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(54) **INCREASED HETEROLOGOUS FE-S ENZYME ACTIVITY IN YEAST**

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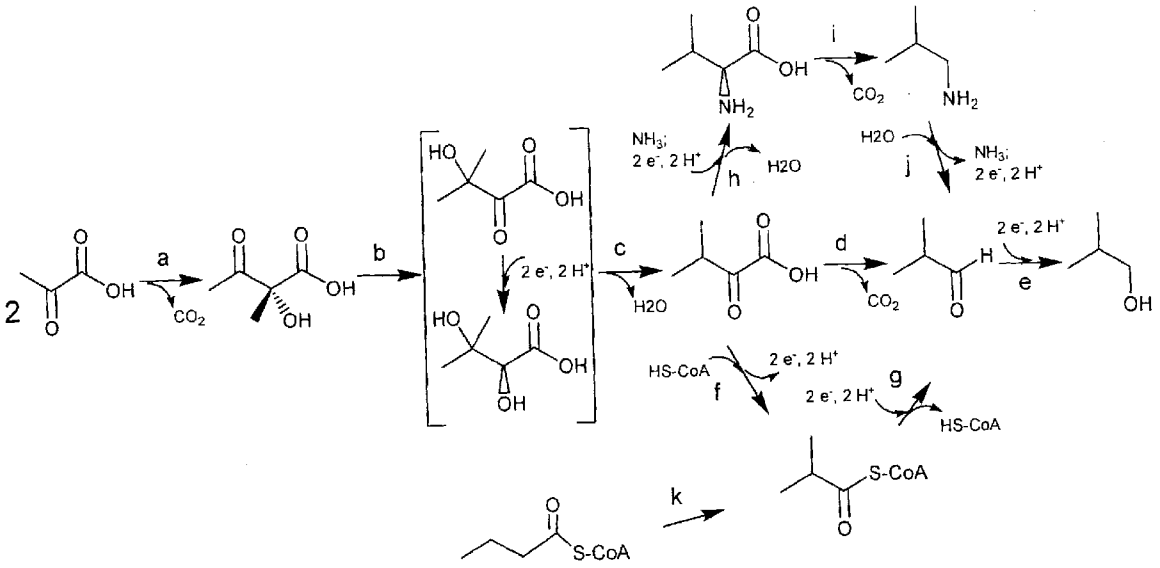
(57) **ABSTRACT**

Yeast strains were engineered that have increased activity of heterologous proteins that require binding of an Fe—S cluster for their activity. The yeast strains have reduced activity of an endogenous Fe—S protein. Activities of heterologous fungal or plant 2Fe-2S dihydroxy-acid dehydratases and Fe—S propanediol dehydratase reactivase were increased for increased production of products made using biosynthetic pathways including these enzymes, such as valine, isoleucine, leucine, pantothenic acid (vitamin B5), isobutanol, 2-butanone and 2-butanol.



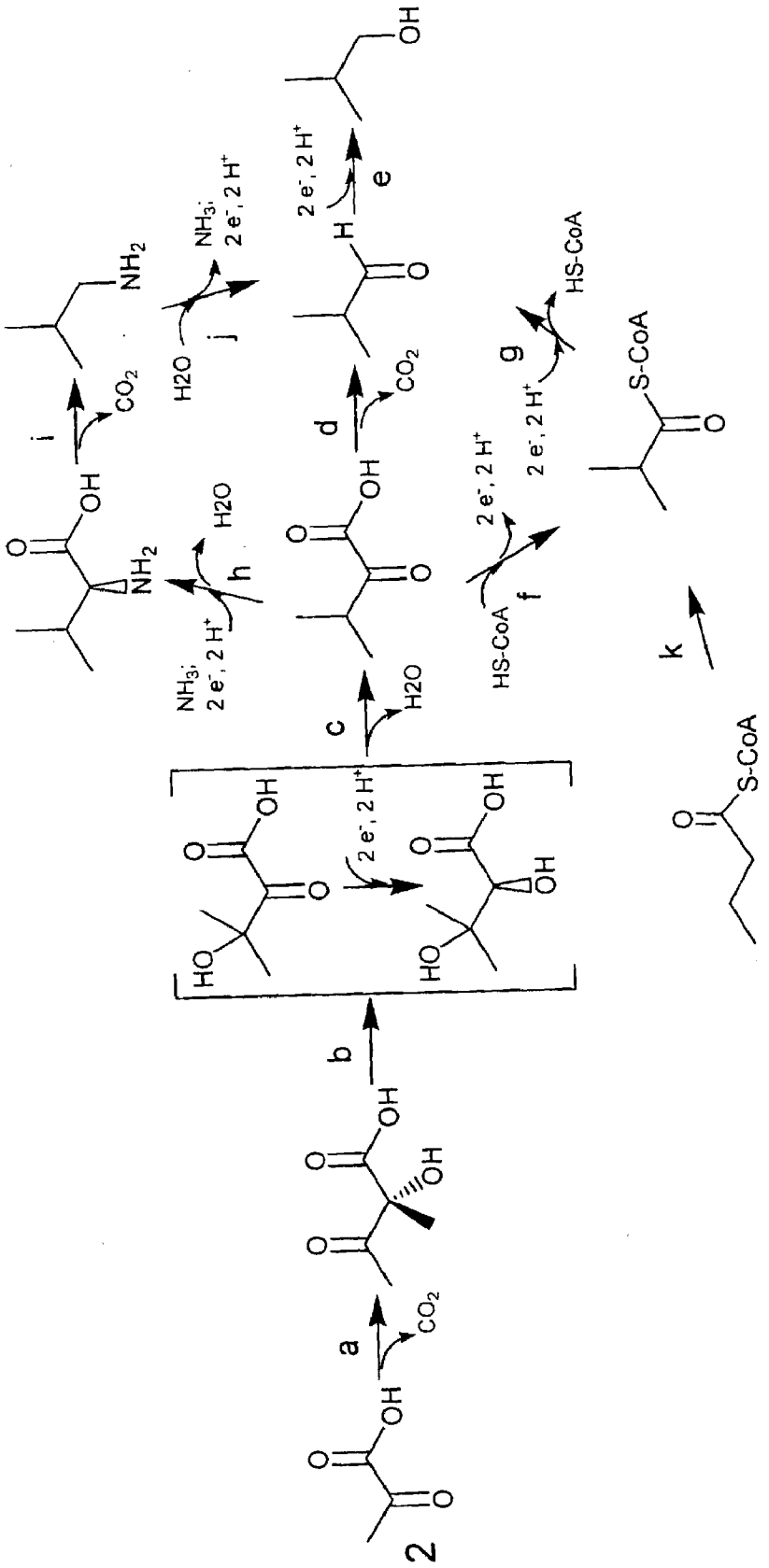


FIG. 1

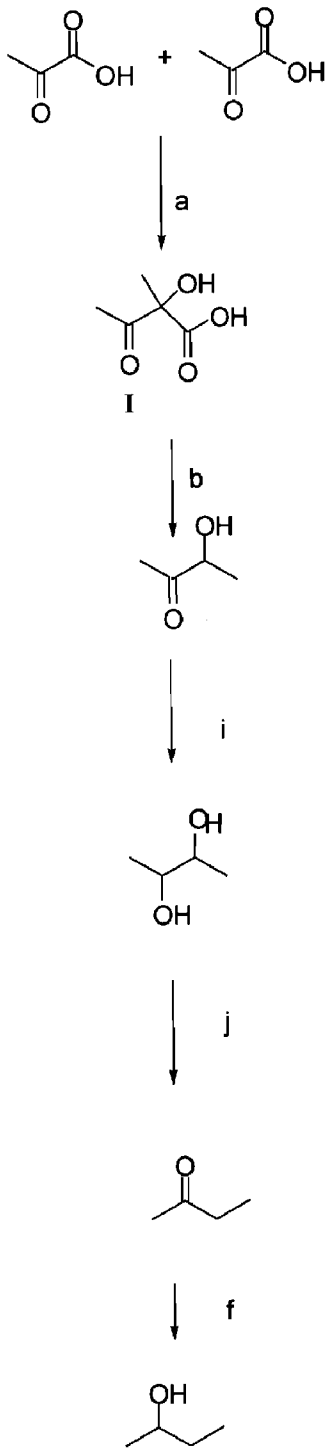


FIG. 2

INCREASED HETEROLOGOUS FE-S ENZYME ACTIVITY IN YEAST

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to and claims the benefit of priority to U.S. Provisional Application Nos. 61/100,801 filed Sep. 29, 2008 and 61/100,806 filed Sep. 29, 2008. The entirety of each is hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] The invention relates to the field of industrial microbiology and the expression of proteins that require an iron-sulfur cluster for activity. More specifically, expression of heterologous Fe—S protein activity in yeast cells is improved through specific host gene inactivation.

BACKGROUND OF THE INVENTION

[0003] Engineering of yeast for fermentative production of commercial products is an active and growing field. Enzymatic pathways engineered for biosynthesis of some products include enzymes that require binding of an iron-sulfur (Fe—S) cluster for activity. Dihydroxy-acid dehydratase (DHAD) is one example. DHAD is part of naturally occurring biosynthetic pathways producing valine, isoleucine, leucine and pantothenic acid (vitamin B5). Increased expression of DHAD activity is desired for enhanced microbial production of branched chain amino acids or of pantothenic acid. In addition, DHAD catalyzed conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate is a common step in the multiple isobutanol biosynthetic pathways that are disclosed in co-pending US Patent Pub No. US 20070092957 A1. Disclosed therein is engineering of recombinant microorganisms for production of isobutanol, which is useful as a fuel additive and whose availability may reduce the demand for petrochemical fuels.

[0004] Diol dehydratase provides an enzyme activity in a biosynthetic pathway for production of 2-butanone and 2-butanol that is disclosed in co-pending US Patent Pub No. US 2007-0292927A1. Disclosed in US Patent Pub No. US20090155870 is a butanediol dehydratase that is useful for expression in this pathway due to its coenzyme B-12 independence. A diol dehydratase reactivase that is an Fe—S cluster protein required for activity of the B12-independent butanediol dehydratase, is also disclosed in US Patent Pub No. US20090155870. 2-Butanone, also referred to as methyl ethyl ketone (MEK), is a widely used solvent, extractant and activator of oxidative reactions, as well as a substrate for chemical synthesis of 2-butanol. 2-butanol is useful as a fuel additive, whose availability may reduce the demand for petrochemical fuels.

[0005] For improved production of compounds synthesized in pathways including an Fe—S cluster containing enzyme, it is desirable to provide a host cell capable of expressing high levels of this enzymatic activity in the production host of interest. Whereas a number of commercially relevant bacteria and yeast can express activity of Fe—S cluster containing proteins, this activity is at levels far below what is commercially useful for enhancing introduced biosynthetic pathways. Consequently a need exists for the discovery of host cells capable of expressing activity of Fe—S

ing high functional expression of heterologous Fe—S cluster containing enzymes is problematic due to the Fe—S cluster requirement, which involves availability and proper loading of the cluster into the apo-protein.

SUMMARY OF THE INVENTION

[0006] Provided herein are recombinant yeast host cells comprising at least one heterologous Fe—S cluster protein wherein the yeast host has reduced expression of at least one endogenous Fe—S cluster protein.

[0007] The recombinant yeast cell may be grown under suitable conditions for the production of products including isobutanol, 2-butanol and 2-butanone.

[0008] In one aspect, the recombinant yeast cell comprises a disruption in the gene encoding the at least one endogenous Fe—S cluster protein.

[0009] In another aspect, the endogenous Fe—S cluster protein is selected from the group consisting of dihydroxy-acid dehydratase, isopropylmalate dehydratase, sulfite reductase, glutamate dehyddrogenase, biotin synthase, aconitase, homoaconitase, lipoate synthase, ferredoxin maturation, NADH ubiquinone oxidoreductase, succinate dehydrogenase, ubiquinol-cytochrome-c reductase, ABC protein Rli1, NTPase Nbp35, and hydrogenase-like protein.

[0010] In another aspect, the yeast is selected from the group consisting of *Saccharomyces, Schizosaccharomyces, Hansenula, Candida, Kluyveromyces, Yarrowia* and *Pichia.*

[0011] In another aspect, the endogenous Fe—S protein is expressed in the mitochondria, and in another embodiment, the endogenous Fe—S cluster protein has an activity selected from the group consisting of: dihydroxy-acid dehydratase and isopropylmalate dehydratase activity.

[0012] In another aspect, the host cell is *Saccharomyces* expressing a gene encoding a polypeptide having the amino acid sequence as set forth in SEQ ID NO:114.

[0013] In some embodiments, the at least one heterologous Fe—S cluster protein is selected from the group consisting of fungal 2Fe-2S dihydroxy-acid dehydratases and plant 2Fe-2S dihydroxy-acid dehydratases. In one embodiment, the heterologous fungal or plant 2Fe-2S cluster dihydroxy-acid dehydratase is expressed in the cytosol. In one embodiment, the heterologous fungal or plant 2Fe-2S cluster dihydroxy-acid dehydratase is a polypeptide having an amino acid sequence that matches the Profile HMM of table 9 with an E value of $<10^{-5}$ wherein the polypeptide additionally comprises all three conserved cysteines, corresponding to positions 56, 129, and 201 in the amino acids sequences of the *Streptococcus mutans* DHAD enzyme corresponding to SEQ ID NO:179. In one embodiment, the heterologous fungal or plant 2Fe-2S cluster dihydroxy-acid dehydratase is a polypeptide having an amino acid sequence that has at least about 95% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150 and 152. In one embodiment, the heterologous fungal or plant 2Fe-2S cluster dihydroxy-acid dehydratase is a polypeptide having an amino acid sequence that is at least about 90% identical to SEQ ID NO:114 using the Clustal W method of alignment using the default parameters of GAP PENALTY=10, GAP LENGTH PENALTY=0.

[0014] In another aspect, a method for the conversion of 2,3-dihydroxyisovalerate to α-ketoisovalerate is provided, said method comprising:

[0015] a) providing (1) a recombinant yeast host cell comprising at least one heterologous gene encoding a 2Fe-2S dihydroxy-acid dehydratase wherein the recombinant yeast host cell has reduced activity of at least one endogenous Fe—S cluster protein; and (2) a source of 2,3-dihydroxyisovalerate; and

[0016] b) growing the recombinant host cell of (a) with said source of 2,3-dihydroxyisovalerate under conditions where the 2,3-dihydroxyisovalerate is converted by the host cell to α-ketoisovalerate.

[0017] In another aspect, a method for the conversion of 2,3-butanediol to 2-butanone is provided, said method comprising:

[0018] a) providing (1) a recombinant yeast host cell comprising at least one heterologus gene encoding a Fe—S propanediol dehydratase reactivase wherein the recombinant yeast host cell has reduced activity of at least one endogenous Fe—S cluster protein; and (2) a source of 2,3-butanediol; and

[0019] b) growing the recombinant host cell of (a) with said source of 2,3-butanediol under conditions where 2,3-butanediol is converted by the hots cell to 2-butanone.

[0020] Also provided is a method for the production of isobutanol comprising growing a recombinant yeast host cell disclosed herein under conditions wherein isobutanol is produced.

[0021] In other embodiments, the at least one heterologous Fe—S cluster protein has Fe—S propanediol dehydratase reactivase activity. In some embodiments, the at least one heterologous Fe—S cluster protein having Fe—S propanediol dehydratase reactivase activity is a propanediol deydratase reactivase having an amino acid sequence that is at least about 90% identical to the amino acid sequence as set forth in SEQ ID NO:44 using 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 over the full length of the protein sequence.

[0022] In some embodiments, the cell produces 2-butanol, and in some embodiments the cell produces 2-butanone. In some embodiments, the cell comprises a 2-butanol biosynthetic pathway, and in some embodiments, the cell comprises a 2-butanone biosynthetic pathway.

BRIEF DESCRIPTION OF THE FIGURES AND SEQUENCE DESCRIPTIONS

[0023] The invention can be more fully understood from the following detailed description, figures, and the accompanying sequence descriptions, which form a part of this application.

[0024] FIG. **1** shows biosynthetic pathways for isobutanol production.

[0025] FIG. **2** shows a biosynthetic pathway for 2-butanone and 2-butanol production.

[0026] Table 9 is a table of the Profile HMM for dihydroxy-acid dehydratases based on enzymes with assayed function prepared as described in Example 1. Table 9 is submitted herewith electronically and is incorporated herein by reference.

[0027] The following sequences conform with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures—the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST. 25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

TABLE 1

Inactivation target Fe—S protein encoding genes

Organism and gene	SEQ ID NO: Nucleic Acid	SEQ ID NO: Peptide
Saccharomyces cerevisiae LEU1	1	2
Schizosaccharomyces pombe LEU1	3	4
Candida galbrata CBS 138 LEU1	5	6
Candida albicans SC 5314 LEU1	7	8
Kluyveromyces lactis LEU1	9	10
Yarrowia lipolytica LEU1	11	12
Pichia stipitis LEU1	13	14
Saccharomyces cerevisiae YJM789 ILV3	111	112
Schizosaccharomyces pombe ILV3	93	94
Candida galbrata CBS 138 ILV3	107	108
Candida albicans SC5314 ILV3	101	102
Kluyveromyces lactis ILV3	113	114
Yarrowia lipolytica ILV3	105	106
Pichia stipitis CBS 6054 ILV3	103	104
Saccharomyces cerevisiae ACO1	153	154
Schizosaccharomyces pombe (chromosome II) ACO1	155	156
Schizosaccharomyces pombe (chromosome I) ACO1	157	158
Kluyveromyces lactis NRRL Y-1140 ACO1	159	160
Candida albicans SC5314 ACO1	161	162
Yarrowia lipolytica CLIB122 ACO1	163	164
Pichia stipitis CBS 6054 ACO1	165	166
Candida glabrata CBS138 (chromosome F) ACO1	167	168
Candida glabrata CBS138 (chromosome D) ACO1	169	170
Candida glabrata CBS138 (chromosome K) ACO1	171	172

TABLE 2

Fungal and plant 2Fe—2S DHADs in addition to those in Table 1

Description	SEQ ID NO: Nucleic acid	SEQ ID NO: Peptide
Chlamydomonas reinhardtii	45	46
Ostreococcus lucimarinus CCE9901	47	48
Vitis vinifera (Unnamed protein product: CAO71581.1)	49	50
Vitis vinifera (CAN67446.1)	51	52
Arabidopsis thaliana	53	54
Oryza sativa (indica cultivar-group)	55	56
Physcomitrella patens subsp. *patens*	57	58
Chaetomium globosum CBS 148.51	59	60
Neurospora crassa OR74A	61	62
Magnaporthe grisea 70-15	63	64
Gibberella zeae PH-1	65	66
Aspergillus niger	67	68
Neosartorya fischeri NRRL 181 (XP_001266525.1)	69	70
Neosartorya fischeri NRRL 181 (XP_001262996.1)	71	72
Aspergillus niger (An03g04520)	73	74
Aspergillus niger (An14g03280)	75	76
Aspergillus terreus NIH2624	77	78
Aspergillus clavatus NRRL 1	79	80
Aspergillus nidulans FGSC A4	81	82

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