Supplementary Information

Versatile selective evolutionary pressure using synthetic defect in universal metabolism

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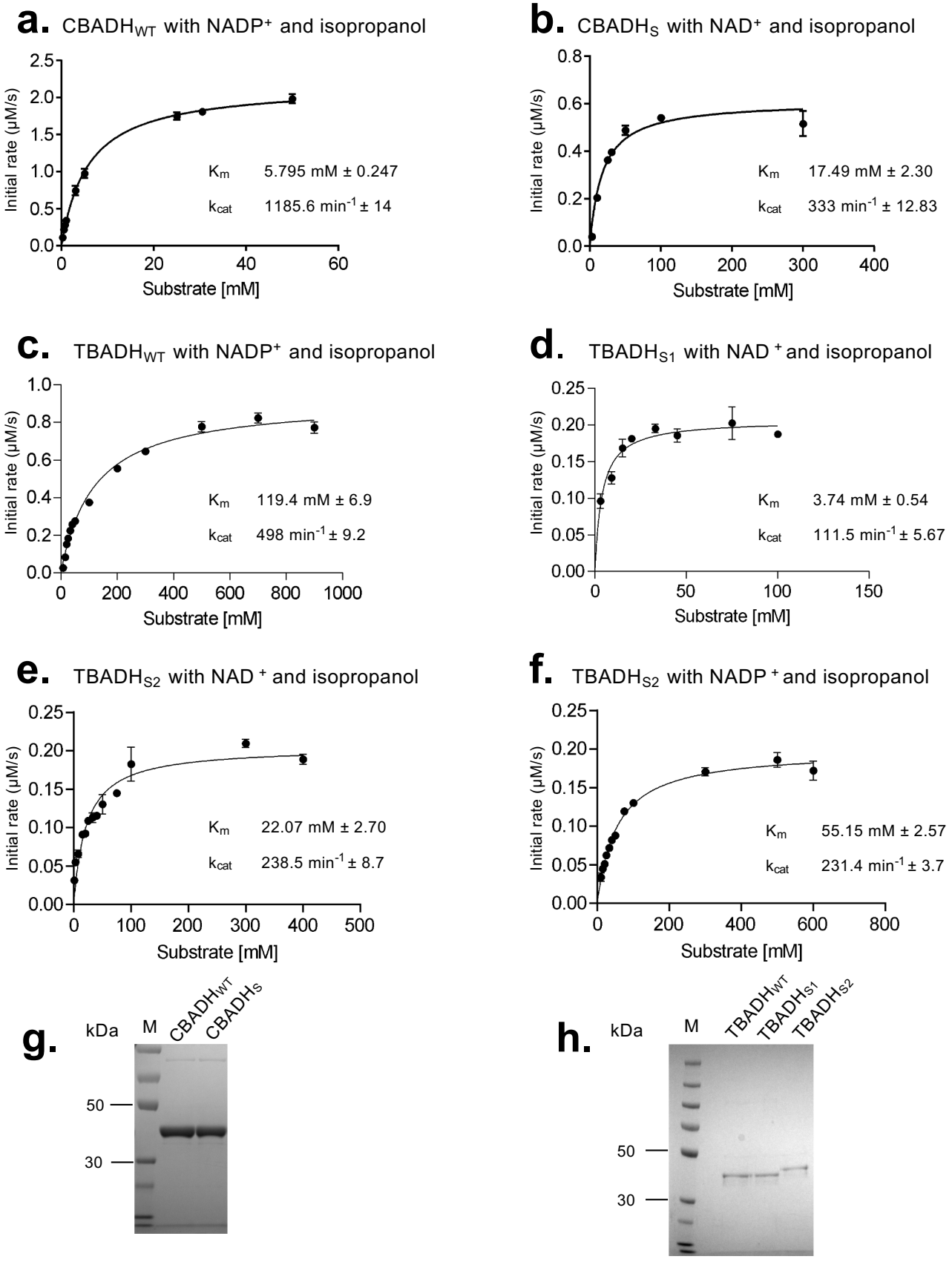
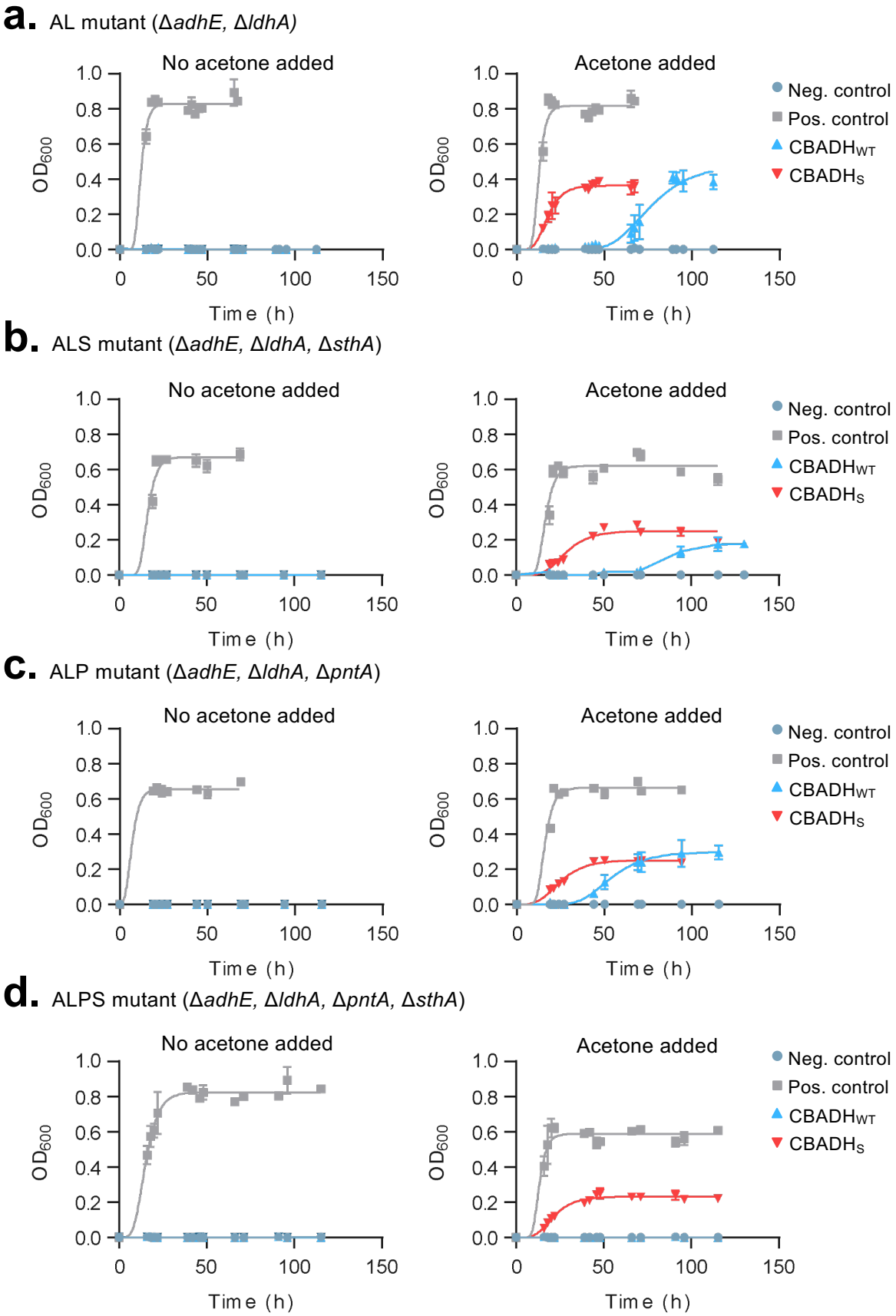
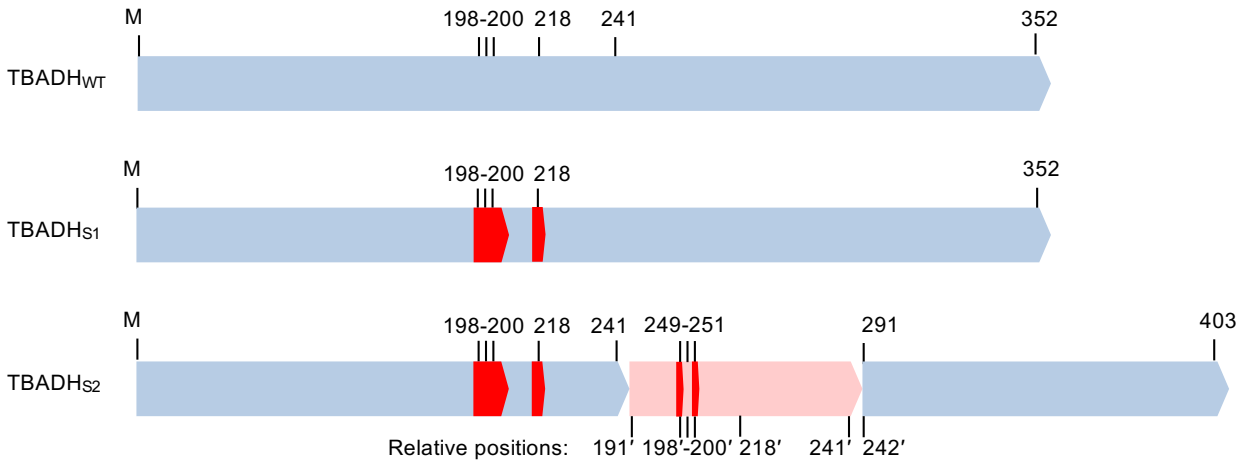


Supplementary Figure 1. Enzymatic activity assays for CBADH and TBADH variants. a-f, Initial reaction rates obtained for each enzyme by varying the isopropanol concentration at a fixed concentration of NAD$^+$ or NADP$^+$. A Michaelis-Menten model was fitted in all cases. Data points represent mean values, with error bars showing standard deviation. n=3 biologically independent assays for all substrate concentrations with both enzymes. Source data are provided as a Source Data file. **g, h**, SDS-PAGE confirming the purity of CBADH$_{WT}$, CBADH$_S$, TBADH$_{WT}$, TBADH$_{S1}$ and TBADH$_{S2}$ used for enzymatic assays. Active, pure proteins were successfully obtained with the described procedure from three independent preparations.

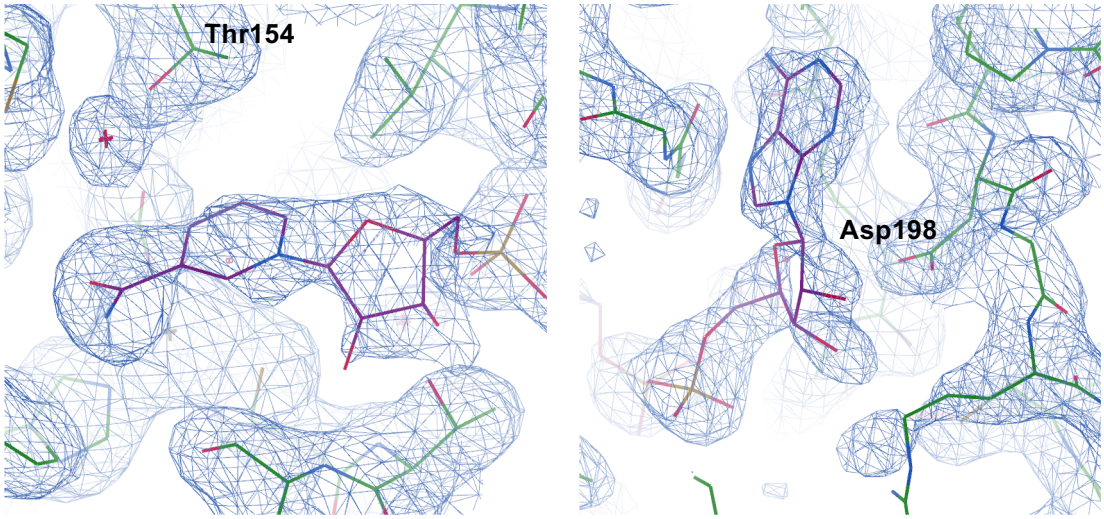


Supplementary Figure 2. Anaerobic cultures of AL, ALS, ALP and ALPS cells containing various NAD and NADP-dependent oxidoreductases. For each *E. coli* strain, anaerobic growth with (right panels) and without (left panels) acetone supplemented to the culture media was followed. **a.** AL mutant (Δ*adhE* Δ*ldhA*). **b.** ALS mutant (Δ*adhE* Δ*ldhA* Δ*sthA*). **c.** ALP mutant (Δ*adhE* Δ*ldhA* Δ*pntA*). **d.** ALPS mutant (Δ*adhE* Δ*ldhA* Δ*pntA* Δ*sthA*). Anaerobic growth of cells with at least one active transhydrogenase was recovered upon transformation of either an NAD or an NADP-dependent oxidoreductase. However, in the case of ALPS cells, where both transhydrogenase genes were deleted, only the NAD-dependent enzyme restored anaerobic growth, indicating that metabolic complementation by NADP-dependent enzymes is mediated by transhydrogenases. Data points of growth curves represent mean values, with error bars showing standard deviation; n=3 biologically independent cultures for all timepoints of growth curves. Source data are provided as a Source Data file.

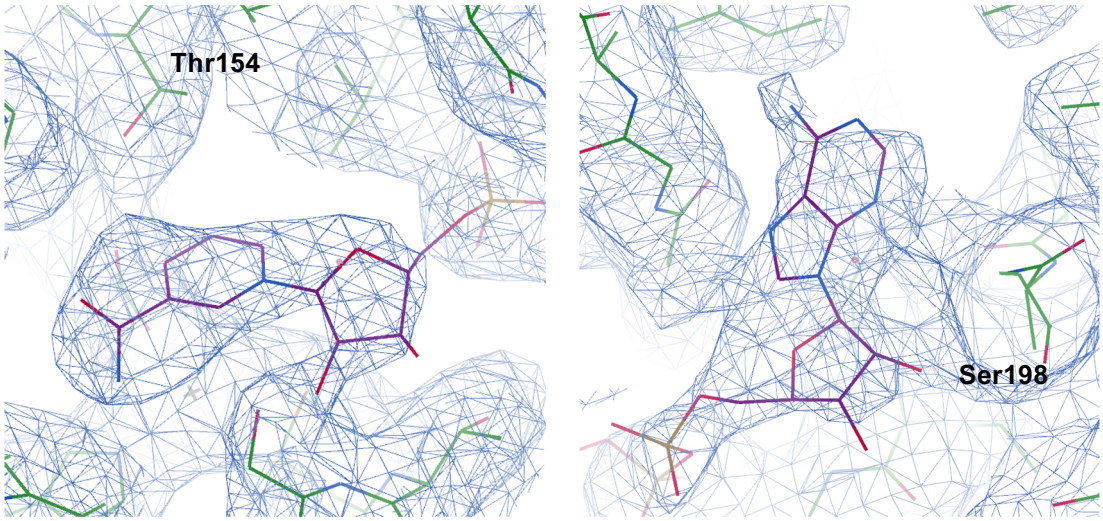


Supplementary Figure 3. Overview of mutations in TBADH$_{S1}$ and TBADH$_{S2}$. Absolute positions are shown above each sequence. Point mutations are shown in red. TBADH$_{S1}$ contains substitutions at all positions targeted for saturation mutagenesis (198, 199, 200 and 218). TBADH$_{S2}$ contains substitutions at all targeted positions and a duplication (shown in pink) of residues 191-241 inserted between positions 241 and 242 of the original sequence. Positions relative to TBADH$_{WT}$ are shown below TBADH$_{S2}$ with a prime symbol. The insertion contains a second copy of the targeted positions, two of which were also substituted. These were positions 249 and 251, corresponding to positions 198' and 200' of TBADH$_{WT}$.

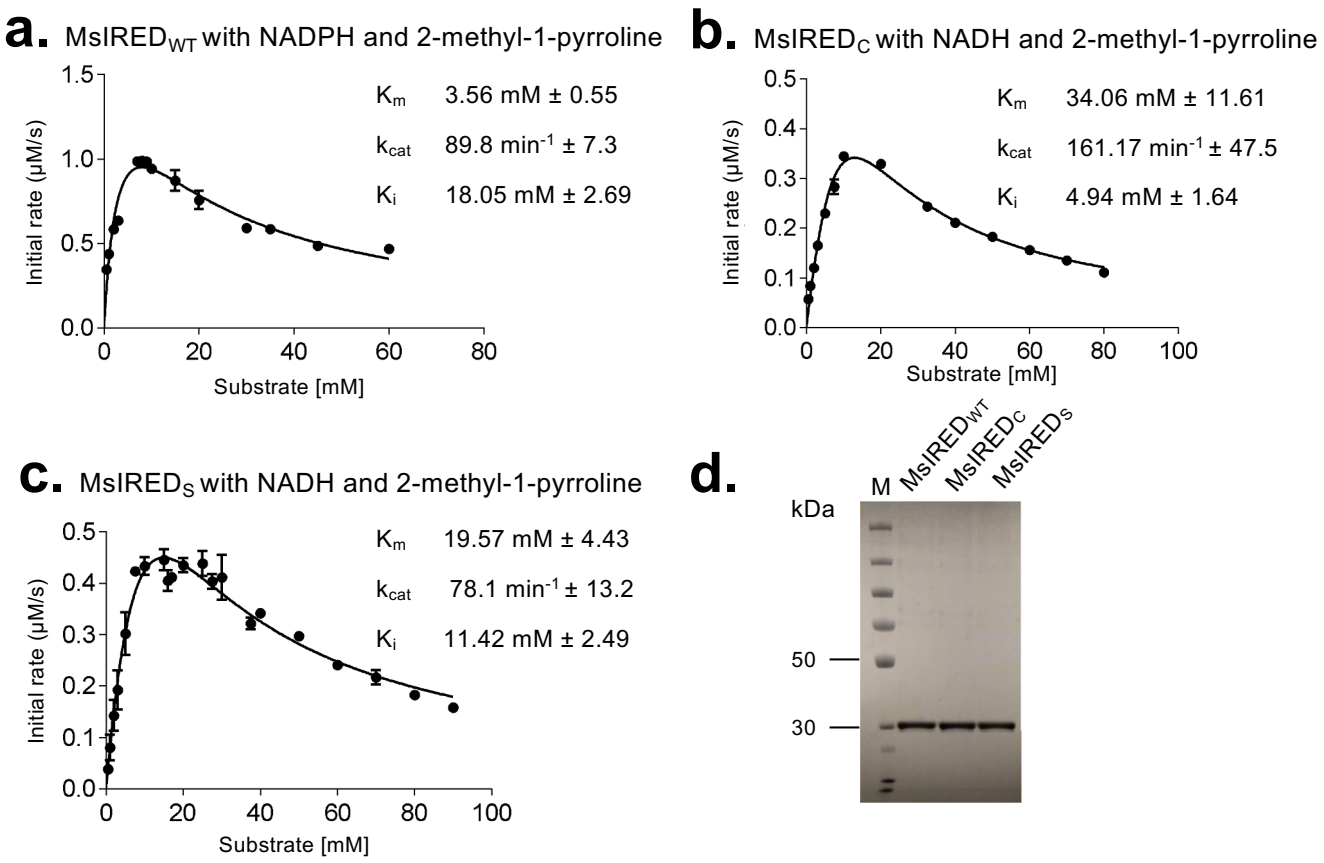
a. $CBADH_S$



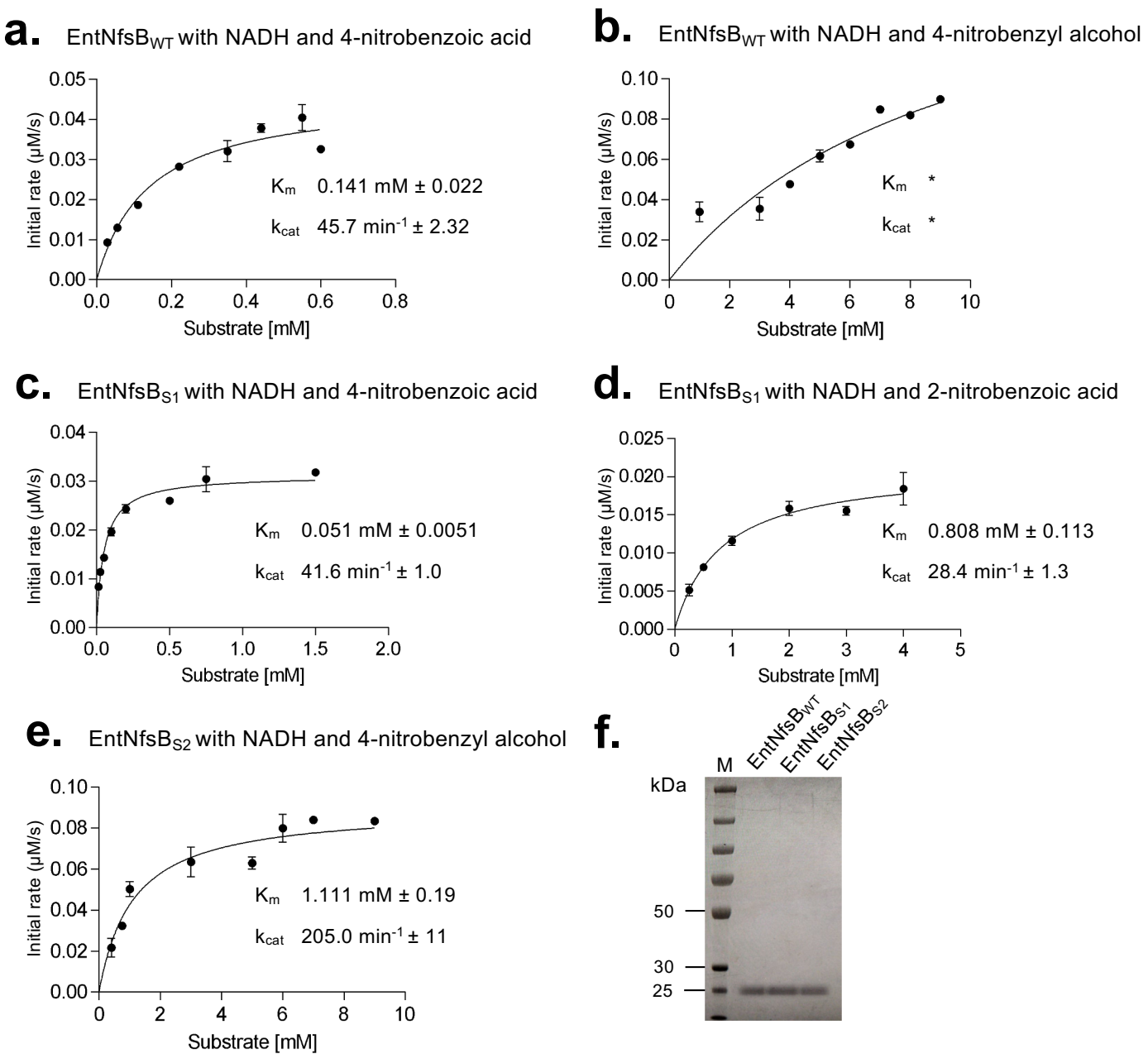
b. $TBADH_{S1}$



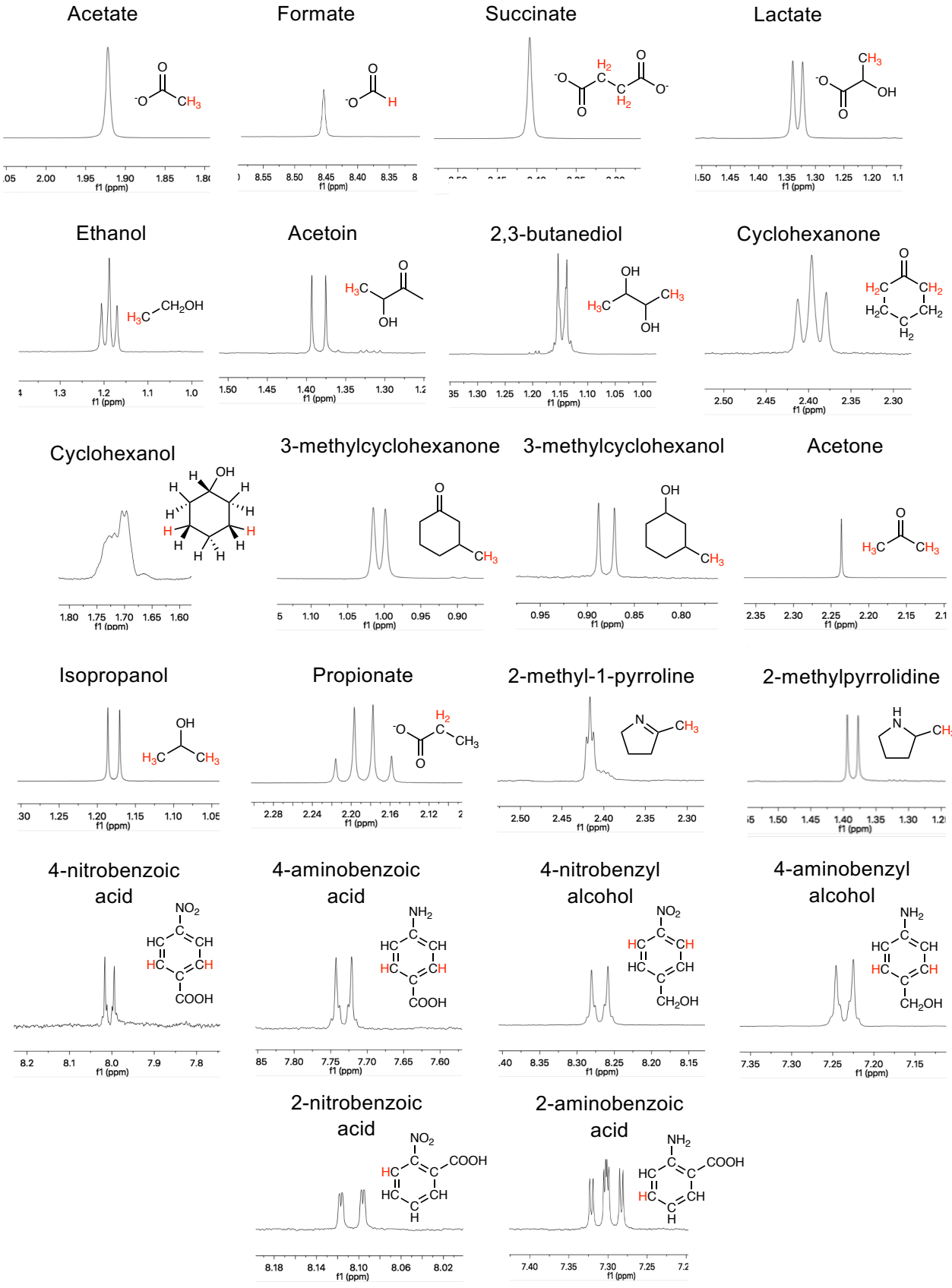
Supplementary Figure 4. Density for the NAD⁺ cofactor in the crystallographic maps of $CBADH_S$ and $TBADH_{S1}$. Left panels display the density for the nicotinamide part of the cofactor, while right panels show the density for the adenine part. The density for the cofactor was stronger in $CBADH_S$ (**a**) than in $TBADH_{S1}$ (**b**), due to partial occupancy of the cofactor in the latter. $2F_o$-F_c maps are shown at sigma level of 1 for $CBADH_S$ and 0.8 for $TBADH_{S1}$. The NAD+ cofactors were refined with an approximate occupancy of 80%.



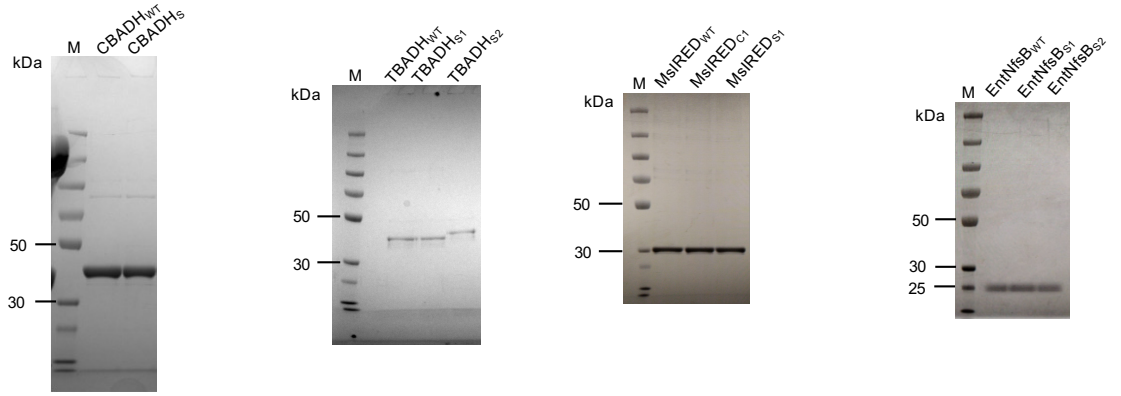
Supplementary Figure 5. Enzymatic activity assays for MsIRED variants. a-c, Initial reaction rates obtained for each enzyme by varying the 2-methyl-1-pyrroline concentration at a fixed concentration of NADH or NADPH. A Michaelis-Menten model equation modified to account for substrate inhibition was fitted in all cases. Data points represent mean values, with error bars showing standard deviation. n=3 biologically independent assays for all substrate concentrations with both enzymes. Source data are provided as a Source Data file. **d,** SDS-PAGE confirming the purity of wild-type MsIRED, MsIRED$_C$ and MsIRED$_S$ used for enzymatic assays. Active, pure proteins were successfully obtained with the described procedure from three independent preparations.



Supplementary Figure 6. Enzymatic activity assays for EntNfsB variants. a-e, Initial reaction rates obtained for each enzyme by varying nitroaromatic substrate (4-NBA, 2-NBA or 4-NBALC) concentration at a fixed concentration of NADH. A Michaelis-Menten model was fitted in all cases. Data points represent mean values, with error bars showing standard deviation. n=3 biologically independent assays for all substrate concentrations with both enzymes. Parameters that could not be determined due to a too low activity are indicated with an asterisk (*). Source data are provided as a Source Data file. **f,** SDS-PAGE confirming the purity of wild-type EntNfsB, EntNfsB$_{S1}$ and EntNfsB$_{S2}$ used for enzymatic assays. Active, pure proteins weresuccessfully obtained with the described procedure from three independent preparations.



Supplementary Figure 7. Characteristic ^{1}H-NMR signals used to quantify each of the analyzed metabolites. Protons contributing to the characteristic signal of each metabolite are highlighted in red.



Supplementary Figure 8. Uncropped gels. The shown gels correspond, from left to right, to Supplementary Fig. 1g, Supplementary Fig. 1h, Supplementary Fig. 5d and Supplementary Fig. 6f.

Supplementary Table 1. ^{1}H-NMR analysis of fermentation broths. Mean values and standard deviations are shown for each metabolite.

Strain	Plasmid	Encoded enzyme	[Substrate] (mM)	[Product] (mM)	[Ethanol] (mM)	[Lactate] (mM)	[Succinate] (mM)	[Acetate] (mM)	[Formate] (mM)
BW25113	-	-	-	-	15.4 ± 0.5	7.1 ± 0.4	2.3 ± 0.1	14.5 ± 0.5	26.2 ± 0.8
AL	pLS1	ADHE	-	-	16.9 ± 0.4	0	2.1 ± 0.1	14.3 ± 0.3	25.6 ± 0.6
AL	pUC19	-	3.2 ± 0.02 (Acetoin)	5.9 ± 0.1 (2,3-butanediol)	0	0	1.2 ± 0.03	6.7 ± 0.1	4.0 ± 0.1
AL	pLS2	BDHA	0 (Acetoin)	8.8 ± 0.2 (2,3-butanediol)	0	0	0.6 ± 0.03	7.5 ± 0.3	4.5 ± 0.1
AL	pLS3	BUDC	0.1 ± 0.002 (Acetoin)	8.5 ± 0.1 (2,3-butanediol)	0	0	0.7 ± 0.1	7.9 ± 0.2	5.7 ± 0.2
AL	pLS12	TADH	0 (Cyclohexanone)	9.1 ± 0.2 (Cyclohexanol)	0	0	0.8 ± 0.05	9.9 ± 0.1	7.9 ± 0.2
AL	pLS12	TADH	4.3 ± 0.1 (3-methylcyclohexanone)	3.8 ± 0.1 (3-methylcyclohexanol)	0	0	0.4 ± 0.01	3.7 ± 0.1	3.1 ± 0.04
AL	pLS6	$CBADH_{WT}$	0.2 ± 0.008 (Acetone)	10.8 ± 0.2 (Isopropanol)	0	0	1.2 ± 0.02	8.3 ± 0.2	6.1 ± 0.1
AL	pLS10_3	$CBADH_{S}$	0.6 ± 0.1 (Acetone)	12.5 ± 0.8 (Isopropanol)	0	0	1.3 ± 0.1	12.8 ± 0.9	9.9 ± 0.7
ALPS	pLS69	$TBADH_{WT}$	8.4 ± 0.2 (Acetone)	0 (Isopropanol)	0	0	0	0	0
ALPS	pLS73_2	$TBADH_{s1}$	0.1 ± 0.01 (Acetone)	14.8 ± 0.3 (Isopropanol)	0	0	1.6 ± 0.1	15.2 ± 0.4	11.2 ± 0.7
ALPS	pLS73_1	$TBADH_{s2}$	0.1 ± 0.04 (Acetone)	15.4 ± 0.4 (Isopropanol)	0	0	1.7 ± 0.1	17.2 ± 0.5	12.5 ± 0.3
AL	pLS130	$MsIRED_{WT}$	5.6 ± 0.1 (2-methyl-1-pyrroline)	6.5 ± 0.1 (2-methylpyrrolidine)	0	0	1.1 ± 0.1	4.3 ± 0.3	2.1 ± 0.1
AL	pLS131	$MsIRED_{C}$	3.3 ± 0.009 (2-methyl-1-pyrroline)	9.3 ± 0.03 (2-methylpyrrolidine)	0	0	1.7 ± 0.01	10.4 ± 0.2	8.5 ± 0.1
AL	pLS133_1	$MsIRED_{S}$	0.8 ± 0.1 (2-methyl-1-pyrroline)	13.3 ± 0.2 (2-methylpyrrolidine)	0	0	1.9 ± 0.1	15.6 ± 0.3	13.6 ± 0.7
AL	pLS168	$EntNfsB_{WT}$	0 (4-nitrobenzoic acid)	0.4 ± 0.008 (4-aminobenzoic acid)	0	0	0.3 ± 0.02	2.9 ± 0.1	2.3 ± 0.2

AL	pLS168	$EntNfsB_{WT}$	8.0 ± 0.02 (2-nitrobenzoic acid)	0 (2-aminobenzoic acid)	0	0	0	0	0
AL	pLS168	$EntNfsB_{WT}$	0.2 ± 0.02 (4-nitrobenzyl alcohol)	0.5 ± 0.03 (4-aminobenzyl alcohol)	0	0	0.5 ± 0.1	6.3 ± 0.1	5.8 ± 0.1
AL	pLS169_1	$EntNfsB_{s1}$	3.2 ± 0.4 (2-nitrobenzoic acid)	3.5 ± 0.04 (2-aminobenzoic acid)	0	0	1.2 ± 0.1	12 ± 0.4	8.9 ± 0.4
AL	pLS169_3	$EntNfsB_{s2}$	0 (4-nitrobenzyl alcohol)	0.8 ± 0.3 (4-aminobenzyl alcohol)	0	0	0.4 ± 0.1	6.1 ± 0.9	5.0 ± 1.2
BW25113	pStA212	-	0 (Acetone)	0 (Isopropanol)	0	0	0	0	0
BW25113	pLS60_1	AtoB/AtoA /AtoD/ ADC/CBA DH_{WT}	12.0 ± 3.6 (Acetone)	62.3 ± 5.1 (Isopropanol)	0	0.8 ± 0.3	2.6 ± 1.6	3.5 ± 1.7	3.6 ± 2.3

Supplementary Table 2. Summary of kinetic parameters of wild-type and evolved enzymes. Parameters that could not be determined due to a too low activity are indicated with an asterisk (*). Cases for which substrate inhibition was not observed are indicated with a hyphen (-).

Enzyme	Substrate	Cosubstrate	K_m	k_{cat} (min^{-1})	k_{cat}/K_m	K_i (mM)
$CBADH_{WT}$	Isopropanol	$NADP^+$	5.80 ± 0.25 mM	1185.6 ± 14.0	204.4 ± 9.1 $min^{-1}mM^{-1}$	-
$CBADH_S$	Isopropanol	NAD^+	17.49 ± 2.30 mM	333.0 ± 12.8	19.0 ± 2.6 $min^{-1}mM^{-1}$	-
$TBADH_{WT}$	Isopropanol	$NADP^+$	119.4 ± 6.9 mM	498.0 ± 9.2	4.2 ± 0.25 $min^{-1}mM^{-1}$	-
$TBADH_{S1}$	Isopropanol	NAD^+	3.74 ± 0.54 mM	111.5 ± 5.7	29.8 ± 4.6 $min^{-1}mM^{-1}$	-
$TBADH_{S2}$	Isopropanol	NAD^+	22.07 ± 2.70 mM	238.5 ± 8.7	10.8 ± 1.4 $min^{-1}mM^{-1}$	-
$TBADH_{S2}$	Isopropanol	$NADP^+$	55.15 ± 2.57 mM	231.4 ± 3.7	4.2 ± 0.2 $min^{-1}mM^{-1}$	-
$MsIRED_{WT}$	2-methyl-1-pyrroline	NADPH	3.56 ± 0.55 mM	89.8 ± 7.3	-	18.05 ± 2.69
$MsIRED_C$	2-methyl-1-pyrroline	NADH	34.06 ± 11.61 mM	161.2 ± 47.5	-	4.94 ± 1.64
$MsIRED_S$	2-methyl-1-pyrroline	NADH	19.57 ± 4.43 mM	78.1 ± 13.2	-	11.42 ± 2.49
$EntNfsB_{WT}$	4-nitrobenzoic acid	NADH	0.141 ± 0.022 mM	45.7 ± 2.3	324.1 ± 53.2 $min^{-1}mM^{-1}$	-
$EntNfsB_{WT}$	4-nitrobenzyl alcohol	NADH	*	*	*	-
$EntNfsB_{S1}$	4-nitrobenzoic acid	NADH	0.051 ± 0.0051 mM	41.6 ± 1.0	815.7 ± 83.9 $min^{-1}mM^{-1}$	-
$EntNfsB_{S1}$	2-nitrobenzoic acid	NADH	0.81 ± 0.113 mM	28.4 ± 1.3	35.1 ± 5.1 $min^{-1}mM^{-1}$	-
$EntNfsB_{S2}$	4-nitrobenzyl alcohol	NADH	1.11 ± 0.19 mM	205.0 ± 11.0	184.5 ± 33.1 $min^{-1}mM^{-1}$	-
$CBADH_{WT}$	$NADP^+$	Isopropanol	55.91 ± 6.58 µM	1072.5 ± 55.17	19.18 ± 2.46 $min^{-1}µM^{-1}$	-
$CBADH_S$	NAD^+	Isopropanol	934.4 ± 136.2 µM	290.58 ± 23.17	0.31 ± 0.05 $min^{-1}µM^{-1}$	-
$TBADH_{WT}$	$NADP^+$	Isopropanol	56.67 ± 11.63 µM	482.19 ± 25.9	8.51 ± 1.81 $min^{-1}µM^{-1}$	-
$TBADH_{S1}$	NAD^+	Isopropanol	1.03 ± 0.16 mM	109.0 ± 4.0	105.6 ± 17.1 $min^{-1}mM^{-1}$	-
$TBADH_{S2}$	NAD^+	Isopropanol	104.4 ± 9.68 µM	220.12 ± 6.17	2.11 ± 0.20 $min^{-1}µM^{-1}$	-
$TBADH_{S2}$	$NADP^+$	Isopropanol	240.7 ± 29.4 µM	234.68 ± 10.57	0.97 ± 0.13 $min^{-1}µM^{-1}$	-

MsIRED$_{WT}$	NADPH	2-methyl-1-pyrroline	24.67 ± 3.68 μM	56.65 ± 2.9	2.30 ± 0.36 $min^{-1}μM^{-1}$	-
MsIRED$_{C}$	NADH	2-methyl-1-pyrroline	23.66 ± 2.5 μM	15.05 ± 0.79	0.64 ± 0.075 $min^{-1}μM^{-1}$	-
MsIRED$_{S}$	NADH	2-methyl-1-pyrroline	19.9 ± 1.71 μM	21.10 ± 0.84	1.06 ± 0.1 $min^{-1}μM^{-1}$	-
EntNfsB$_{WT}$	NADH	4-nitrobenzoic acid	29.78 ± 1.3 μM	46.48 ± 1.03	1.56 ± 0.076 $min^{-1}μM^{-1}$	-
EntNfsB$_{WT}$	NADH	4-nitrobenzyl alcohol	30.65 ± 3.96 μM	297.7 ± 18.23	9.71 ± 1.39 $min^{-1}μM^{-1}$	-
EntNfsB$_{S1}$	NADH	4-nitrobenzoic acid	28.90 ± 1.87 μM	41.88 ± 1.14	1.45 ± 0.10 $min^{-1}μM^{-1}$	-
EntNfsB$_{S1}$	NADH	2-nitrobenzoic acid	26.37 ± 3.49 μM	28.57 ± 1.20	1.08 ± 0.15 $min^{-1}μM^{-1}$	-
EntNfsB$_{S2}$	NADH	4-nitrobenzyl alcohol	28.86 ± 2.73 μM	219.16 ± 9.0	7.59 ± 0.78 $min^{-1}μM^{-1}$	-

Supplementary Table 3. Plasmids used in this work.

Plasmid	Description	Antibiotic resistance	Source
pUC19	High copy expression vector (pMB1 origin of replication) with lacZα – **Negative control** for anaerobic growth complementation experiments	AmpR	Heap Laboratory (Norrander, Kempe, and Messing, 1983)
pJET1.2	Cloning vector	AmpR	Invitrogen
pET28a	Bacterial expression vector with 6xHis-tag	KanR	Invitrogen
pMAK705	*E. coli* vector for directed mutagenesis by allele exchange	CmR	Heap Laboratory (Hamilton, *et al.*, 1989)
pStA0	Start-Stop Assembly Level 0 storage vector	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pStA1AB	Start-Stop Assembly Level 1 vector (A and B fusion sites)	TetR	Heap Laboratory (Taylor *et al.*, 2019)
pStA1BC	Start-Stop Assembly Level 1 vector (B and C fusion sites)	TetR	Heap Laboratory (Taylor *et al.*, 2019)
pStA1CD	Start-Stop Assembly Level 1 vector (C and D fusion sites)	TetR	Heap Laboratory (Taylor *et al.*, 2019)
pStA1DE	Start-Stop Assembly Level 1 vector (D and E fusion sites)	TetR	Heap Laboratory (Taylor *et al.*, 2019)
pStA1EZ	Start-Stop Assembly Level 1 vector (E and Z fusion sites)	TetR	Heap Laboratory (Taylor *et al.*, 2019)
pGT323	pStA0 containing BBa_J23100 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT324	pStA0 containing BBa_J23102 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT326	pStA0 containing BBa_J23107 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT327	pStA0 containing BBa_J23116 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT328	pStA0 containing BBa_J23113 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT336	pStA0 containing BBa_J23118 promoter	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT330	pStA0 containing RBSc13	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT331	pStA0 containing RBSc33	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT332	pStA0 containing RBSc44	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT333	pStA0 containing RBSc58	AmpR	Heap Laboratory (Taylor *et al.*, 2019)
pGT334	pStA0 containing RBSc36	AmpR	Heap Laboratory (Taylor *et al.*, 2019)

pGT335	pStA0 containing RBSc42	AmpR	Heap Laboratory (Taylor *et al*., 2019)
pGT337	pStA0 containing Terminator 1 (L3S2P55)	AmpR	Heap Laboratory (Taylor *et al*., 2019)
pGT338	pStA0 containing Terminator 2 (L3S2P21)	AmpR	Heap Laboratory (Taylor *et al*., 2019)
pGT339	pStA0 containing Terminator 3 (ECK120033737)	AmpR	Heap Laboratory (Taylor *et al*., 2019)
pGT340	pStA0 containing Terminator 4 (ECK1200196000)	AmpR	Heap Laboratory (Taylor *et al*., 2019)
pStA212	Start-Stop Assembly Level 2 vector (fusion sites 1 and 2) – **Negative control** for isopropanol pathway	KanR	Heap Laboratory (Taylor *et al*., 2019)
pLS1	pUC19 containing sequence encoding ADHE of *E. coli* BW25113 (AIN31697.1) – **Positive control** for anaerobic growth experiments	AmpR	This work
pLS2	pUC19 containing sequence encoding BDHA of *Bacillus subtilis* 168 (CAB12443.1)	AmpR	This work
pLS3_1	pJET1.2 storage plasmid containing sequence encoding BUDC of *Klebsiella pneumoniae* (AAC78679.1)	AmpR	This work
pLS3	pUC19 containing sequence encoding BUDC of *Klebsiella pneumoniae* (AAC78679.1)	AmpR	This work
pLS6	pUC19 containing sequence encoding CBADH of *Clostridium beijerinckii* (AAA23199.2)	AmpR	This work
pLS10	Library equivalent to pLS6 with saturation mutagenesis of amino acid positions 198, 199, 200 and 218 of CBADH ($CBADH_{Lib}$)	AmpR	This work
pLS10_3	Plasmid from library pLS10 encoding variant $CBADH_S$ (G198D, S199Y, R200R, Y218P)	AmpR	This work
pLS12_1	pJET1.2 storage plasmid containing sequence encoding TADH of *Thermus* sp. ATN-1 (ACD50896.1)	AmpR	This work

pLS12	pUC19 containing sequence encoding TADH of *Thermus* sp. ATN-1 (ACD50896.1)	AmpR	This work
pLS39	pMAK705 with 500 bp upstream and 500 bp downstream of *sthA* from *E. coli*	CmR	This work
pLS40	pMAK705 with 500 bp upstream and 500 bp downstream of *pntA* from *E. coli*	CmR	This work
pLS46	pStA0 containing *atoB* gene of *E. coli* (AAC75284.1)	AmpR	This work
pLS47	pStA0 containing *atoD* gene of *E. coli* (AAC75281.1)	AmpR	This work
pLS48	pStA0 containing *atoA* gene of *E. coli* (AAC75282.1)	AmpR	This work
pLS49	pStA0 containing *adc* gene of *Clostridium acetobutylicum* (AAA63761.1)	AmpR	This work
pLS50	pStA0 containing *cbadh* gene of *Clostridium beijerinckii* (AAA23199.2)	AmpR	This work
pLS53	pStA1AB containing full transcription unit coding for acetyl-CoA acetyltransferase from *E. coli* (*atoB*) (library of six promoters and six RBS, Terminator 1)	TetR	This work
pLS54	pStA1BC containing full transcription unit coding for acetate CoA-transferase subunit alpha from *E. coli* (*atoD*) (library of six promoters and six RBS, Terminator 2)	TetR	This work
pLS55	pStA1CD containing full transcription unit coding for acetate CoA-transferase subunit beta from *E. coli* (*atoA*) (library of six promoters and six RBS, Terminator 3)	TetR	This work
pLS56	pStA1DE containing full transcription unit coding for acetoacetate decarboxylase from *Clostridium acetobutylicum* (*adc*) (library of six promoters and six RBS, Terminator 4)	TetR	This work

pLS57	pStA1EZ containing full transcription unit coding for NADP-dependent isopropanol dehydrogenase from *Clostridium beijerinckii* (*cbadh*) (library of six promoters and six RBS, Terminator 1)	TetR	This work
pLS60	pStA212 containing combinatorial isopropanol pathway library MP_{Lib}	KanR	This work
pLS60_1	Plasmid from library MP_{Lib} (pLS60) encoding isopropanol pathway variant MP_{S1}	KanR	This work
pLS60_2	Pathway from library MP_{Lib} (pLS60) encoding isopropanol pathway variant MP_{S2}	KanR	This work
pLS63	pMAK705 with 500 bp upstream and 500 bp downstream of *ldhA* from *E. coli*	CmR	This work
pLS67	pJET1.2 storage plasmid containing sequence encoding TBADH of *Thermoanaerobacter brockii* (CAA46053.1)	AmpR	This work
pLS69	pUC19 containing sequence encoding TBADH of *Thermoanaerobacter brockii* (CAA46053.1)	AmpR	This work
pLS73	Library equivalent to pLS69 with saturation mutagenesis of amino acid positions 198, 199, 200 and 218 of TBADH ($TBADH_{Lib}$)	AmpR	This work
pLS73_1	Plasmid from library pLS73 encoding variant $TBADH_{S2}$ (G198H, S199R, R200A, Y218M and a 153 bp insertion between residues 241 and 242)	AmpR	This work
pLS73_2	Plasmid from library pLS73 encoding variant $TBADH_{S1}$ (G198S, S199K, R200P, Y218V)	AmpR	This work
pLS90	pET28a containing sequence encoding $TBADH_{S2}$ with N-terminal 6xHis-tag	KanR	This work

pLS91	pET28a containing sequence encoding $TBADH_{S1}$ with N-terminal 6xHis-tag	KanR	This work
pLS97	pET28a containing sequence encoding wild-type TBADH with N-terminal 6xHis-tag	KanR	This work
pLS98	pET28a containing sequence encoding $CBADH_S$ with N-terminal 6xHis-tag	KanR	This work
pLS99	pET28a containing gene encoding wild-type CBADH with N-terminal 6xHis-tag	KanR	This work
pLS129	pJET1.2 storage plasmid containing sequence encoding MsIRED of *Myxococcus stipitatus* (AGC43099.1)	AmpR	This work
pLS130	pUC19 containing sequence encoding MsIRED of *Myxococcus stipitatus* (AGC43099.1)	AmpR	This work
pLS131	pUC19 containing sequence encoding $MsIRED_C$ variant of *Myxococcus stipitatus* MsIRED (N32E, R33Y, T34E, K37R, L67I, T71V)	AmpR	This work
pLS133	Library equivalent to pLS131 with saturation mutagenesis of amino acid positions 32, 33, 34 and 37 of MsIRED ($MsIRED_{Lib}$)	AmpR	This work
pLS133_1	Plasmid from library pLS133 encoding variant $MsIRED_S$ (N32E, R33V, T34R, K37R)	AmpR	This work
pLS161	pUC19 containing sequence encoding wild-type MsIRED with C-terminal 6xHis-tag	AmpR	This work
pLS162	pUC19 containing sequence encoding $MsIRED_C$ with C-terminal 6xHis-tag	AmpR	This work
pLS164	pUC19 containing sequence encoding $MsIRED_S$ with C-terminal 6xHis-tag	AmpR	This work
pLS168_1	pJET1.2 storage plasmid containing sequence encoding EntNfsB of *Enterobacter cloacae* (AAA62801.1)	AmpR	This work
pLS168	pUC19 containing sequence encoding EntNfsB of	AmpR	This work

	Enterobacter cloacae (AAA62801.1)		
pLS169	Library equivalent to pLS168 with saturation mutagenesis of amino acid positions 40, 41, 68 and 124 of EntNfsB ($EntNfsB_{Lib}$)	AmpR	This work
pLS169_1	Plasmid from library pLS133 encoding variant $EntNfsB_{S1}$ (S40A, T41I, Y68Y, F124A)	AmpR	This work
pLS169_3	Plasmid from library pLS133 encoding variant $EntNfsB_{S2}$ (S40S, T41L, Y68L, F124L)	AmpR	This work
pLS180	pUC19 containing sequence encoding EntNfsB with C-terminal 6xHis-tag	AmpR	This work
pLS181	pUC19 containing sequence encoding $EntNfsB_{S1}$ with C-terminal 6xHis-tag	AmpR	This work
pLS182	pUC19 containing sequence encoding $EntNfsB_{S2}$ with C-terminal 6xHis-tag	AmpR	This work

Supplementary Table 4. Oligonucleotides used in this work. Restriction sites are highlighted in green.

Purpose	Template	Oligo ID	Description	Sequence (5' – 3')
Amplification of pUC19 for Golden Gate Assembly	pUC19	oligoLS315	Fw - *BbsI*	TCTCTGAAGACCCTAAGGATCCCCGGGTACC
Amplification of pUC19 for Golden Gate Assembly	pUC19	oligoLS314	Rv - *BbsI*	TCTCTGAAGACTCCATGTGTTCGTACCTCCTGC ATG
Construction of pLS1	*adhE* of *E. coli* BW25113	oligoLS19	Fw - *SphI*	CCGTTCGCATGCAGGAGGTACGAACACATGGC TGTTACTAATGT
Construction of pLS1	*adhE* of *E. coli* BW25113	oligoLS20	Rv - *BamHI*	GCTGAAGGATCCTTAAGCGGATTTTTTCG
Verification of *adhE* KO	*adhE* of *E. coli* BW25113	oligoLS1	Fw - internal	CCTGTGGTGTTCTGTCTG
Verification of *adhE* KO	*adhE* of *E. coli* BW25113	oligoLS2	Rv - internal	TAGATTTCGGAATACCCA
Verification of *adhE* KO	*adhE* of *E. coli* BW25113	oligoLS3	Fw - external	GGCGAAAAGCGATGCTG
Verification of *adhE* KO	*adhE* of *E. coli* BW25113	oligoLS4	Rv - external	CGGTGGGAAGGTGTTCTGC
Verification of *ldhA* KO	*ldhA* of *E. coli* BW25113	oligoLS5	Fw - internal	GCCGCCCGGTGCTGGAAG
Verification of *ldhA* KO	*ldhA* of *E. coli* BW25113	oligoLS6	Rv - internal	GGCGACGGAATACGTCAT
Verification of *ldhA* KO	*ldhA* of *E. coli* BW25113	oligoLS7	Fw - external	GAAGGTTGCGCCTACACT
Verification of *ldhA* KO	*ldhA* of *E. coli* BW25113	oligoLS8	Rv - external	CACCAAAGCTGATTTCTG
Construction of pLS2	Sequence encoding BDHA of *Bacillus subtilis* 168	oligoLS23	Fw - *SphI*	CCGTTCGCATGCAGGAGGTACGAACACATGAA GGCAGCAAGATG
Construction of pLS2	Sequence encoding BDHA of *Bacillus subtilis* 168	oligoLS24	Rv - *BamHI*	GCTGAAGGATCCTTAGTTAGGTCTAACAAGGAT TTTGACT
Construction of pLS6	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS87	Fw - *SphI*	GTTCGCATGCATTCGGATCTATACAGATAAGGA GAAAGAGATGAAAGGCTTTGCCATGCTGGG
Construction of pLS6	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS88	Rv - *BamHI*	CTTCCATGGATCCTCACTATTAGAGGATAACTA CGGCC
Construction of pLS10 library ($CBADH_{Lib}$)	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS112	Rv	CTTGGCGGCCTCAACGCAAATAGGNNNNNNNN NGACACCAATAATCCGACCTGC

Construction of pLS10 library ($CBADH_{Lib}$)	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS113	Fw	TTCTACGGCGCGACCGACATTCTGAATNNNAAA AATGGCCATATTGTGGAC
Construction of pLS39	500 bp upstream and downstream of *sthA*	oligoLS208	Fw - *BamHI*	TTCAGCGGATCCAATGTATCTGCATGAAGCACA GACCCACCAGTTACTGG
Construction of pLS39	500 bp upstream and downstream of *sthA*	oligoLS212	Rv	AACAGGTAAGCCCTACCATGTAAAACTTTATCG AAATGGCCATCCATTCTTGCGCGG
Construction of pLS39	500 bp upstream and downstream of *sthA*	oligoLS213	Fw	GCCATTTCGATAAAGTTTTACATGGTAGGGCTT ACCTGTTCTTATACATAAAAGCAACAGAATGG
Construction of pLS39	500 bp upstream and downstream of *sthA*	oligoLS209	Rv - *HindIII*	TTCAGCAAGCTTCATTAAACCGCTCTCATCAAC CATGGTCAGACCCAGTTCG
Verification of *sthA* KO	*sthA* of *E. coli* BW25113	oligoLS194	Fw - internal	GATGGAACAAAATTTTCAGCGTGCC
Verification of *sthA* KO	*sthA* of *E. coli* BW25113	oligoLS193	Rv - internal	ATAGTAATAGGTTCCGGCCC
Verification of *sthA* KO	*sthA* of *E. coli* BW25113	oligoLS195	Fw - external	CAGGCAATGGGTTTCTGTTTTG
Verification of *sthA* KO	*sthA* of *E. coli* BW25113	oligoLS196	Rv - external	CGAACTGGGTCTGACCATGGTTGATGAGAGCG GTTTAATG
Construction of pLS40	500 bp upstream and downstream of *pntA*	oligoLS216	Fw - *BamHI*	TTCAGCGGATCCGAAACGACCAGAGCCGCCAG GTTCA
Construction of pLS40	500 bp upstream and downstream of *pntA*	oligoLS217	Rv	CCGATGGAAGGGAATATCATGTAAGGGGTAAC ATATGTCTGGAGGATTAGTTACAGCTGCATACA TTGTTGCCGC
Construction of pLS40	500 bp upstream and downstream of *pntA*	oligoLS219	Fw	CCAGACATATGTTACCCCTTACATGATATTCCC TTCCATCGGTTTTATTGATG
Construction of pLS40	500 bp upstream and downstream of *pntA*	oligoLS218	Rv - *HindIII*	TTCAGCAAGCTTCAGGAGGGTGTTCTTAAGCTT CATAAAAATAATCCTTCGCCTTGCGCAAA
Verification of *pntA* KO	*pntA* of *E. coli* BW25113	oligoLS223	Fw - internal	GTGCTCCGACAACAATAATCC
Verification of *pntA* KO	*pntA* of *E. coli* BW25113	oligoLS224	Rv - internal	TGATGGTGATTGGTGCGGGTG
Verification of *pntA* KO	*pntA* of *E. coli* BW25113	oligoLS216	Fw - external	GAAACGACCAGAGCCGCCAGGTTCA
Verification of *pntA* KO	*pntA* of *E. coli* BW25113	oligoLS221	Rv - external	TTTGCGCAAGGCGAAGGATTATTTTTATGAAGC
Construction of pLS46	*atoB* of *E. coli* BW25113	oligoLS230	Fw - *BsaI*	AAGGGGTTGGTCTCATGTGCTCTTCGATGAAAA ATTGTGTCATCGTCAGTGCGGTACG
Construction of pLS46	*atoB* of *E. coli* BW25113	oligoLS231	Rv - *BsaI*	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATT AATTCAACCGTTCAATCACCATCGCAATTCCC
Construction of pLS47	*atoD* of *E. coli* BW25113	oligoLS234	Fw - *BsaI*	AAGGGGTTGGTCTCATGTGGCTCTTCGATGAAA ACAAAATTGATGACATTACAAGACG
Construction of pLS47	*atoD* of *E. coli* BW25113	oligoLS243	Rv - *BsaI*	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATT ATTTGCTCTCCTGTGAAACGATGATGTG
Construction of pLS48	*atoA* of *E. coli* BW25113	oligoLS235	Fw - *BsaI*	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATT ATAAATCACCCCGTTGCGTATTC
Construction of pLS48	*atoA* of *E. coli* BW25113	oligoLS242	Rv - *BsaI*	AAGGGGTTGGTCTCATGTGGCTCTTCGATGGA TGCGAAACAACGTATTGCGC

Construction of pLS49	*adc* of *Clostridium acetobutylicum* ATCC 824	oligoLS228	Fw - *BsaI*	AAGGGGTTGGTCTCATGTGGCTCTTCGATGTTAAAGGATGAAGTAATTAAACAAATTAGCACG
Construction of pLS49	*adc* of *Clostridium acetobutylicum* ATCC 824	oligoLS229	Rv - *BsaI*	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATTACTTAAGATAATCATATATAACTTCAGCTCTAGGC
Construction of pLS50	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS232	Fw - *BsaI*	AAGGGGTTGGTCTCATGTGGCTCTTCGATGAAAGGCTTTGCCATGCTGGGTATTAAC
Construction of pLS50	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS233	Rv - *BsaI*	AAGGGGTTGGTCTCTGGTCTTACGCTCTTCATTAGAGGATAACTACGGCCTTAATGAGATCTTTAGG
Construction of pLS63	500 bp upstream and downstream of *ldhA*	oligoLS244	Fw - *BamHI*	TTCAGCGGATCCTGTCTGTTTTGCGGTCGCCAG
Construction of pLS63	500 bp upstream and downstream of *ldhA*	oligoLS247	Rv	CACTGGAGAAAGTCTTATGTAATCTTGCCGCTCCCCTGCATTCCAG
Construction of pLS63	500 bp upstream and downstream of *ldhA*	oligoLS246	Fw	CAGGGGAGCGGCAAGATTACATAAGACTTTCTCCAGTGATGTTGAATC
Construction of pLS63	500 bp upstream and downstream of *ldhA*	oligoLS245	Rv - *HindIII*	TTCAGCAAGCTTCAAGCAGAATCAAGTTCTACCGTGC
Construction of pLS73 library ($TBADH_{Lib}$)	Sequence encoding TBADH of *Thermoanaerobacter brockii*	oligoLS258	Rv	AGCGTCGACACAGACTGGNNNNNNNNNAACAGCAATGATACGTCCTGC
Construction of pLS73 library ($TBADH_{Lib}$)	Sequence encoding TBADH of *Thermoanaerobacter brockii*	oligoLS259	Fw	GCCAAGTATTACGGAGCAACCGACATCGTGAACNNNAAGGATGGGCC
Construction of pLS90	Sequence encoding $TBADH_{S2}$	oligoLS288	Fw - *NdeI*	GCAGCCATATGATGAAGGGGTTCGCAATGCTGTCTATTGG
Construction of pLS90	Sequence encoding $TBADH_{S2}$	oligoLS289	Rv - *BlpI*	TTATTGCTCAGCTTAAGCCAGAATAACCACTGGTTTGATAAGGTCCTTTGG
Construction of pLS91	Sequence encoding $TBADH_{S2}$	oligoLS288	Fw - *NdeI*	GCAGCCATATGATGAAGGGGTTCGCAATGCTGTCTATTGG
Construction of pLS91	Sequence encoding $TBADH_{S2}$	oligoLS289	Rv - *BlpI*	TTATTGCTCAGCTTAAGCCAGAATAACCACTGGTTTGATAAGGTCCTTTGG
Construction of pLS97	Sequence encoding TBADH of *Thermoanaerobacter brockii*	oligoLS288	Fw - *NdeI*	GCAGCCATATGATGAAGGGGTTCGCAATGCTGTCTATTGG
Construction of pLS97	Sequence encoding TBADH of *Thermoanaerobacter brockii*	oligoLS289	Rv - *BlpI*	TTATTGCTCAGCTTAAGCCAGAATAACCACTGGTTTGATAAGGTCCTTTGG
Construction of pLS98	Sequence encoding $CBADH_S$	oligoLS294	Fw - *NdeI*	GCAGCCATATGATGAAAGGCTTTGCCATGCTGGGTATTAACAAATTAGG
Construction of pLS98	Sequence encoding $CBADH_S$	oligoLS295	Rv - *BlpI*	TTATTGCTCAGCTTAGAGGATAACTACGGCCTTAATGAGATCTTTAGGTTTATCTTTCATGAG

Construction of pLS99	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS294	Fw - *NdeI*	GCAGCCATATGATGAAAGGCTTTGCCATGCTG GGTATTAACAAATTAGG
Construction of pLS99	Sequence encoding CBADH of *Clostridium beijerinckii*	oligoLS295	Rv - *BlpI*	TTATTGCTCAGCTTAGAGGATAACTACGGCCTT AATGAGATCTTTAGGTTTATCTTTCATGAG
Construction of pLS131	Sequence encoding $MsIRED_C$	oligoLS344	Rv	ACGATAATATCGCTGCGTTTAAC
Construction of pLS131	Sequence encoding $MsIRED_C$	oligoLS345	Fw	CTGGCAAAACTGGGCGCACATC
Construction of pLS131	Sequence encoding $MsIRED_C$	oligoLS342	Rv	/5'Phos/CGGTTCGCTACGGGCTTTTTCATATTCC CACACCGTGGTCG
Construction of pLS131	Sequence encoding $MsIRED_C$	oligoLS343	Fw	/5'Phos/GGTTAATGTGATTGATTATGACGTTTCT GATCAGCTGCTG
Construction of pLS133 library ($MsIRED_{Lib}$)	Sequence encoding MsIRED of *Myxococcus stipitatus*	oligoLS337	Fw	GCTGAGAAGACCGACCACGGTGTGGNNNNNN NNNAAAGCCNNNAGCGAACCGCTGGCAAAACT G
Construction of pLS133 library ($MsIRED_{Lib}$)	Sequence encoding MsIRED of *Myxococcus stipitatus*	oligoLS338	Rv	GCTGAGAAGACCGTGGTCGTGTAGCCAGATTG CAGGAATGCTTTAATCAGTGCGGAGCCCATAC GGCC
Construction of pLS161	Sequence encoding wild-type MsIRED with C-terminal 6xHis-tag	oligoLS358	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTG GTGTTTCAGGAAGCGGGTCAGAATTGCAAAG
Construction of pLS161	Sequence encoding wild-type MsIRED with C-terminal 6xHis-tag	oligoLS359	Fw - *BbsI*	TCTCTGAAGACAACATGAAACCGACCCTGACC GTTATTGGC
Construction of pLS162	Sequence encoding $MsIRED_C$ with C-terminal 6xHis-tag	oligoLS358	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTG GTGTTTCAGGAAGCGGGTCAGAATTGCAAAG
Construction of pLS162	Sequence encoding $MsIRED_C$ with C-terminal 6xHis-tag	oligoLS359	Fw- *BbsI*	TCTCTGAAGACAACATGAAACCGACCCTGACC GTTATTGGC
Construction of pLS164	Sequence encoding $MsIRED_S$ with C-terminal 6xHis-tag	oligoLS358	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTG GTGTTTCAGGAAGCGGGTCAGAATTGCAAAG
Construction of pLS164	Sequence encoding $MsIRED_S$ with C-terminal 6xHis-tag	oligoLS359	Fw - *BbsI*	TCTCTGAAGACAACATGAAACCGACCCTGACC GTTATTGGC
Construction of pLS169 library ($EntNfsB_{Lib}$)	Sequence encoding EntNfsB of *Enterobacter cloacae*	oligoLS363	Rv - *BbsI*	TCTCTGAAGACTCGGTGCTGGCTACAATGAAGT GCCACGGCTGGGAGTTNNNNNNGGACGGGCT GTACTGC
Construction of pLS169	Sequence encoding EntNfsB of *Enterobacter cloacae*	oligoLS366	Fw - *BbsI*	CTCTGAAGACCAGTGGATGGCGAAGCAGGTTT ACCTGAACGTCGG

library ($EntNfsB_{Lib}$)				
Construction of pLS169 library ($EntNfsB_{Lib}$)	Sequence encoding EntNfsB of *Enterobacter cloacae*	oligoLS364	Fw - *BbsI*	CTCTGAAGACAGCACCGAGGAAGGAAAAGCGCGCGTGGCGAAGTCCGCTGCGGGCACCNNNGTGTTCAACGAACG
Construction of pLS169 library ($EntNfsB_{Lib}$)	Sequence encoding EntNfsB of *Enterobacter cloacae*	oligoLS365	Rv - *BbsI*	TCTCTGAAGACATCCACTGGTCGTCATCTTTCAGATCCACGCGGTGCATGTCGGCNNNGTAGGTGCGGCC
Construction of pLS180	Sequence encoding EntNfsB with C-terminal 6xHis-tag	oligoLS385	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTGGTGGCACTCGGTCACAATCGTGCTCAGC
Construction of pLS180	Sequence encoding EntNfsB with C-terminal 6xHis-tag	oligoLS386	Fw - *BbsI*	TCTCTGAAGACAACATGGATATCATTTCTGTCGCCCTG
Construction of pLS181	Sequence encoding $EntNfsB_{S1}$ with C-terminal 6xHis-tag	oligoLS385	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTGGTGGCACTCGGTCACAATCGTGCTCAGC
Construction of pLS181	Sequence encoding $EntNfsB_{S1}$ with C-terminal 6xHis-tag	oligoLS386	Fw - *BbsI*	TCTCTGAAGACAACATGGATATCATTTCTGTCGCCCTG
Construction of pLS182	Sequence encoding $EntNfsB_{S2}$ with C-terminal 6xHis-tag	oligoLS385	Rv - *BbsI* 6xHis-tag	TCTCTGAAGACTCCTTAGTGGTGGTGGTGGTGGTGGCACTCGGTCACAATCGTGCTCAGC
Construction of pLS182	Sequence encoding $EntNfsB_{S2}$ with C-terminal 6xHis-tag	oligoLS386	Fw - *BbsI*	TCTCTGAAGACAACATGGATATCATTTCTGTCGCCCTG
Sequencing	pUC19	M13	Rv	CAGGAAACAGCTATGACC
Sequencing	pUC19	M13	Fw	TGTAAAACGACGGCCAGT
Sequencing	pET28a	T7	Fw	TAATACGACTCACTATAGGG
Sequencing	pLS60	oligoLS275	Fw	CATCCTATGGAACTGCCTCG
Sequencing	pLS60	oligoLS276	Fw	GAAAGTGAATGTCAACGGCG
Sequencing	pLS60	oligoLS277	Fw	CATCGTTGCGACACACTTGGC
Sequencing	pLS60	oligoLS278	Fw	CTGCACCATGCCACTCACTG
Sequencing	pLS60	oligoLS279	Fw	CCGTACATGAAGCTTGGACAGG

Supplementary Table 5. Synthetic DNA sequences used in this project. All synthetic genes were codon-adapted suitably for expression in *E. coli* and chemically synthesized (IDT or DNA2.0). Restriction sites are highlighted in green and start and stop codons in bold.

Synthetic DNA	Sequence (5'- 3')	RBS and Restriction sites
Sequence encoding 2,3-butanediol dehydrogenase BUDC of *Klebsiella pneumoniae*	CCGTTCGCATGCCAATCTTAATCAA ATCAGACAGAGAGAGTACAAT**ATG**A AAAAAGTCGCACTTGTTACCGGCGC CGGCCAGGGGATTGGTAAAGCTAT CGCCCTTCGTCTGGTGAAGGATGG ATTTGCCGTGGCCATTGCCGATTAT AACGACACCACCGCCAAAGCGGTC GCCTCCGAAATCAACCAGGCCGGC GGCCGCGCCATGGCGGTGAAAGTG GATGTCTCCGACCGCGATCAGGTG TTTGCCGCCGTCGAACAGGCGCGC AAAACGCTGGGCGGCTTCGACGTC ATCGTCAACAACGCCGGCGTGGCG CCGTCCACGCCGATCGAGTCCATT ACCCCGGAGATTGTCGATAAAGTCT ACAACATCAACGTTAAAGGGGTGAT CTGGGGCATTCAGGCGGCGGTCGA GGCCTTTAAGAAAGAGGGTCACGG CGGGAAAATCATCAACGCCTGTTCC CAGGCCGGCCACGTCGGCAACCCG GAGCTGGCGGTATATAGCTCGAGT AAATTCGCCGTACGCGGCTTAACCC AGACCGCCGCTCGCGACCTCGCGC CGCTGGGCATCACAGTCAACGGCT ACTGCCCGGGGATTGTCAAAACGC CAATGTGGGCCGAAATTGACCGCC AGGTGTCCGAAGCCGCCGGTAAAC CGCTGGGTTACGGTACCGCCGAGT TCGCCAAACGCATCACCCTCGGCC GCCTGTCCGAGCCGGAAGATGTCG CCGCCTGCGTCTCCTATCTTGCCAG CCCGGATTCTGATTATATGACCGGT CAGTCATTGCTGATCGACGGCGGG ATGGTGTTTAAC**TAA**GGATCCGCTG AA	*SphI* (5'end) *BamHI* (3'end) Synthetic RBS sequence: CAATCTTAATCAAATCAGACAGAGA GAGTACAAT
Sequence encoding NAD-dependent alcohol dehydrogenase TADH of *Thermus* sp. ATN-1	CCGTTCGCATGCAGGAGGTACGAA CAC**ATG**AAGGGGTTCGCAATGCTG TCTATTGGAAAAGTTGGCTGGATTG AAAAGGAGAAGCCAGCGCCAGGGC CTTTCGACGCAATTGTTCGCCCTTT GGCAGTCGCACCTTGCACGTCTGA CATCCACACCGTTTTCGAAGGAGCC ATTGGTGAACGTCATAACATGATCT TGGGACACGAAGCGGTAGGTGAGG TTGTAGAGGTCGGTTCTGAAGTTAA GGACTTTAAACCTGGAGACCGCGT GGTGGTGCCCGCGATTACGCCTGA CTGGCGTACTTCAGAGGTCCAACG TGGATATCACCAACATAGCGGCGG TATGCTGGCGGGTTGGAAGTTCTC CAATGTGAAGGACGGTGTTTTCGGA GAATTCTTCCATGTTAATGACGCCG ACATGAATTTGGCGCACCTTCCGAA GGAGATTCCGTTAGAAGCCGCGGT AATGATCCCCGACATGATGACCACC GGCTTTCATGGAGCGGAGCTGGCG GACATCGAGTTGGGCGCTACCGTG GCTGTACTTGGCATCGGTCCTGTC GGTCTGATGGCGGTGGCAGGGGC CAAGTTGCGTGGTGCAGGACGTAT CATTGCTGTTGGTTCTCGTCCAGTC TGTGTCGACGCTGCCAAGTATTACG GAGCAACCGACATCGTGAACTATAA GGATGGGCCAATTGAGTCACAGATT ATGAACCTTACAGAAGGGAAGGGA GTTGATGCAGCTATTATTGCAGGCG GGAATGCGGATATCATGGCGACAG CCGTCAAGATCGTGAAGCCCGGTG	*SphI* (5'end) *BamHI* (3'end) Shine-Dalgarno RBS: AGGAGGTACGAACAC

	GAACTATTGCTAATGTGAATTACTTT GGTGAGGGAGAAGTTTTGCCGGTG CCTCGCCTGGAATGGGGTTGTGGG ATGGCCCACAAAACGATCAAGGGA GGTCTGTGTCCAGGGGGACGTCTG CGCATGGAACGCTTGATTGACCTTG TCTTTTACAAACGTGTGGACCCGAG TAAATTGGTCACACACGTATTCCGT GGCTTTGATAACATTGAAAAGGCGT TCATGTTGATGAAGGATAAACCAAA GGACCTTATCAAACCAGTGGTTATT CTGGCT**TAA**GGATCCTTCAGC	
Sequence encoding NADP-dependent isopropanol dehydrogenase CBADH of *Clostridium beijerinckii*	GTTCGCATGCATTCGGATCTATACA GATAAGGAGAAAGAGATGAAAGGC TTTGCC**ATG**CTGGGTATTAACAAAT TAGGATGGATTGAAAAAGAACGCCC CGTCGCGGGTTCCTATGATGCGATT GTACGACCCTTAGCCGTTTCCCCGT GCACTAGCGATATTCATACAGTATT TGAAGGGGCTCTCGGCGATCGAAA GAATATGATTTTAGGCCATGAAGCC GTTGGCGAAGTCGTTGAAGTGGGC TCCGAAGTGAAAGATTTCAAACCGG GTGACCGTGTCATCGTGCCCTGTA CTACCCCAGATTGGCGCTCTCTGG AGGTTCAAGCTGGTTTTCAACAACA TAGTAATGGTATGTTGGCCGGCTG GAAGTTTTCCAACTTCAAAGATGGA GTATTTGGGGAGTATTTTCATGTGA ACGATGCGGATATGAATTTGGCCAT CCTGCCAAAAGACATGCCCTTGGA GAATGCTGTAATGATCACCGATATG ATGACCACCGGATTTCATGGGGCC GAGTTGGCCGATATCCAGATGGGT AGTTCTGTCGTTGTGATTGGTATCG GGGCAGTTGGGTTAATGGGAATTG CTGGGGCCAAATTACGCGGAGCAG GTCGGATTATTGGTGTCGGCAGTC GGCCTATTTGCGTTGAGGCCGCCA AGTTCTACGGCGCGACCGACATTCT GAATTACAAAAATGGCCATATTGTG GACCAGGTAATGAAGCTAACCAATG GGAAAGGCGTGGACCGTGTGATTA TGGCTGGAGGTGGGAGTGAAACAC TGAGCCAAGCAGTGAGCATGGTGA AACCTGGGGGAATTATCAGCAATAT CAACTATCACGGCTCTGGTGACGCT TTGTTAATTCCCCGCGTGGAATGGG GATGTGGCATGGCGCACAAGACGA TCAAAGGCGGTTTGTGTCCCGGAG GCCGTTTACGGGCCGAAATGCTAC GGGATATGGTGGTGTACAACCGTG TGGATTTGTCCAAGCTGGTGACTCA CGTTTATCACGGTTTTGACCATATT GAAGAAGCCTTGCTACTCATGAAAG ATAAACCTAAAGATCTCATTAAGGC CGTAGTTATCCTC**TAA**TAGTGAGGA TCCATGGAAG	*SphI* (5'end) *BamHI* (3'end) Synthetic RBS sequence: ATTCGGATCTATACAGATAAGGAGA AAGAGATGAAAGGCTTTGCC
Sequence encoding NADP-dependent isopropanol dehydrogenase TBADH of *Thermoanaerobacter brockii*	CCGTTCGCATGCAGGAGGTACGAA CAC**ATG**AAGGGGTTCGCAATGCTG TCTATTGGAAAAGTTGGCTGGATTG AAAAGGAGAAGCCAGCGCCAGGGC CTTTCGACGCAATTGTTCGCCCTTT GGCAGTCGCACCTTGCACGTCTGA CATCCACACCGTTTTCGAAGGAGCC ATTGGTGAACGTCATAACATGATCT TGGGACACGAAGCGGTAGGTGAGG TTGTAGAGGTCGGTTCTGAAGTTAA GGACTTTAAACCTGGAGACCGCGT GGTGGTGCCCGCGATTACGCCTGA CTGGCGTACTTCAGAGGTCCAACG TGGATATCACCAACATAGCGGCGG TATGCTGGCGGGTTGGAAGTTCTC CAATGTGAAGGACGGTGTTTTCGGA GAATTCTTCCATGTTAATGACGCCG	*SphI* (5'end) *BamHI* (3'end) Shine-Dalgarno RBS: AGGAGGTACGAACAC

	ACATGAATTTGGCGCACCTTCCGAA GGAGATTCCGTTAGAAGCCGCGGT AATGATCCCCGACATGATGACCACC GGCTTTCATGGAGCGGAGCTGGCG GACATCGAGTTGGGCGCTACCGTG GCTGTACTTGGCATCGGTCCTGTC GGTCTGATGGCGGTGGCAGGGGC CAAGTTGCGTGGTGCAGGACGTAT CATTGCTGTTGGTTCTCGTCCAGTC TGTGTCGACGCTGCCAAGTATTACG GAGCAACCGACATCGTGAACTATAA GGATGGGCCAATTGAGTCACAGATT ATGAACCTTACAGAAGGGAAGGGA GTTGATGCAGCTATTATTGCAGGCG GGAATGCGGATATCATGGCGACAG CCGTCAAGATCGTGAAGCCCGGTG GAACTATTGCTAATGTGAATTACTTT GGTGAGGGAGAAGTTTTGCCGGTG CCTCGCCTGGAATGGGGTTGTGGG ATGGCCCACAAAACGATCAAGGGA GGTCTGTGTCCAGGGGGACGTCTG CGCATGGAACGCTTGATTGACCTTG TCTTTTACAAACGTGTGGACCCGAG TAAATTGGTCACACACGTATTCCGT GGCTTTGATAACATTGAAAAGGCGT TCATGTTGATGAAGGATAAACCAAA GGACCTTATCAAACCAGTGGTTATT CTGGCT**TAA**GGATCCTTCAGC	
Sequence encoding NADP-dependent (R)-selective imine reductase MsIRED of *Myxococcus stipitatus*	CCGTTCGCATGCAGGAGGTACGAA CAC**ATG**AAACCGACCCTGACCGTTA TTGGCGCTGGCCGTATGGGCTCCG CACTGATTAAAGCATTCCTGCAATC TGGCTACACGACCACGGTGTGGAA CCGTACCAAAGCCAAAAGCGAACC GCTGGCAAAACTGGGCGCACATCT GGCTGATACGGTGCGTGACGCCGT TAAACGCAGCGATATTATCGTGGTT AATGTGCTGGATTATGACACCTCTG ATCAGCTGCTGCGCCAAGACGAAG TGACGCGTGAACTGCGCGGCAAAC TGCTGGTTCAGCTGACCAGCGGTT CTCCGGCACTGGCTCGTGAACAGG AAACGTGGGCGCGCCAACATGGCA TTGATTATCTGGACGGTGCGATCAT GGCCACCCCGGATTTTATTGGCCA GGCAGAATGCGCTCTGCTGTACAG TGGTTCCGCGGCCCTGTTCGAAAA ACACCGTGCTGTCCTGAATGTGCTG GGCGGTGCCACCAGCCATGTCGGC GAAGATGTTGGTCATGCCTCAGCAC TGGACAGCGCCCTGCTGTTTCAGAT GTGGGGCACCCTGTTCGGTACGCT GCAAGCACTGGCTATTTCTCGCGCA GAAGGCATCCCGCTGGAAAAAACC ACGGCGTTTATCAAACTGACCGAAC CGGTCACCCAGGGTGCCGTTGCAG ATGTCCTGACCCGTGTTCAGCAAAA TCGCCTGACCGCAGACGCTCAGAC GCTGGCAAGTCTGGAAGCTCATAA CGTGGCGTTCCAACACCTGCTGGC CCTGTGTGAAGAACGTAATATCCAT CGCGGTGTTGCGGATGCCATGTAC TCCGTTATTCGTGAAGCGGTCAAAG CCGGCCACGGTAAAGATGACTTTG CAATTCTGACCCGCTTCCTGAAA**TA** **A**GGATCCTTCAGC	*SphI* (5'end) *BamHI* (3'end) Shine-Dalgarno RBS: AGGAGGTACGAACAC
Sequence encoding oxygen-insensitive NAD(P)-dependent nitroreductase EntNfsB (NfsB) of *Enterobacter cloacae*	CATCTGAAGACAAC**ATG**GATATCAT TTCTGTCGCCCTGAAACGCCACTCT ACCAAGGCGTTCGACGCAAGCAAA AAACTGACCGCGGAAGAAGCGGAA AAAATCAAAACCCTGCTGCAGTACA GCCCGTCCAGCACCAACTCCCAGC CGTGGCACTTCATTGTAGCCAGCAC CGAGGAAGGAAAAGCGCGCGTGGC GAAGTCCGCTGCGGGCACCTATGT	*BbsI* (5'end) *BbsI* (3'end)

	GTTCAACGAACGCAAAATGCTGGAT GCTTCCCACGTGGTGGTGTTCTGC GCGAAAACCGCGATGGATGACGCC TGGCTGGAGCGCGTCGTGGATCAG GAAGAGGCCGATGGCCGTTTCAAC ACGCCGGAAGCCAAAGCCGCAAAC CATAAGGGCCGCACCTACTTCGCC GACATGCACCGCGTGGATCTGAAA GATGACGACCAGTGGATGGCGAAG CAGGTTTACCTGAACGTCGGCAACT TCCTGCTGGGCGTGGGCGCGATGG GTCTGGACGCGGTACCAATTGAAG GTTTCGACGCCGCTATTCTCGACGA AGAGTTTGGCCTGAAAGAGAAAGG CTTCACCAGCCTGGTGGTGGTACC GGTTGGGCACCACAGCGTGGAAGA TTTCAACGCCACGCTGCCGAAATCT CGCCTGCCGCTGAGCACGATTGTG ACCGAGTGC**TAA**GGAGTCTTCAGA GA	

Supplementary Table 6. Sequence of saturated positions for 10 random variants of the CBADH library and the selected variant.

Enzyme / variant	Position			
	198	199	200	218
WT	GGC	AGT	CGG	TAC
R1	TCC	CGG	ACC	TTG
R2	TTA	TTC	TAA	Deletion of 210 bp
R3	TTC	CGT	ATA	CGG
R4	TCA	GCT	GTA	TAG
R5	AAT	GTG	ACA	GGG
R6	CTA	GCG	AAC	GGG
R7	CAT	CTC	CAA	ACC
R8	AAC	TCC	TCA	GTG
R9	AAA	CCA	TCA	GTT
R10	GCC	CCC	AGG	CGT
CBADHs	GAC	TAT	AGA	CCG

Supplementary Table 7. Sequence of saturated positions for 10 random variants of the TBADH library and selected variants. $TBADH_{S2}$ contained a duplication of residues 191 to 241, in addition to substitutions in the targeted residues both in the positions of the original sequence and in the corresponding positions of the duplication. 198', 199', 200' and 218' denote positions in the duplication equivalent to the original residues 198, 199, 200 and 218. These positions only exist in $TBADH_{S2}$.

Enzyme / variant	Position							
	198	199	200	218	198'	199'	200'	218'
WT	GGT	TCT	CGT	TAT	-	-	-	-
R1	CTT	TCC	CGC	TTA	-	-	-	-
R2	TGA	CGC	CGA	GTT	-	-	-	-
R3	CTC	TCC	CGC	GTG	-	-	-	-
R4	AAT	CAA	AAG	AGG	-	-	-	-
R5	TTA	ACA	CGA	CGC	-	-	-	-
R6	AGC	ACC	CGA	CGA	-	-	-	-
R7	AAC	TGA	ACT	GTA	-	-	-	-
R8	CCC	CAG	AGG	AGA	-	-	-	-
R9	Insertion 153 bp GCA	TCA	CGT	TAC	-	-	-	-
R10	GAC	TAT	AGA	CCG	-	-	-	-
$TBADH_{S1}$	TCA	AAA	CGG	GTA	-	-	-	-
$TBADH_{S2}$	CAC	CGC	GCC	ATG	GCA	TCA	AAA	TAC

Supplementary Table 8. Sequence of saturated positions for 10 random variants of the MsIRED library and the selected variant.

Enzyme / variant	Position					
	32	33	34	37	67	71
WT	AAC	CGT	ACC	AAA	ATC	ACC
R1	GGT	GGT	GAT	GGG		
R2	GGT	GGG	GGG	GGG		
R3	GTG	TCT	GCT	GGG		
R4	CTT	CGT	ACT	TTT		
R5	CAT	GAT	GCG	CAG		
R6	TTG	CTT	TAT	GTG		
R7	CTA	ATT	GTT	ACT		
R8	CCG	AAG	TAT	TGT		
R9	ATT	GTT	TAG	ATG		
R10	GAG	CGG	GTG	TTG		
MSIRED$_C$	GAA	TAT	GAA	CGT	ATT	GTT
MSIRED$_S$	GAG	GTG	CGG	CGG		

Supplementary Table 9. Sequence of saturated positions for 10 random variants of the EntNfsB library and the selected variants.

Enzyme / variant	Position			
	40	41	68	124
WT	AGC	ACC	TAT	TTC
R1	TTA	TTA	GTG	CAA
R2	AAC	GTA	TAG	CCC
R3	TCA	GTA	AAT	GTA
R4	TAC	ATA	CCT	ACC
R5	GTA	TTA	TGT	TGC
R6	AGC	TTC	AAG	TTA
R7	TCC	TTC	TTT	CAA
R8	CGA	ATA	TAG	TGC
R9	CTA	TAA	TCG	CCC
R10	CAA	AAC	ATG	CCA
EntNfsB$_{S1}$	GCA	ATA	TAT	GCA
EntNfsB$_{S2}$	TCA	CTA	CTT	CTC

Supplementary Table 10. Conditions for anaerobic growth experiments.

Mutant strain	Transformed plasmid/library	Antibiotic	Added external substrate	Generated reduced product
AL	pUC19	Ampicillin	As required for the complementation experiment	-
AL	pLS1	Ampicillin	-	-
AL	pLS2	Ampicillin	15 mM acetoin	2,3-butanediol
AL	pLS3	Ampicillin	15 mM acetoin	2,3-butanediol
AL	pLS6	Ampicillin	15 mM acetone	Isopropanol
AL	pLS10 (library)	Ampicillin	15 mM acetone	Isopropanol
AL	pLS10_3	Ampicillin	15 mM acetone	Isopropanol
AL	pLS12	Ampicillin	10 mM cyclohexanone or 3-methylcyclohexanone	Cyclohexanol or 3-methylcyclohexanol
AL	pLv2	Kanamycin	-	-
AL	pLS60	Kanamycin	-	Isopropanol
ALPS	pLS69	Ampicillin	15 mM acetone	Isopropanol
ALPS	pLS73 (library)	Ampicillin	15 mM acetone	Isopropanol
ALPS	pLS73_1	Ampicillin	15 mM acetone	Isopropanol
ALPS	pLS73_2	Ampicillin	15 mM acetone	Isopropanol
AL	pLS130	Ampicillin	15 mM 2-methyl-1-pyrroline	2-methylpyrrolidine
AL	pLS131	Ampicillin	15 mM 2-methyl-1-pyrroline	2-methylpyrrolidine
AL	pLS133 (library)	Ampicillin	15 mM 2-methyl-1-pyrroline	2-methylpyrrolidine
AL	pLS133_1	Ampicillin	15 mM 2-methyl-1-pyrroline	2-methylpyrrolidine
AL	pLS168	Ampicillin	2.5 mM 4-nitrobenzoic acid	4-aminobenzoic acid
AL	pLS168	Ampicillin	8 mM 2-nitrobenzoic acid	2-aminobenzoic acid
AL	pLS168	Ampicillin	2.5 mM 4-nitrobenzyl alcohol	4-aminobenzyl alcohol
AL	pLS169 (library)	Ampicillin	8 mM 2-nitrobenzoic acid	2-aminobenzoic acid
AL	pLS169_1	Ampicillin	8 mM 2-nitrobenzoic acid	2-aminobenzoic acid
AL	pLS169 (library)	Ampicillin	2.5 mM 4-nitrobenzyl alcohol	4-aminobenzyl alcohol
AL	pLS169_3	Ampicillin	2.5 mM 4-nitrobenzyl alcohol	4-aminobenzyl alcohol

Supplementary Table 11. Characteristic ^{1}H-NMR signals used to quantify each compound of interest. The multiplicity of each signal (s – singlet, d – doublet, t – triplet, q – quartet, m – multiplet) and the number of contributing protons is shown between brackets.

Metabolite	δ for characteristic signal of the metabolite (ppm)
Ethanol	1.19 (t,3)
Lactate	1.32 (d,3)
Succinate	2.41 (s,4)
Acetate	1.92 (s,3)
Formate	8.46 (s,1)
Acetoin	1.38 (d, 3)
2,3-butanediol	1.15 (d,6)
Cyclohexanone	2.40 (t,4)
Cyclohexanol	1.72 (m, 2)
3-methylcyclohexanone	1.01 (d,3)
3-methylcyclohexanol	0.88 (d,3)
Acetone	2.24 (s,6)
Isopropanol	1.18 (d,6)
Propionate	2.19 (q, 2)
2-methyl-1-pyrroline	2.42 (s,3)
2-methylpyrrolidine	1.38 (d,3)
4-nitrobenzoic acid	8.01 (d,2)
4-aminobenzoic acid	7.73 (d,2)
2-nitrobenzoic acid	8.11 (d,1)
2-aminobenzoic acid	7.30 (t,1)
4-nitrobenzyl alcohol	8.27 (d,2)
4-aminobenzyl alcohol	7.23 (d,2)

Supplementary Table 12. Conditions for enzymatic activity assays.

Enzyme	Substrate	Cofactor	Enzyme concentration	Absorbance (nm)
$CBADH_{WT}$	Isopropanol	1 mM $NADP^+$	110 nM	340
$CBADH_{WT}$	Isopropanol	10 mM NAD^+	110 nM	340
$CBADH_S$	Isopropanol	1 mM $NADP^+$	110 nM	340
$CBADH_S$	Isopropanol	10 mM NAD^+	110 nM	340
$TBADH_{WT}$	Isopropanol	1.2 mM $NADP^+$	110.8 nM	340
$TBADH_{WT}$	Isopropanol	10 mM NAD^+	110.8 nM	340
$TBADH_{S1}$	Isopropanol	1.2 mM $NADP^+$	110 nM	340
$TBADH_{S1}$	Isopropanol	10 mM NAD^+	110 nM	340
$TBADH_{S2}$	Isopropanol	2.35 mM $NADP^+$	51.55 nM	340
$TBADH_{S2}$	Isopropanol	2.35 mM NAD^+	51.55 nM	340
$MsIRED_{WT}$	2-methyl-1-pyrroline	0.25 mM NADPH	1.2 μM	340
$MsIRED_{WT}$	2-methyl-1-pyrroline	0.25 mM NADH	1.2 μM	340
$MsIRED_C$	2-methyl-1-pyrroline	0.25 mM NADH	1.26 μM	340
$MsIRED_S$	2-methyl-1-pyrroline	0.25 mM NADH	1.25 μM	340
$EntNfsB_{WT}$	4-nitrobenzoic acid	0.3 mM NADH	60.8 nM	370
$EntNfsB_{WT}$	2-nitrobenzoic acid	0.3 mM NADH	60.8 nM	370
$EntNfsB_{WT}$	4-nitrobenzyl alcohol	0.3 mM NADH	26.3 nM	370
$EntNfsB_{S1}$	4-nitrobenzoic acid	0.3 mM NADH	45 nM	370
$EntNfsB_{S1}$	2-nitrobenzoic acid	0.3 mM NADH	45 nM	370
$EntNfsB_{S2}$	4-nitrobenzyl alcohol	0.3 mM NADH	26.3 nM	370

Supplementary Table 13. Data collection parameters and refinement statistics for the crystal structures of $CBADH_S$ and $TBADH_{S1}$.

	$CBADH_S$ variant	**$TBADH_{S1}$ variant**
Beamline	Diamond I03	Diamond I04
PDB code	6SCH	6SDM
Wavelength	0.9762	0.9795
Resolution range	59.36 - 2.199 (2.278 - 2.199)	71.68 - 2.85 (2.952 - 2.85)
Space group	P 1 21 1	P 21 21 21
Unit cell	75.8115 99.5695 114.089 90 102.768 90	79.1234 123.946 169.24 90 90 90
Total reflections	265743 (16967)	258210 (26258)
Unique reflections	82558 (7335)	39642 (3923)
Multiplicity	3.2 (2.3)	6.5 (6.7)
Completeness (%)	98.45 (87.66)	99.87 (99.62)
Mean I/sigma(I)	7.57 (0.71)	6.92 (1.11)
Wilson B-factor	34.89	69.95
R-merge	0.09525 (1.064)	0.1918 (1.671)
R-meas	0.114 (1.345)	0.2086 (1.811)
R-pim	0.06202 (0.8095)	0.08117 (0.6942)
CC1/2	0.996 (0.345)	0.991 (0.554)
CC*	0.999 (0.716)	0.998 (0.844)
Reflections used in refinement	82766 (7315)	39597 (3913)
Reflections used for R-free	4037 (357)	2015 (182)
R-work	0.1639 (0.2548)	0.1746 (0.3201)
R-free	0.2068 (0.2927)	0.2390 (0.3647)
CC(work)	0.970 (0.619)	0.955 (0.750)
CC(free)	0.960 (0.587)	0.940 (0.651)
Number of non-hydrogen atoms	11354	10622
macromolecules	10666	10618
ligands	332	4
solvent	356	0
Protein residues	1417	1416
RMS(bonds)	0.007	0.009
RMS(angles)	0.93	1.06
Ramachandran favored (%)	95.87	95.10
Ramachandran allowed (%)	4.13	4.69
Ramachandran outliers (%)	0.00	0.21
Rotamer outliers (%)	0.97	0.27
Clashscore	5.11	4.48
Average B-factor	45.12	77.08
macromolecules	44.13	77.04
ligands	78.13	174.49
solvent	43.81	-
Number of TLS groups	18	15