Supplementary Materials

Functional Reconstitution of a Pyruvate Dehydrogenase in the Cytosol of *Saccharomyces cerevisiae* through Lipoylation Machinery Engineering

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SUPPLEMENTARY RESULTS AND DISCUSSION

Octonoyl-CoA as the Lipoylation Substrate

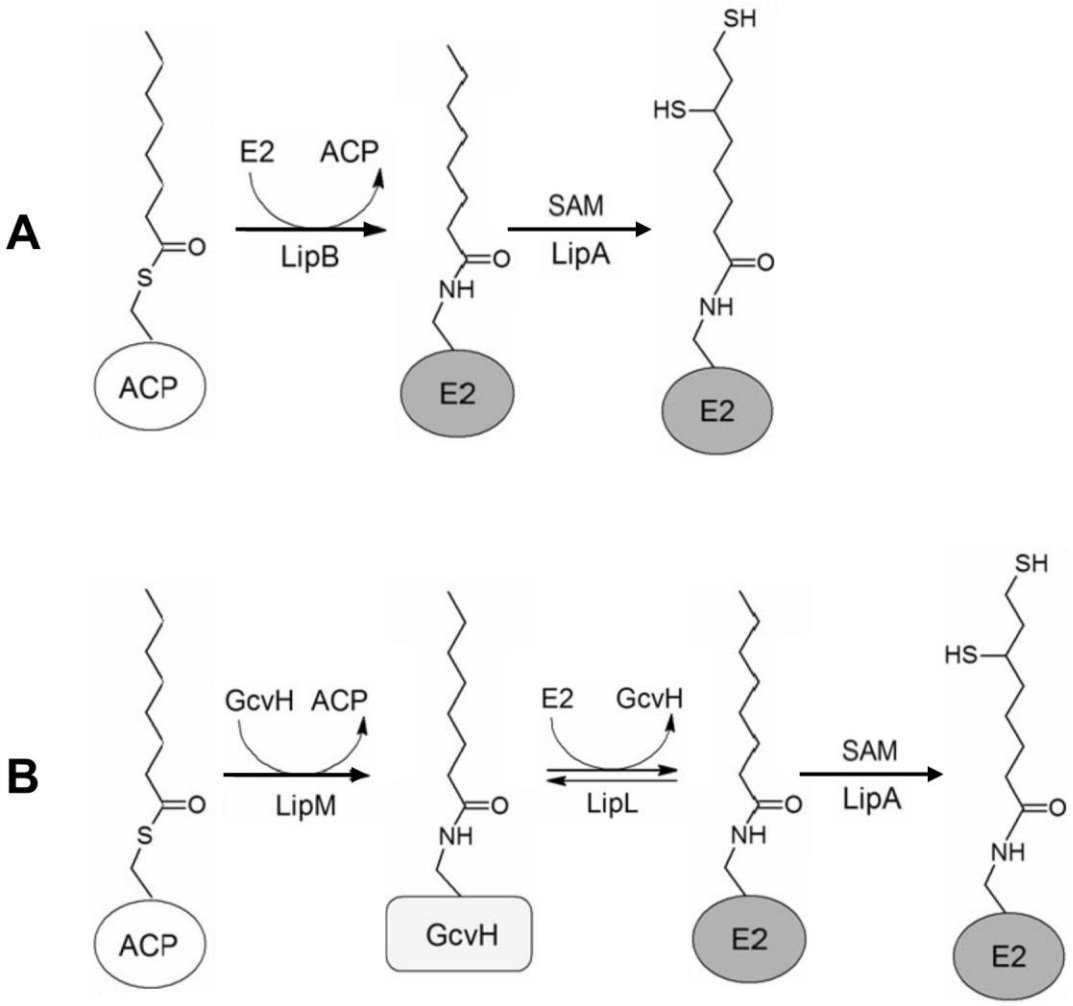
Previous studies have been shown that both LipM from *B. subtilis* and Lip3p from *S. cerevisiae* have octanoyl-CoA transferase activity (*1, 2*), indicating that *B. subtilis* and *S. cerevisiae* may be able to accept octanoyl-CoA as the substrate for protein lipoylation. To test whether octanoyl-CoA could be accepted as the substrate, Faa2p (*3*), a medium chain fatty acyl-CoA synthetase, was relocated to the cytosol by removing the peroxisome targeting sequences (cyto*FAA2*). The peroxisomal targeting sequence was predicted using the PTSs Predictor from PeroxisomeDB 2.0 (http://www.peroxisomedb.org) (*4*). As shown in Supplementary Figures S8, no significant cell growth could be observed for all cyto*FAA2* expressing strains, even when octanoic acid was supplemented. Our results indicated that octanoyl-CoA could not be accepted as the lipoylation precursor to activate PDH in the cytosol of yeast.

De novo Lipoylation with the Type II FAS from the Mitochondria of *S. cerevisiae*

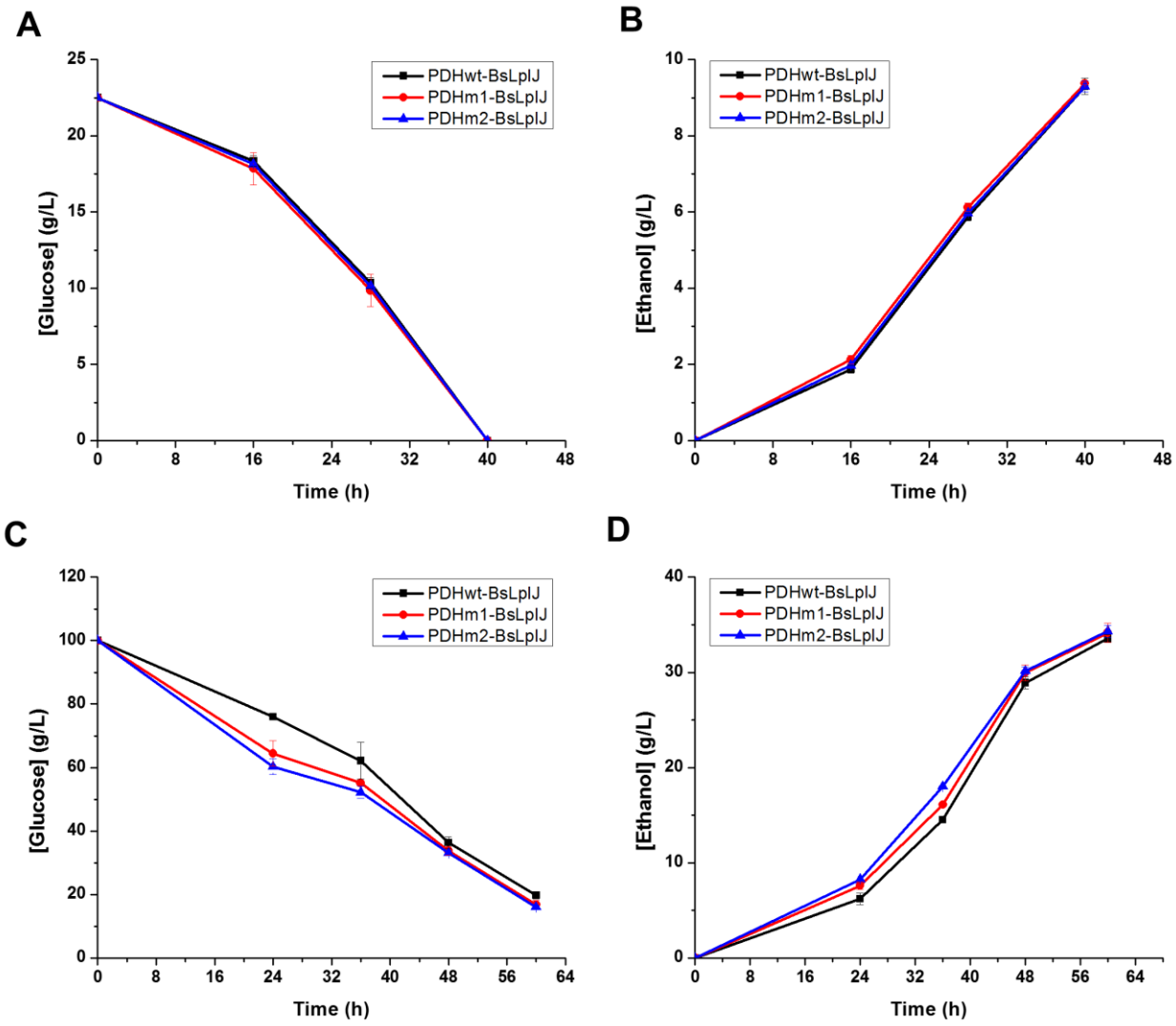
A type II FAS should be functionally reconstituted in the cytosol to provide octanoyl-ACP for the *de novo* lipoylation machinery. Initially, the type II FAS (Cem1p-Oar1p-Htd2p-Etr1p-Acp1p-Ppt2p-Mct1p) from the mitochondria of *S. cerevisiae* (Supplementary Figure S3) was tested. The whole pathway enzymes were relocated to the cytosol by removing the mitochondrial targeting sequences (cytoFAS1). Unfortunately, no cell growth was observed (data not shown) with the co-expression of M2J and ScLA in the Acs^- strain when cultured in synthetic medium with glucose as the sole carbon source, indicating a lack of enough octanoyl-ACP for lipoylation. One possibility is due to the poorly characterized property of Htd2p, whose mitochondrial targeting sequence could not be predicted by most of publicly accessible programs. Thus, the *E.*

coli homologs, EcFabA and EcFabZ, which were confirmed to complement the *htd2*Δ strain (*5*), were included instead to construct cytoFAS2 and cytoFAS3. Unfortunately, still no growth was observed (data not shown), indicating that the mitochondrial FAS system was not functional in the cytosol. Nevertheless, the mitochondrial FAS is still not well characterized (*6*). For example, the enzyme and the corresponding substrate for the initial condensation step are still unknown, which is one major concern with this FAS system. Therefore, the type II FAS from *E. coli* (*7*), the most well-characterized FAS system, was investigated next for *de novo* lipoylation in the cytosol of yeast (Supplementary Figure S3) in the present study.

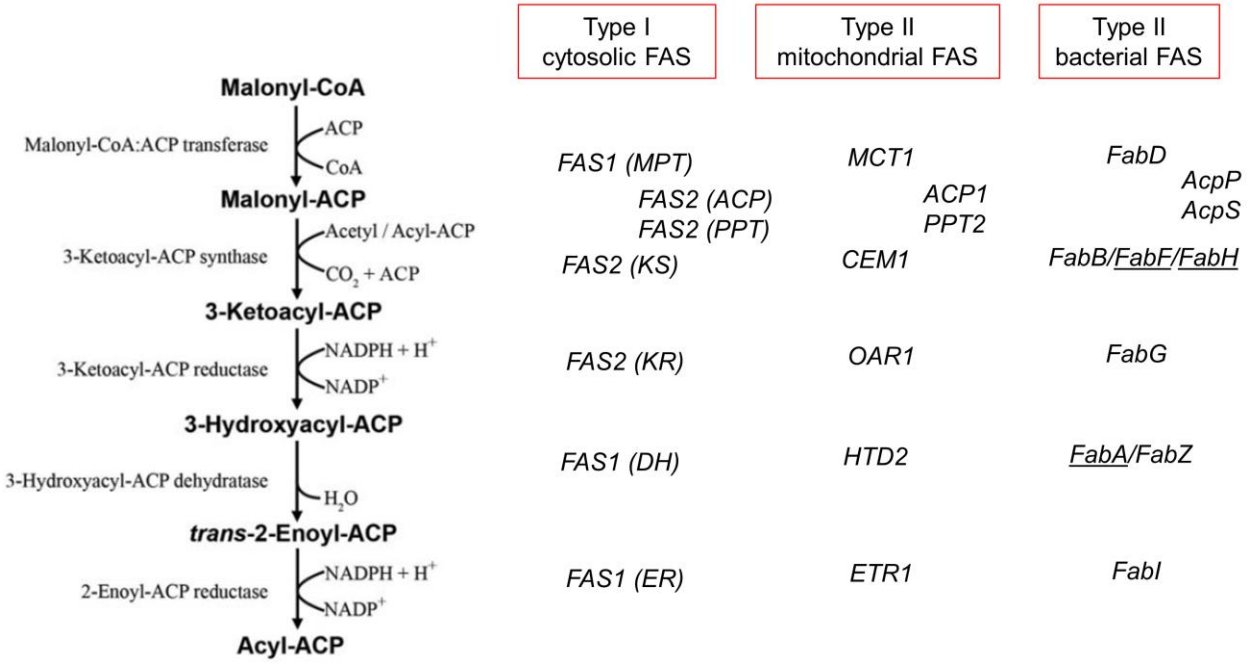
Supplementary Figure S1. *De novo* lipoylation machineries. Octanoyl-ACP, an intermediate of fatty acid biosynthesis by a type II FAS, is used as the substrate for *de novo* lipoylation. (A) In *Escherichia coli*, the *de novo* synthetic pathway proceeds by two consecutive reactions: the octanoyltransferase (LipB) transfers the octanoyl moiety from octanoyl-ACP to the target apo-proteins; and then the octanoyl moiety is converted to lipoic acid by the lipoyl synthase (LipA) to generate lipoylated proteins (*8*). (B) In *Bacillus subtilis*, LipM is an octanoyltransferase that specifically transfers the octanoyl moiety from octanoyl-ACP to GcvH, whereas LipL is an amido-transferase that transfers the octanoyl moiety from GcvH to PDH (*9, 10*). In *Saccharomyces cerevisiae*, the lipoylation pathway is not well characterized, and a lipoylation machinery similar to that of *B. subtilis* is proposed (*2, 11, 12*). Both *B. subtilis* and *S. cerevisiae* require GCV (GcvH and Gcv3p, respectively) for PDH lipoylation, which is not the case for *E. coli*.



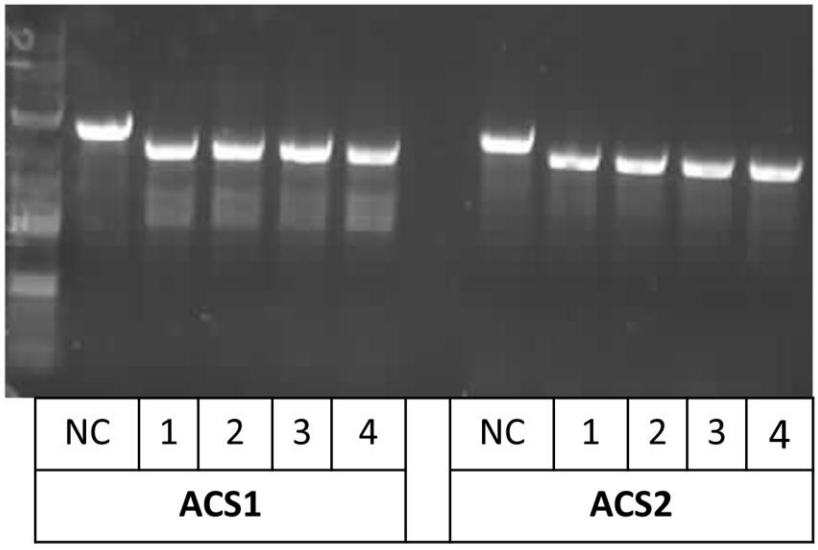
Supplementary Figure S2. Fermentation performance of *acs2*Δ yeast strains containing PDH mutants with low (A and B) and high (C and D) glucose concentrations under anaerobic conditions. Error bars represented SD of biological duplicates.

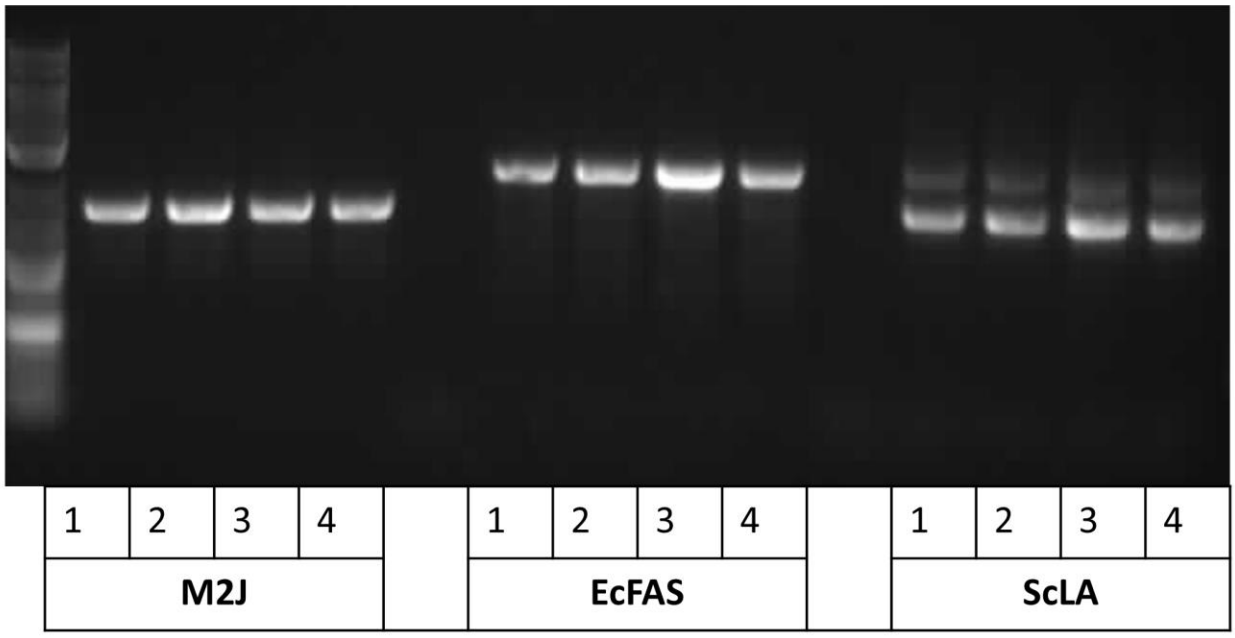


Supplementary Figure S3. Fatty acid biosynthesis and the fatty acid synthase (FAS) systems in *E. coli* (Type II) and cytosol (Type I) and mitochondria (Type II) of yeast. As for the *E. coli* FAS, the genes underlined (if there are several analogs catalyzing the same biochemical reaction) were cloned and expressed in the cytosol of yeast in this study. In terms of the *E. coli* 3-Ketoacyl-ACP synthase, FabF demonstrated broad substrate specificities and higher activities than FabB (*13*). For the 3-Hydroxyacyl-ACP dehydratase, FabA was most active on intermediate chain length substrates and also possessed significant activity toward both short and long chain substrates (*14*). Therefore, *FabF* and *FabA* rather than *FabB* and *FabZ* were chosen to construct EcFAS in the cytosol of yeast in this study.

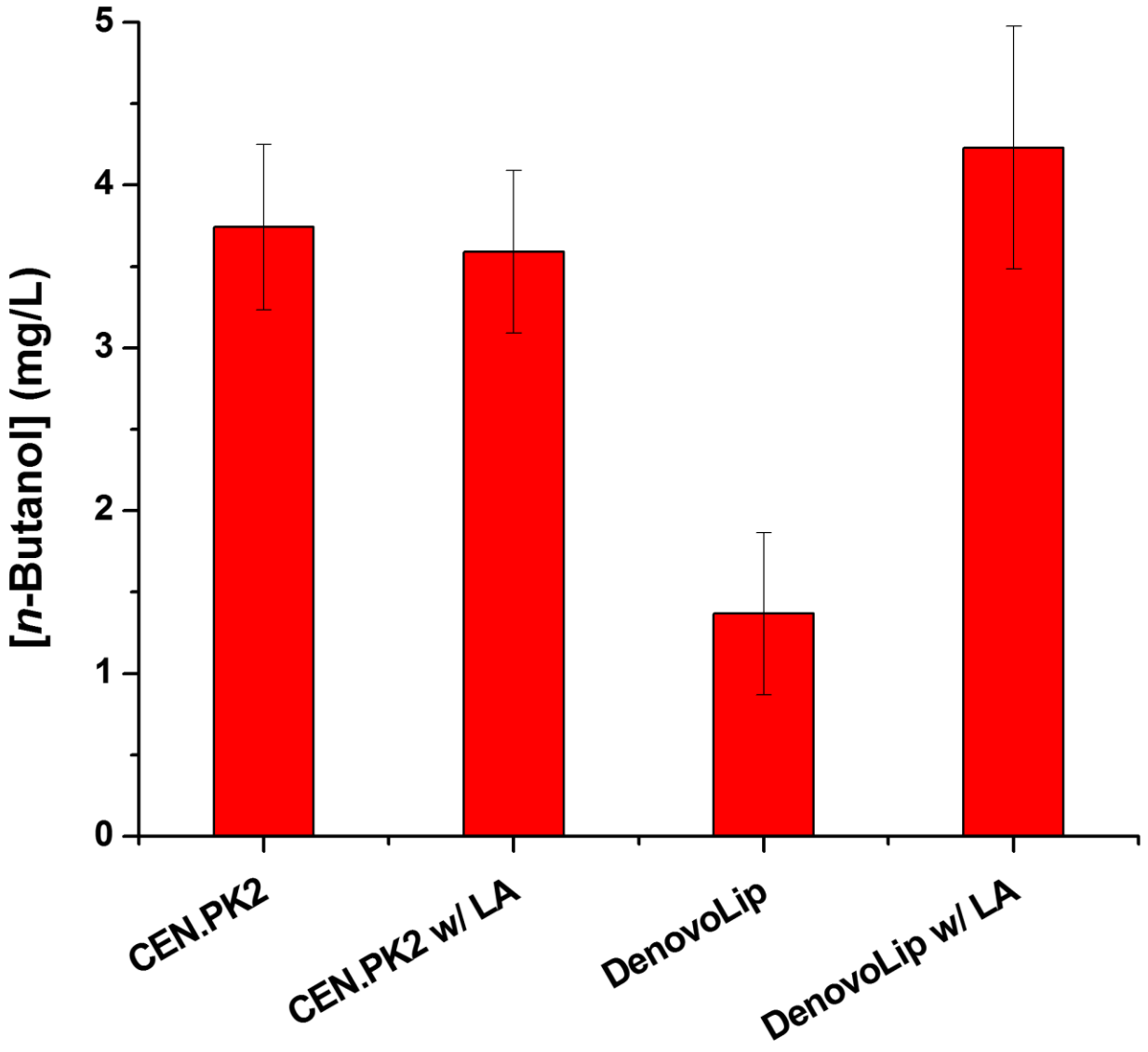


Supplementary Figure S4. PCR verification of the deletion of both *ACS1* and *ACS2* and the presence of all the three plasmids, M2J, EcFAS, and ScLA. 1, 2, 3, and 4 represented the 4 randomly picked up colonies mentioned in Figure 5B. CEN.PK2 (*ACS1*$^{+}$ *ACS2*$^{+}$) was included as a negative control for PCR verification. Primers that specifically amplified Ec*LpdA* cassette, Ec*FabA*+Ec*FabD* cassettes, and *GCV3* cassette (also including 500bp upstream sequences of the vector) were used to verify the presence of M2J, EcFAS, and ScLA, respectively.

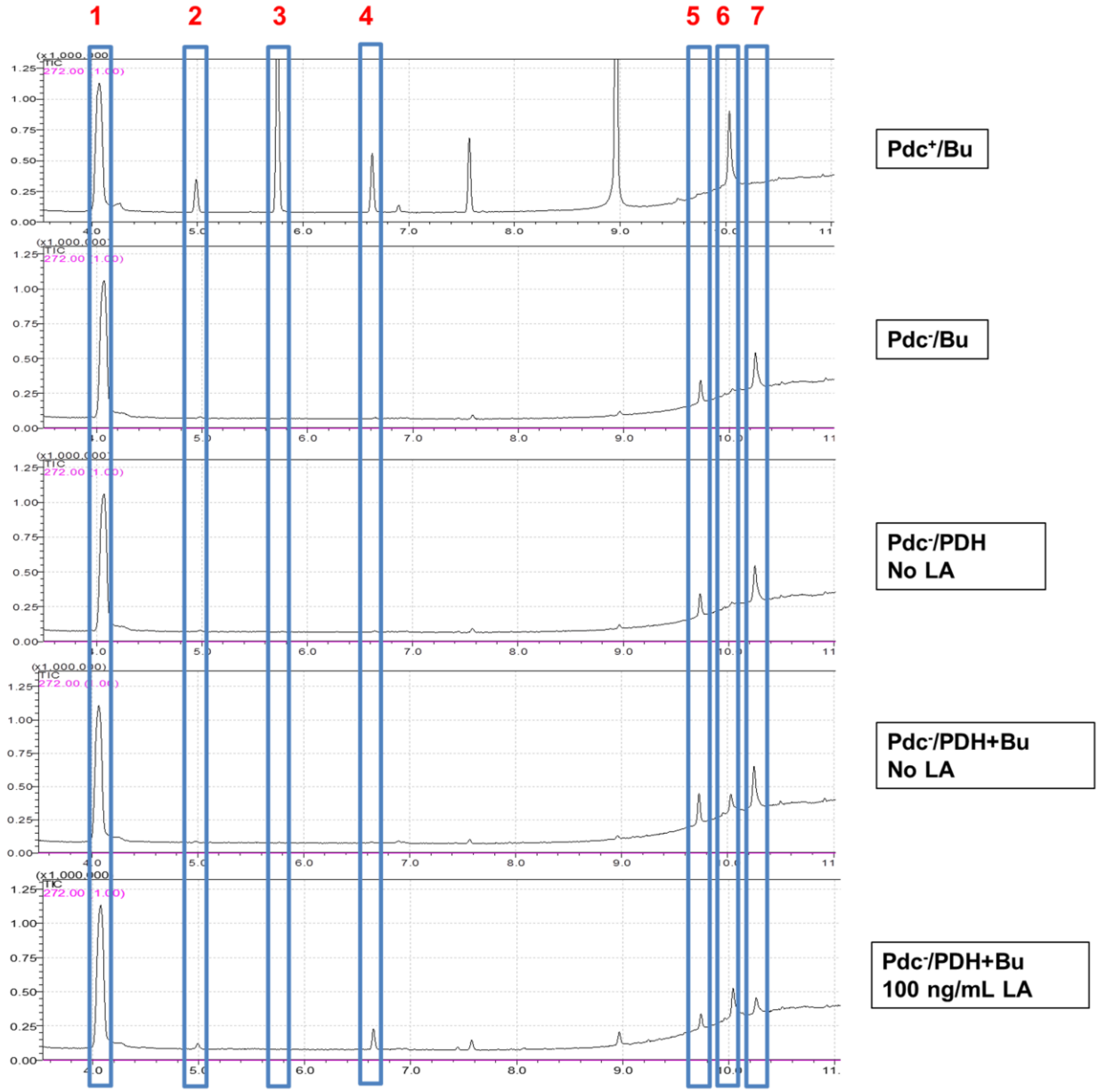




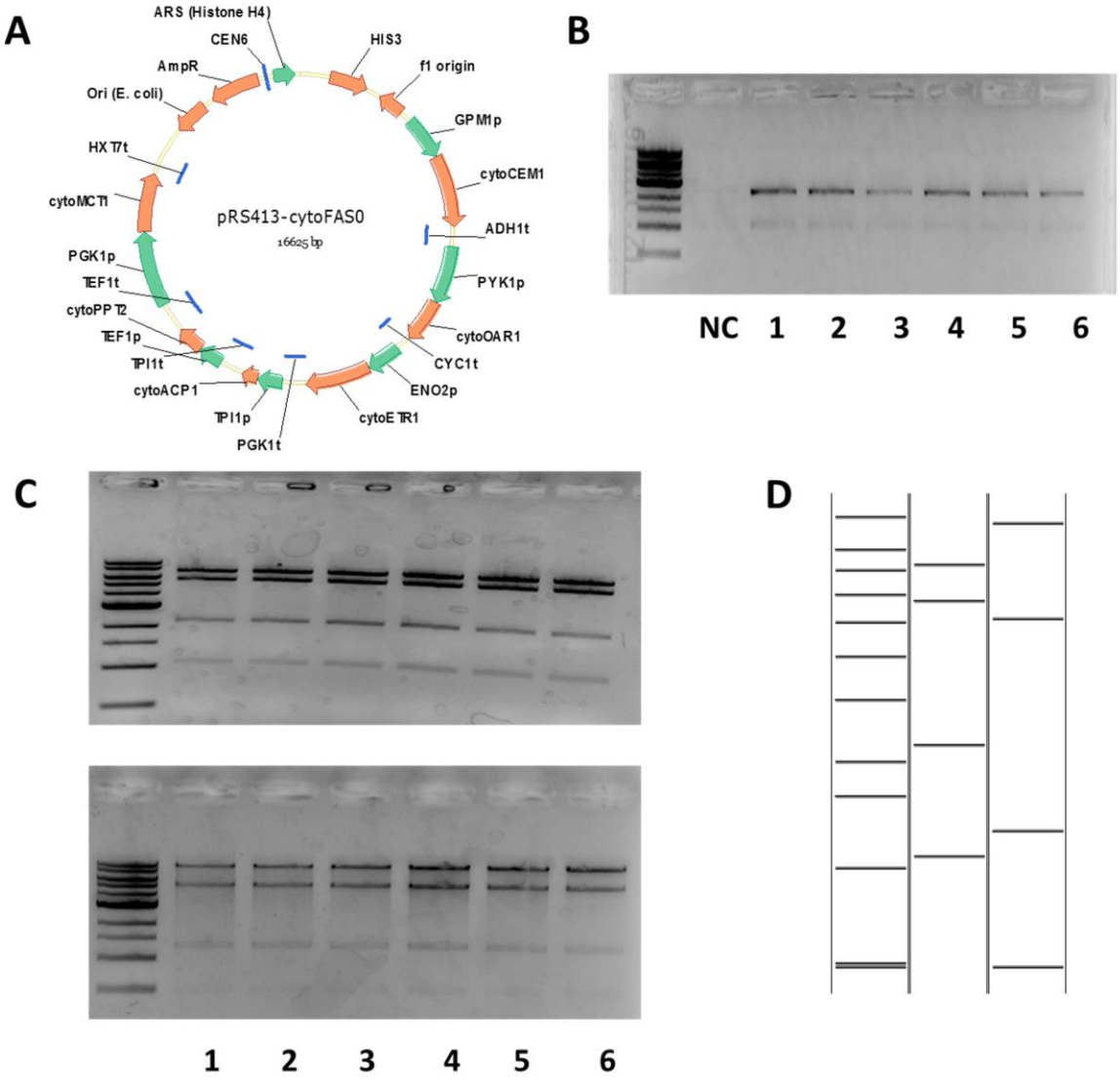
Supplementary Figure S5. Production of *n*-butanol in engineered yeast strains with alternative acetyl-CoA biosynthesis. BuPa28, a CoA-dependent *n*-butanol biosynthetic pathway constructed in our previous studies (*15*, *16*), was introduced as a reporter of the intracellular acetyl-CoA levels. Wild-type CEN.PK2 (*ACS1*$^{+}$ *ACS2*$^{+}$) strain was also included as a positive control. The CEN.PK2/BuPa28 and DenovoLip/BuPa28 strains were pre-cultured in SCD-URA medium under aerobic conditions (30^{o}C, 250 rpm) until saturation, and then inoculated into 5 mL fresh SCD-URA in sealed 14 mL culture tube (micro-anaerobic conditions) at an initial OD around 0.1 for *n*-butanol fermentation. Lipoic acid was supplemented at a final concentration of 100 μg/L as indicated. DenovoLip and DenovoLip w/ LA represented the yeast strains with alternative acetyl-CoA biosynthesis by the *de novo* lipoylation pathway and the scavenging lipoylation pathway, respectively. Error bars represented SD of biological duplicates.



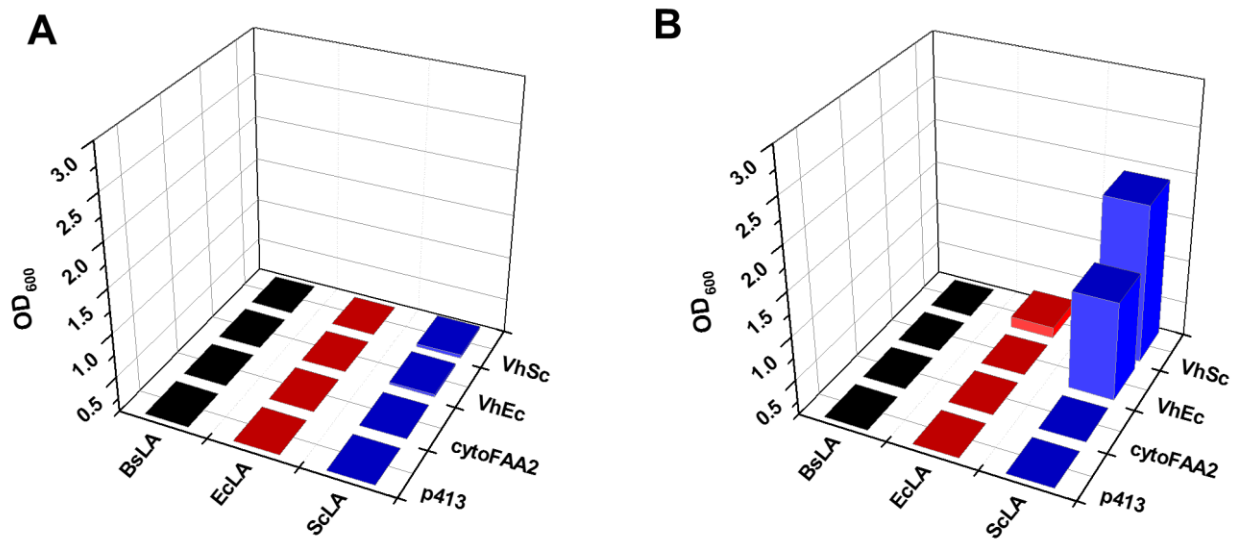
Supplementary Figure S6. Activity of PDH in the Pdc$^-$ yeast strain with or without the supplementation of lipoic acid. *n*-Butanol biosynthesis was employed as a reporter of cytosolic acetyl-CoA levels in engineered yeast strains (*15*). A Pdc$^+$ yeast strain with an *n*-butanol biosynthetic pathway constructed in our previous studies was included for comparison (*15*). Yeast strains were pre-cultured in the corresponding SCD dropout media and then inoculated into fresh media with or without lipoic acid (as indicated) at an initial OD around 0.1. Fermentation was carried out under oxygen-limited conditions (30^{o}C and 100rpm) and the samples were taken two days (Pdc$^+$ yeast strain) or four days (Pdc$^-$ strain) after inoculation and analyzed using GC-MS as described in our previous studies, with 100 mg/L *2*-butanol included as an internal standard (*15*, *16*). GC-MS peaks: 1, 2-butanol; 2, *iso*-butanol; 3, *n*-butanol; 4, *3*-methyl-*1*-butanol; 5, *iso*-butyric acid; 6, *n*-butyric acid; 7, *3*-methyl-*1*-butyric acid.



Supplementary Figure S7. Verification of large plasmids containing multiple genes, with that of cytoFAS0 (pRS413-*GPM1p*-cyto*CEM1-ADH1t-PYK1p*-cyto*OAR1-CYC1t-ENO2p*-cyto*ETR1-PGK1t-TPI1p*-cyto*ACP1-TPI1t-TEF1p*-cyto*PPT2-TEF1t-PGK1p*-cyto*MCT1-HXT7t*) as an example. (A) Plasmid map of cytoFAS0. (B) The constructed plasmids were first verified by diagnostic PCR, with the primers targeting the promoter region of the third expression cassette (ENO2p-Seq-F) and the terminator region of the fourth expression cassette (TPT1t-Seq-R). The expected size of the PCR amplicon (cyto*ETR1-PGK1t-TPI1p*-cyto*ACP1*) was around 2.5 kb. Generally, the presence of two consecutive expression cassettes, which were located in the middle of the whole assembled synthetic pathway, was a strong indicator that the whole pathway was correctly assembled. A negative control (NC, pRS413) and six samples were included for analysis in this case. (C) The constructed plasmids were then verified by restriction digestions with at least two different enzymes, such as *Bam*HI (top panel) and *Xho*I (bottom panel) in this case. (D) The expected restriction digestion patterns of cytoFAS0 by *Bam*HI (Lane 2) and *Xho*I (Lane 3). NEB 1 kb DNA Ladder (Lane 1) was included as DNA standards. If necessary, the constructed plasmid was further verified by DNA sequencing.



Supplementary Figure S8. Reconstitution of a semi-synthetic lipoylation pathway using cytoFAA2. Recombinant yeast strains were pre-cultured on SCD-HWLU medium supplemented with 100 µg/L lipoic acid, washed three times in ddH_2O, and inoculated into 5 mL fresh SCD-HWLU medium in 14 mL round-bottom culture tubes without (A) or with (B) the supplementation of 10 mg/L octanoic acid. Cell densities were measured 5-7 days after inoculation. The results represented the average of biological duplicates, whose variation was less than 10%. Most of the data were the same as those in Figure 4A and 4B and included for easier comparison.



Supplementary Table S1. List of oligonucleotides used in this study. The restriction sites were underlined and the homology arms for DNA assembler or Gibson Assembly were shown in red.

Primer	Sequence (5'-->3')	Individual gene	Whole pathway
ACS2-KO-F	atgacaatcaaggaacataaagtagtttatgaagctcacattcgtacgctgcaggtcgac	Knockout of ACS2	
ACS2-KO-R	ctacagcagaaatgatggcaggtacaacttctgggttggcgcataggccactagtggatc		
ACS2-OF	gtgactgaacttcttcgtcactg		
ACS2-OR	ggataacaggggcgattgtgtac		
ACS1-KO-F	atgtcgccctctgccgtacaatcatcaaaactagaagaacttcgtacgctgcaggtcgac	Knockout of ACS1	
ACS1-KO-R	caacttgaccgaatcaattagatgtctaacaatgccagggcgcataggccactagtggatc		
ACS1-OF	ggtattgcagttggggtatagttcctc		
ACS1-OR	tagcgatgcggcagttgttcctgaag		
EcLplA-F	gttcgcggatccatgtccacattacgcctgctcatc	pH5	
EcLplA-R	cacccgctcgagctaccttacagcccccgccatc		
BsLplJ-F	gttcgcggatcc atgttatttatagacaatcaaaatatcaatgatc	pH5	
BsLplJ-R	cacccgctcgag ttagtaaatcagatcaaggaaatcctctttc		
Lpl-Gib-F	gatgcagtaatatacacagattccgtgtcgac atagcttcaaaatgtttctactc	---	M2J Gibson
Lpl-Gib-R	gtaaagaaccctttctatacccgcagcgtcgac atagcgccgatcaaagtatttg		
EcLipB-F	gttcgcggatcc atgtatcaggataaaattcttgtc	pH4	EcLA DNA Assembler
EcLipB-R	cacccgctcgag ttaagcggtaatatattcgaagtcc		
EcLipA-F	gttcgcggatcc atgagtaaacccattgtgatg	pH5	
EcLipA-R	cacccgctcgag ttacttaacttccatccctttc		
BsGcvH-F	gttcgcggatcc atgagcataccaaaagatttg	pH3	BsLA DNA Assembler
BsGcvH-R	cacccgctcgag ttagtcttcttgtgtcatctcttc		
BsLipM-F	gttcgcggatcc atgcaaaaagaaacttggcg	pH4	
BsLipM-R	cacccgctcgag ttaccgcttataattccattc		
BsLipA-F	gttcgcggatcc atggcaaagaaagacgaacacctcagaaag	pH5	
BsLipA-R	cacccgctcgag ttatgcttgtgcttgacgctttttagatg		
BsLipL-F	gttcgcggatcc atggcaaaccaaccgattgatttac	pH6	
BsLipL-R	gagtcccaagctt tcacccaaataccttgcattccg		
ScGCV3-F	gttcgcggatccatg acttcccaacatgagtggatag	pH3	ScLA DNA Assembler
ScGCV3-R	cacccgctcgag tcagtcatcatgaaccagtgtcttttc		
ScLIP3-F	gttcgcggatccatg gtgggacagcgaaacctcatac	pH4	
ScLIP3-R	cacccgctcgag ttatgtgtaactgtcaatattctc		
ScLIP5-F	gttcgcggatccatg aatgcattgaatactgattc	pH5	
ScLIP5-R	cacccgctcgag ttatttcatgtttctttttcttc		
ScLIP2-F	gttcgcggatccatg ttcaatgtctgtaggcgacaatg	pH6	
ScLIP2-R	gagtcccaagctt tcacggattctttttcaaaatattg		

lpdA-F1	gcacgtagcaaaagttatcgaagaag	---	PDHm1 PDHm2 Gibson
lpdA-R1	gtataggcgatggacgggataactttc		
lpdA-F2	cgaaagttatcccgtccatcgcctataccaaaccagaagttgcatgggtag		
lpdA-R2	ggtggtatccgagagaattgtgtgattgcttttaattaatttcggagaatctcacatgc		
lpdA-F2N	cgaaagttatcccgtccatcgcctataccggtccagaagttgcatgggtag		
cytoCEM1-F	gttggaagatctatg ctctcttccaaaaatggac	pH1	cytoFAS0/1/2/3 DNA Assembler Gibson
cytoCEM1-R	cacccgctcgag ttaactcccttcccatttc		
cytoOar1-F	acaagacaccaatcaaaacaaataaaacatcatcacaatg ttatttcaaaagggtttaag	pH2	
cytoOar1-R	gagggcgtgaatgtaagcgtgacataactaattacatgatttacgtctccaaagcagttc		
cytoETR1	tcataacaccaagcaactaatactataacatacaataata atgtcgtcctcagctcatca	pH3	
cytoETR1	aaagaaaaaaattgatctatcgatttcaattcaattcaattaccattctaaaacaacca		
cytoACP1-F	gttcgcggatccatg tctgcaaacttgagcaaag	pH4	
cytoACP1-R	cacccgctcgag ttagtttgcgtcgggattg		
cytoPPT2-F	gttcgcggatccatg gtatacttgccaagatttg	pH5	
cytoPPT2-R	cacccgctcgag ttactctctttctaccaag		
cytoMCT1-F	gttcgcggatccatg aaatcaagagaattccaaac	pH6	
cytoMCT1-R	gagtcccaagctt tcaatctttgttctcctctg		
cytoHtd2-F	cacaaaaacaaaaagttttttttaattttaatcaaaaaatg catcttcaactgggagaaca	HXT7p-CDS-HXT7t	
cytoHtd2-R	aattagagcgtgatcatgaattaataaaagtgttcgcaaatcaacagaaaacatcagctt		
FabA-F	aaacacaaaaacaaaaagttttttttaattttaatcaaaaaatggtagataaacgcgaatc		
FabA-R	aattagagcgtgatcatgaattaataaaagtgttcgcaaatcagaaggcagacgtatcct		
FabZ-F	aaacacaaaaacaaaaagttttttttaattttaatcaaaaa atgactactaacactcatac		
FabZ-R	aattagagcgtgatcatgaattaataaaagtgttcgcaaatcaggcctcccggctacgag		
HTD2-Gib-F	gtaatacgactcactatagggcgaattgaggacttctcgtaggaacaatttcg	---	
HTD2-Gib-R	caaatcttaaagtcatacattgcacgactaaggataactgactcattagacac		
FabD-F	gttcgcggatcc atgacgcaatttgcatttgtgttc	pH1	EcFAS DNA Assembler Gibson
FabD-R	cacccgctcgag ttaaagctctagcgccgctgccatc		
FabG-F	tttacaagacaccaatcaaaacaaataaaacatcatcaca atgaattttgaaggaaaaat	pH2	
FabG-R	gagggcgtgaatgtaagcgtgacataactaattacatgattcagaccatgtacatcccgc		
FabI-F	tcataacaccaagcaactaatactataacatacaataataatgggttttctttccggtaa	pH3	
FabI-R	aaagaaaaaaattgatctatcgatttcaattcaattcaattttatttcagttcgagttcgt		
AcpS-F	gttggaagatct atggcaatattaggtttagg	pH4	
AcpS-R	cacccgctcgag ttaactttcaataattacc		
AcpP-F	gttcgcggatcc atgagcactatcgaagaacg	pH5	
AcpP-R	cacccgctcgag ttacgcctggtggccgttg		
FabF-F	gttcgcggatcc atgtctaagcgtcgtgtagttgtgac	pH6	
FabF-R	gagtcccaagctt ttagatctttttaaagatcaaagaac		
FabH-F	gttcgcggatcc atgtatacgaagattattg	pH3	
FabH-R	cacccgctcgag ctagaaacgaaccagcgcg		
FabH-Gib-F	gatccagtatagtgtattcttcctg gcggccgc gtgtcgacgctgcgggtatag	---	
FabH-Gib-R	accaacctgatgggttcctagatata gcggccgc cag		

cytoFAA2-F	acacaaaaacaaaaagttttttaattttaatcaaaaaatggccgctccagattatgcac	pH5	
cytoFAA2-R	aattagagcgtgatcatgaattaataaaagtgttcgcaaactatgtcttgactagtgaac		
VhAasS-F	gttcgcggatcc atgaaccagtatgtaaatgatc	pH6	
VhAasS-R	gagtcccaagctt ttacagatgaagtttacgcagttc		
TE-Gib-F	ctttagatccagtatagtgtattcttcctggcggccgc tagcttcaaaatgtttctactc	---	rP48 Gibson
TE-Gib-R	caatatcgaataacgatggcttccccgctatacttaag atagcgccgatcaaagtatttg		
PDC1p-Seq-F	catatttcttgtcatattccttctc	Sequencing primers for genes cloned into the helper plasmids	
ADH1t-Seq-R	cttttcgtaaatttctggcaaggtag		
PYK1p-Seq-F	cttaacttgtttattattctctcttgtttc		
CYC1t-Seq-R	cttcaggttgtctaactccttcctttc		
ENO2p-Seq-F	gtatcttttcttcccttgtctcaatc		
PGK1t-Seq-R	ctattattttagcgtaaaggatgggg		
TPI1p-Seq-F	catttactattttcccttcttacg		
TPI1t-Seq-R	caatataaaaaagctttccgtagtcatc		
TEF1p-Seq-F	gtttcatttttcttgttctattacaac		
TEF1t-Seq-R	ccagactatatataaggataaattac		
HXT7p-Seq-F	catcaagaacaaacaagctcaacttgtc		
HXT7t-Seq-R	caagatatcattaaaaatataaaattag		
PGK1p-Seq-F	ctaattcgtagtttttcaagttcttagatgc		
GPM1P-Seq-F	cctttcttaattctgttgtaattaccttcc		
GPDp-Seq-F	ggtaggtattgattgtaattctgtaaatc		

Supplementary Table S2. Mitochondrial targeting sequences (MTS) of the lipoylation pathway and type II FAS pathway from the mitochondria of yeast. MTSs were mainly predicted using the MITOPROT on-line program and removed during cloning to relocate the whole pathway enzymes into the cytosol. The transit peptides annotated on NBCI with experimental evidence were also included for comparison (in brace).

Proteins	MTS	Cleaved Sequences
Gcv3p (P39726)	1-47 (1-47)	MLRTTRLWTTRMPTVSKLFLRNSSGNALNKNKLPFLYSSQGPQAVRY
Lip2p (Q06005)	1-12	MSRCIRQSVCTN
Lip3p (DAA08754)	1-14	MSMMLSNWALSPRY
Lip5p (P32875)	1-34 (1-18)	MYRRSVGVLFVGRNTRWISSTIRCGTSATRPIRS
Acp1p (P32463)	1-36 (1-36)	MFRSVCRISSRVAPSAYRTIMGRSVMSNTILAQRFY
Ppt2p (Q12036)	1-19	MSFASRNIGRKIAGVGVDI
Cem1p (P39525)	1-27	MSRRVVITGLGCVTPLGRSLSESWGNL
Oar1p (P35731)	1-23	MHYLPVAIVTGATRGIGKAICQK
Htd2p (P38790) *	1-35	MKSKTWIFRDVLSSHRTKAFDSLLCRRLPVSKAT
Etr1p (P38071)	---(1-9)	MLPTFKRYM

*Predicted by TargetP ***(*17*)**

Supplementary References

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