433   | -772  | 1411  | -299  | -2507 | -1836  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 318(A)   | 2474  | -2397 | -916  | -367  | -7397 | -273  | -722   | -2529 | 555   | -2507 | -1610 | 592   | -2110 | 837   | -405  | -138  | -1006 | -2082 | -2899 | -2039  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 319(M)   | -154  | -986  | -2485 | -337  | 1024  | -375  | -1233  | 325   | -444  | 867   | 1235  | -1752 | -2474 | -1419 | 1020  | -1456 | 670   | -411  | 831   | 535    |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 320(R)   | -1311 | -2432 | -1349 | -724  | -2724 | -2272 | -794   | -2361 | 513   | 644   | 1079  | 976   | 597   | -382  | 2908  | -1246 | -1219 | -2044 | -2579 | -2061  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 321(R)   | 897   | -2364 | -833  | 905   | -2678 | -1930 | -568   | -2405 | 1293  | -2366 | 1485  | -575  | -2020 | -117  | 2045  | 95    | -893  | -1984 | -2543 | -1892  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 322(N)   | 505   | -2300 | -750  | 523   | -2598 | 121   | -525   | -594  | 485   | 95    | -1395 | 1720  | -1957 | 348   | 224   | 551   | -821  | -1910 | -2497 | -1828  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 323(E)   | 444   | -2266 | -766  | 1488  | -2651 | -1871 | -533   | -2276 | 589   | -2266 | 1474  | 1478  | -1903 | 692   | -629  | -781  | 12    | -279  | -2472 | 475    |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 324(N)   | 1511  | -1770 | -728  | -204  | -2244 | -1781 | -426   | 282   | 601   | -2121 | -1133 | 1769  | -1827 | 1398  | 447   | -748  | -766  | -1713 | -1779 | -1595  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 325(N)   | 1053  | -3109 | 1756  | 1735  | -3424 | -2143 | -1074  | -3191 | -946  | -3124 | 2254  | 2158  | -2417 | -657  | -1430 | -1361 | 197   | -2727 | -3303 | -2540  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 326(H)   | -2064 | -3071 | -1245 | -1267 | -3352 | -2570 | 471    | -3611 | -1060 | -3528 | -2812 | -1479 | -2961 | 1286  | -1287 | 670   | -2133 | -3161 | -3310 | -2601  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |
| 327(Q)   | -891  | -2294 | 332   | 948   | -2585 | -739  | -537   | -2316 | -131  | -138  | -1391 | -518  | 1404  | 2021  | 634   | -785  | 619   | -561  | -2495 | -1830  |
| -        | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249   |
| -        | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |        |

Table Z

|         |       |       |       |       |       |       |        |       |       |       |       |       |       |       |       |       |       |       |       |       |        |
|---------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| 3328(I) | -1632 | -1661 | -2846 | -86   | -1626 | -2983 | -1663  | 3240  | -1327 | -191  | -722  | -2160 | -3016 | -1615 | 822   | -2097 | -1566 | -560  | -2157 | -1805 | 44500% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3329(E) | -2734 | -3605 | -1382 | 3593  | -3624 | -2966 | -2317  | -3317 | -2175 | -3167 | 1983  | -1898 | -3440 | -2054 | -2556 | -2649 | -2920 | -3234 | -3920 | -3370 | 44600% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -2336 | -8139 | -325  | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3330(W) | -1530 | -1265 | -2068 | -1964 | 479   | -1810 | -483   | -1181 | -1470 | -988  | -802  | -1648 | -2104 | -1454 | -1405 | -1157 | -1583 | -1218 | 5462  | 838   | 44700% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -38   | -5840 | -6882 | -894  | -1115 | -109  | -3775* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3331(K) | 8     | -2067 | -905  | 437   | -2275 | -1941 | -611   | -307  | 2031  | -2031 | -1189 | -636  | -2030 | 1425  | -709  | -864  | -350  | 1337  | -2323 | -1722 | 44800% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3332(V) | -2445 | -2012 | -5067 | -4708 | -2628 | -4682 | -4456  | 1596  | -4690 | -1533 | -1402 | -4414 | -4675 | -4156 | -4653 | -3600 | 1117  | 3227  | -4065 | -3597 | 44900% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3333(G) | -4435 | -4203 | -5092 | -5462 | -5693 | 3834  | -5028  | -6627 | -5765 | -6297 | -5970 | -5141 | -4804 | -5546 | -5385 | -4727 | -4815 | -5862 | -4924 | -5849 | 45000% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3334(E) | 1477  | -2744 | -762  | 2410  | -3114 | -2113 | -884   | -2850 | -445  | -2792 | -1912 | 619   | -2297 | -451  | 1346  | -1191 | -1280 | -2412 | -2952 | -2288 | 45100% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3335(K) | -1204 | -2643 | 366   | 1309  | -2998 | -2086 | -724   | -2722 | 2626  | -2637 | -1741 | -718  | -2200 | 1198  | 862   | -1073 | -1133 | -2287 | -2710 | -2133 | 45200% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3336(L) | -3571 | -3023 | -5954 | -5375 | -1321 | -5646 | -4390  | -632  | -5138 | 2962  | 1671  | -5358 | -4852 | 4044  | -4689 | -4963 | -3436 | 742   | -3082 | -3239 | 45300% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 3337(R) | -4845 | -4446 | -5107 | -4682 | -5537 | -4412 | -3791  | -5946 | -2789 | -5502 | -5118 | -4521 | -4754 | -3572 | 4219  | -4869 | -4832 | -5644 | -4538 | -4993 | 45400% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 338(E)  | 943   | -2422 | 1002  | 1200  | -2741 | 377   | -572   | -2493 | 982   | -2439 | -1517 | -522  | -1998 | -117  | -681  | 1129  | -534  | -2044 | -2609 | -1921 | 45500% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 339(M)  | -3391 | -2886 | -5774 | -5202 | -1338 | -5407 | -4210  | -576  | -4943 | 1721  | 4369  | -5109 | -4742 | 3963  | -4548 | -4669 | -3273 | 771   | -3038 | -3137 | 45600% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 340(M)  | -1812 | -1501 | -3997 | -3375 | 696   | -3401 | -2222  | -516  | -2983 | 441   | 4360  | -2591 | -3334 | 2559  | 641   | -2503 | -1740 | 404   | -1934 | -1561 | 45700% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -8    | -8139 | -9181 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 341(P)  | 102   | -1769 | -1729 | -1313 | -428  | -2112 | -1410  | -2021 | -1236 | -2235 | -1470 | -1394 | 3205  | 695   | -1607 | 703   | -1207 | -1700 | -2613 | -2121 | 45800% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |
| -       | -259  | -8139 | -2637 | -894  | -1115 | -701  | -1378* | *     |       |       |       |       |       |       |       |       |       |       |       |       |        |
| 342(W)  | -3486 | -3022 | -4312 | -4121 | 1891  | -4228 | -1173  | -2749 | 310   | -2389 | -2250 | -3147 | -4206 | -2971 | -3005 | -3484 | -3396 | -2818 | 5644  | 611   | 45900% |
| -       | -149  | -500  | 233   | 43    | -381  | 399   | 106    | -626  | 210   | -466  | -720  | 275   | 394   | 45    | 96    | 359   | 117   | -369  | -294  | -249  |        |

CA 2831130 2018-08-03

270

## CLAIMS

### What is claimed is:

1. A recombinant host cell comprising an engineered isobutanol production pathway and at least one of
  - i. a heterologous polypeptide with ketol-acid reductoisomerase (KARI) activity selected from the group consisting of:
    1. a polypeptide having at least 90% identity to a KARI enzyme derived and expressed from *Anaerostipes caccae*, or an active fragment thereof; and
    2. a polypeptide having at least 90% identity or at least 95% identity to SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65, wherein said polypeptide has a  $K_M$  for NADH less than that of SEQ ID NO: 27;
  - or
  - ii. a heterologous polynucleotide encoding the heterologous polypeptide with KARI activity of i),wherein the engineered isobutanol production pathway comprises the following substrate to product conversions:
  - a. pyruvate to acetolactate
  - b. acetolactate to 2,3-dihydroxyisovalerate
  - c. 2,3-dihydroxyisovalerate to 2-ketoisovalerate
  - d. 2-ketoisovalerate to isobutyraldehyde; and
  - e. isobutyraldehyde to isobutanol.
2. The recombinant host cell of claim 1, wherein the polypeptide is SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, or 65.
3. The recombinant host cell of claim 1, wherein the heterologous polypeptide with KARI activity comprises substitutions in amino acids corresponding to S56 and S58 of SEQ ID NO: 27.
4. The recombinant host cell of claim 3, wherein the heterologous polypeptide with KARI activity further comprises a substitution of one or more of the amino acids corresponding to I86, N87, T131, or T191 of SEQ ID NO: 27.

5. The recombinant host cell of claim 1, wherein the heterologous polypeptide with KARI activity comprises an amino acid substitution at one or more of the positions corresponding to amino acids A41, S56, S58, N87, T131, T191, R227, or Q246 of SEQ ID NO: 27.
6. The recombinant host cell of claim 1 comprising a heterologous polynucleotide encoding a polypeptide having KARI activity comprising SEQ ID NO: 33 or 35 or an active fragment thereof.
7. The recombinant host cell of any one of claims 1-6, wherein the heterologous polypeptide with KARI activity has a  $K_M$  for NADH less than 100, less than 50, or less than 10  $\mu$ M at pH 6.8.
8. The recombinant host cell of any one of claims 1-7 further comprising at least one host cell modification that enhances performance of the engineered isobutanol production pathway, wherein the host cell modification comprises reduced or eliminated aldehyde dehydrogenase expression or activity and/or reduced or eliminated acetolactate reductase expression or activity.
9. The recombinant host cell of claim 8, wherein the host cell modification comprising reduced or eliminated aldehyde dehydrogenase expression or activity comprises at least one of a deletion, mutation, or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde dehydrogenase activity.
10. The recombinant host cell of any one of claims 1-9, wherein said host cell comprises at least one or more of a deletion, mutation, or substitution in an endogenous polynucleotide encoding a polypeptide having aldehyde oxidase activity wherein said deletion, mutation, or substitution reduces or eliminates aldehyde oxidase expression or activity compared to a host cell without said deletion, mutation, or substitution.
11. The recombinant host cell of any one of claims 1-10, wherein said host cell comprises at least one or more deletion, mutation, or substitution in an endogenous polynucleotide encoding a polypeptide having acetolactate reductase activity wherein said deletion, mutation, or substitution reduces or eliminates acetolactate reductase expression or activity compared to a host cell without said deletion, mutation, or substitution.
12. The recombinant host cell of claim 9, wherein said recombinant host cell is *Saccharomyces cerevisiae* and said polypeptide having aldehyde dehydrogenase activity is ALD2, ALD3, ALD4, ALD5, or ALD6.



13. The recombinant host cell of claim 9, wherein said recombinant host cell is *Kluyveromyces lactis* and said polypeptide having aldehyde dehydrogenase activity is SEQ ID NO: 743, SEQ ID NO: 745, SEQ ID NO: 747, or SEQ ID NO: 749.
14. The recombinant host cell of claim 9, wherein said recombinant host cell is *Pichia stipitis* and said polypeptide having aldehyde dehydrogenase activity is SEQ ID NO: 751, SEQ ID NO: 753, SEQ ID NO: 755, SEQ ID NO: 757, or SEQ ID NO: 759.
15. The recombinant host cell of claim 9, wherein said recombinant host cell is *Lactobacillus plantarum* and said polypeptide having aldehyde dehydrogenase activity is AldH.
16. The recombinant host cell of claim 9, wherein said recombinant host cell is *E. coli* and said polypeptide having aldehyde dehydrogenase activity is SEQ ID NO: 761, SEQ ID NO: 763, or SEQ ID NO: 765.
17. The recombinant host cell of claim 10, wherein said polypeptide having aldehyde oxidase activity is AOX1, AOX2, or a combination thereof.
18. The recombinant host cell of claim 11, wherein said recombinant host cell is *Saccharomyces cerevisiae* and the endogenous polynucleotide is selected from the group consisting of SEQ ID NO: 676, SEQ ID NO: 678, SEQ ID NO: 680, SEQ ID NO: 682, SEQ ID NO: 684, SEQ ID NO: 686, SEQ ID NO: 688, SEQ ID NO: 690, SEQ ID NO: 692, SEQ ID NO: 694, SEQ ID NO: 696, SEQ ID NO: 702, SEQ ID NO: 704, SEQ ID NO: 706, SEQ ID NO: 708, SEQ ID NO: 710, SEQ ID NO: 712, SEQ ID NO: 714, SEQ ID NO: 716, SEQ ID NO: 718, SEQ ID NO: 720, SEQ ID NO: 722, SEQ ID NO: 724, SEQ ID NO: 726, SEQ ID NO: 728, and SEQ ID NO: 730.
19. The recombinant host cell of claim 18, wherein the polypeptide having acetolactate reductase activity is SEQ ID NO: 677 (YMR226C).
20. The recombinant host cell of any one of claims 1-11, wherein the host cell is a yeast host cell.
21. The recombinant host cell of claim 20, wherein the yeast is *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Kluyveromyces*, *Yarrowia*, *Issatchenkia*, or *Pichia*.
22. The recombinant host cell of any one of claims 1-11, wherein the host cell is *Saccharomyces cerevisiae*.

23. The recombinant host cell of any one of claims 1-22, wherein each substrate to product conversions is catalyzed by an enzyme that is recombinantly expressed by the host cell.
24. The recombinant host cell of claim 23, wherein all of the substrate to product conversions are catalyzed by enzymes heterologous to the host cell.
25. The recombinant host cell of claim 24, wherein at least one heterologous polynucleotide encoding at least one of the enzymes heterologous to the host cell is chromosomally integrated into the host cell.
26. The recombinant host cell of any one of claims 23-25, wherein the substrate to product conversions are catalyzed by enzymes substantially localized to the cytosol.
27. The recombinant host cell of any one of claims 23-26, wherein the substrate to product conversion for isobutyraldehyde to isobutanol is catalyzed by an alcohol dehydrogenase enzyme which utilizes NADH as a cofactor.
28. The recombinant host cell of any one of claims 1-27, wherein said host cell further comprises reduced, disrupted or eliminated ability to convert acetolactate to 2,3-dihydroxy-2-methylbutyrate.
29. The recombinant host cell of any one of claims 1-28, wherein the host cell has reduced, disrupted or eliminated pyruvate decarboxylase expression or activity.
30. The recombinant host cell of claim 29, wherein the host cell has reduced, disrupted, or eliminated PDC1, PDC5, or PDC6 activity or a combination thereof.
31. The recombinant host cell of any one of claims 1-30, wherein the host cell has reduced or eliminated NAD-dependent glycerol-3-phosphate dehydrogenase expression or activity.
32. The recombinant host cell of claim 31, wherein the host cell has reduced GPD2 expression or activity.
33. The recombinant host cell of any one of claims 1-32, wherein the host cell has reduced or eliminated FRA2 expression or activity.
34. The recombinant host cell of any one of claims 1-33, wherein the host cell produces isobutanol under anaerobic conditions and wherein the molar ratio of isobutanol to glycerol is greater than 1.
35. The recombinant host cell of any one of claims 1-34, wherein the polypeptide having KARI activity matches the profile HMM provided in Table Z with a profile HMM E value of  $<10^{-3}$ .

36. The recombinant host cell of any one of claims 1-35, wherein said host cell produces isobutanol at a yield greater than 25%, 50%, 75%, or 90% of theoretical yield.
37. The recombinant host cell of any one of claims 1-36, wherein the host cell has an effective isobutanol productivity of at least 3, at least 4, or at least 5 grams per gram of cells after 48 hours, wherein the last 24 of the 48 hours are under anaerobic conditions.
38. The recombinant host cell of any one of claims 1-37, wherein isobutanol is produced and wherein ethanol is also produced.
39. A method for producing isobutanol comprising:
  - a) providing the recombinant host cell of any one of claims 1-38, and
  - b) contacting the host cell of a) with a carbon substrate under conditions whereby isobutanol is produced.
40. The method of claim 39, wherein at least a portion of the contacting occurs under anaerobic conditions.
41. The method of claim 39 or 40, wherein the contacting occurs in the presence of an extractant.
42. The method of claim 41, wherein the contacting occurs in the presence of a sufficient quantity of organic extractant to form a two-phase system comprising an aqueous phase and an organic phase.
43. A fermentative composition comprising the host cell and isobutanol produced according to the method of any one of claims 39-42.
44. A composition comprising isobutanol and a recombinant host cell of any one of claims 1-38.

1/25

Figure 1

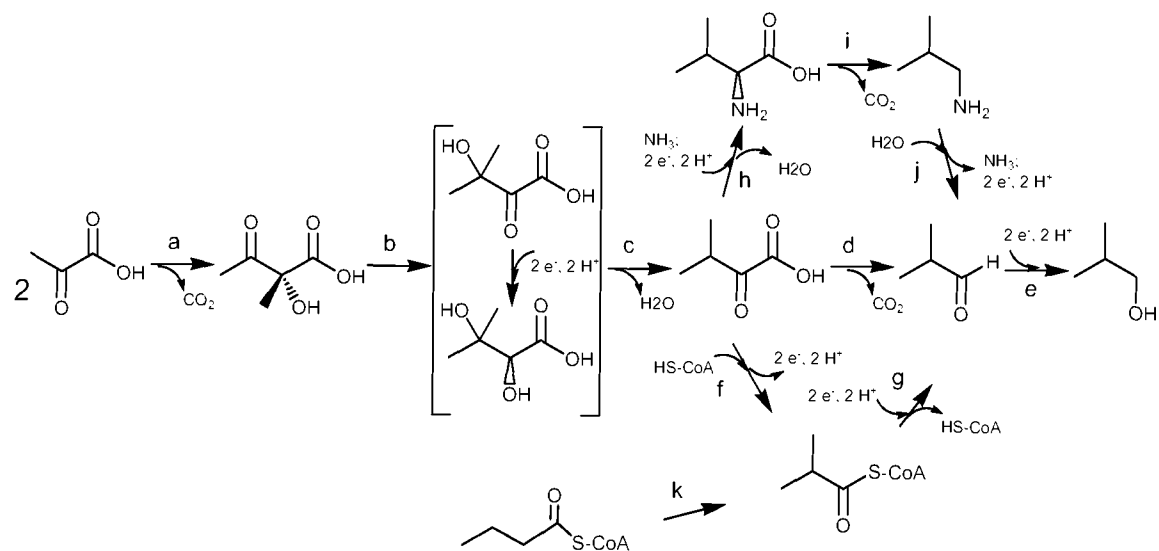


FIGURE 2.

|    |     |       |                                                                                   |
|----|-----|-------|-----------------------------------------------------------------------------------|
| 5  | K9  | (1)   | MEECKMAKIYYQEDCNLSLLDGKTI <del>AVIGY</del> <b>Y</b> GSQGHAAH <b>L</b> NAKESGCNVII |
|    | PF5 | (1)   | -----MKVFYDKDCDLSIIQGGKVAIT <b>Y</b> GSQGHAA <b>C</b> NLKDSDVDVTV                 |
| 10 | K9  | (51)  | GLYEGSKSWKRAEEQ <b>F</b> EVYTAEEAAAKADIIMIL <b>I</b> NDEKQATMYKNDIE               |
|    | PF5 | (45)  | GLRKGSAIVAKAEAH <b>GL</b> KVTDVAAAAGADLVMI <b>L</b> T <b>P</b> DEFQSQLYKNEIE      |
| 15 | K9  | (101) | PNLEAGNMLMFAHGFIHFGCI <del>VPPKDV</del> DTMIAPKPGHTVRSEYEEGK                      |
|    | PF5 | (95)  | PNIKKGATLAFSHGFATHYNQVVPRA <del>LD</del> LVIMIAPKAPGHTVRSEFVKGG                   |
| 20 | K9  | (151) | GVPCLVAVEQD <b>A</b> TGKALDMALAYAL <b>A</b> IGGARAGVLETTERTETETDLFGE              |
|    | PF5 | (145) | GIPDLIAIYQD <b>A</b> SGNAKNVALSYAA <b>G</b> VGGGRTGIETTFKDETETDLFGE               |
|    | K9  | (201) | QAVLCGGVCALMQAGFETLVEAGYDPRNAYFECIHEMKLIVDLIYQSGFS                                |
|    | PF5 | (195) | QAVLCGGTVELVKAGFETLVEAGYAPEMAYFECLELKLIVDLMYEGGIA                                 |
|    | K9  | (251) | GMRYISNTAEYGDYITGPKIITEDTKAMKKILSDIQDGTFAKDFLVDM                                  |
|    | PF5 | (245) | NMYSISNNAEYGEYVTGPPEVINAESRQAMRNALKRIQDGEYAKMFISEG                                |
|    | K9  | (301) | SDAGSQVHFKA <del>MR</del> KLASEHPAEVVGEEIRSLYSWDEDKLINN---                        |
|    | PF5 | (295) | --ATGYP <del>S</del> MTAKRRNNAAHGIEIIGEQLRSMMPWIGANKIVDKAKN                       |

3/25

FIGURE 3A.

|           |       |                           |                                   |                                   |
|-----------|-------|---------------------------|-----------------------------------|-----------------------------------|
| K9        | (1)   | MEECKMARLYYQEDCNLSLLDGKTI | AVIGYGSQGHAAHNA <b>A</b> KESG-CNV | I                                 |
| K1        | (1)   | ---WAATWYEKDA             | DLVSFEGKRVAVIGYGSQGHAAHNLNRD      | SG-VDVV                           |
| K2        | (1)   | ---MAAQIWIYEND            | DLVLDGKVKVAIGYGSQGHAAHNLNRD       | SG-VDVV                           |
| 5         | (1)   | ---MFXMYYEKDT             | DLNLRGKKVAIGYGSQGHAAHNLNRD        | SG-VDVV                           |
| S2        | (1)   | -QEELMKVYYDS              | DADGLIKSKKIALIGYGSQGHAAQNLRD      | SGVAEVA                           |
| K25       | (1)   | ---MVKVYYNEVT             | N--LGDKTIALIGYGSQGHAAQNLRD        | TG-HNV                            |
| K26       | (1)   | ---MAVQMEYEK              | VKVPALDGKTI                       | AVIGYGSQGHAAQNLRD                 |
| L. Lactis | (1)   | ---MAVTMYED               | DEVSALAGKQIAVIGYGSQGHAAQNLRD      | SG-HNVI                           |
| 10        | (50)  | IGLYEGSKSWKRAEE           | QGFVYTAAEA                        | AKKADIIMILIN <b>D</b> EKQATMYKNDI |
| K1        | (46)  | VGLRPTSKSVEYAKE           | QGLEVQSVADATAEAD                  | VVMILLPDQYQAAVYKSEI               |
| K2        | (46)  | VGLRPTSKSVEQAKE           | QGLEVKSVP                         | EAADIIIMILAPDQYQRTIWANDI          |
| K7        | (45)  | VGLYNGSKSWAKAE            | AAAGLQVATVADAAK                   | ADLIMILLPDEKQAKIYNEEI             |
| 15        | (50)  | IALRPDSASVKKAQ            | DAGFKVLTNAEAAK                    | WADLIMILAPDEHQAAIYAEDL            |
| K25       | (43)  | IGIRAG-KSAENAK            | NDGFTVLPVSEAV                     | QQANVVMILAPDEIQGELYEKEI           |
| K26       | (46)  | IGVRPG-KSFDKAK            | EDGFTYTVAEATK                     | LA                                |
| L. Lactis | (46)  | IGVRHG-KSFDKAK            | EDGFTTEVGEAVAK                    | ADVIMVLAPDEIQQSIYEEDI             |
| 20        | (100) | EPNLEAGNMLMFAH            | GNIFHFGCIVPPK                     | DVDV <b>T</b> MIAPKPGHTVRSEYEEG   |
| K1        | (96)  | EPNLKPGAALAF              | AHGNFIHYGIKPS                     | EDHPVFMVAPKPGGHI                  |
| K2        | (96)  | EPNIKPGAAIAFA             | HGNFIHYGIKPS                      | EDHPVFMVAPKPGGHI                  |
| K7        | (95)  | APNLEEGNALVFA             | HGNIFHGQVPPSV                     | DVFMVAPKPGGHLVRRTYTEG             |
| S2        | (100) | KDNLRPGSALAF              | AHGLNIFHGLIE                      | PKRIDVFMIA                        |
| K25       | (92)  | APNLSAGDALAF              | AHGNIFHFEVIT                      | PPKEVDVFLVAPKPGGHLVRRTFSEG        |
| K26       | (95)  | APNLEAGNAVGF              | AHGNIFHFEFIK                      | VPADVDFMCA                        |
| L. Lactis | (95)  | KPNLKAGSALGF              | AHGNIFHFGYIK                      | VPEDVDVFMVAPKAPGHLVRRTYTEG        |
| 30        | (150) | KGVPCLVAVEQDA             | TGKALD                            | MALAYALAI                         |
| K1        | (146) | RGVPVWVAVEQD              | PDGKTWPLCLAY                      | AKALGALRAGAIKTTFTTEETD            |
| K2        | (146) | RGVPVWVAVEQD              | PRGDWDITLAY                       | AKALGALRAGAIKTTFKKEETD            |
| K7        | (145) | AGVPCLIAVHQDA             | TGKAKQYALAY                       | ANGIGGARAGVLETTFKDETE             |

4/25

FIGURE 3B.

S2 (150) GGVPCLVAVDQDASGNAHDIALAYASGIGGGSGVETTFREEVEETDLFG  
 K25 (142) FAVPALFAVYQDATGNAQETALSAYAKGIGATRGVLETTTFKEETETDLFG  
 K26 (145) FGVPALYAVYQDATGNAKDIAWDCKGIGAAARVGLLETTYKEETEEDLFG  
 5 L. Lactis (145) FGTPALFVSHQNASGHARELIAMDWAKGIGCARVGIIETTFKEETEEDLFG  
  
 K9 (200) EQAVLCGGVCALMQAGFETIVEAGYDPRNAYFECIHEMKLIIVDLIYQSGF  
 K1 (196) EQDVLMGGINHLCDMGFDVLTAEAGYQPEIAYFEVFFHELKMLVDLANEGGL  
 K2 (196) EQNVLMGGVKNLIVEMGFELTDAGYQPEIAYFEVCHLKMVLVDLMNEGGL  
 K7 (195) EQAVLCGGVSELIKAGFETIVEAGYAPENAYFECMHEMKLIIVDLIYQGGGL  
 S2 (200) EQAVLCGGLTALITAGFETITEAGYAPENAYFECMHEMKLIIVDLIYEAGI  
 K25 (192) EQAVLCGGLTSMIETGFTETIVEAGYQPEIAYFEVCHLKMVLVDLIYEGGF  
 K26 (195) EQAVLCGGLTALITETGFTETIVEAGYAPENAYFEVCHLKMVLVDLIYEGGF  
 10 L. Lactis (195) EQAVLCGGLTALVEAGFETITEAGYAGELAYFEVCHLKMVLVDLIYEGGF  
  
 K9 (250) SGMRYISINTAEYGDYITGPKIITEDTKAMKKIILSDIQDGTFAKDFLVD  
 K1 (246) NKARWSCSDTAQYGDYTS--TVITEETKRMQYQLKRIQDGSFAKEFMDD  
 K2 (246) NKARWSCSDTAQYGDYTN--TVINEDCRKRMQYHLGRIQDGSFAKEFIDD  
 K7 (245) SMMRYISINTAEYGDYIGRRIITDETKEMKKVLTETIQDGTFAKNWLE  
 S2 (250) ANMRYISINTAEYGDIVSGPRVINEESKAMKAILDDIQSGRFVSKFVLD  
 K25 (242) AKMRDSISINTAEYGDYVSGSRIITEQTKANMKNVLDIQNGTFAKGFAD  
 K26 (245) KMRQSIINTAEFGDYVSGPRVITEQVKENMKAVLADIQNGKFAFDFVND  
 20 L. Lactis (245) TKMRQSIINTAEFGDYVTGPRIITDEVKKNMKLVLIADIQSGKFAQDFVDD  
  
 K9 (300) MSDAGSQVHFKAAMKRLASEHPAEVVGEEIRSLYSWSEDKLINN-----  
 K1 (294) QAAG--APKFKKLQEEYSHPHLETVPKLRAMFSWNNQVDADADMAESFN  
 K2 (294) QDAG--APKFKELQEEYGNVRIETVPKLRAMFSWNNQDQDDADMAT-FT  
 K7 (295) NQTN--RPGFNARRRMEAEHPIEKVGKELRGMSWI DTAKVD-----  
 S2 (300) NRAAG--QPELKAARKRMAAHPTEQVGARLRKMPMPWIASNKLVDKARN-KL  
 K25 (292) NQAG--FPEFKKMRQNGNHPIEKVGSELKRMMPFVTKD-----  
 K26 (295) YKAG--RPKLTAYREAAANLEIEKVGAE LRKAMPFVGQNDDDDAFKIYN--  
 30 L. Lactis (295) FRAG--RPKLIAYREAAKNLEIEKIGAE LRQAMPFTQSGDDDAFKIYQ--

5/25

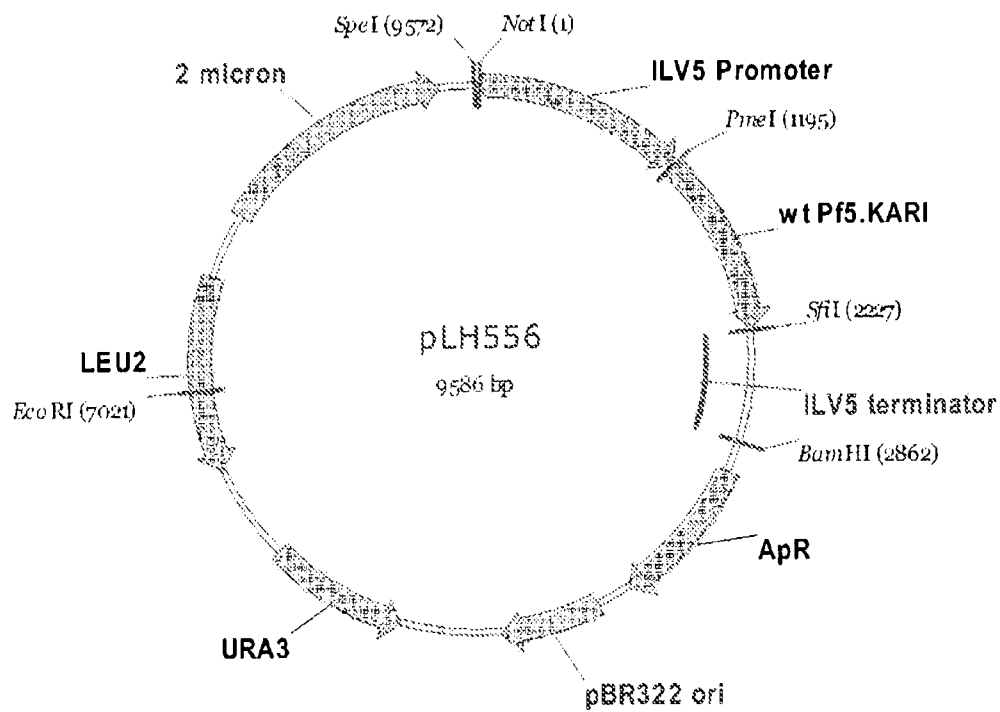
FIGURE 3C.

|    |           |       |           |
|----|-----------|-------|-----------|
|    | K9        | (344) | -----     |
|    | K1        | (342) | GKIARTQVQ |
| 5  | K2        | (341) | GKIARSQVQ |
|    | K7        | (335) | -----     |
|    | S2        | (347) | GCF-----  |
|    | K25       | (329) | -----     |
|    | K26       | (341) | -----     |
| 10 | L. Lactis | (341) | -----     |



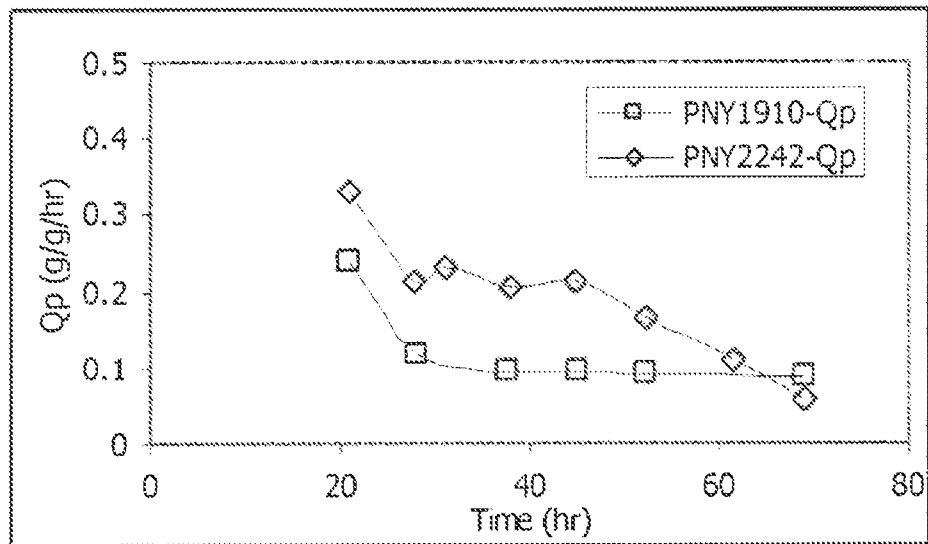
6/25

FIGURE4. (pLH556)



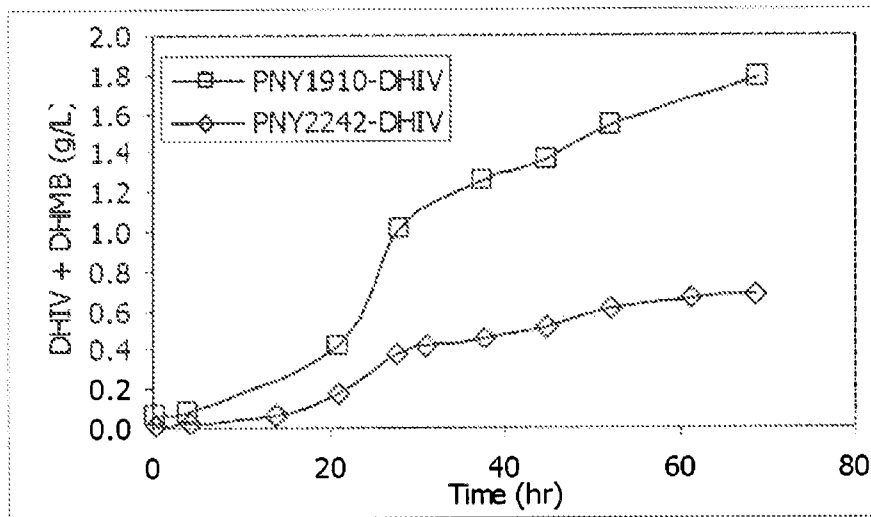
7/25

FIGURE 5



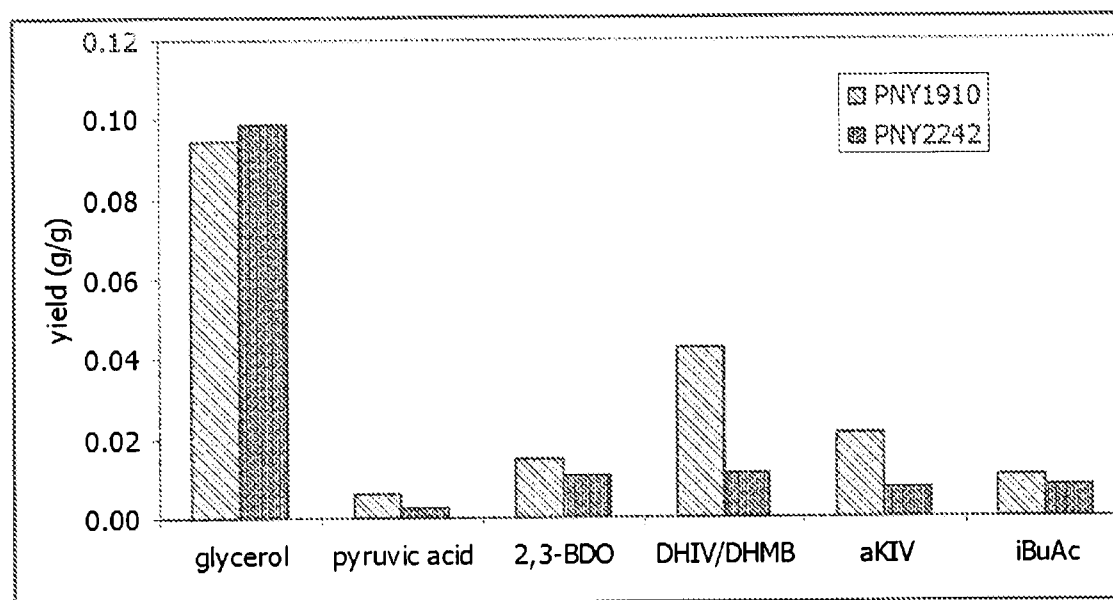
8/25

FIGURE 6.



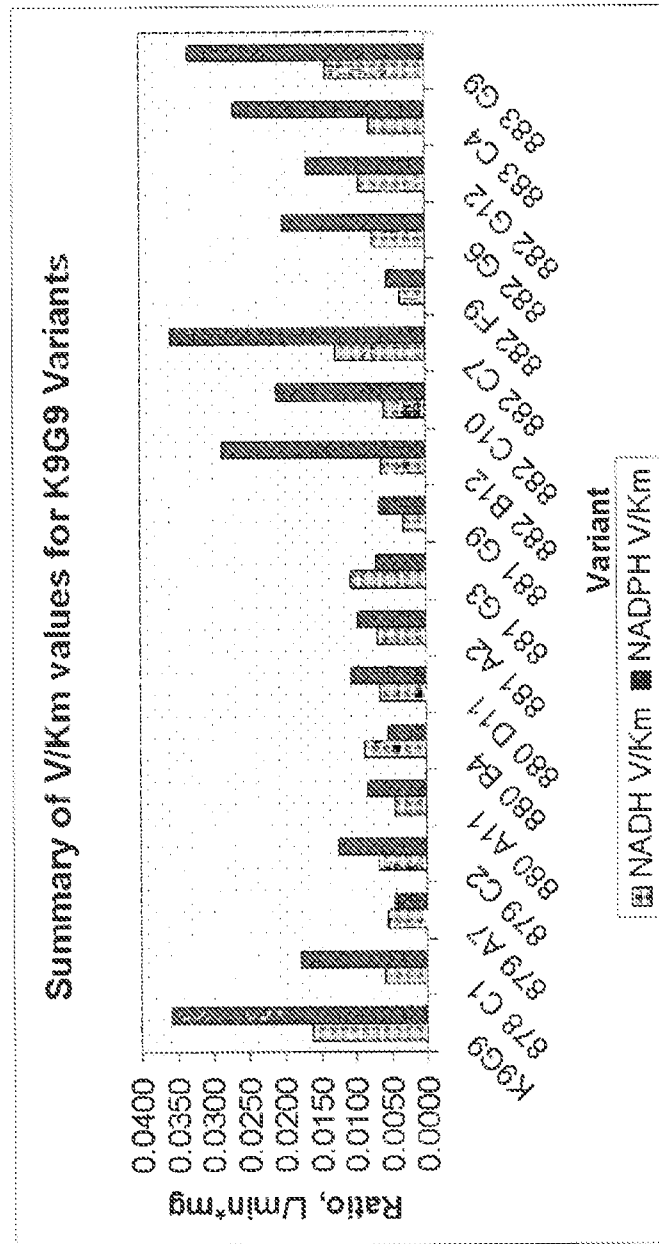
9/25

FIGURE 7



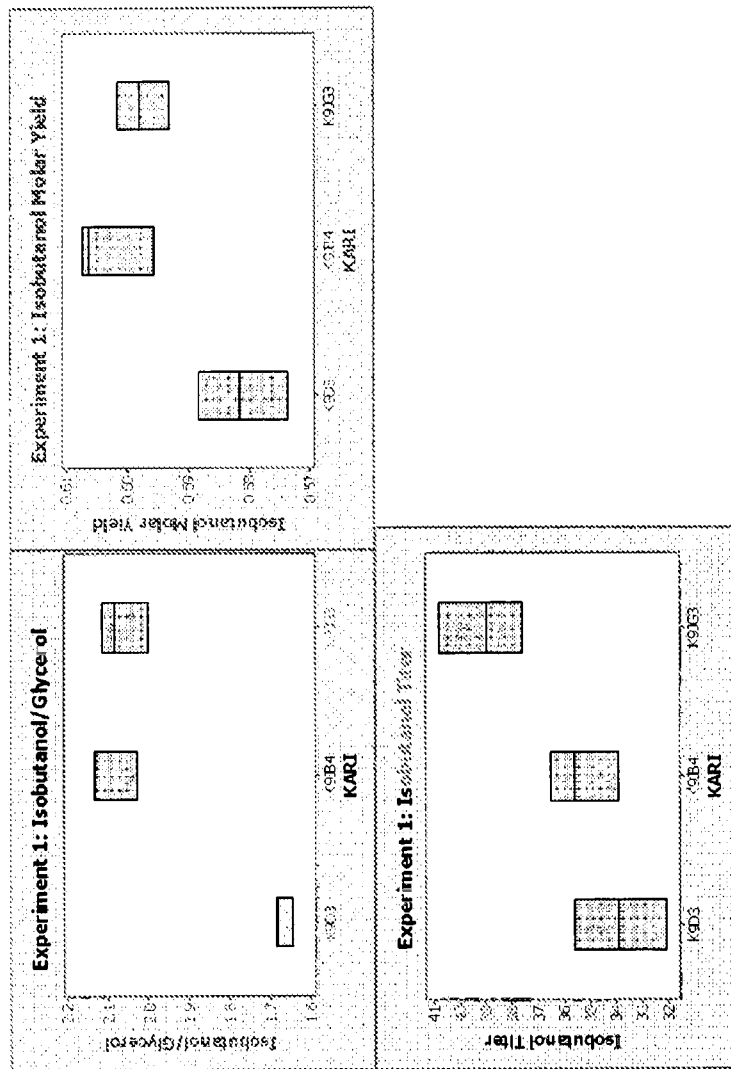
10/25

FIGURE 8



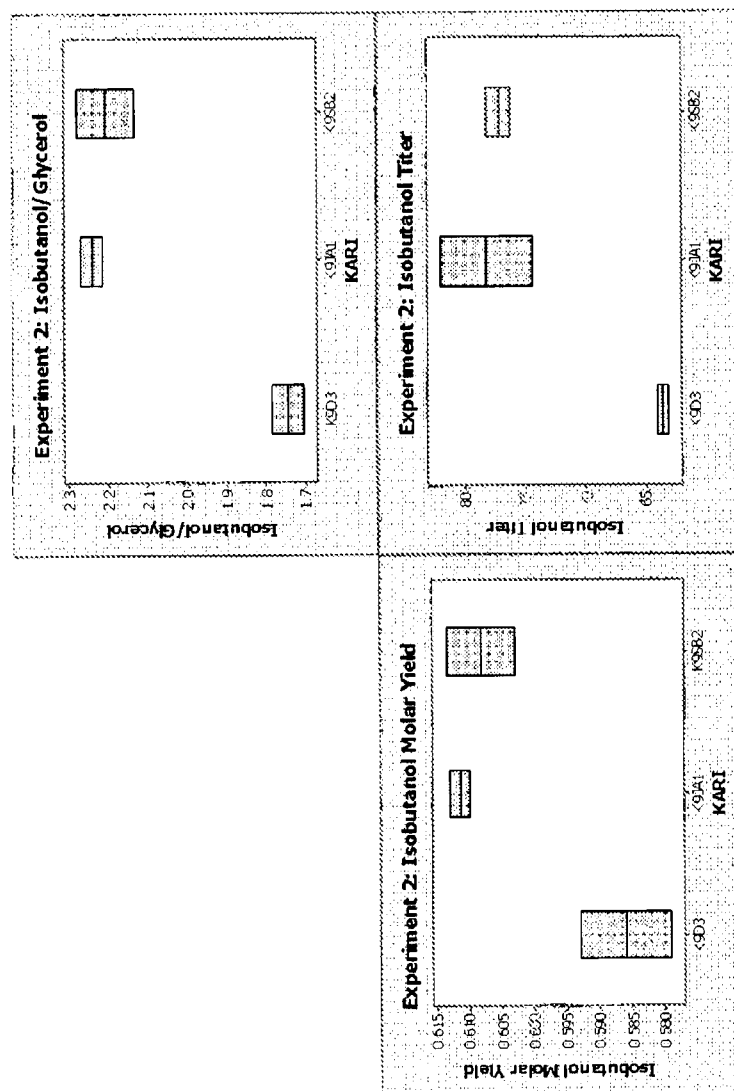
11/25

FIGURE 9A



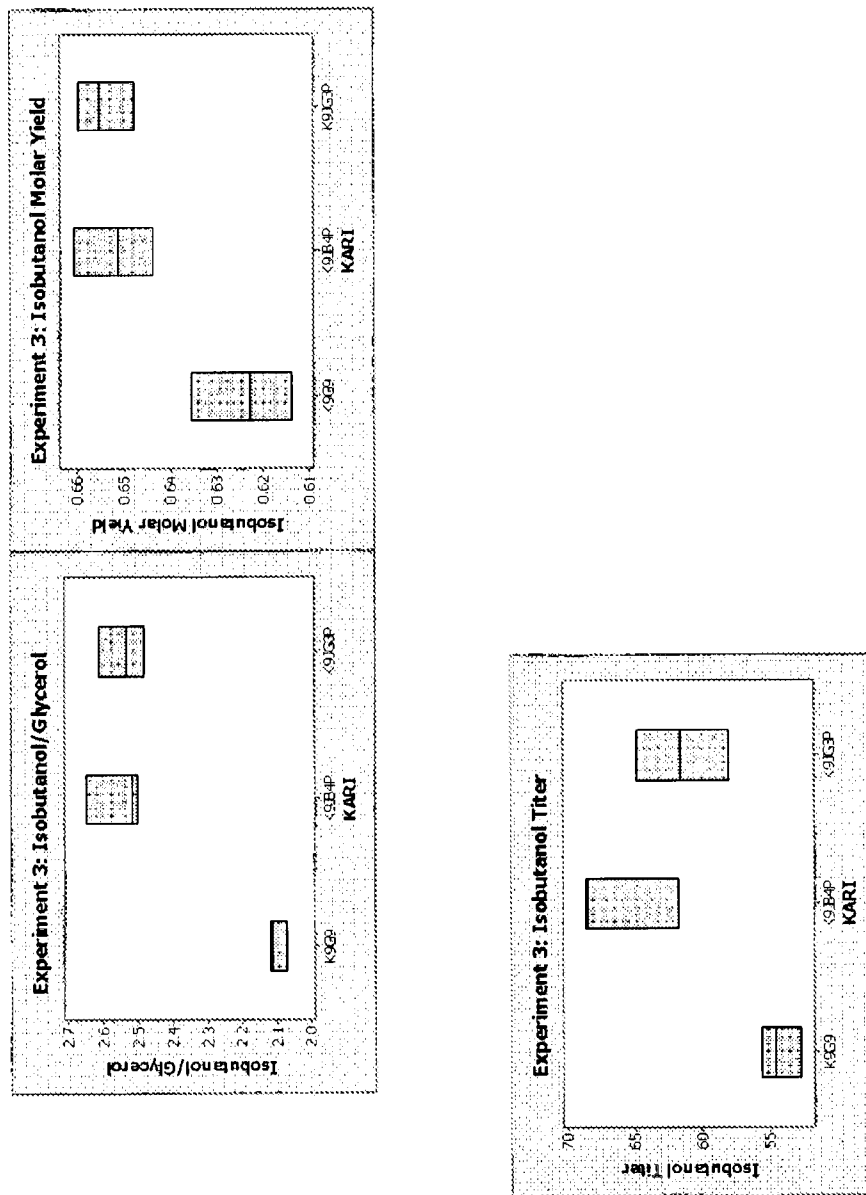
12/25

FIGURE 9B



13/25

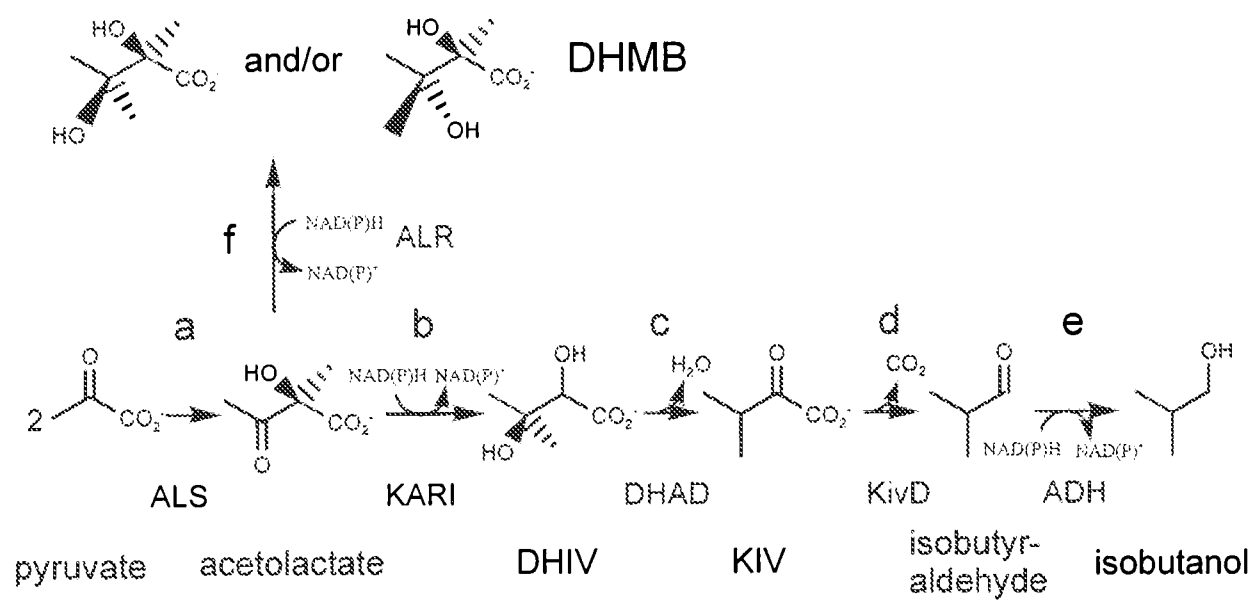
FIGURE 9C





14/25

Figure 10



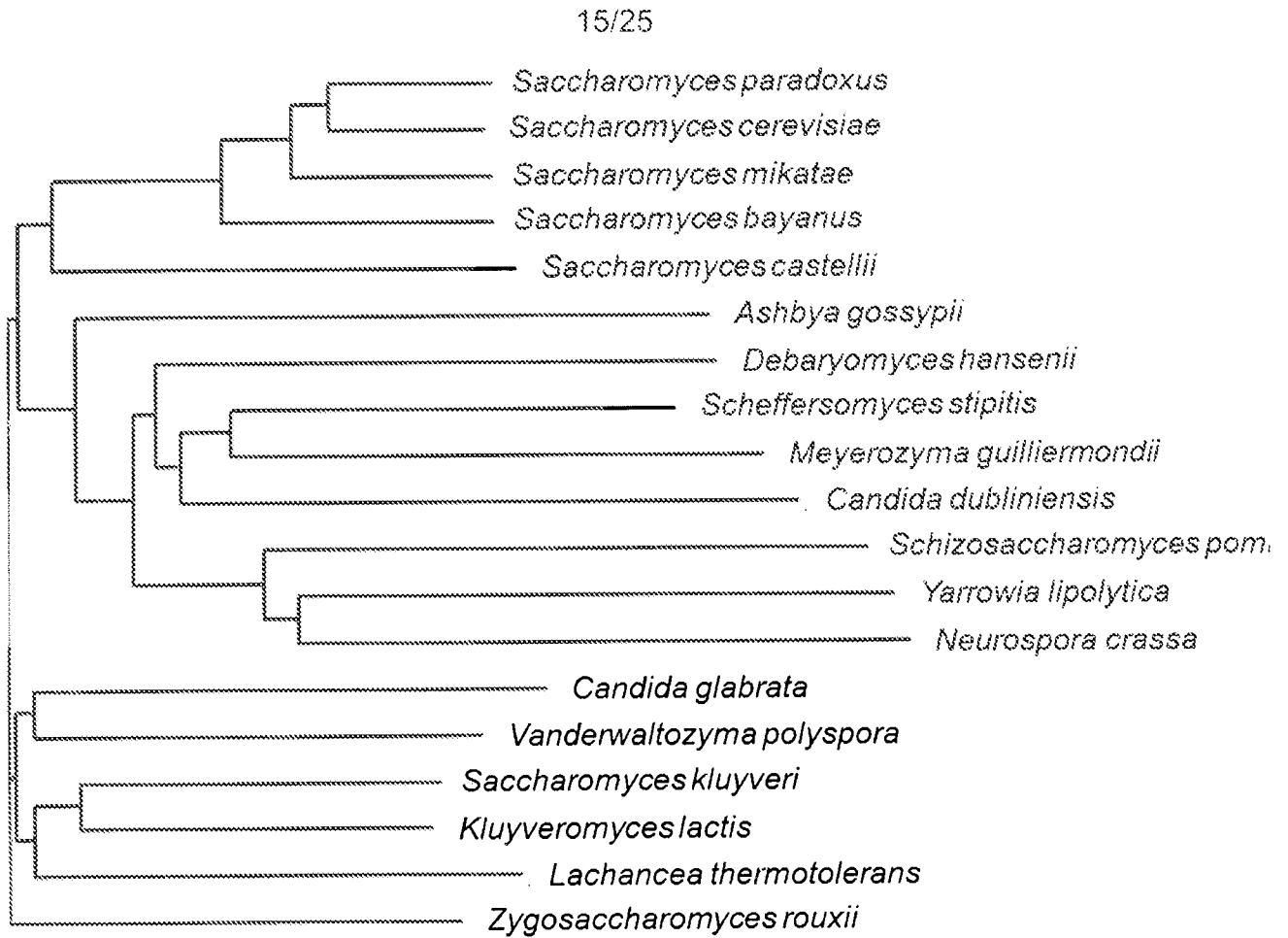


Figure 11

16/25

Figure 12

|              |            |            |             |            |             |
|--------------|------------|------------|-------------|------------|-------------|
|              | 1          |            |             |            | 50          |
| AABY01000127 | .....      | .....      | .....       | .....      | .....       |
| AACH01000019 | .....      | .....      | .....       | .....      | .....       |
| AACA01000631 | .....      | .....      | .....       | .....      | .....       |
| AACF01000116 | .....      | .....      | .....       | .....      | .....       |
| AE016819     | .....      | .....      | .....       | .....      | .....       |
| CR382139     | .....      | .....      | .....       | .....      | .....       |
| XM_001387479 | .....      | .....      | .....       | .....      | .....       |
| XM_001482184 | ATGTGCCTCT | TACCAGCCGG | TAGCACTGTA  | TTATGTCATC | ACCCAGTAGT  |
| XM_002419771 | .....      | .....      | .....       | .....      | .....       |
| CR380959     | .....      | .....      | .....       | .....      | .....       |
| XM_001645671 | .....      | .....      | .....       | .....      | .....       |
| SAKLOH04730  | .....      | .....      | .....       | .....      | .....       |
| XM_451902    | .....      | .....      | .....       | .....      | .....       |
| XM_002553230 | .....      | .....      | .....       | .....      | .....       |
| XM_002494574 | .....      | .....      | .....       | .....      | .....       |
| YMR226C      | .....      | .....      | .....       | .....      | .....       |
| NM_001018495 | .....      | .....      | .....       | .....      | .....       |
| XM_501554    | .....      | .....      | .....       | .....      | .....       |
| XM_957621    | .....      | .....      | .....       | .....      | .....       |
|              | 51         |            |             |            | 100         |
| AABY01000127 | .....      | .....      | .....AT     | GTCCCAAGGT | AGAAAAGCTG  |
| AACH01000019 | .....      | .....      | .....AT     | GTCTCAAGGT | AGAAAAGCTG  |
| AACA01000631 | .....      | .....      | .....AT     | GTCCCAAGGT | AGAAAAGCTG  |
| AACF01000116 | .....      | .....      | .....AT     | GTCTCAAGGT | CCTAAAGCTG  |
| AE016819     | .....      | .....      | .....AT     | GTCCCTAGGA | AGAAAAGCAG  |
| CR382139     | .....      | .....      | .....AT     | GTCGTACGGA | TCTAAAGCTG  |
| XM_001387479 | .....      | .....      | .....AT     | GTCGTTTGGA | AAAAAAGCTG  |
| XM_001482184 | GAGTGTGGAG | ATTAAATCCT | CAATCTTCAT  | GTCTTTCGGT | GCCAAAAGCCG |
| XM_002419771 | .....      | .....      | .....AT     | GTCATTTGGT | AGAAAAGCTG  |
| CR380959     | .....      | .....      | .....AT     | GTCTCAAGGA | AGAAAAGCTG  |
| XM_001645671 | .....      | .....      | .....AT     | GTCACAGGGT | AGAAAAGGCTT |
| SAKLOH04730  | .....      | .....      | .....AT     | GTCTCAAGGT | AGAAGGGCTG  |
| XM_451902    | .....      | .....      | .....AT     | GTCTCAAGGT | AGAAAAGGCTG |
| XM_002553230 | .....      | .....      | .....AT     | GTCACAGGGA | AGAAGAGCAG  |
| XM_002494574 | .....      | .....      | .....AT     | GTCACAAGGT | GTCAAAGCTG  |
| YMR226C      | .....      | .....      | .....AT     | GTCCCAAGGT | AGAAAAGCTG  |
| NM_001018495 | .....      | .....      | .....       | .....      | .....       |
| XM_501554    | .....      | .....      | .....AT     | GTCTTTCGGA | GATAAAGCTG  |
| XM_957621    | .....      | .....      | .....AT     | GTCTTCAGC. | .....AGTAG  |
|              | 101        |            |             |            | 150         |
| AABY01000127 | CAGAAA.GAT | TGGCTAACAA | GACCGTGCTC  | ATTACGGGTG | CCTCTGCTGG  |
| AACH01000019 | CAGAAA.GAT | TGGCTGGCAA | AACCGTTCTC  | ATCACGGGTG | CCTCTGCTGG  |
| AACA01000631 | CAGAAA.GAT | TGGCCAACAA | GACGGTGCTC  | ATTACAGGCG | CTTCTGCTGG  |
| AACF01000116 | CCGAAA.GAT | TGAATGAGAA | GATTGTGTTT  | ATCACTGGTG | CTTCAGCTGG  |
| AE016819     | CTGAAA.GAT | TAGCCAACAA | AATTGTGCTT  | GTGACTGGTG | CCTCTGCGGG  |
| CR382139     | CTGAAC.GTG | TTGCCAATAA | GATTGTCTTA  | ATCACTGGTG | CTTCATCTGG  |
| XM_001387479 | CTGAAA.GAC | TTGCCAACAA | AATCATTCCT  | ATCACCGGGG | CTTCGTCGCG  |
| XM_001482184 | CTGAAC.GCC | TTGCCAACAA | GATCATATTG  | ATCACTGGGG | CATCGTCGCG  |
| XM_002419771 | CTGAAA.GAT | TAGCCAATAG | ATCCATTCCT  | ATCACTGGTG | CTTCATCTGG  |
| CR380959     | CTGAGA.GGT | TACAAGGGAA | GATTGCCTTT  | ATTACGGGTG | CCTCTGCGGG  |
| XM_001645671 | CAGAAA.GGT | TGGCTGGTAA | AACGTGTATTA | ATTACAGGTG | CTTCATCAGG  |
| SAKLOH04730  | CAGAAA.GAC | TAGCBAACAA | GACCGTTTTT  | ATAACTGGCG | CCTCTGCGCG  |

17/25

|              |            |            |            |            |            |
|--------------|------------|------------|------------|------------|------------|
| XM_451902    | CTGAAA.GAT | TGCAAAACAA | GACAATTTTC | ATTACCGGTG | CTTCTGCAGG |
| XM_002553230 | CTGAAA.GAC | TGGCAGGAAA | GACTGTCTTC | ATCACAGGCG | CATCAGCCGG |
| XM_002494574 | CTGAAA.GAC | TAGCTGGTAA | GACTGTATTC | ATTACAGGTG | CTTCTGCAGG |
| YMR226C      | CAGAAA.GAT | TGGCTAAGAA | GACTGTCCTC | ATTACAGGTG | CATCTGCTGG |
| NM_001018495 | ATGAGCCGTT | TGGATGGAAA | AACGATTTTA | ATCACTGGTG | CCTCTTCTGG |
| XM_501554    | CTGCTC.GAC | TTGCGGGCAA | GACCGTCTTT | GTTACCGGCG | CCTCGTCCGG |
| XM_957621    | CCAAGC.GCC | TCGCGGGCAA | GACCATTGTC | ATAACCGGAG | CGTCCTCAGG |

|              |             |            |                                   |
|--------------|-------------|------------|-----------------------------------|
|              | 151         |            | 200                               |
| AABY01000127 | TATTGGTAAG  | GCCACCGCAT | TAGAGTATTT G...GAGGCA TCCAATGGTG  |
| AACH01000019 | TATTGGCAAA  | GCCACTGCAT | TAGAGTATTT G...GAGGCA TCCAATGGCG  |
| AACA01000631 | TATTGGTAAG  | GCCACCGCAT | TGGAGTATTT G...GAAGCA TCCAATGGAA  |
| AACF01000116 | TATTGGGCAA  | GCCACCGCTT | TGGATATACAT G...GATGCG TCGAACGGTA |
| AE016819     | CATTGGCCGT  | GCTACAGCCA | TTAACTATGC A...GACGCG ACGGACGGGG  |
| CR382139     | AATTGGTGAA  | GCAACTGCCA | AAGAAATTGC A...TCAGCC GCTAATGGCA  |
| XM_001387479 | TATTGGTGAA  | GCTACAGCTA | GAGAGTTTGC A...TCTGCT GCCAATGGGA  |
| XM_001482184 | TATAGGCGAG  | GCTACCGCCA | GAGAATTCGC T...GCTGCT GCCAATGGAA  |
| XM_002419771 | GATTGGTGAA  | GCATGTGCTA | AAGTTTTTCG T...GAAGCA TCTAATGGTC  |
| CR380959     | CATCGGTAAA  | GCTACAGCCA | TTGAGTATTT G...GATGCT TCCAATGGTA  |
| XM_001645671 | GATTGGGAAA  | GCCACTGCAT | TAGAATATCT A...GATGCC TCCAATGGTC  |
| SAKLOH04730  | CATTGGCCAA  | GCTACTGCTT | TGGAATACTG T...GATGCT TCTAACGGTA  |
| XM_451902    | TATTGGTCAA  | GCCACAGCAT | TGGAATATCT A...GATGCT GCTAACGGTA  |
| XM_002553230 | TATCGGTCAAG | GCCACTGCGC | AAGAATACCT G...GAAGCA TCCGAAGGCA  |
| XM_002494574 | TATCGGTCAA  | GCAACTGCAA | AGGAATATTT G...GATGCA TCCAATGGTC  |
| YMR226C      | TATTGGTAAG  | GCGACCGCAT | TAGAGTACTT G...GAGGCA TCCAATGGTG  |
| NM_001018495 | AATTGGAAAA  | AGCACTGCTT | TTGAAATTGC C...AAAGTT GCCAAAG...  |
| XM_501554    | CATTGGCCAG  | GCCACTGTTC | TCGCTCTAGC C...GAAGCT GCCAAGGGCG  |
| XM_957621    | AATCGGGCGT  | AGCACAGCCT | TCGAGTTTGC GCGGACCGCG CCCAACCACG  |

|              |            |             |                                   |
|--------------|------------|-------------|-----------------------------------|
|              | 201        |             | 250                               |
| AABY01000127 | ATATGAAACT | GGTCTTAGCT  | GCTAGAAGAT TAGAAAAGCT CGAGGAATTA  |
| AACH01000019 | ATATGAAATT | AATCTTAGCC  | GCTAGAAGAT TAGAAAAGCT CGAGGAATTG  |
| AACA01000631 | ACATGAAACT | GATCTTGGCT  | GCGAGGAGAT TGGAGAAGCT AGAGGAGCTG  |
| AACF01000116 | CTGTGAAATT | GGTTCTAGTT  | GCCAGAAGAT TGGAGAAATT ACAACAATTG  |
| AE016819     | CAATCAAGTT | GATTTTGGTG  | GCAAGACGCG CAGAAAAGCT CACCAGCTTG  |
| CR382139     | ATTTAAATT  | AGTGTTGTGT  | GCTAGACGAA AAGAAAAGTT GGATAATTTA  |
| XM_001387479 | ATATCAGATT | GATTTTGACA  | GCCAGAAGAA AAGAAAAGTT GGCTCAATTG  |
| XM_001482184 | AAATTCTGTT | GATTTTGACC  | GCTCGGAGAG AAGACAACT CAAGTCTCTC   |
| XM_002419771 | AAGTTAAATT | AGTTT TAGGA | GCAAGAAGAA AAGAACGATT AGTTAAATTA  |
| CR380959     | GTGTGAAGCT | AGTTCTTGGT  | GCACGTAGAA TGGAGAAATT GGAGGAGTTG  |
| XM_001645671 | ATATGAAGTT | AATTTTAGTT  | GCAAGAAGAT TAGAAAAATT GCAAGAGTTG  |
| SAKLOH04730  | AAATAAACTT | GGTGTTAAGT  | GCCAGAAGGC TGGAAAAATT GCAAGAGTTA  |
| XM_451902    | ATGTCAAATT | GATCTTAGCA  | GCAAGAAGGT TGGCTAAGTT GGAAGAATTG  |
| XM_002553230 | AAATCAAGTT | GATCCTTGCA  | GCAAGAAGAC TCGACAAGCT GGAGGAAATC  |
| XM_002494574 | AAATTAAATT | GATCTTGGCT  | GCAAGAAGAT TAGAGAAAATT ACACGAGTTT |
| YMR226C      | ATATGAACT  | GATCTTGGCT  | GCTAGAAGAT TAGAAAAGCT CGAGGAATTG  |
| NM_001018495 | ...TAAACT  | TATTTTGGCT  | GCTCGCAGAT TTTCTACCGT TGAAGAAATT  |
| XM_501554    | ACCTCAAGTT | TGTGCTTGCT  | GCCCCGACGAA CCGACCGTCT GGACGAGCTC |
| XM_957621    | GCCTCAAGCT | GATCTTGACG  | GCCCCCGCG TCGATGCCCT GGAGCAGATT   |

|              |             |            |                                  |
|--------------|-------------|------------|----------------------------------|
|              | 251         |            | 300                              |
| AABY01000127 | AAGAAAACCTA | TTGATCAGG. | AGTTTCCA.. AACGCCAAAG TTCATGTGGC |
| AACH01000019 | AAGAAGACTA  | TCGATGAAG. | AGTTTCCA.. AACGCAAAGG TCCATGTGAC |
| AACA01000631 | AAGAAGACCA  | TCGACGAGG. | AGTTTCCC.. AATGCAAAGG TTCACGTTGG |
| AACF01000116 | AAGGAAGTCA  | TTGAGGCAA. | AATACCCT.. AAGAGTAAAG TCTATATTGG |
| AE016819     | AAACAGGAGA  | TCGA.AAGCA | AGTATCCC.. AACGCCAAGA TCCATGTCCG |
| CR382139     | TCT.AAAGAA  | TTGACTGACA | AATATTCA.. TCCATCAAGG TTCATGTTGC |

18/25

|              |            |            |             |             |            |
|--------------|------------|------------|-------------|-------------|------------|
| XM_001387479 | TCA.GACTCA | TTGACCAAGG | AATTTCCA..  | ACTATCAAAA  | TCCATTCTGC |
| XM_001482184 | TCG.CAACAA | TTGAGCCTCA | TTTACCCG..  | CAAATTAATA  | TCCATTCTGC |
| XM_002419771 | TCT.GATACT | TTAATTAAAC | AAATATCCT.. | AATATTAATA  | TTCATCATGA |
| CR380959     | AAG.AAGGAA | TTGCTGGCTC | AATATCCT..  | GATGCAAGA   | TTCATATAGG |
| XM_001645671 | AAGGAAACAA | TTTGTAAG.  | AATATCCA..  | GAATCTAAGG  | TTCATGTTGA |
| SAKL0H04730  | AAGGACAAAA | TCACCAAGG. | AGTATCCT..  | GAAGCCAAGG  | TTTATATTGG |
| XM_451902    | AAGGAAAAAA | TCAATGCTG. | AATACCCA..  | CAAGCTAAAG  | TATATATCGG |
| XM_002553230 | AAAGCCAAGG | TTTCTAAAG. | ACTTCCCT..  | GAAGCACAGG  | TGCATATCGG |
| XM_002494574 | AAAGAACAAA | CTAC.AAAGA | GTTACCCA..  | AGCGCTCAAG  | TCCACATTGG |
| YMR226C      | AAGAAGACCA | TTGATCAAG. | AGTTTCCA..  | AACGCAAAAAG | TTCATGTGGC |
| NM_001018495 | GCAAAGGAGT | TAGA.ATCGA | AATAT....   | GAAGTATCGG  | TTCTTCCTCT |
| XM_501554    | AAGAAGAAGC | TGGAGACCG. | ACTACAAG..  | GGTATCCAGG  | TGCTGCCTTT |
| XM_957621    | GCCAAGGAGA | TCCGCCAAGA | GGTTGGCGAG  | GGCGTGCAAG  | TGCTGCCTGT |

|              |            |            |            |            |
|--------------|------------|------------|------------|------------|
|              | 301        |            |            | 350        |
| AABY01000127 | CCAAGTGGAT | ATCACTCAAG | CAGAAAAGAT | CAAGCCCTTT |
| AACH01000019 | CAAAGTGGAC | ATCACACAGA | CAGAAAAGAT | CAAGCCCTTT |
| AACA01000631 | CCAAGTGGAT | ATCACACAGG | CCGAGAAGAT | CAAGCCCTTC |
| AACF01000116 | GAAGTTGGAT | GTGACAGAGC | TTGAGACCAT | TCAACCATTC |
| AE016819     | ACAATTGGAT | GTGACCCAAC | TGGACCAGAT | CCGCCCATTT |
| CR382139     | TCAACTAGAT | GTATCTAAGC | TCGAGACTAT | CAAGCCATTT |
| XM_001387479 | CAAATTGGAT | GTGACCGAAC | ATGATGGCAT | CAAGCCCTTC |
| XM_001482184 | TCGTCTTGAT | GTCTCTGAGT | TTTCGTCACT | TAAGCCGTTT |
| XM_002419771 | TTTTTTGGAT | GTTACTATTA | AAGATTCAAT | TTCAAAATTC |
| CR380959     | TAAACTGGAT | GTTACAGACT | TTGAAAACGT | CAAGCAGTTT |
| XM_001645671 | AGAATTAGAT | ATTTCTGATA | TTAATAGAAT | CCCAGAATTT |
| SAKL0H04730  | TGTGCTTGAT | GTGACCGAAA | CGGAAAAAAT | CAAACCATTC |
| XM_451902    | TCAATTGGAC | GTCACTGAAA | CTGAGAAGAT | TCAACCTTTC |
| XM_002553230 | CCAGCTAGAT | GTGACTCAGA | CGGACAAAAT | CCAGCCTTTT |
| XM_002494574 | TAAATTGGAC | GTCACTGCAA | TTGACACCAT | AAAACCATTT |
| YMR226C      | CCAGCTGGAT | ATCACTCAAG | CAGAAAAAAT | CAAGCCCTTC |
| NM_001018495 | TAAATTGGAT | GTTTCTGATT | TGAAGTCTAT | TCCTGGGGTA |
| XM_501554    | CAAGCTGGAC | GTGTCCAAGG | TCGAGGAGAC | CGAGAACATT |
| XM_957621    | CAAGCTGGAC | GTCAGCCAGC | CGGAGGAGGT | CAGGGGGTTT |

|              |            |            |            |            |
|--------------|------------|------------|------------|------------|
|              | 351        |            |            | 400        |
| AABY01000127 | TGCCAAAGGA | GTTCAAAGAC | ATTGACATTT | TGGTGAACAA |
| AACH01000019 | TGCCAGAGGA | GTTCAAAGAC | ATTGATATTC | TGGTGAACAA |
| AACA01000631 | TGCCGAGGAC | ATTCAAGGAT | ATTGACATCC | TGATAAACAA |
| AACF01000116 | TTCTTGAGGA | ATTTAAGGAT | ATTGATATCT | TGATTAATAA |
| AE016819     | TACCTGAGGA | GTTCCGAGAC | ATTGATATTT | TAATTAACAA |
| CR382139     | TACCGAAAGA | ATTCTCTGAC | GTGGATGTAT | TAGTCAACAA |
| XM_001387479 | TACCCAAGGA | TTTCGCCGAC | ATCGATGTGT | TGATCAACAA |
| XM_001482184 | TGCCAAAGGA | TTTTGCTAGC | ATCGACGTTT | TGGTGAATAA |
| XM_002419771 | TTCTCATGA  | ATTTG..AAC | CT.GATGTAT | TAATTAATAA |
| CR380959     | TGCCAGAAGA | GTTCAAGGAC | ATCGACATCC | TGATCAATAA |
| XM_001645671 | TACCTGAAGA | ATTCAAAGAT | ATTGATATAT | TGATTAACAA |
| SAKL0H04730  | TACCGAAGA  | ATTTAAAGAT | ATTGACATCT | TGATCAATAA |
| XM_451902    | TGCCGAAGA  | ATTCAAGGAT | ATCGATATTT | TGATTAACAA |
| XM_002553230 | TGCCGAAGA  | GTTCAAAGAC | ATCGACATCC | TGATCAACAA |
| XM_002494574 | TACCAAGGA  | ATTTCAAGAT | ATCGATATTT | TGATCAACAA |
| YMR226C      | TGCCACAAGA | GTTCAAGGAT | ATTGACATTC | TGGTGAACAA |
| NM_001018495 | TGCCAAAGGA | ATTTGCTGAT | ATCGATGTCT | TGATTAATAA |
| XM_501554    | TGCCCAAGGA | GTTTTCCGAG | GTGGACGTGC | TTATCAACAA |
| XM_957621    | TGCCGAGGA  | GTGGAGGGAT | ATCCATGTGC | TGGTCAATAA |

401

450

19/25

|              |            |            |            |             |            |
|--------------|------------|------------|------------|-------------|------------|
| AABY01000127 | GCCCTTGGTA | CCGACCGTGT | GGGGGAGATT | GCAACACAAG  | ATATCCAGGA |
| AACH01000019 | GCTCTTGGTA | CGGACCGTGT | TGGGGAGATT | GATACACAGG  | ACGTCCAGGA |
| AACA01000631 | GCCCTGGGCT | CCGAACGTGT | CGGGGAAATT | GCCACACAGG  | ACATCCAGGA |
| AACF01000116 | GCATTAGGTT | CCGATCGTGT | AGGTGATATT | GATATAAAAG  | ATGTGAAGGG |
| AE016819     | GCCCTCGGCA | CTGAGAGGGT | GGGGGAAATC | TCGATGGACG  | ATATCCAGGA |
| CR382139     | GCTTTGGGCC | GTGATGAAGT | TGGAACCAT  | GACACAGATG  | ATATGTTATC |
| XM_001387479 | GCTCTTGGAA | AAGCATCTGT | TGGTGAAATC | AGTGACAGTG  | ATATCCAAGG |
| XM_001482184 | GCATTGGGAA | GAGCCAATGT | TGGTGAAATT | TCCCAAGAGG  | AAATCAATGG |
| XM_002419771 | GCCTTGGGGA | AAGAAGAAGT | TGGAGAATTG | AAAGATGAAG  | ATATTACGGA |
| CR380959     | GCGTTGGGGT | CTGACAAAGT | TGGAGACATT | GACCCTGAGG  | ATATCGCAGG |
| XM_001645671 | GCATTAGGAA | GTGATACTAT | TGGTAATATC | GAGAATGAGG  | ATATTAAAGG |
| SAKLOH04730  | GCGTTAGGCT | CTGATCCTGT | TGGTACCATC | AAAACCTGAAG | ATATTGAAGG |
| XM_451902    | GCTTTGGGAT | CTGATGTTGT | CGGTACCATC | AGTAGCGAGG  | ACATCAAAGG |
| XM_002553230 | GCGCTCGGAT | CCGACCCCGT | GGGCACAATC | GACCCCAATG  | ATATTCAAGG |
| XM_002494574 | GCATTAGGTA | CTGATAAAGT | TGGTGATATT | GCAGATGAAG  | ACGTGGAAGG |
| YMR226C      | GCTCTTGGCA | GTGACCGTGT | GGGCCAGATC | GCAACGGAGG  | ATATCCAGGA |
| NM_001018495 | GCTCTAGGTA | CCGATAAAGT | CATTGATCTT | AATATTGATG  | ACGCCGTTAC |
| XM_501554    | GTCCACGGCA | CCGAAAAGGT | TGGCTCCATC | AACCAGAACG  | ACATTGAGAT |
| XM_957621    | GTAAAAGG.. | .....A..GC | TCCCTCCATC | GCCGAAGAAG  | ACATCAACGT |

451

500

|              |             |            |            |            |            |
|--------------|-------------|------------|------------|------------|------------|
| AABY01000127 | TGTGTTTGAC  | ACCAACGTCA | CAGCTTTAAT | TAATATCACT | CAAGCTGTGC |
| AACH01000019 | CGTGTTTCGAC | ACCAACGTCT | CGGCTTTGAT | TAATGTCACA | CAGGCTGTTC |
| AACA01000631 | CGTGTTTCGAC | ACCAACGTCA | CGGCGTTGAT | CAACGTCACG | CAAGCAGTGC |
| AACF01000116 | AATGATGGAT  | ACCAATGTCT | TGGGGTTGAT | CAATGTGACG | CAAGCTGTGT |
| AE016819     | GGTTTTCAAC  | ACTAATGTTA | TCGGCTTGGT | GCACTTGACT | CAGGAGGTTC |
| CR382139     | GATGTTTCAA  | ACTAATGTTT | TAGGGTTAAT | TACCATCACA | CAGGCTGTTT |
| XM_001387479 | CATGATGCAA  | ACGAATGTCT | TGGGACTCAT | CAACATGACT | CAGGCTGTGA |
| XM_001482184 | CATGTTCCAT  | ACCAATGTTT | TTGGGTTGAT | AAACTTAACT | CAGGAGGTGT |
| XM_002419771 | AATGTTTGAT  | ACTAATGTCA | TTGGAGTCAT | TCGTATGACT | CAAGCAGTTT |
| CR380959     | AATGGTTAAC  | ACCAACGTCC | TTGCATTGAT | CAATTTAACA | CAATTGTTGT |
| XM_001645671 | TATGTTTGAG  | ACTAACGTTT | TTGGATTAAT | CTGTTTAACA | CAAGCTGTAC |
| SAKLOH04730  | AATGATCAAC  | ACCAATGTCT | TAGCTCTTAT | CAATATTACT | CAAGCTGTCT |
| XM_451902    | TATGATAGAT  | ACTAACGTTG | TTGCCCTTAT | CAACGTTACC | CAAGCTGTTT |
| XM_002553230 | CATGATCCAG  | ACTAACGTTA | TCGGGCTTAT | AAATGTTACC | CAAGCCGTTT |
| XM_002494574 | TATGTTTCGAC | ACCAATGTCT | TGGGGTTAAT | CAAAGTTACT | CAAGCTGTTT |
| YMR226C      | CGTGTTTGAC  | ACCAACGTCA | CGGCTTTAAT | CAATATCACA | CAAGCTGTAC |
| NM_001018495 | CATGATTACT  | ACCAATGTTT | TTGGTATGAT | GGCTATGACT | CGTGCGGTTT |
| XM_501554    | CATGTTCCAC  | ACAAACGTGC | TCGGACTCA  | TTCTGTCACT | CAGCAGTTTG |
| XM_957621    | CATGTTTGCC  | ACCAACGTCA | CCGGCCTGAT | CAACATGACC | CAAGCCATCC |

501

550

|              |            |             |             |        |            |
|--------------|------------|-------------|-------------|--------|------------|
| AABY01000127 | TGCCCATTTT | TCAAGCCAAG  | AACTCAG...  | .....G | GGATATTGTG |
| AACH01000019 | TGCCCATCTT | CCAAGCTAAG  | AACTCAG...  | .....G | GGATATTGTG |
| AACA01000631 | TGCCAATTTT | CCAAGCCAAG  | AACTCAG...  | .....G | GGACATCGTC |
| AACF01000116 | TGCACATTTT | CCAAAAGAAG  | AACTCCG...  | .....G | TGATATTGTG |
| AE016819     | TACCTATTAT | GAAAGCCAAG  | AATTCCG...  | .....G | GGACATTGTC |
| CR382139     | TGCCAATCAT | GAAAAAGAAG  | AACAGCG...  | .....G | AGATGTTGTT |
| XM_001387479 | TTCCCATTTT | TAAGGCTAAA  | AATTCTG...  | .....G | AGATATCGTC |
| XM_001482184 | TACCCATCTT | CAAAAAGAAA  | AATGCTG...  | .....G | AGATATTGTG |
| XM_002419771 | TACCTTTACT | TAAAAAAGAAA | CCTTATG...  | .....C | TGATGTGGTT |
| CR380959     | TGCCATTATT | CAAGAAGAAG  | AACAGTG...  | .....G | TGATATCGTC |
| XM_001645671 | TTCCAATATT | CAAGGCTAAA  | AATGGTG...  | .....G | TGATATTGTC |
| SAKLOH04730  | TGCCAATCTT | CAAAGCCAAG  | AATTTCTG... | .....G | TGATATCGTA |
| XM_451902    | TGCCTATTTT | CAAAGCAAAG  | AATTCCG...  | .....G | TGACATCGTT |
| XM_002553230 | TGCCCATCTT | CAAGGCCAAA  | AACTCTG...  | .....G | TGATATCGTG |
| XM_002494574 | TACCTATCTT | CAAAAAGAAA  | AATTCTG...  | .....G | TGATGTCGTT |

20/25

|              |            |            |            |            |            |
|--------------|------------|------------|------------|------------|------------|
| YMR226C      | TGCCCATATT | CCAAGCCAAG | AATTCAG... | .....G     | AGATATTGTA |
| NM_001018495 | TTCCTATATT | CTACAGCAAA | AACAAGG... | .....G     | TGATATTTTG |
| XM_501554    | TCGGCGAGAT | GCGAAAGCGA | AACAAGG... | .....G     | CGACATTGTC |
| XM_957621    | TGCCCATCTT | CAAGGCCCGC | GGCTCCGAGG | GTGGTTCGGG | AGACATTGTG |

551

600

|              |            |            |            |             |            |
|--------------|------------|------------|------------|-------------|------------|
| AABY01000127 | AACCTGGG.T | TCGGTGGCTG | GCAGGGATGC | ATACCCAACG  | GGTCCATCT  |
| AACH01000019 | AACCTGGG.C | TCGGTAGCTG | GCAGAGATGC | ATACCCAACG  | GGTCCATCT  |
| AACA01000631 | AACCTGGGGC | TCGGTGGCCG | GCAGAGACGC | ATACCCCAACA | GGTCCATCT  |
| AACF01000116 | AACCTAGG.T | TCAGTTGCTG | GAAGAGATGC | ATACCCAACA  | GGGTCCATTT |
| AE016819     | AATGTTGG.G | TCGATTGCCG | GCCGCGAAGC | CTACCCTGGT  | GGTCTATTT  |
| CR382139     | AATATAGG.T | TCAATTGCTG | GAAGAGACTC | TTACCCTGGA  | GGTGGAATTT |
| XM_001387479 | AACATCGG.T | TCGATTGCTG | GAAGAGACCC | TTACCCTGGG  | GGATCGATCT |
| XM_001482184 | AACATTGG.C | TCAGTGGCCG | GTAGAGAACC | TTACCCTGGA  | GGTGCAGTAT |
| XM_002419771 | TTCATTGG.A | AGTATTGCTG | GACGTGTTCC | TTATAAAAAAT | GGAGGTGGTT |
| CR380959     | AACCTGGG.A | TCGATTGCTG | GTAGAGACGC | ATACCCAACG  | GGTGCTATAT |
| XM_001645671 | AATTTAGG.G | TCAATTGCAG | GCATAGAAGC | TTACCCAACA  | GGATCTATAT |
| SAKL0H04730  | AACCTGGG.G | TCTGTCGCTG | GTAGAGATGC | TTATCCAACC  | GGTGCAATCT |
| XM_451902    | AACCTAGG.T | TCTGTTGCCG | GTAGAGATGC | ATATCCAACT  | GGTCTATCT  |
| XM_002553230 | AACCTGGG.T | TCTGTCGCTG | GCAGAGAAGC | TTACCCTACA  | GGATCTATTT |
| XM_002494574 | AACATTAG.T | TCGGTTGCTG | GTAGAGAGGC | ATACCCAGGT  | GGTCCATTT  |
| YMR226C      | AATTTGGG.T | TCAATCGCTG | GCAGAGACGC | ATACCCAACA  | GGTCTATCT  |
| NM_001018495 | AACGTTGG.C | AGTATTGCCG | GCAGAGAATC | ATACGTAGGC  | GGCTCCGTTT |
| XM_501554    | AACATTGG.C | TCCATCGCCG | GACGAGAGCC | CTACGTTGGA  | GGAGGAATCT |
| XM_957621    | AACATCGG.T | TCTATTGCCG | GGAGAGAACC | ATACGCGGGA  | GGTCCATCT  |

601

650

|              |            |            |             |            |             |
|--------------|------------|------------|-------------|------------|-------------|
| AABY01000127 | ATTGTGCCTC | CAAGTTTGCC | GTGGGGGCGT  | TCACTGATAG | TTTAAGAAAG  |
| AACH01000019 | ATTGTGCATC | TAAGTTTGCC | GTCGGGGCTT  | TCACTGAGAG | TTTGAGAAATG |
| AACA01000631 | ACTGTGCTTC | CAAGTTTGCC | GTCGGTGCGT  | TCACTGACAG | TTTGAGAAAG  |
| AACF01000116 | ACTGTGCTTC | TAAATTTGCC | GTGAGGGCCT  | TTACTGAAAG | TTTGAGAAAG  |
| AE016819     | ACTGTGCCAC | GAAACATGCG | GTCGAAGGCTT | TCACCAAGGC | CATGCGGAAG  |
| CR382139     | ACTGTCCAAC | TAAGGCAAGT | GTCGAAGTCGT | TTTCGCAAGT | TTTAAGAAAG  |
| XM_001387479 | ACTGTGCCTC | CAAGGCTGCT | GTTAAGTTCT  | TCTCGCATTC | TTTGAGAAAG  |
| XM_001482184 | ACTGTGCTTC | AAAGGCAGCA | GTTAACTACT  | TTTCTCATTC | TTTGAGAAAG  |
| XM_002419771 | ATTGTGCATC | TAAAGCTGCT | GTTTCGTAGT  | TCACCGATAC | ATTTAGAAAA  |
| CR380959     | ACTGTGCAAC | AAAACATGCT | GTCAGGGCAT  | TCACACAATC | CTTAAGGAAAG |
| XM_001645671 | ATTGTGCAAC | TAAATTTGCA | GTTAAAGCAT  | TCACTGAAAG | TTTAAGAAAG  |
| SAKL0H04730  | ACTGTGCTAG | CAAACATGCA | GTCAGAGCCT  | TCACTCAAAG | TTTGAGGAAG  |
| XM_451902    | ATTGTGCTTC | GAAGCATGCT | GTCAGAGCGT  | TCACTCAGTC | TTTGAGAAAA  |
| XM_002553230 | ACTGCGCTAC | GAAGCACGCG | GTGCGTGCTT  | TCACCCAGAG | CCTGCGCAAG  |
| XM_002494574 | ACTGTGCTAC | TAAACACGCT | GTTAAGGCAT  | TCACTGAAAG | TTTGCGTAAG  |
| YMR226C      | ATTGTGCCTC | TAAGTTTGCC | GTGGGGGCGT  | TCACTGATAG | TTTGAGAAAG  |
| NM_001018495 | ACTGCTCTAC | CAAGTCTGCC | CTTGCTCAAT  | TCACTTCCGC | TTTGCGTAAG  |
| XM_501554    | ACTGTGCCAC | CAAGGCCGCC | GTGCGATCTT  | TCACTGAGAC | TCTCCGAAAA  |
| XM_957621    | ACTGCGCCAC | CAAGGCTGCT | GTGCGCAGCT  | TCACCGACGC | GCTGCGCAAG  |

651

700

|              |             |            |            |            |            |
|--------------|-------------|------------|------------|------------|------------|
| AABY01000127 | GAGCTTATCA  | ACACCAAGAT | CAGAGTCATC | CTAATCGCAC | CAGGGCTAGT |
| AACH01000019 | GAACCTATAA  | ACACTAAGAT | TAGAGTCATT | CTAATTGCAC | CAGGGTTAGT |
| AACA01000631 | GAACCTGATCA | ACACGAAGAT | CAGAGTTATC | TTGATCGCGC | CGGGGCTGGT |
| AACF01000116 | GAATTAATTA  | ATACCAAGAT | TAGGGTGATA | TTGATAGCCC | CGGGTATCGT |
| AE016819     | GAGCTCATTA  | GCACCAAGAT | CCGGGTCTTC | GAAATTGCGC | CGGGCTCTGT |
| CR382139     | GAATTGATTA  | GCACCAAGAT | TAGAGTTCTT | GAGGTTGACC | CTGGTAATGT |
| XM_001387479 | GAACCTATTA  | ACACCAGAAT | CAGAGTTTTG | GAAGTTGATC | CAGGTGCTGT |
| XM_001482184 | GAAACTATCA  | ATTCCAAAAT | CAGGGTCATG | GAGGTGGATC | CTGGGGCAGT |
| XM_002419771 | GAAACTATTA  | ATACTGGTAT | TAGAGTCATT | GAAGTTGATC | CAGGTGCAGT |

21/25

|              |            |            |            |            |             |
|--------------|------------|------------|------------|------------|-------------|
| CR380959     | GAATTGATCA | ACACCGACAT | TAGAGTAATT | GAAATTGCTC | CTGGTATGGT  |
| XM_001645671 | GAATTGATTA | ATACAAAGAT | CAGAGTTATT | GAAATTGCAC | CAGGTATGGT  |
| SAKL0H04730  | GAATTGGTGA | ACACCAATAT | CAGAGTGATT | GAAATTGCTC | CGGGTAATGT  |
| XM_451902    | GAATTAATCA | ATACTGGTAT | TAGGGTCATT | GAGATTGCTC | CAGGTAAATGT |
| XM_002553230 | GAAGTATCA  | ACACAAACAT | CAGGGTTATT | GAGGTCGCTC | CAGGTAAACGT |
| XM_002494574 | GAATTAGTCG | ATACAAAAAT | CAGAGTCATG | AGTATTGATC | CTGGTAATGT  |
| YMR226C      | GAGCTCATCA | ACACTAAAAT | TAGAGTCATT | CTAATTGCAC | CAGGGCTAGT  |
| NM_001018495 | GAGACTATTG | ACACTCGCAT | TCGTATTATG | GAGGTTGATC | CTGGCTTGGT  |
| XM_501554    | GAGAACATCG | ACACTCGAAT | CCGAGTCATT | GAGGTTGATC | CTGGAGCCGT  |
| XM_957621    | GAGCTGATCG | CCACGCGCAT | CCGTGTCATG | GAGATTGACC | CTGGCCAGGT  |

|              |            |            |             |
|--------------|------------|------------|-------------|
|              | 701        |            | 750         |
| AABY01000127 | CGAAACTGAA | TTTTCACTGG | TTAGATACAG  |
| AACH01000019 | CGAAACTGAG | TTTTCCCTGG | TTAGATACAG  |
| AACA01000631 | TGAGACCGAG | TTCTCACTGG | TCAGATACAG  |
| AACF01000116 | CGAAACTGAA | TTCTCAGTTG | TTAGATACAA  |
| AE016819     | AGAAACGGAA | TTCTCCATGG | TTCTGATGCG  |
| CR382139     | TGAAACTGAA | TTTTCAAATG | TCAGATTCAA  |
| XM_001387479 | GTTGACCGAG | TTCTCTTTGG | TTCTGTTTCCA |
| XM_001482184 | AGAGACAGAG | TTCTCGTTGG | TTCTGTTTGG  |
| XM_002419771 | ACTTACTGAA | TTTAGTGTTG | TTCTGTTATAA |
| CR380959     | CGAAACCGAG | TTTTCTGTGG | TCAGGTACAA  |
| XM_001645671 | TAACACTGAA | TTTTCTGTAA | TTAGATATAA  |
| SAKL0H04730  | TGAAACCGAG | TTTTCCCTAG | TTAGATATAA  |
| XM_451902    | CGAAACTGAG | TTCTCTCTAG | TTAGATACAA  |
| XM_002553230 | GGAGACCGAG | TTTTCTCTGG | TTAGATACAA  |
| XM_002494574 | AGAGACCGAA | TTTTCTATGG | TTAGATTCCG  |
| YMR226C      | CGAGACTGAA | TTTTCACTAG | TTAGATACAG  |
| NM_001018495 | CGAAACCGAA | TTCAGCGTTG | TGAGATTCCA  |
| XM_501554    | TGAGACCGAG | TTCTCCGTGG | TGCGATTCCG  |
| XM_957621    | CGAGACCGAG | TTCAGCGTGG | TGAGGTTCTA  |

|              |            |            |             |
|--------------|------------|------------|-------------|
|              | 751        |            | 800         |
| AABY01000127 | AGAATGTCTA | CAA.GGACAC | CACCCCATTA  |
| AACH01000019 | AGAATGTTTA | CAA.GGACAC | CACCTCCGTTG |
| AACA01000631 | AAAACGTCTA | CAA.GGACAC | TACGCCGTTG  |
| AACF01000116 | AATCTGTCTA | CGATGGAGTT | CACCCCTTGG  |
| AE016819     | AGAAAGTGTA | CCA.GGGATT | TGAACCCCTA  |
| CR382139     | AGCTGGTTTA | CGC.GGGTAC | TGAACCATTA  |
| XM_001387479 | ATGCTGTTTA | TGA.AGGTAC | CCAACCTTTG  |
| XM_001482184 | AAAAGGTGTA | TGA.GGGAAC | CGAGCCTTTG  |
| XM_002419771 | ATGCTGTTTA | TAC.TGGTAC | TGAACCATTA  |
| CR380959     | ACGACGTCTA | CAG.AGGTAC | AACACCACTA  |
| XM_001645671 | ATAAAGTTTA | TGA.AAACAC | TACTCCTTTA  |
| SAKL0H04730  | AAAAGGTTTA | TGA.AGGTAC | TAACCCATTA  |
| XM_451902    | AACAGGTTTA | CAA.AGGTAC | TACTCCTCTA  |
| XM_002553230 | AGAAGGTTTA | CGA.AGGCAC | ACAACCCCTT  |
| XM_002494574 | AGAAGGTTTA | CCA.AGACAC | TGTCCCATTA  |
| YMR226C      | AGAATGTTTA | CAA.GGATAC | TACCCCATTT  |
| NM_001018495 | ATAATGTTTA | CAA.AAATAG | TGAGCCTTTG  |
| XM_501554    | ACGCTGTTTA | CGC.TGGAAC | CGAGCCTCTG  |
| XM_957621    | ATGCCGTCTA | TGC.CGGTGT | CGATCCCTTG  |

|              |            |            |            |
|--------------|------------|------------|------------|
|              | 801        |            | 850        |
| AABY01000127 | TTTGATCGTG | TACGCAACTT | CCAGGAAACA |
| AACH01000019 | TTTGATTGTG | TATGCGACTT | CAAGGAAGCA |
| AACA01000631 | CTTAATCGTA | TATTCCACTT | CCAGAAAGCA |



22/25

|              |             |            |            |            |            |
|--------------|-------------|------------|------------|------------|------------|
| AACF01000116 | TTTAATTGTA  | TACACCACTT | CAAGAAAACA | GAACACAGTA | ATTGCTGACA |
| AE016819     | TACAATTGTC  | TATGCCACAT | CCAGAAGATC | CAACACCGTA | GTTGCAGAGA |
| CR382139     | GGTTGTCGTA  | TTCGGACTTA | CAAGAAAGCA | AAATACCGTT | ATTGCTGAGA |
| XM_001387479 | AGTTATCGTG  | TTTGGTATCA | CCAGAAAGCA | GAACACCGTC | ATAGCCGAAA |
| XM_001482184 | AATCATTGTG  | TTTGCTGTGT | CGAGAAAGC  | CAAAACTGTC | ATTGCGGAAA |
| XM_002419771 | AGTGGTTGTT  | TTTGCATCTT | CAAGAAAACA | AAATACCGTT | ATTGCTGATA |
| CR380959     | TTTGATTGTG  | TACTCTACCA | GCAGAAAGCC | AAACATGGTG | GTAGCAGATG |
| XM_001645671 | TTTGATAGTT  | TACACCACTT | CTAGAAAATC | GAATACCGTT | ATCGCTGATG |
| SÄKLOH04730  | CCTTATTGTG  | TATGCTACTT | CTAGAAAGCC | TAATACTGTC | ATTGCGGATG |
| XM_451902    | CTTGATCGTT  | TATGCCACTT | CAAGAAAACC | TAATACTGTC | ATCGCTGATG |
| XM_002553230 | CCTAATCGTT  | TACGCAACCT | CGAGAAAACC | AAACACCGTC | ATCGCGGACG |
| XM_002494574 | TTTAATCGTC  | TATGCAACCT | CTAGAAAGCA | AAACACTGTC | ATTGCTGACA |
| YMR226C      | TCTGATCGTC  | TATGCAACTT | CCAGAAAACA | AAATACTGTA | ATTGCAGACA |
| NM_001018495 | GGTGATTCTT  | TTTGCCCTCA | CTCGCAGAGA | AAACGTCGTT | ATTGCCGATA |
| XM_501554    | GTTTCATCACC | TACACTCTCA | CTCGACGAGA | GAATGTCGTC | ATTGCCGATA |
| XM_957621    | GATCGTGGTG  | TTCGTCGTAA | CACGACGGGA | GAACGTTGTT | GTTGCTGATA |

851

900

|              |            |            |            |            |            |
|--------------|------------|------------|------------|------------|------------|
| AABY01000127 | CGCTAATCTT | TCCAACCAAC | CAAGCATCGC | CTCACCACAT | CTTCCGTGGA |
| AACH01000019 | CACTAATCTT | TCCTACCAAC | CAAGCGTCAC | CTTACCATAT | CTTTCGCGGG |
| AACA01000631 | CCCTGATCTT | CCCCACCAAC | CAAGCCTCGC | CCTACCACAT | CTTTCGCGGT |
| AACF01000116 | CTTTGATATT | CCCAACCTCT | CAAGGTTCCG | CATTCCACGT | CCATCGCGAT |
| AE016819     | TGGTCGTTTA | CCCATCCGCG | CAAGGTTCTC | TGTACGATAC | TCACCGCAAC |
| CR382139     | CATTAGTCTT | TTCAACCAAT | CAAGCCAGCT | CATCTCACTT | ATACCGTGAA |
| XM_001387479 | CCTTGGTATT | CCCAAGTCAC | CAGGCTTCTG | CCTCTCATGT | TTACAAGGCT |
| XM_001482184 | CTTTGGTGTT | TCCTACCCAT | CAGGCTGGAG | CAGTTCATGT | TCATAGAGGG |
| XM_002419771 | CTTTGATTTT | CCCAAATCAT | CAAGCTTCTC | CAGATCATGT | TTATAGAAAA |
| CR380959     | TCCTGGTCTT | CCCAACACAC | CAGGCATCGG | CTTCGCACAT | CTACAGGGGC |
| XM_001645671 | TTTTGGTATT | CCCAACATGC | CAAGCTTCTG | CATCCCATAT | CTATCGTGGA |
| SÄKLOH04730  | TTTTGGTTTT | TGCTTCAAAC | CAAGCATCCC | CTTACCATAT | CTATCGCGGT |
| XM_451902    | TTTTGGTATT | TGCTTCAAAC | CAAGCATCTC | CTTACCACAT | TTACCGTGGC |
| XM_002553230 | TTTTGGTTTT | CGCTTCGAAC | CAGGCTTCGC | CTTACCACAT | TTACCGTGGT |
| XM_002494574 | CTTTGATCTT | CTCTTCTAAC | CAGGCATCAC | CATACCACCT | CTACAGAGGC |
| YMR226C      | CTTTAATCTT | TCCAACAAC  | CAAGCGTCAC | CTCATCATAT | CTTCCGTGGA |
| NM_001018495 | CACCTGTTTT | CCCATCCCAT | CAAGGTGGTG | CCAATCATGT | GTACAGAAAG |
| XM_501554    | CTCTCATTTT | CCCCAACCAC | CAGGCTTCTC | CTACTCACGT | CTACCGAAAG |
| XM_957621    | CGTTGGTCTT | CCCTAGCCAT | CAGGCTGGCG | CTGGTATTAT | GCACCGCAAG |

901

921

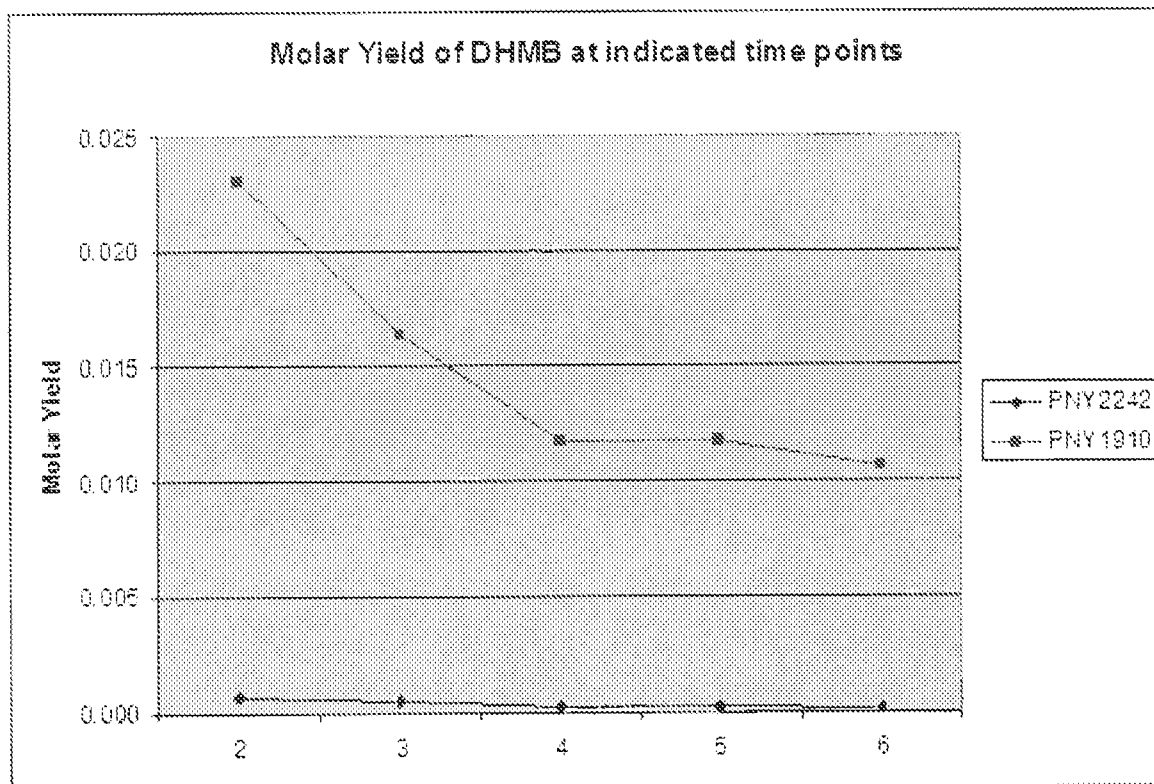
|              |            |            |       |                |
|--------------|------------|------------|-------|----------------|
| AABY01000127 | TGA.....   | .....      | ..... | (SEQ ID NO:41) |
| AACH01000019 | TGA.....   | .....      | ..... | (SEQ ID NO:47) |
| AACA01000631 | TAA.....   | .....      | ..... | (SEQ ID NO:43) |
| AACF01000116 | TAA.....   | .....      | ..... | (SEQ ID NO:45) |
| AE016819     | TAA.....   | .....      | ..... | (SEQ ID NO:49) |
| CR382139     | AGCGATAAAT | AA.....    | ..... | (SEQ ID NO:53) |
| XM_001387479 | CCTAAGTAG. | .....      | ..... | (SEQ ID NO:55) |
| XM_001482184 | CCGCTTGAGT | GA.....    | ..... | (SEQ ID NO:57) |
| XM_002419771 | CCTAATTAA. | .....      | ..... | (SEQ ID NO:61) |
| CR380959     | GACTAA.... | .....      | ..... | (SEQ ID NO:51) |
| XM_001645671 | TAA.....   | .....      | ..... | (SEQ ID NO:59) |
| SÄKLOH04730  | GACTAA.... | .....      | ..... | (SEQ ID NO:69) |
| XM_451902    | GAATAG.... | .....      | ..... | (SEQ ID NO:67) |
| XM_002553230 | TAG.....   | .....      | ..... | (SEQ ID NO:65) |
| XM_002494574 | TCTCAAGACA | AAACCAATTG | A     | (SEQ ID NO:63) |
| YMR226C      | TAA.....   | .....      | ..... | (SEQ ID NO:24) |
| NM_001018495 | CAAGCGTAG. | .....      | ..... | (SEQ ID NO:73) |
| XM_501554    | AACTGA.... | .....      | ..... | (SEQ ID NO:71) |

23/25

NM\_957621 TCGACATGA. .... (SEQ ID NO:75)

24/25

Figure 13



25/25

Figure 14

