Supporting Information for:

Mitochondrial Compartmentalization Confers Specificity to the 2-Ketoacid Recursive Pathway: Increasing Isopentanol Production in *Saccharomyces cerevisiae*

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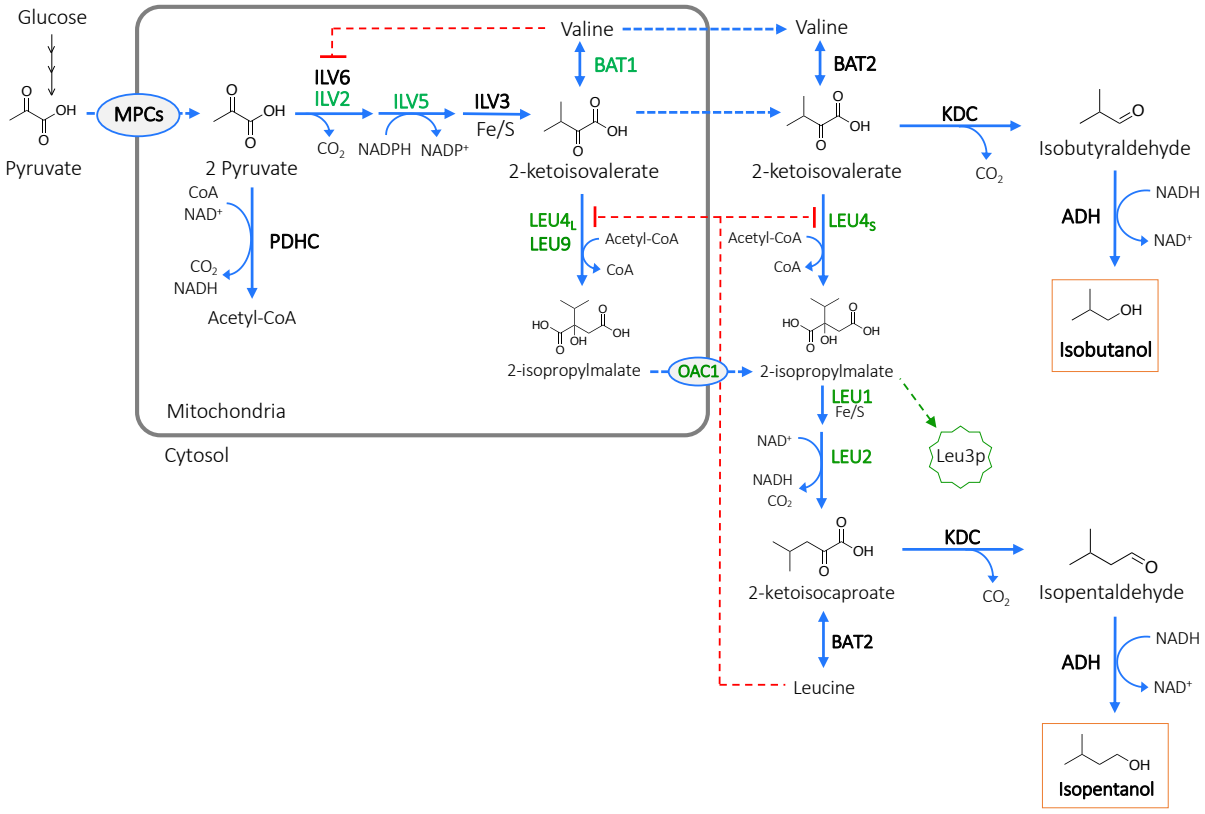
Supplementary Table S1. Previous efforts to engineer *Saccharomyces cerevisiae* for isopentanol production.

Location of *LEU* Genes	Titer (mg/L)	Yield (mg product per g sugar)	Carbon Source	Ratio of Isopentanol to Isobutanol	Supplementary Reference
Native*	130 ± 21	1.30	Glucose	0.27	1
Native	765.7	7.66	Glucose	2.60	2
Cytosol	523 ± 39	13.07	Galactose	0.97	3
Native	561.2	14.18	Galactose	1.74	4
Mitochondria[#]	482 ± 18	4.82	Glucose	14.3	This Study
Mitochondria[+]	1241 ± 55	11.48	Glucose	6.12	This Study

* Termination enzymes KDC and ADH are targeted to mitochondria.

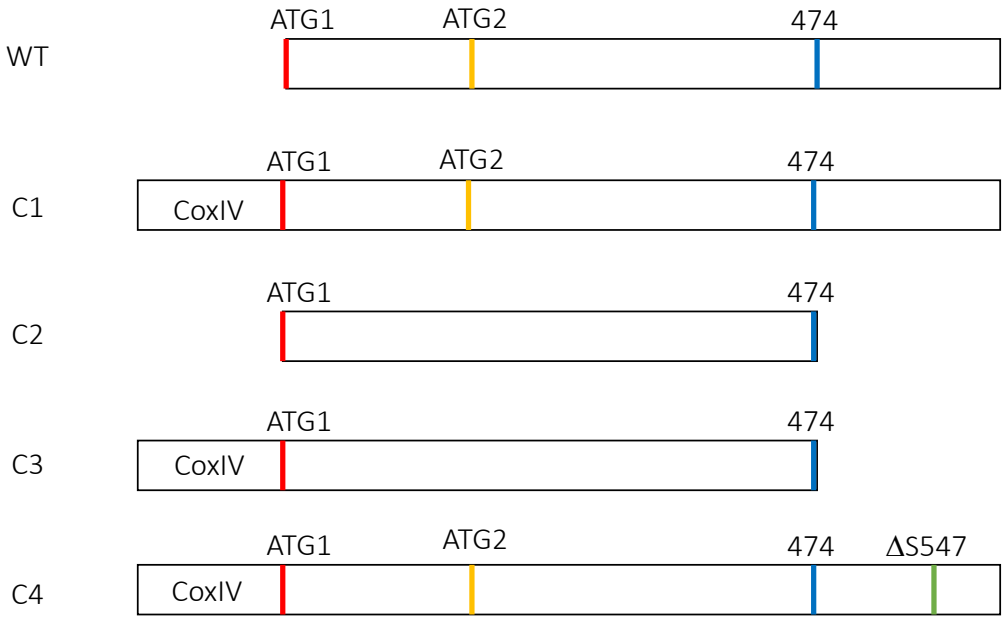
[#] Expressing *LEU* gene products in mitochondria of a *leu4Δ leu9Δ* strain (SHy76). Highest ratio of isopentanol to isobutanol reported in *S. cerevisiae*.

[+] Expressing *LEU* gene products in mitochondria and the cytosol of a *bat1Δ leu4Δ leu9Δ oac1Δ* strain (SHy252). Highest isopentanol titer as well as yield from glucose reported in *S. cerevisiae*.

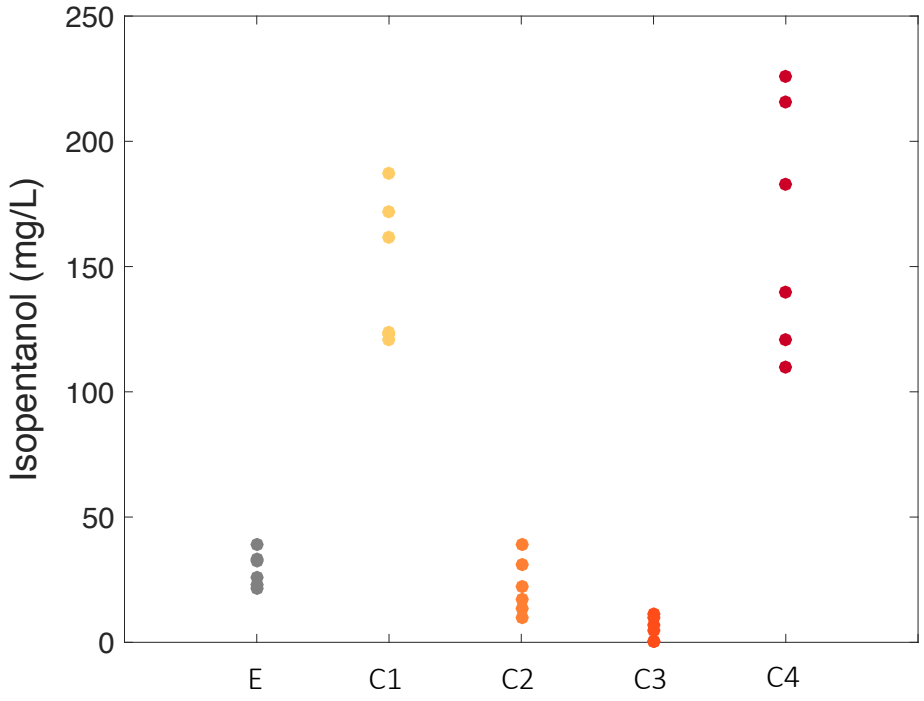


Supplementary Figure S1. Schematic of isobutanol and isopentanol biosynthesis from glucose in *Saccharomyces cerevisiae*. Acetolactate synthase (*ILV2*), ketol-acid reductoisomerase (*ILV5*), and dihydroxyacid dehydratase (*ILV3*) convert two molecules of pyruvate to one molecule of 2-ketoisovalerate (KIV), which can either be converted to isobutanol by a ketoacid decarboxylase (KDC) and an alcohol dehydrogenase (ADH), or elongated by 2-isopropylmalate synthase (*LEU4* or *LEU9*), isopropylmalate isomerase (*LEU1*), and 3-isopropylmalate dehydrogenase (*LEU2*) to 2-ketoisocaproate (KIC). Transcription and translation of *LEU4* results in short (*LEU4*$_S$) and long (*LEU4*$_L$) forms of the protein, located in the cytosol and the mitochondria, respectively.[5] Alternatively, pyruvate can be converted to acetyl-CoA in the mitochondria by the pyruvate dehydrogenase complex (PDHC). While PDHC activity reduces the amount of pyruvate channeled toward isopentanol, Leu4p also requires acetyl-CoA as a cofactor, making PDHC activity important for mitochondrial Leu4p activity, and therefore isopentanol production. Genes labeled in green are regulated by transcriptional activator Leu3p. Red dashed lines indicate feedback inhibition.

a.

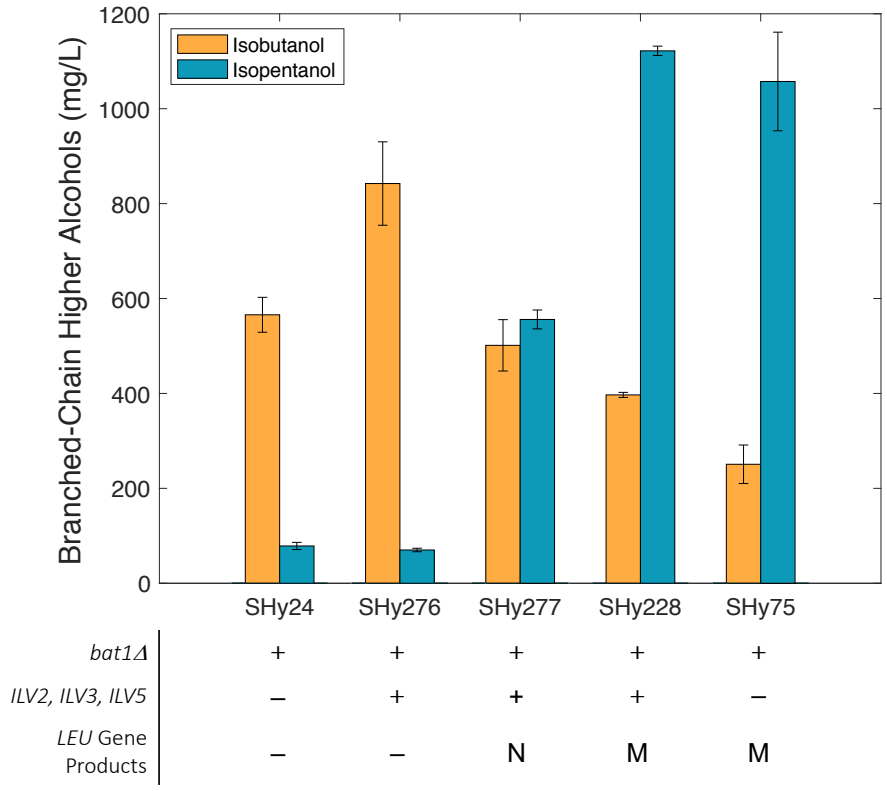

WT
ATG1
ATG2
474
C1
CoxIV
ATG1
ATG2
474
C2
ATG1
474
C3
CoxIV
ATG1
474
C4
CoxIV
ATG1
ATG2
474
ΔS547

b.

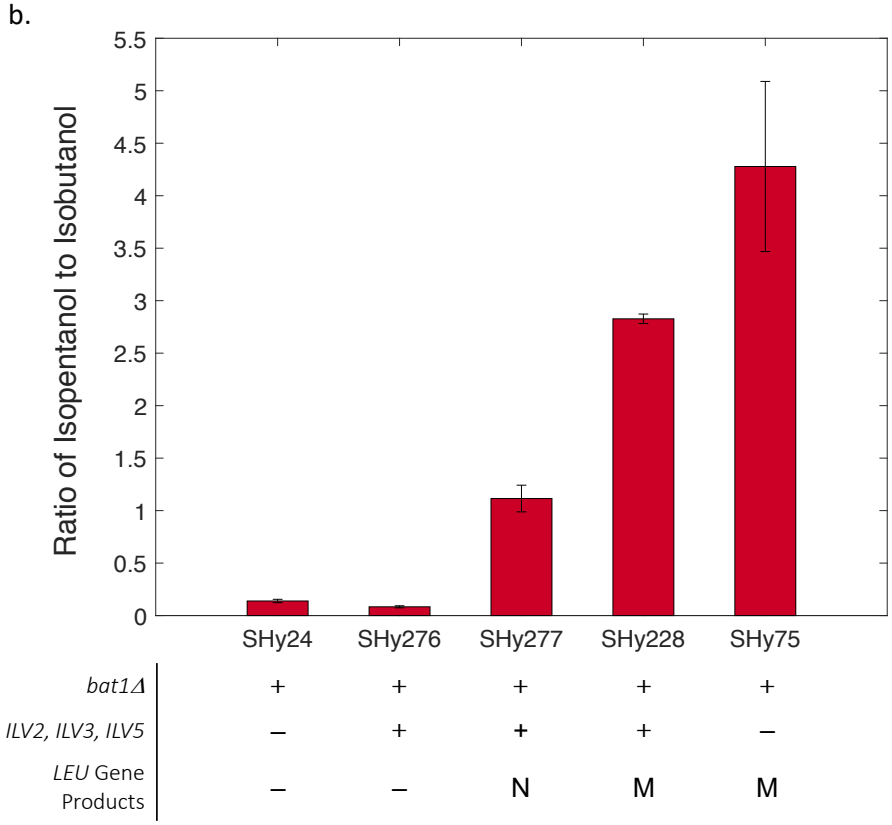

Isopentanol (mg/L)
250
200
150
100
50
0
E
C1
C2
C3
C4

Supplementary Figure S2. Design and testing of putative leucine insensitive Leu4p variants. (a) Schematic of constructs created to achieve a leucine feedback insensitive and mitochondrially localized 2-IPMS. Constructs 1, 2, 3, and 4 correspond to plasmids pSH40, pSH39, pSH41, and pSH42, respectively (Supplementary Table S2). ATG2 corresponds to Methionine 31, which is mutated to isoleucine in C2 and C3. (b) Isopentanol titers of CEN.PK2-1C *Δleu4Δleu9* transformants – SHy70, SHy69, SHy71, and SHy261 – harboring 2μ plasmids constitutively expressing C1, C2, C3, or C4 respectively, as shown in Figure 3a. The *Δleu4Δleu9* transformants harboring empty 2μ plasmids (E) are not viable in the absence of leucine. Thus, the wild type CEN.PK2-1C background was used for empty plasmid controls (E). Six colonies were screened from each transformation. Isopentanol titers were measured after 48-h high-cell-density fermentations in SC-Leu medium containing 10% glucose.

a.

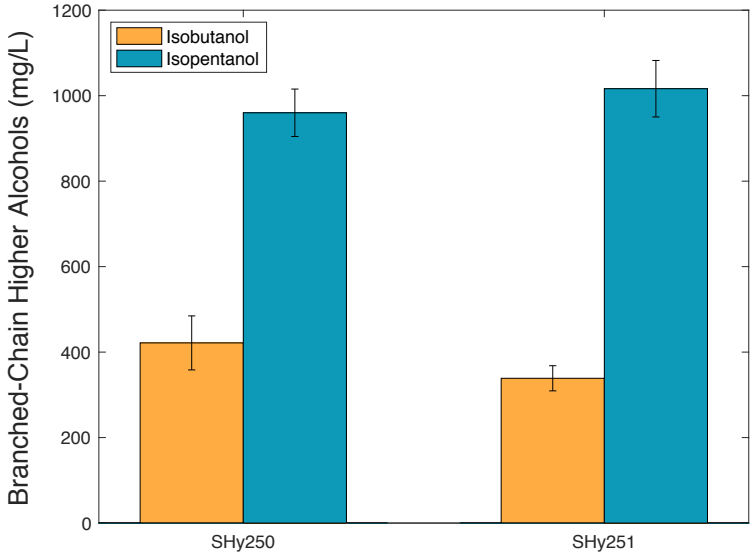

Isobutanol
Isopentanol
Branched-Chain Higher Alcohols (mg/L)
1200
1000
800
600
400
200
0
SHy24
SHy276
SHy277
SHy228
SHy75
bat1Δ + + + + +
ILV2, ILV3, ILV5 – + + + –
LEU Gene Products – – N M M

b.


Ratio of Isopentanol to Isobutanol
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
SHy24
SHy276
SHy277
SHy228
SHy75
bat1Δ + + + + +
ILV2, ILV3, ILV5 – + + + –
LEU Gene Products – – N M M

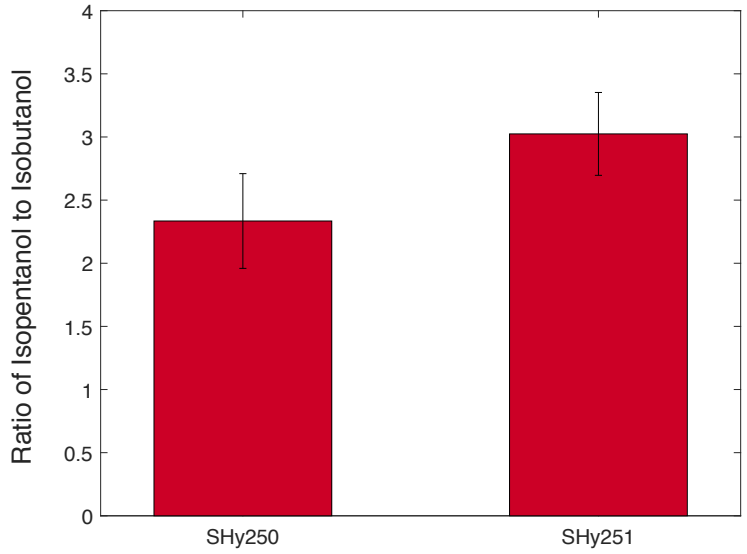
Supplementary Figure S3. Effects of overexpressing *ILV2, ILV3,* and *ILV5* on BCHA production in *bat1Δ* strains. (a) Isobutanol and isopentanol titers resulting from overexpression of *LEU* gene products in addition to *ILV2, ILV3,* and *ILV5* in *bat1Δ* strains. Isobutanol (orange) and isopentanol (blue) titers were determined after 48-h high-cell-density fermentations in SC-Ura-Val medium containing 10% glucose. Error bars represent the standard deviation of three or more independent fermentations. (b) Ratios of isopentanol to isobutanol titers of the strains detailed in (a). Error bars represent the propagated error calculated using the standard deviations shown in (a). Strains either harbor an empty 2μ plasmid (−), or one overexpressing $LEU4^{S547\Delta}$, *LEU1,* and *LEU2* gene products in their native subcellular locations (N) or in mitochondria (M).

a.



bat1Δ leu4Δ leu9Δ	+	+
oac1Δ	–	+
LEU Gene Products	M	M

b.



bat1Δ leu4Δ leu9Δ	+	+
oac1Δ	–	+
LEU Gene Products	M	M

Supplementary Figure S4. Effects of *OAC1* deletion on isobutanol and isopentanol titers. Both strains contain a 2μ plasmid overexpressing $LEU4^{S547\Delta}$, *LEU1,* and *LEU2* in mitochondria (M). (a) Isobutanol (orange) and isopentanol (teal) titers of *bat1Δ leu4Δ leu9Δ* strains with or without *OAC1* deletion. BCHA titers were measured after 48-h high-cell-density fermentations in SC-Ura-Val medium containing 10% glucose. Error bars represent the standard deviation of three or more independent fermentations. (b) Ratios of isopentanol to isobutanol titers of the strains detailed in (a). Error bars represent the propagated error calculated using the standard deviations shown in (a).

Supplementary Table S2. Plasmids used in this study

Plasmid	Description	Source
pAG26	Plasmid containing *hphMX6* deletion cassette	6
pUG6	Plasmid containing *loxP-kanMX-loxP* deletion cassette	7
p173	pRS426 (2µ plasmid, *URA3* marker)	8
p222	CEN plasmid, *URA3* marker, P_{GAL1}-Cre-T_{CYC1}	7
p553	Integration vector, *TRP1* marker	This study
p558	Integration vector, *HIS3* marker	This study
p715	pRSII425 (2µ plasmid, *LEU2* marker)	9
pYZ23	Delta integration vector containing *lox66-BleMX-lox71* cassette	10
pYZ33	Delta integration vector containing *lox66-BleMX-lox71* cassette, P_{THD3}-*ILV2*- HA-T_{ADH1} P_{PGK1}-*ILV3*-His-T_{CYC1} P_{TEF1}-*ILV5*-Myc-T_{ACT1}	This study
pYZ84	Plasmid containing *lox66-natMX6-lox71* deletion cassette	11
pSH39	2µ plasmid, *LEU2* marker, P_{THD3}-$LEU4^{M31I/\Delta(E475–A619)}$-HA-$T_{ADH1}$	This study
pSH40	2µ plasmid, *LEU2* marker, P_{THD3}-CoxIV-*LEU4*-HA-T_{ADH1}	This study
pSH41	2µ plasmid, *LEU2* marker, P_{THD3}-CoxIV-$LEU4^{M31I/\Delta(E475–A619)}$HA-$T_{ADH1}$	This study
pSH42	2µ plasmid, *LEU2* marker, P_{THD3}-CoxIV-$LEU4^{S547\Delta}$-HA-T_{ADH1}	This study
pSH47	2µ plasmid, *URA3* marker, P_{TEF1}-Cdc9-*LEU1*-Myc-T_{ACT1} P_{PGK1}-CoxVI-*LEU2*-His-T_{CYC1} P_{TDH3}-CoxIV- $LEU4^{S547\Delta}$-HA-T_{ADH1}	This study
pSH54	2µ plasmid, *URA3* marker, P_{TEF1}-*LEU1*-Myc-T_{ACT1} P_{PGK1}-*LEU2*-His-T_{CYC1} P_{TDH3}- $LEU4^{S547\Delta}$-HA-T_{ADH1}	This study
pSH132	Integration vector, *HIS3* marker, P_{TEF1}-*LEU1*-Myc-T_{ACT1} P_{PGK1}-*LEU2*-His-T_{CYC1} P_{TDH3}- $LEU4^{S547\Delta}$-HA-T_{ADH1}	This study

Supplementary Table S3. Yeast strains used in this study

Strain	Description	Genotype	Source
CEN.PK2-1C	Wild type	*MATa ura3–52 trp1–289 leu2–3,112 his3Δ1 MAL2–8^{C} SUC2*	12
FY4/5	Wild type	S288C MATa/α (prototroph)	13
SHy1	*bat1Δ*	CEN.PK2-1C *bat1Δ::hphMX6*	11
SHy23	Wild type (p173)	CEN.PK2-1C (empty plasmid)	11
SHy24	*bat1Δ* (p173)	SHy1 (empty plasmid)	11
SHy51	*leu4Δ*	CEN.PK2-1C *leu4Δ::loxP-kanMX-loxP*	This study
SHy60	*leu4Δ leu9Δ*	SHy51*, leu9Δ::lox66-natMX6-lox71*	This study
SHy69	*leu4Δ leu9Δ* (pSH39)	SHy60 (*LEU4$^{M31I/\Delta(E475–A619)}$*)	This study
SHy70	*leu4Δ leu9Δ* (pSH40)	SHy60 (CoxIV-*LEU4*)	This study
SHy71	*leu4Δ leu9Δ* (pSH41)	SHy60 (CoxIV-*LEU4$^{M31I/\Delta(E475–A619)}$*)	This study
SHy74	Wild type (pSH47)	CEN.PK2-1C (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4$^{S547\Delta}$*)	This study
SHy75	*bat1Δ* (pSH47)	SHy1 (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4$^{S547\Delta}$*)	This study
SHy76	*leu4Δ leu9Δ* (pSH47)	SHy60 (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4$^{S547\Delta}$*)	This study
SHy77	*bat1Δ leu4Δ leu9Δ* (pSH47)	YZy117 (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4$^{S547\Delta}$*)	This study
SHy96	*bat1Δ* (pSH54)	SHy1 (*LEU1*, *LEU2, LEU4$^{S547\Delta}$*)	This study
SHy97	*bat1Δ leu4Δ leu9Δ* (pSH54)	YZy117 (*LEU1*, *LEU2, LEU4$^{S547\Delta}$*)	This study
YZy117	*bat1Δ leu4Δ leu9Δ*	CEN.PK2-1C *bat1Δ::hphMX6, leu4Δ::loxP-kanMX-loxP, leu9Δ::lox66-natMX6-lox71*	This study
SHy126	*bat1Δ leu4Δ leu9Δ* (p173)	YZy117 (empty plasmid)	This study

SHy128	Wild type (pSH54)	CEN.PK2-1C (*LEU1*, *LEU2, LEU4*$^{S547\Delta}$)	This study
SHy129	*leu4Δ leu9Δ* (p173)	SHy60 (empty plasmid)	This study
SHy130	*leu4Δ leu9Δ* (pSH54)	SHy60 (*LEU1*, *LEU2, LEU4*$^{S547\Delta}$)	This study
SHy149	*bat1Δ leu4Δ leu9Δ oac1Δ*	CEN.PK2-1C *bat1Δ::hphMX6, leu4Δ::loxP, leu9Δ::lox66, oac1Δ::loxP-kanMX-loxP*	This study
SHy172	*bat1Δ leu4Δ leu9Δ oac1Δ* (pSH47)	SHy149 (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4*$^{S547\Delta}$)	This study
SHy209	*bat1Δ* (pYZ33)	SHy1 (*ILV2, ILV3, ILV5*)	This study
SHy228	*bat1Δ* (pYZ33, pSH47)	SHy209 (Cdc9-*LEU1*, CoxVI-*LEU2,* CoxIV-*LEU4*$^{S547\Delta}$)	This study
SHy246	*bat1Δ leu4Δ leu9Δ oac1Δ* (pSH47, pSH132)	SHy172 (*LEU4*$^{S547\Delta}$, *LEU1*, *LEU2*)	This study
SHy250	*bat1Δ leu4Δ leu9Δ* (pSH47, p553)	SHy77 (empty integration vector)	This study
SHy251	*bat1Δ leu4Δ leu9Δ oac1Δ* (pSH47, p553)	SHy172 (empty integration vector)	This study
SHy252	*bat1Δ leu4Δ leu9Δ oac1Δ* (pSH47, pSH132, p553)	SHy246 (empty integration vector)	This study
SHy255	*bat1Δ leu4Δ leu9Δ oac1Δ* (pSH47, p553, p558)	SHy251 (empty integration vector)	This study
SHy258	Wild type (p715)	SHy20 (empty plasmid)	This study
SHy260	*leu4Δ leu9Δ* (p715)	SHy60 (empty plasmid)	This study
SHy261	*leu4Δ leu9Δ* (pSH42)	SHy60 (CoxIV-*LEU4*$^{S547\Delta}$*)*	This study
SHy276	*bat1Δ* (pYZ33, pSH54)	SHy209 (empty plasmid)	This study
SHy277	*bat1Δ* (pYZ33, p173)	SHy209 (*LEU1*, *LEU2, LEU4*$^{S547\Delta}$)	This study

Supplementary References

[1] Avalos, J. L., Fink, G. R., and Stephanopoulos, G. (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols, *Nat. Biotechnol. 31*, 335-341.

[2] Park, S. H., Kim, S., and Hahn, J. S. (2014) Metabolic engineering of Saccharomyces cerevisiae for the production of isobutanol and 3-methyl-1-butanol, *Appl. Microbiol. Biotechnol. 98*, 9139-9147.

[3] Yuan, J., Mishra, P., and Ching, C. B. (2017) Engineering the leucine biosynthetic pathway for isoamyl alcohol overproduction in Saccharomyces cerevisiae, *J. Ind. Microbiol. Biotechnol. 44*, 107-117.

[4] Yuan, J., Chen, X., Mishra, P., and Ching, C.-B. (2017) Metabolically engineered Saccharomyces cerevisiae for enhanced isoamyl alcohol production, *Appl. Microbiol. Biotechnol. 101*, 465-474.

[5] Beltzer, J. P., Morris, S. R., and Kohlhaw, G. B. (1988) Yeast LEU4 encodes mitochondrial and nonmitochondrial forms of alpha-isopropylmalate synthase, *J. Biol. Chem. 263*, 368-374.

[6] Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae, *Yeast 15*, 1541-1553.

[7] Güldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast, *Nucleic Acids Res. 24*, 2519-2524.

[8] Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors, *Gene 110*, 119-122.

[9] Chee, M. K., and Haase, S. B. (2012) New and Redesigned pRS Plasmid Shuttle Vectors for Genetic Manipulation of Saccharomyces cerevisiae, *G3 2*, 515-526.

[10] Zhao, E. M., Zhang, Y., Mehl, J., Park, H., Lalwani, M. A., Toettcher, J. E., and Avalos, J. L. (2018) Optogenetic regulation of engineered cellular metabolism for microbial chemical production, *Nature 555*, 683.

[11] Hammer, S. K., and Avalos, J. L. (2017) Uncovering the role of branched-chain amino acid transaminases in Saccharomyces cerevisiae isobutanol biosynthesis, *Metab. Eng. 44*, 302-312.

[12] Entian, K.-D., and Kötter, P. (2007) 25 Yeast Genetic Strain and Plasmid Collections, *Method. Microbiol. 36*, 629-666.

[13] Baker Brachmann, C., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications, *Yeast 14*, 115-132.