Supplementary Information

A molecular rheostat maintains ATP levels to drive a synthetic biochemistry system

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Supplementary Results

Supplementary Note

Equations used for COPASI modeling

The copasi enzymatic equations 1-15 were used to model the production of Isobutanol from glucose using the described metabolic pathway consisting of glycolysis catabolic pathway and the isobutanol anabolic pathway. The equations were derived based off of established rate equations used to model yeast glycolysis.

Hexokinase (1)

$$\frac{\text{vmax} \cdot A \cdot B}{\text{Kma} \cdot \text{Kmb} + A \cdot \text{Kmb} + B \cdot \text{Kma} + A \cdot B}$$

PGI (2)

$$\frac{\text{vmax} \cdot A \cdot B}{\text{Kma} \cdot \text{Kmb} + A \cdot \text{Kmb} + B \cdot \text{Kma} + A \cdot B}$$

PfkA (3)

$$\frac{\frac{\text{Vf} \cdot \text{substrate}}{\text{Kms}} - \frac{\text{Vr} \cdot \text{product}}{\text{Kmp}}}{1 + \frac{\text{substrate}}{\text{Kms}} + \frac{\text{product}}{\text{Kmp}}}$$

FBA (4)

$$\frac{\text{Vf} \cdot \left(\text{substrate} - \frac{\text{productp} \cdot \text{productq}}{\text{Keq}}\right)}{\text{Kms} + \text{substrate} \cdot \left(1 + \frac{\text{productp}}{\text{Kip}}\right) + \frac{\text{Vf}}{\text{Vr} \cdot \text{Keq}} \cdot (\text{Kmq} \cdot \text{productp} + \text{Kmp} \cdot \text{productq} + \text{productp} \cdot \text{productq})}$$

GapN (5)

$$\frac{Vf \cdot \left(substratea \cdot substrateb - \frac{productp \cdot productq}{Keq}\right)}{substratea \cdot substrateb + Kmb \cdot substratea + Kma \cdot substrateb \cdot \left(1 + \frac{productq}{Kiq}\right) + \frac{Vf}{Vr \cdot Keq} \cdot \left(Kmq \cdot productp \cdot \left(1 + \frac{substratea}{Kia}\right) + productq \cdot (Kmp + productp)\right)}$$

GapM6 (6)

$$\frac{\frac{Vmax \cdot \left(A \cdot B \cdot C - \frac{P \cdot Q}{Keq}\right)}{Kgap \cdot Knadp \cdot Kppi}}{\left(1 + \frac{A}{Kgap}\right) \cdot \left(1 + \frac{B}{Knadp}\right) \cdot \left(1 + \frac{C}{Kppi}\right) + \frac{P}{Kbpg} \cdot \left(1 + \frac{Q}{Knadph}\right) - 1}$$

PGK (7)

$$\frac{Vf \cdot \left(substratea \cdot substrateb - \frac{productp \cdot productq}{Keq}\right)}{substratea \cdot substrateb + Kmb \cdot substratea + Kma \cdot substrateb \cdot \left(1 + \frac{productq}{Kiq}\right) + \frac{Vf}{Vr \cdot Keq} \cdot \left(Kmq \cdot productp \cdot \left(1 + \frac{substratea}{Kia}\right) + productq \cdot (Kmp + productp)\right)}$$

PGM (8)

$$\frac{\frac{Vf \cdot substrate}{Kms} - \frac{Vr \cdot product}{Kmp}}{1 + \frac{substrate}{Kms} + \frac{product}{Kmp}}$$

Enolase (9)

$$\frac{Vf \cdot \left(substrate - \frac{product}{Keq}\right)}{substrate \cdot \left(1 + \frac{product}{Kii}\right) + Kms \cdot \left(1 + \frac{product}{Kmp}\right)}$$

Pyk (10)

$$\frac{vmax \cdot A \cdot B}{Kma \cdot Kmb + A \cdot Kmb + B \cdot Kma + A \cdot B}$$

AlsS (11)

$$\frac{vmax \cdot A \cdot B}{Kma \cdot Kmb + A \cdot Kmb + B \cdot Kma + A \cdot B}$$

IlvC (12)

$$\frac{Vf \cdot \left(substratea \cdot substrateb - \frac{productp \cdot productq}{Keq}\right)}{substratea \cdot substrateb + Kmb \cdot substratea + Kma \cdot substrateb \cdot \left(1 + \frac{productq}{Kiq}\right) + \frac{Vf}{Vr \cdot Keq} \cdot \left(Kmq \cdot productp \cdot \left(1 + \frac{substratea}{Kia}\right) + productq \cdot (Kmp + productp)\right)}$$

IlvD (13)

$$\frac{Vf \cdot \left(substrate - \frac{product}{Keq}\right)}{substrate \cdot \left(1 + \frac{product}{Kii}\right) + Kms \cdot \left(1 + \frac{product}{Kmp}\right)}$$

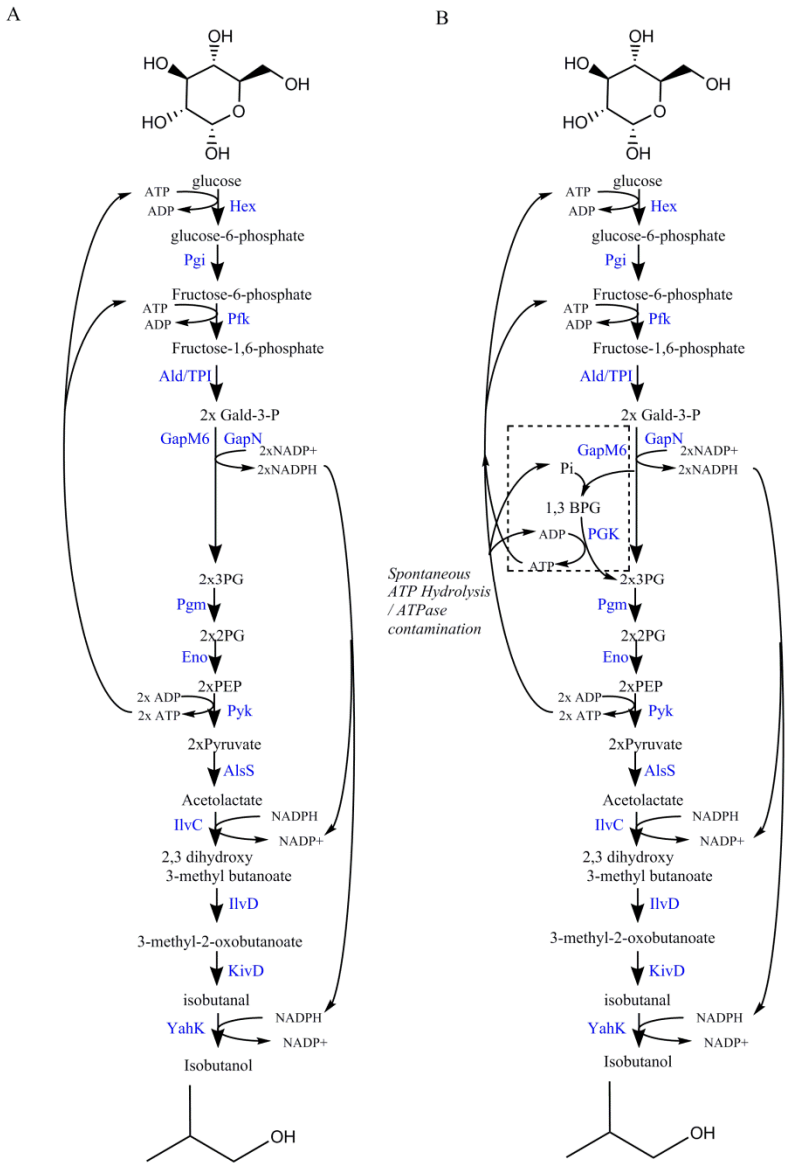
KivD (14)

$$\frac{V \cdot substrate}{Km + substrate}$$

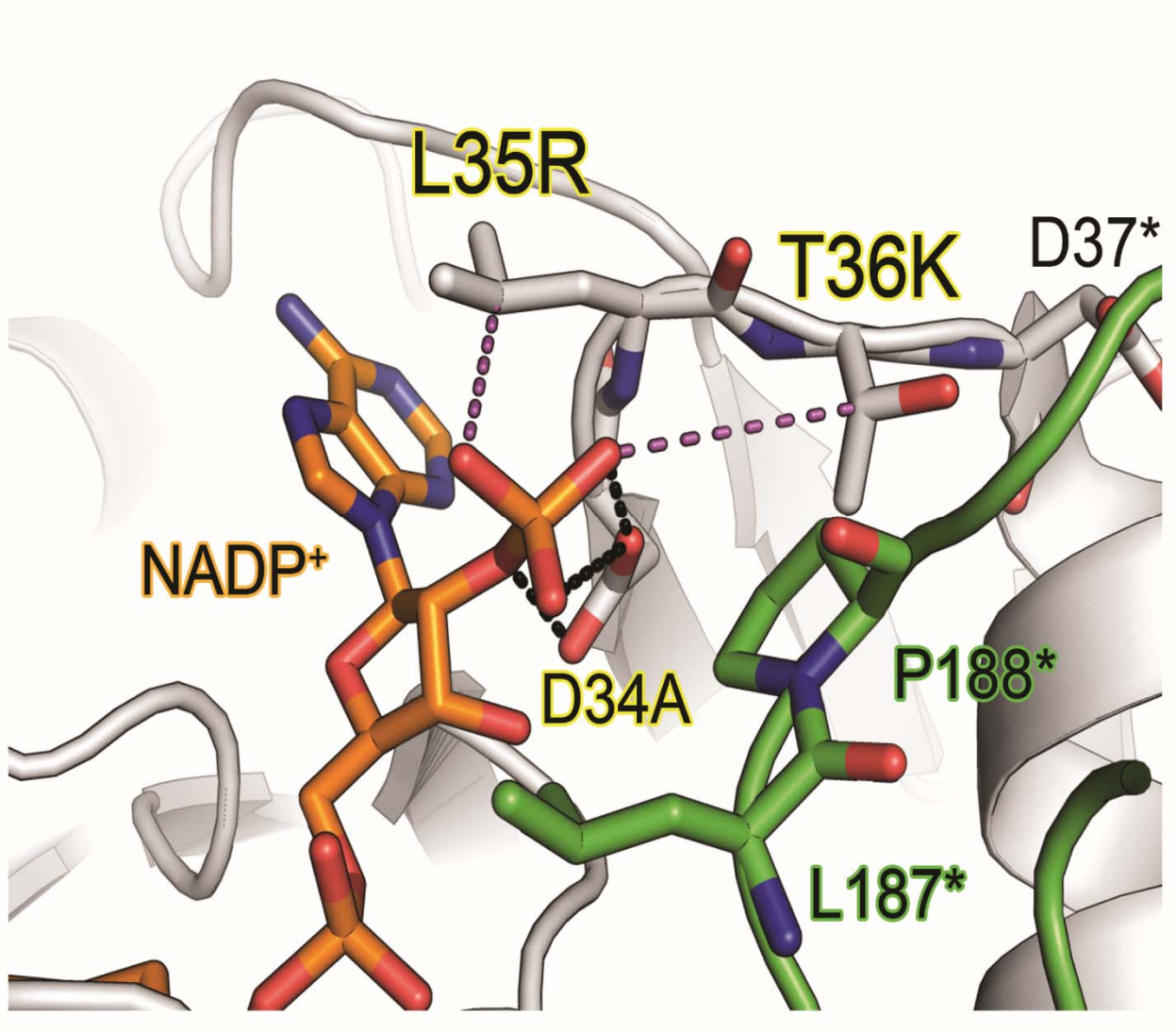
YahK (15)

$$\frac{Vf \cdot \left(substratea \cdot substrateb - \frac{productp \cdot productq}{Keq}\right)}{substratea \cdot substrateb + Kmb \cdot substratea + Kma \cdot substrateb \cdot \left(1 + \frac{productq}{Kiq}\right) + \frac{Vf}{Vr \cdot Keq} \cdot \left(Kmq \cdot productp \cdot \left(1 + \frac{substratea}{Kia}\right) + productq \cdot (Kmp + productp)\right)}$$

Supplementary Figures



Supplementary Figure 1. *Detailed schematic of the stoichiometric pathway and rheostat pathway*



Supplementary Figure 2. *Engineering of mGAPDH.* A crystal structure of GsGapDH bound to NAD+ is shown with a modeled phosphate at the 2' position to indicate how $NADP^+$ could bind.

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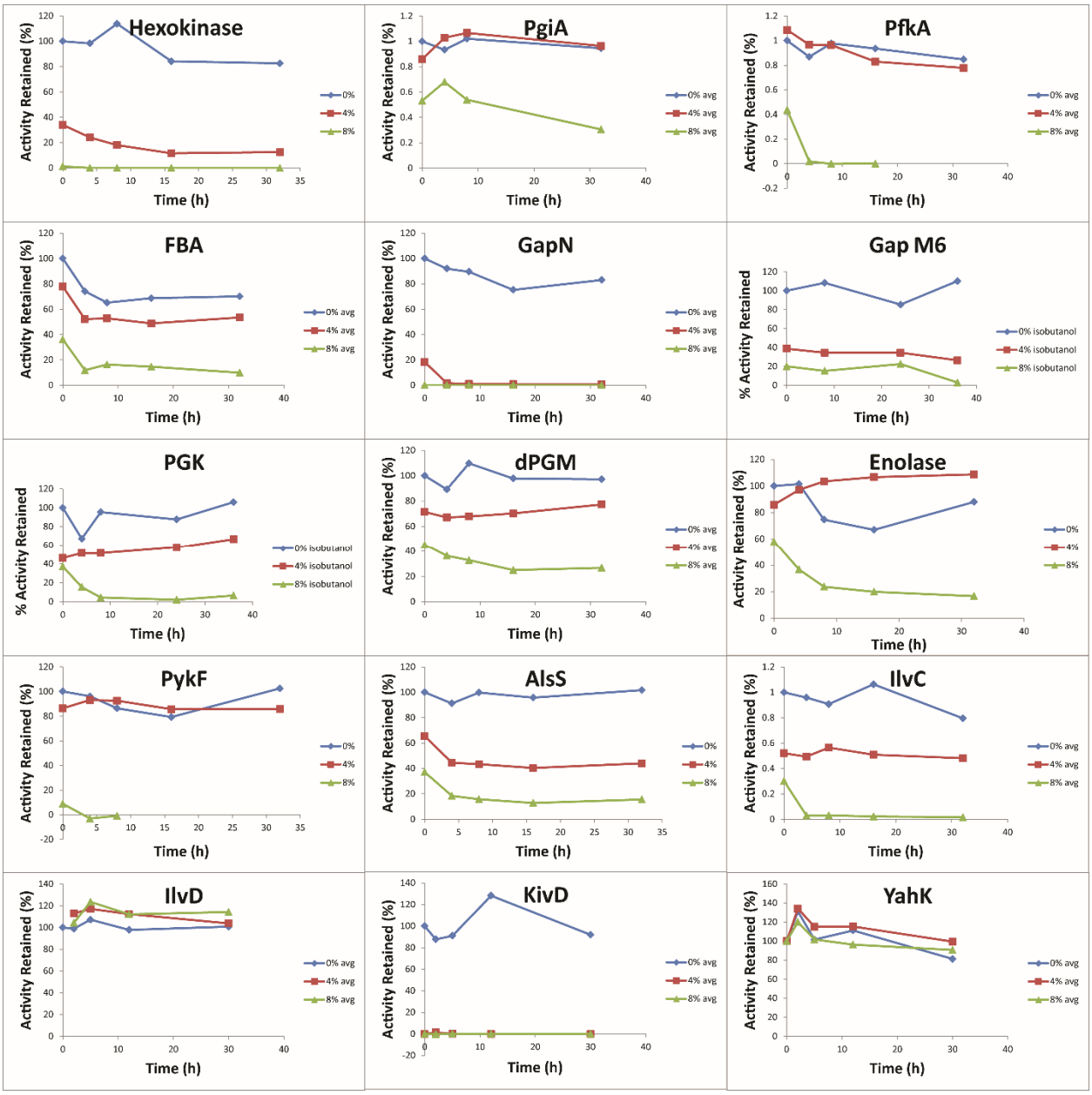
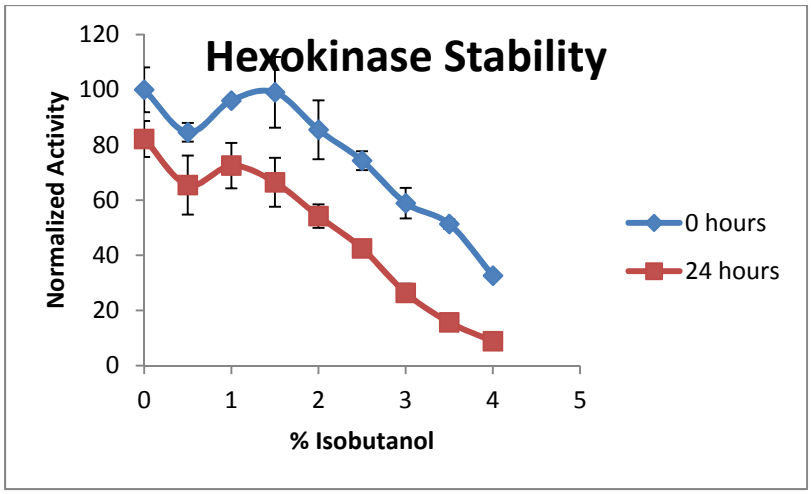
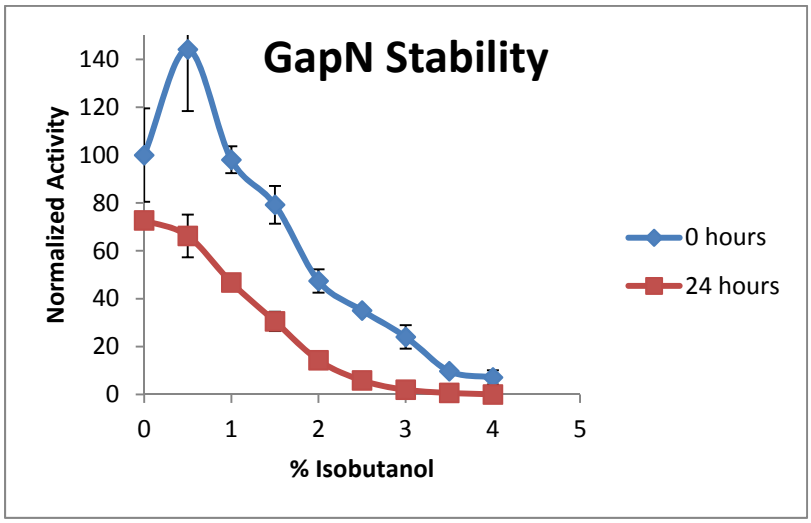
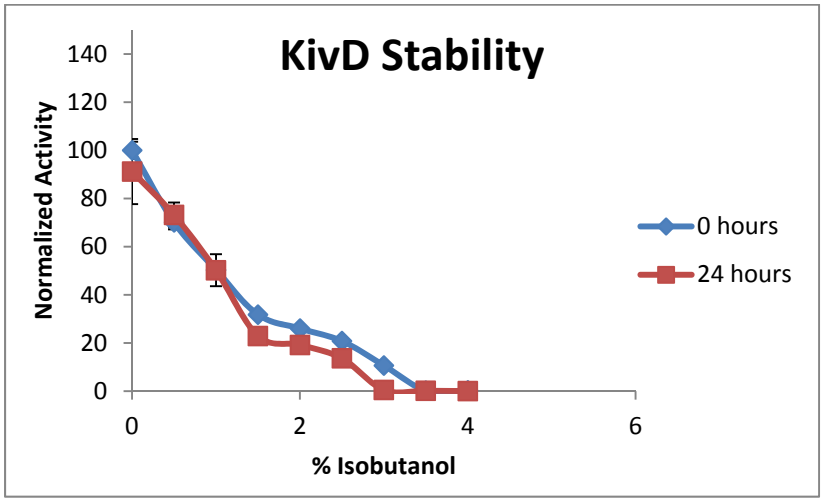


Supplementary Figure 3. *Enzyme activity over time in 0, 4, and 8% isobutanol*







Supplementary Figure 4. Activity of *Hexokinase, GapN and KivD incubated in various concentrations of isobutanol.*

Supplementary Tables

Supplementary Table 1. ATPase activity in enzyme preparations.

Enzyme	ATPase 2Activity* (nm/μg/hour)
Sc Hk	0.71
Gt PgiA	0.12
Gs PfkA	0.18
Sa FBA	0.15
Gs TPI	0.06
Sm GapN	0.07
Gs PGK	6.28
Gs PGM	0.09
Ec Enolase	0.17
Ec PykF	0.06
Bs AlsS	0.15
Ec IlvC	0.36
Re IlvD	0.10
Ll KivD	0.19
Ec YahK	0.35

* The background ATPase (no enzyme control) at room temperature is 0.8 nm/hour

Supplementary Table 2. Kinetic parameters for the wild-type and mGapDH enzymes

Enzyme	Cofactor	kcat (μM/min/mg)	KM (mM)	k_{cat}/K_m
gapDH WT	NAD+	21.8 ± 2.6	1.5 ± 0.3	14.5
	NADP+	1.2 ± 0.1	1.6 ± 0.5	2.1
mGapDH	NAD+	0.2 ± 0.04	0.6 ± 0.4	0.4
	NADP+	3.2 ± 0.1	0.3 ± 0.04	11.9

Supplementary Table 3. Enzymes and Expression conditions.

Enzyme	Name	Accession number	Source Organism	Expression Time	expression Temp (C˚)
Sc Hk	Hexokinase	-	*S. Cerevisiae*	N/A	N/A
Gt PgiA	Phosphoglucoisomerase	ABO68222	*G. thermodenitrificans NG80-2*	Overnight	37
Gs PfkA	Phosphoglucokinase	KOR92562	*G. stearothermophilus ATCC12980*	Overnight	18
Sa FBA	Fructose 1,6 bisphosphate aldolase	BAR10119	*S. aureus subsp. Aureus*	Overnight	18
Gs TPI	Triose Phosphate isomerase	KOR95273	*G. stearothermophilus ATCC12980*	Overnight	37
Sm GapN	Non- phosphorylating Glyceraldehyde 3 phosphate dehydrogenase	NP_J721104	*S. mutans*	Overnight	18
Gs mGapDH	Phosphorylating Glyceraldehyde-3-phosphate Dehydrogenase	WP_033015082	*G. stearothermophilus ATCC12980*	Overnight	18
Gs PGK	Phosphoglycerate Kinase	WP_033015089	*G. stearothermophilus ATCC12980*	Overnight	18
Gs PGM	Phosphoglycerate Mutase	KOR95274	*G. stearothermophilus ATCC12980*	Overnight	37
Ec Enolase	Enolase	NP_417259	*E. coli K12 sp MG1655*	Overnight	18
Ec PykF	Pyruvate Kinase	NP_416191	*E. coli K12 sp MG1655*	Overnight	18
Bs AlsS	Acetolactate Synthase	NP_391482	*B. Subtilis subtilis 168*	Overnight	18
Ec IlvC	Ketol-Acid Reductoisomerase	AKK14493	*E. coli K12 sp MG1655*	Overnight	18
Re IlvD	Dihydroxy-Acid Dehydratase	Q0K4J3	*R. eutropha*	Overnight	18
Ll KivD	Keto-Isovalerate Decarboxylase	CAG34226	*L. lactis subsp. Lactis*	Overnight	18
Ec YahK	Alcohol Dehydrogenase	NC_000913.3	*E. coli K12*	3 hours	*37*

Supplementary Table 4. Enzyme assay conditions

enzyme	coupled enzymes	cofactors	metabolites
hexokinase	zwf	0.5mM NADP+	5mM glucose
Pgi	Pfk, Pyk, LDH	0.25mM NADH, 5mM PEP, 1mM ATP	5mM glucose-6-phosphate
Pfk	Pyk, LDH	0.25mM NADH, 5mM PEP, 1mM ATP	5mM fructose-6-phosphate
Ald	GapN, Tpi	0.5mM NADP+	5mM fructose-1,6-bisphosphate
GapN	Ald, Tpi	0.5mM NADP+	5mM fructose-1,6-bisphosphate
GapM6	Ald, Tpi, PGK	0.5mM NADP+	5mM fructose-1,6-bisphosphate
PGK	GapM6,	0.25mM NADPH, 0.5mM, 1mM ATP	2.5mM 3-phosphoglycerate
Pgm	PGK, GapM6	0.25mM NADPH, 0.5mM, 1mM ATP, .25 mM 2,3 BPG*	2.5mM 2-phosphoglycerate
eno	Pyk, LDH	0.25mM NADH, 5mM PEP,1mM ATP	2.5mM 2-phosphoglycerate
Pyk	LDH	0.25mM NADH, 1mM ATP	5mM PEP
AlsS	IlvC	0.25mM NADPH	5mM pyruvate
IlvC	AlsS	0.25mM NADPH	5mM pyruvate
IlvD	KivD, YahK	0.25mM NADPH	2.5mM 2,3-dihydroxyisovalerate
KivD	YahK	0.25mM NADPH	2.5 mM 2-ketoisovalerate
YahK	KivD	0.25mM NADPH	2.5 mM 2-ketoisovalerate

*2,3 BPG is an allosteric activator

Supplementary Table 5. Enzyme activity and units added for the final reaction

Enzyme	Stock concentration (mg/mL)	Enzyme Activity (uM/min/mg)	Units/mL added to reaction
Sc Hk	2.73	7.2 ± 0.1	0.025
Gt PgiA	5.7	19.2 ± 0.2	1.094
Gs PfkA	8.49	9.6 ± 0.2	3.260
Sa FBA	9.84	21.1 ± 0.3	8.305
Gs TPI	12.75	96.6 ± 5.4	12.317
Sm GapN	16.09	20.6 ± 2.7	9.944
Gs Gap M6	4.47	7.9 ± 0.4	0.177
Gs PGK	6.28	102.3 ± 2.9	3.212
Gs PGM	12.16	97.2 ± 13.0	17.729
Ec Enolase	26.09	82.5 ± 0.1	53.485
Ec PykF	16.39	365.3 ± 37.9	299.363
Bs AlsS	6.22	6.94 ± 1.22	1.727
Ec IlvC	21.43	0.88 ± 0.23	1.509
Re IlvD	11.58	3.4 ± 0.3[1]	1.575
Ll KivD	6.27	13.39 ± 2.28	0.672
Ec YahK	2.18	3.64 ± 0.37	0.635

Supplementary Table 6. COPASI modeling parameters used

Enzyme	Vmax	Km (forward)	Km (reverse)	Keq
Sc Hk	7.2	glucose: 0.08 ATP: 0.15	g6p: 30 ADP: 0.2	2000
Gt PgiA	19.2	g6p: 1.4	f6p: 0.3	0.29
Gs PfkA	9.6	f6p: 0.1 ATP: 0.7	f1,6bp: 0.1 ADP: 0.7	5
Sa FBA	21.1	F1,6bp: 0.3	g3p: 0.3	0.1
Sm GapN	20.6	g3p: 0.1 NADP+: 0.2	3pg: 0.5 NADPH: 0.3	10
Gs Gap M6	7.9	g3p: 0.3 NADP+: 0.3	1,3bpg: 0.3 NADPH: 0.3	10
Gs PGK	6.28	1,3bpg: 0.03 ADP: 0.2	3pg: 0.5 ATP: 0.3	3200
Gs PGM	12.16	3pg: 0.2	2pg: 0.08	0.2
Ec Enolase	26.09	2pg: 0.04	pep: 0.5	6.7
Ec PykF	16.39	pep: 0.14 ATP: 2.1		irreversible
Bs AlsS	6.22	pyr: 3.2		irreversible
Ec IlvC	0.88	aclac: 0.5 NADPH: 0.3	2,3dhv: 0.5 NADP+: 0.3	0.2
Re IlvD	11.58	2,3dhv: 0.3	2kiv: 0.5	0.3
Ll KivD	6.27	2kiv: 0.1		Irreversable
Ec YahK	3.64	isobual: 0.4 NADPH: 0.1	Isobuol: 0.3 NADP: 0.3	5

Supplementary Table 7. List of primers used in this work

	Enzyme	Primer Sequence (F: Forward Primer, R: Reverse Primer, M: Mutagenic Primer)
1	Hex	From sigma
2	Pgi	F: 5' GGTGCCGCGCGGCAGCCATATGACCCATATTCGTTTCGAC 3' R: 5' CAGTGGTGGTGGTGGTGGTGCTCGAGTTATTTCAACCGTTTCTCCAGTTC 3'
3	Pfk	F: 5' CCTGGTGCCGCGCGGCAGCCATATGAAACGCATTGGTGTGTTG 3' R: 5' CAGTGGTGGTGGTGGTGGTGCTCGAGTTAAATGGACAATTCTTTCGAC 3'
4	Fba	F: 5' GCCGCGCGGCAGCCATATGAATAAAGAGCAATTAGAAAAAATGAAAAATGGAAAAGGC 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTTAGTTTTTGTTTACAGATGCGTCGTAG 3'
5	Tpi	F: 5' GGTGCCGCGCGGCAGCCATATGAGAAAACCGATCATTGCAGG 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTTACTCATGACGCCCCGC 3'
6	GapN	F: 5' TTAACTTTAAGAAGGAGATATACCATGGGCATGACAAAACAATATAAAAATTATGTC 3' R: 5' GTGGTGGTGGTGGTGGTGCTCGAGTTTGATATCAAATACGACGGATTTAAC 3'
7	mGap	F: 5' CCTGGTGCCGCGCGGCAGCCATATGGCAGTCAAAGTGGGAATCAAC 3' R: 5' CAGTGGTGGTGGTGGTGGTGCTCGAGTTACAGCCCTTTCGAGGCGGTG 3' M: 5' TGGTGGCGGTGAACGCGCGTAAAGATGCGAATACGCTT 3'
8	Pgk	F: 5' GGTGCCGCGCGGCAGCCATATGAACAAGAAGACGATCCGC 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTTATTTGTCTTCGAGTGCGACG 3'
9	Pgm	ASKA Clone
10	Eno	F: 5' GGTGCCGCGCGGCAGCCATATGTCCAAAATCGTAAAAATCATCGGTCGTGAAATC 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTTATGCCTGGCCTTTGATCTCTTTACGACC 3'
11	Pyk	F: 5' GGTGCCGCGCGGCAGCCATATGAAAAAGACCAAAATTGTTTGCACCATCGGACC 3' R: 5' CAGTGGTGGTGGTGGTGGTGCTCGAGTTACAGGACGTGAACAGATGCGGTG 3'
12	AlsS	F: 5' GGTGCCGCGCGGCAGCCATATGTCAGAACGTTTCCCAAATGACGTGG 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTTACGCCAGACGCGGGTTAACTTTATC 3'
13	IlvC	ASKS clone
14	IlvD	F: 5' GCCTGGTGCCGCGCGGCAGCCATATGATGGCATTCAACAAACGCTCGCAGAAC 3' R: 5' GGTGGTGGTGGTGGTGCTCGAGTCAGTCCGTCACTGCCCCCTTG 3'
15	KivD	F: 5' GCCTGGTGCCGCGCGGCAGCCATATGATGGCATTCAACAAACGCTCGCAGAAC 3' **R:** 5' GGTGGTGGTGGTGGTGCTCGAGTCAGTCCGTCACTGCCCCCTTG 3'
16	YahK	ASKA Clone

1. Lu, J., Brigham, C. J., Plassmeier, J. K. & Sinskey, A. J. Characterization and modification of enzymes in the 2-ketoisovalerate biosynthesis pathway of Ralstonia eutropha H16. *Appl. Microbiol. Biotechnol.* **99,** 761–774 (2015).