Isobutanol production freed from biological limits using synthetic biochemistry

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Supplementary Table 1. Summary of production parameters obtained using cell-based methods.

Organism	Substrate	Titer (g/L)	Yield (%)	Productivity (g/L/h)	Comments	Ref.
Magnusiomyces magnusii	Glucose and 2-oxoisovalerate	0.76	nd	0.01*		1
Saccharomyces cerevisiae	Glucose	0.26	nd	0.003		2
Saccharomyces cerevisiae	Xylose	3.1	9.4	0.04	fed-batch fermentation	3
Saccharomyces cerevisiae	Glucose	8.4	13	0.04*	Fed-batch fermentation	4
Saccharomyces cerevisiae	Glucose	0.15	nd	nd		5
Saccharomyces cerevisiae	Glucose	0.64	1.6*	nd		6
Saccharomyces cerevisiae	Glucose	1.62	3.9*	0.25*		7
Saccharomyces cerevisiae	Glucose	0.68	8.3*	nd		8
Saccharomyces cerevisiae	xylose	0.09	nd	0.002*		9
Clostridium thermocellum	cellulose	5.1	nd	nd		10
Clostridium thermocellum	cellulose	5.4	41	0.07*		11
Clostridium cellulolyticum	cellulose	0.66	nd	nd		12
Geobacillus thermoglucosidasius	cellobiose	0.6	8.3	0.01*		13
Bacillus subtilis	Glucose	6.1	63	0.17*	fed-batch fermentation	14
Bacillus subtilis	Aminoacids	0.09	47	nd		15
Bacillus megaterium	2-ketoisovalerate	0.3	80	0.01*	cultures grown and product extracted under supercritical carbon dioxide	16
Synechococcus elongatus	carbon dioxide	0.45	nd	0.003*		17
Ralstonia eutropha	carbon dioxide	0.85	nd	nd	bioreactor	18
Escherichia coli	Glucose	50	68	0.7	bioreactor with *in situ* product removal (gas stripping)	19
Escherichia coli	Glucose	22	86	0.31*		20
Escherichia coli	Aminoacids	2	56	0.02*		21

nd – not determined
* - the values were not reported but were estimated from graphs in the publication if available.

Supplementary Table 2. Summary of genome mining screen.

	Enzyme	Organism	Accession number	Codon optimized?	Expression level	Activity at 25 °C	Isobutanol stability
Hex	Hexokinase	*T. maritima*	AAD36537	No	+++	+++	+++
Glk	Glucokinase	*G. stearothermophilus*	KOR95707	No	+++	++++	
Pgi	Glucose-6-phosphate isomerase	*T. maritima*	*AAD36455*	No	+++	+++	+++
	Glucose-6-phosphate isomerase	*P. aerophilum*	*HII46470*	No	+	+	
Pfk	Phosphofructokinase B	*E. coli*	*ACT43547*	No	+++	++++	++
	Phosphofructokinase A	*G. stearothermophilus*	*KOR92562*	No	+++	++++	-
	Phosphofructokinase	*P. aerophilum*	*HII48166*	No	+	+	-
	Phosphofructokinase	*T. thermophilus*	*BAD71785*	No	++	++	
Fba	Fructose-1,6-bisphosphate aldolase	*T. maritima*	*AAD35362*	No	+	+	+++
	Fructose-1,6-bisphosphate aldolase	*G. stearothermophilus*	*KOR93738*	No	+++	++	
	Fructose-1,6-bisphosphate aldolase	*T. thermophilus*	*BAD71596*	No	++	++	
Tpi-Pgk	Triose Phosphate Isomerase - Phosphoglycerate Kinase Fusion	*T. maritima*	*AAD35771*	No	+++	+++	+++
GapN	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*T. kodakarensis*	*BAD84894*	No	++	++	+++
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*T. tenax*	*CCC81810*	No	++	+	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*S. solfataricus*	*AAK43292*	No	++	+	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*A. pernix*	*BAA80789*	No	-	-	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*M. jannaschii*	*AAB99418*	No	++	-	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*T. takaii*	*BAT70977*	No	++	-	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*M. sedula*	*ABP95456*	No	++	-	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*P. furiosus*	*AAL80879*	No	-	-	
	Glyceraldehyde-3-phosphate Dehydrogenasnon-phosphorylating)	*T. neapolitana*	*ACM23724*	No	-	-	
GapDH	Glyceraldehyde-3-phosphate Dehydrogenasphosphorylating)	*A. fulgidus*	*AAB89515*	No	++	++	+++
	Glyceraldehyde-3-phosphate Dehydrogenasphosphorylating)	*G. stearothermophilus*	*KOR95271*	No	+++	+++	
Pgk	Phosphoglycerate Kinase	*T. maritima*	*AAD35771*	No	+++	+++	+++
	Phosphoglycerate Kinase	*G. stearothermophilus*	*KOR95272*	No	+++	+++	
GpmA	Phosphoglycerate Mutase (2,3-bisphosphoglycerate dependent)	*E. coli*	*ACT42583*	No	+++	+++	-
GpmI	Phosphoglycerate Mutase (2,3-bisphosphoglycerate independent)	*G. stearothermophilus*	*KOR95229*	No	+++	+++	+++
Eno	Phosphoenolpyruvate hydratase	*A. fulgidus*	*AIG98006*	No	++	++	
	Phosphoenolpyruvate hydratase	*T.thermophilus*	*BAD69825*	No	++	++	+++
	Phosphoenolpyruvate hydratase	*G. stearothermophilus*	*KOR95275*	No	+++	+++	

Pyk	Pyruvate Kinase	*T. maritima*	*AAD35300*	No	++	++	
	Pyruvate Kinase	*T.thermophilus*	*BAD69826*	No	++	++	+++
	Pyruvate Kinase A	*G. stearothermophilus*	*KOR92563*	No	+++	+++	
IlvC	Ketol-acid Reductoisomerase	*T. thermophilus*	*BAD71034*	Yes	++	+	+++
	Ketol-acid Reductoisomerase	*G. stearothermophilus*	*KOR94737*	Yes	+++	++	+++
	Ketol-acid Reductoisomerase	*Thermococcus chitonophagus*	*ASJ16613*	Yes	+++	++	++
	Ketol-acid Reductoisomerase	*Caldicellulosiruptor bescii* DSM 6725	*ACM59630*	Yes	+	+	+++
IlvD	Dihydroxyacid Dehydratase	*S. mutans*	*AJD56310*	No	+++	++	++
	Dihydroxyacid Dehydratase	*G. stearothermophilus*	*KOR95295*	No	+++	O_2 sensitive	
	Dihydroxyacid Dehydratase	*T. thermophilus*	*BAD71057*	No	++	O_2 sensitive	

Supplementary Table 3. Data collection and refinement statistics.

PDB identification number	**6VGS**
Ligand bound	Thiamine pyrophosphate (TTP) with Mg ion
Data collection temperature (K)	100
Data Collection Statistics	
Resolution (Å, high limit)	1.80
Resolution (Å, low limit)	100.00
Space group	$P2_12_12_1$
Unit cell parameters	
a (Å)	128.20
b (Å)	128.27
c (Å)	147.74
α, β, γ (°)	90.00, 90.00, 90.00
Radiation source	APS 24-ID-C
Radiation wavelength (Å)	0.97930
Measured reflections	1,972,360
Unique reflections	223,094
Overall completeness (%)	99.40
Last shell completeness (%) (1.80-1.85 Å)	97.20
Overall redundancy	8.8
Last shell redundancy	7.9
Overall R_{merge} [a]	0.151
Last shell R_{merge} (1.80-1.85 Å)	1.055
Overall I/σ(I)	12.3
Last shell I/σ(I) (1.80-1.85 Å)	2.1
Refinement Statistics	
R_{work} [b]	18.3
R_{free} [c]	21.0
Number of residues (protein/water)	2164/1181
RMSD bond lengths (Å)	0.008
RMSD bond angles (°)	1.423

[a] $R_{merge}(I) = \Sigma_{hkl} \Sigma_i |I_i(hkl) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_i I_i(hkl)$

[b] $R_{work} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$

[c] $R_{free} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$, where all reflections belong to a test set of 5% randomly selected data

Supplementary Table 4. Enzyme assay conditions.

	Coupling enzymes*	Cofactors and substrates
TmHex	PK/LDH	1 mM ATP, 2.5 mM PEP, 1 mM NADH, 3 mM Glucose
TmPgi	EcPfkB, PK/LDH	1 mM ATP, 2.5 mM PEP, 1 mM NADH, 3 mM Glucose-6-Phosphate
EcPfkB	PK/LDH	1 mM ATP, 2.5 mM PEP, 1 mM NADH, 3 mM Fructose-6-Phosphate
TtFba	TmTpi, TkGapN	1 mM $NADP^+$, 0.75 mM Glucose-1-Phosphate, 2.5 mM Fructose 1,6-bis-Phosphate
TmTpi	TkGapN	1 mM $NADP^+$, 0.75 mM Glucose-1-Phosphate, 2.5 mM Dihydroxyacetone Phosphate
TkGapN	TmTpi	1 mM $NADP^+$, 0.75 mM Glucose-1-Phosphate, 2.5 mM Dihydroxyacetone Phosphate
AfGapDH	TmTpi, TmPgk	1 mM $NADP^+$, 2.5 mM ADP, 4 mM PO_4, 2.5 mM Dihydroxyacetone Phosphate
TmPgk	PK/LDH	1 mM ATP, 1 mM NADH, 2.5 mM PEP, 2.5 mM Glycerate 3-Phosphate
Gs iPgm	TtEno, PK/LDH	2.5 mM ADP, 2 mM NADH, 4 mM PO_4, 0.5 mM $MnCl_2$, 2.5 mM Glycerate 3-Phosphate
TtEno	Gs iPgm, PK/LDH	2.5 mM ADP, 2 mM NADH, 4 mM PO_4, 0.5 mM $MnCl_2$, 2.5 mM Glycerate 3-Phosphate
TtPyk	LDH	1.5 mM ADP, 1 mM NADH, 3 mM PO_4, 1 mM Fructose 1,6-bis-Phosphate, 3 mM PEP
AlsS	GsIlvC	1 mM NADPH, 1 mM TPP, 5 mM Pyruvate
GsIlvC	AlsS	1 mM NADPH, 1 mM TPP, 5 mM Pyruvate
SmIlvD	KivD, EcYahK	1 mM NADPH, 1 mM TPP, 2.5 mM (R)-2,3-dihydroxy-isovalerate
KivD	EcYahK	1 mM NADPH, 1 mM TPP, 5 mM 2-ketoisovalerate
EcYahK		1 mM NADPH, 5 mM Isobutyraldehyde

*Enzyme concentrations given in Supplementary Table 5.

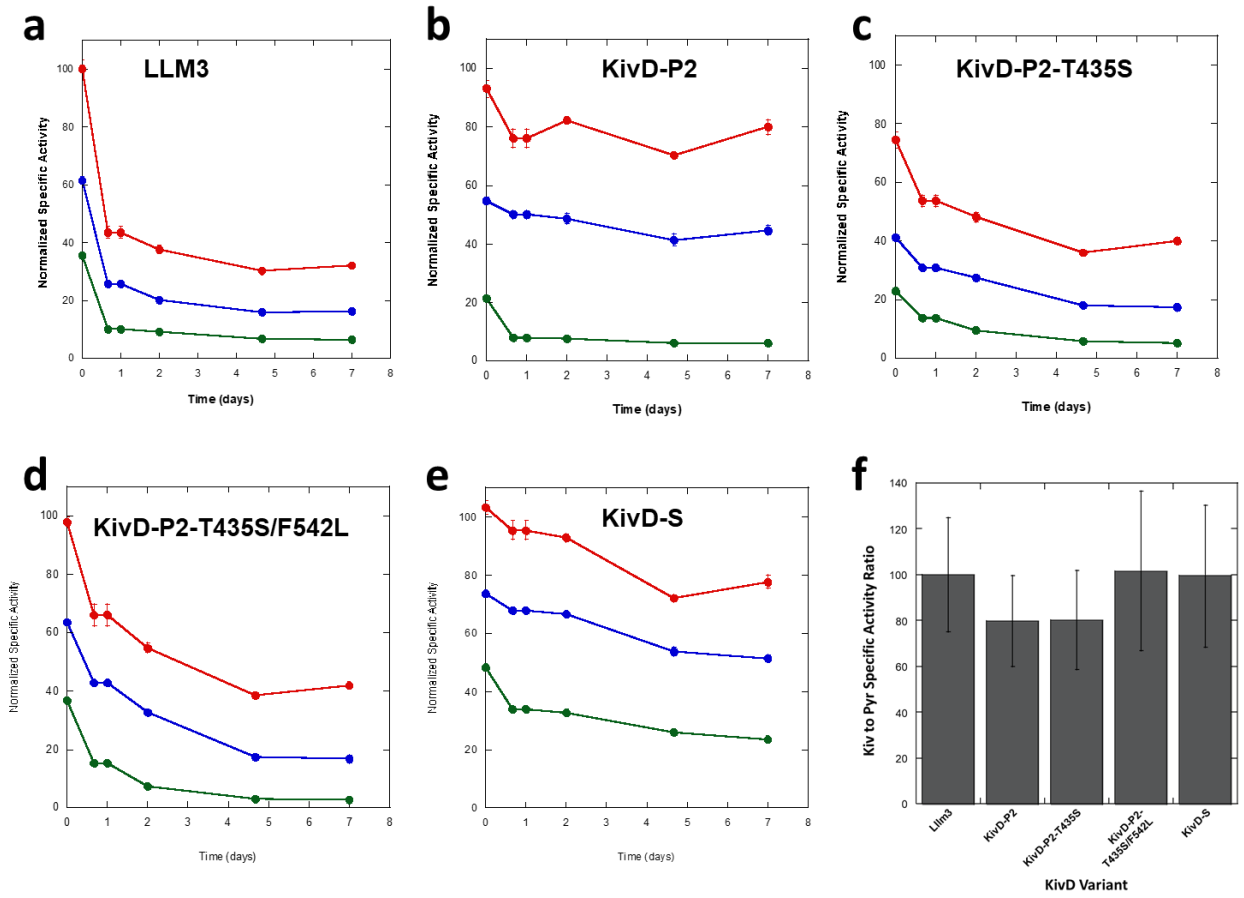
Supplementary Table 5. Enzyme amounts per assay.

Enzyme assayed	Amount added (µg)	Coupling enzymes amount added
TmHex	0.49	PK/LDH* (2 µL)
TmPgi	0.55	EcPfkB (76 µg)/PK/LDH (2 µL)
EcPfkB	1.52	PK/LDH (2 µL)
TtFba	10.47	TmTpi (43.15 µg)/TkGapN' (330 µg)
TmTpi	0.48	TkGapN' (114 µg)
TkGapN'	1.66	TmTpi (43.15 µg)
AfGapDH	122.2	TmTpi (43.15 µg)/TmPgk (390 µg)
TmPgk	1.56	PK/LDH (30 µL)
Gs iPgm	3.53	TtEno (216 µg)/PK/LDH (10 µL)
TtEno	1.08	Gs iPgm (348.25 µg)/PK/LDH (10 µL)
TtPyk	1.67	LDH** (2 µL)
AlsS-P	1.10	GsIlvC (183 µg)
GsIlvC	12.2	AlsS-P (169 µg)
SmIlvD	42.7	KivD-S (155 µg)/EcYahK (271 µg)
KivD-S	2.64	EcYahK (271 µg)
EcYahK	84	none

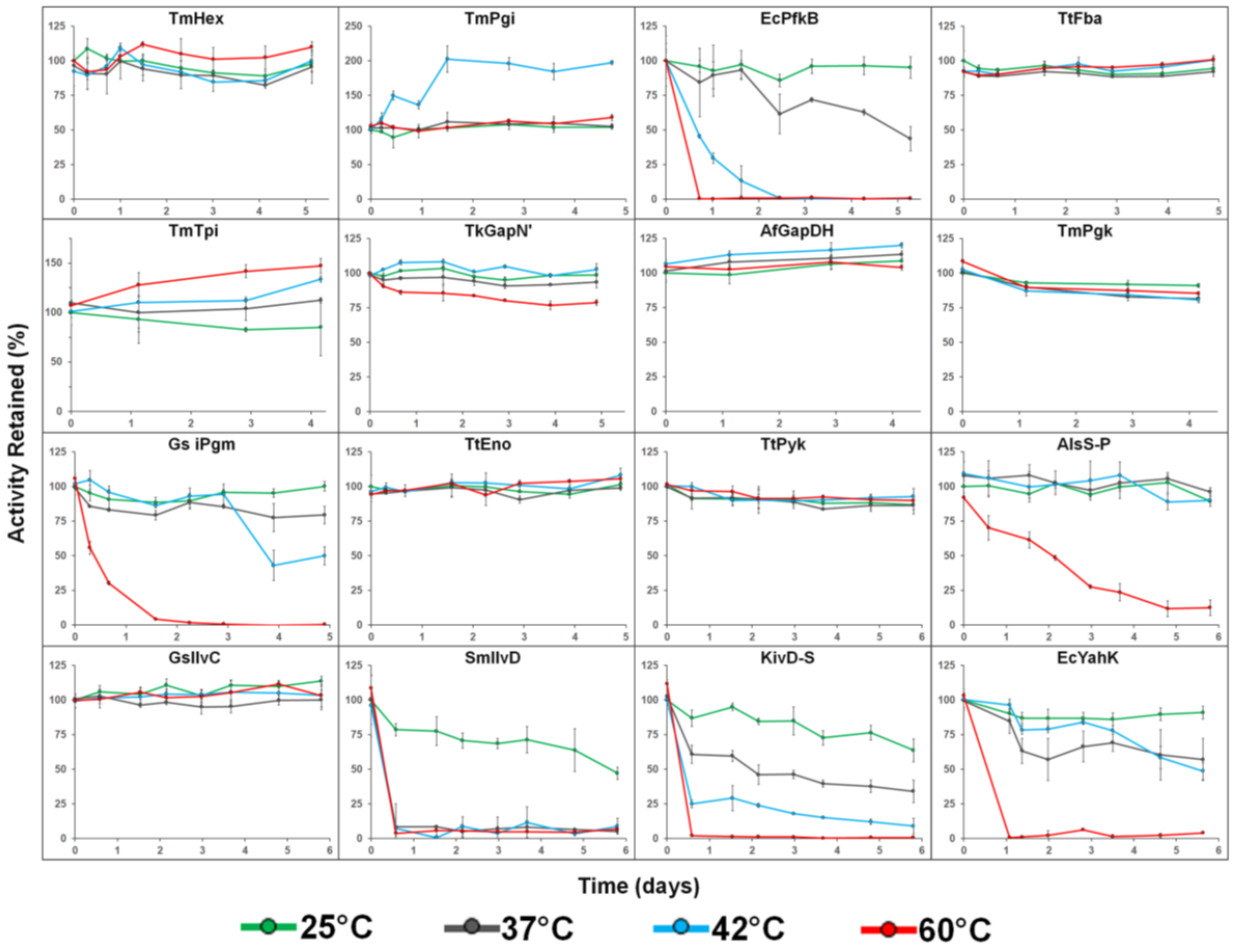
* PK/LDH solution was purchased from Sigma-Aldrich (catalog number: P0294)
** LDH was purchased from Millipore Sigma and resuspended in 50% glycerol solution containing 10 mM HEPES, pH 7.0, 100 mM KCl and 0.1 mM EDTA at a concentration of 0.2 units/µL (catalog number: 427217)

Supplementary Table 6. Enzyme specific activities and loadings for isobutanol production system.

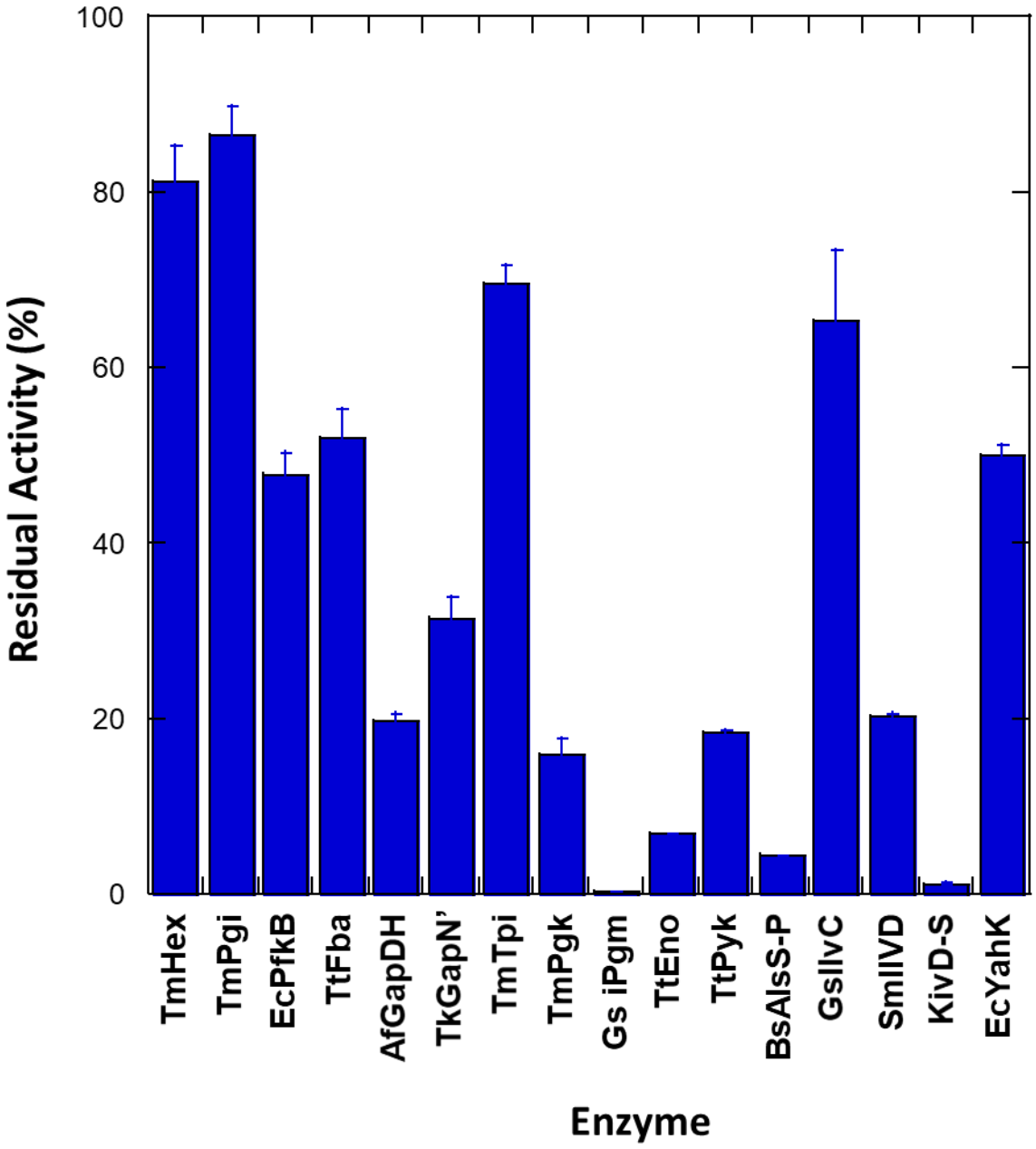
Enzyme	Stock (mg/mL)	Activity (units/mg)	Loading concentration (mg/mL)	Units added (per mL)
TmHex	4.87	14.5±0.3	0.14	2.03
TmPgi	6.08	29.6±1.1	0.30	8.82
EcPfkB	13.47	8.3±0.1	0.22	1.82
TtFba	2.59	4.7±0.1	0.35	1.64
TmTpi	2.85	210±0.7	0.14	30.44
TkGapN’	9.73	7.6±0.8	0.46	3.45
AfGapDH	8.87	10.9±0.6	0.05	0.53
TmPgk	2.23	8.4±0.5	0.20	1.68
Gs iPgm	13.93	49.4±2.2	0.18	8.88
TtEno	6.75	117±5	0.66	77.18
TtPyk	4.82	23.8±0.8	0.20	4.76
BsAlsS-P	7.49	4.9±0.2	0.10	0.50
GsIlvC	9.86	0.9±0.1	1.13	1.01
SmIlvD	4.27	2.7±0.2	0.46	1.27
KivD-S	45.73	18.0±1.6	0.15	2.70
EcYahK	17.29	4.5±0.4	0.27	1.20



Supplementary Fig. 1. Stabilizing KivD. **a-e,** The activity of the indicated enzymes over time during incubation in 0% (red), 4% (blue) or 8% (green) isobutanol. In these screening assays, the enzyme solution was only diluted 2-fold in the final assay mix. Hence, the final assays contained 0%, 2% or 4% isobutanol, which accounts for the different initial activities. **f,** Ratio of specific activity for the substrates α-ketoisovalerate and pyruvate with the indicated enzymes. Errror bars represent standard deviations of biological triplicates. Source data are provided as a Source Data file.



Supplementary Fig. 2. Thermal tolerance of enzymes used in this work. Enzymes were incubated at the indicated temperatures and aliquots assayed over time. Activity retained is the percentage of activity remaining compared to the activity at time zero and 25°C. Error bars represent the standard deviation of biological triplicates. Source data are provided as a Source Data file.



Supplementary Fig. 3. Residual activity of enzymes after 120 hours in bioreactor reaction that included addition of second aliquot of BsAlsS-P and KivD-S. The progress of the reaction is shown in Fig. 5. Error bars represent the mean and standard deviation of triplicates. Source data are provided as a Source Data file.

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