Supplementary Information

Expanding ester biosynthesis in *Escherichia coli*

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Supplementary Table 1 Genotype of *E. coli* host strains and plasmids used in this study

***E. coli* strain**	**Genotype**	**Reference**
BW25113	*rrnB*$_{T14}$ Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LC78}$	1
JCL16	BW25113/F' [*traD*36, *proAB*$^{+}$ *lacI*^{q} ZΔM15 Tn10(tet^{r})]	2
JCL88	Same as JCL16 but with Δ*adhE* Δ*frd* Δ*ldhA* Δ*pta* Δ*fnr*	3
JCL260	Same as JCL16 but with Δ*adhE* Δ*frd-ldhA* Δ*pta* Δ*pflB* Δ*fnr*	3
AL704	Same as JCL260 but with Δ*yqhD* Δ*adhP* Δ*eutG* Δ*yiaY* Δ*yjgB* Δ*fucO*	4
AL1050	F- lambda- *ilvG*- *rfb*-50 *rph*-1 *lacI*^{q} *tetR spec*^{R}	This work
Plasmid Name	**Genotype**	**Reference**
pGR03	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *alsS-ilvCD*	4
pSA40	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$:	2
pSA69	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *alsS-ilvCD*	3
pSA138	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *kivd-yqhD*	3
pZE12-luc	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *luc*	5
pAL495	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *mRFP1*	This work
pAL497	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *luxCDE*	This work
pAL603	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *alsS-ilvCD, P*$_{L}$lacO$_{1}$: *kivd-adhA*	This work
pAL609	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *kivd-mhpF*	This work
pAL622	ColE1 ori; Kan^{R}; *ATF1*	This work
pAL633	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *bkdA1-bkdA2-bkdB-lpdV*	This work
pAL670	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *EHT1*	This work
pAL675	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *EEB1*	This work
pAL676	p15A ori; Cm^{R}; *P*$_{L}$lacO$_{1}$: *ATF1*	This work
pAL679	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *kivd-ATF1*	This work
pAL682	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *mRFP1*	This work
pAL683	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *EEB1*	This work
pAL684	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *EHT1*	This work
pAL685	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *ATF1*	This work
pAL689	p15A ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *cat*	This work
pAL692	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *EHT1, P*$_{L}$lacO$_{1}$: *bkdA1-bkdA2-bkdB-lpdV*	This work
pAL693	ColE1 ori; Amp^{R}; *P*$_{L}$lacO$_{1}$: *cat, P*$_{L}$lacO$_{1}$: *bkdA1-bkdA2-bkdB-lpdV*	This work
pAL723	ColE1 ori; Kan^{R}; *P*$_{L}$lacO$_{1}$: *ATF1*	This work

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Supplementary Table 2 Yield of isobutyl acetate from glucose by strain 5

Isobutyl acetate ($g\ l^{-1}$)	Glucose consumed ($g\ l^{-1}$)	Yield (g Isobutyl acetate/g Glucose)	% of Max (0.42 g/g)	D_{600} at 96 h
17.2 ± 0.4	51.6 ± 1.3	0.334 ± 0.016	79.6 ± 3.8	6.63 ± 0.22

Errors are s.d. (n = 3).

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Supplementary Table 3 Degradation test of isobutyl acetate in *E. coli* culture

	Isobutyl acetate	Isobutanol
No cell	99.9 ± 0.031%	0.12 ± 0.031%
JCL260	99.9 ± 0.023%	0.14 ± 0.027%
strain 4	99.8 ± 0.023%	0.22 ± 0.023%

Cells were grown in 5 ml M9P with 50 g l^{-1} glucose in 15 ml-screw-cap tubes at 37°C until D_{600} ~0.4, then 1 mM IPTG was added and tubes were transferred to 30°C. Then, 1 h after induction, 1 g l^{-1} isobutyl acetate was added. Remaining isobutyl acetate and formation of isobutanol was measured after 24 h by GC-FID analysis. Errors are s.d. (n = 3).

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Supplementary Table 4 Degradation test of isobutyl isobutyrate in *E. coli* culture

	Isobutyl isobutyrate	Isobutanol
No cell	99.3 ± 0.22%	0.7 ± 0.22%
AL704	98.8 ± 0.18%	1.2 ± 0.18%
strain 9	98.5 ± 0.48%	1.5 ± 0.48%
strain 16	97.5 ± 0.48%	2.5 ± 0.48%
strain 18	98.8 ± 0.40%	1.2 ± 0.40%

Cells were grown in 5 ml M9P with 50 g l^{-1} glucose in 15 ml-screw-cap tubes at 37°C until D_{600} ~0.4, then 1 mM IPTG was added and tubes were transferred to 30°C. Then, 1 h after induction, 1 g l^{-1} isobutyl isobutyrate was added. Remaining isobutyl isobutyrate and formation of isobutanol was measured after 24 h by GC-FID analysis. Errors are s.d. (n = 3).

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Supplementary Table 5 Oligonucleotides used in this study

Name	Sequence
GR387	TCTAGAGGCATCAAATAAAACGAAA
GR388	GTGACCTTTCTCCTGCATGC
GR689	GCATGCAGGAGAAAGGTCACATGAGTAAGCGTAAAGTCGCCATTATC
GR698	TCTAGAGGCATCAAATAAAACGAAAGGC
GR699	GGTACCTTTCTCCTCTTTAATGAATTCGG
GR701	TTTTATTTGATGCCTCTAGATCATGCCGCTTCTCCTGCCTT
GR720	GCATGCAGGAGAAAGGTCACATGAACGAAATCGACGAAAAGAATCAAG
GR721	TTTTATTTGATGCCTCTAGATTACGGACCCAGCAGCAGTG
GR724	TTAAAGAGGAGAAAGGTACCATGAACGAGTACGCCCCCCTG
GR725	TTTTATTTGATGCCTCTAGATCAGATATGCAAGGCGTGGC
SD67	GAACGCCGTACGCGAGCGGTATCAGCTCACTCAAA
SD68	GCCTCGTCCTAGGTCTAGGGCGGCGGATTTGTC
SD69	CGCCGCCCTAGACCTAGGACGAGGCCCTTTCGTCTTCACCTCGAG
SD70	GAGCTGATACCGCTCGCGTACGGCGTTCACCGACAAACAACAGAT
YT018	TAAACGCGTGCTAGAGGCATCAAAT
YT040	CATGGTACCTTTCTCCTCTTTAATGAATTCGGTCA
YT087	CATTGTACCTTTCTCCTCTTTAATGAATTC
YT193	CATTAAAGAGGAGAAAGGTACCATGGCTTCCTCCG
YT194	TTATTTGATGCCTCTAGAGTCATTAAGCACCGGTGGAGT
YT253	CATTAAAGAGGAGAAAGGTACAATGGAAAAACACTTACCTTTAATAATAAAT
YT255	ATCGTTTAAACGAACATTTCCTTATTTGTTGGTATTAC
YT256	TAAGGAAATGTTCGTTTAAACGATGCTGAAG
YT257	ATTTGATGCCTCTAGCACGCGTTTAGTTGCCTCCTTCATTCTTAG
YT438	CATTAAAGAGGAGAAAGGTACAATGTTTCGCTCGGGTTACTATCCAAC
YT439	ATTTGATGCCTCTAGCACGCGTTTATAAAACTAACTCATCAAAGCTGCCCAAGA
YT440	CATTAAAGAGGAGAAAGGTACAATGTCAGAAGTTTCCAAATGGCCAG
YT441	TTTGATGCCTCTAGCACGCGTTTATACGACTAATTCATCAAACTTAGTGAAAAATTCTGC
YT442	CATTAAAGAGGAGAAAGGTACAATGAACGAAATCGACGAAAAGAATCAAG
YT443	ATTTGATGCCTCTAGCACGCGTTTACGGACCCAGCAGCAGTG
YT466	TCTCACCAATAAAAAACGCCCGGCGAATTGTGAGCGGATAACAATTGACATT
YT467	GGATTTGTTCAGAACGCTCGGTTGCCTAGCACGCGTTTATACGACTAATTCATCA
YT468	GCAACCGAGCGTTCTGAACAAATC
YT469	CGCCGGGCGTTTTTTATTGGT
YT470	GAATTCATTAAAGAGGAGAAAGGTACAATGGAGAAAAAAATCACTGGATATACCACCG
YT471	ATTTGATGCCTCTAGCACGCGTTTACGCCCCGCCCTGC
YT479	GGATTTGTTCAGAACGCTCGGTTGCCTAGCACGCGTTTACGCCCC

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Supplementary Table 6 Plasmid construction by SLIC

	Vector PCR			Insert PCR			
Plasmid	**Primer 1**	**Primer 2**	**Template[a]**	**Primer 1**	**Primer 2**	**Template**	**Gene of Interest[f]**
pAL495	YT087	YT018	pGR03	YT193	YT194	BBa_E1010[b]	*mRFP1*
pAL497	YT087	YT018	pGR03	YT253	YT255	gDNA *V. harveyi*[c]	*luxCD*
				YT256	YT257		*luxE*
pAL603	SD67	SD68	pSA138	SD69	SD70	pSA69	*alsS-ilvCD*
pAL609	GR387	GR388	pSA138	GR689	GR701	gDNA *E.coli* JCL16	*mhpF*
pAL633	GR698	GR699	pSA138	GR724	GR725	gDNA *P. putida* g7	KDHC operon
pAL670	YT087	YT018	pGR03	YT440	YT441	gDNA *S. cerevisiae*[d]	*EHT1*
pAL675	YT087	YT018	pGR03	YT438	YT439	gDNA *S. cerevisiae*[d]	*EEB1*
pAL676	YT087	YT018	pGR03	YT442	YT443	pAL622	*ATF1*[e]
pAL679	GR387	GR388	pSA138	GR720	GR721	pAL622	*ATF1*[e]
pAL682	YT087	YT018	pSA69	YT193	YT194	BBa_E1010[b]	*mRFP1*
pAL683	YT087	YT018	pSA69	YT438	YT439	gDNA *S. cerevisiae*[d]	*EEB1*
pAL684	YT087	YT018	pSA69	YT440	YT441	gDNA *S. cerevisiae*[d]	*EHT1*
pAL685	YT087	YT018	pSA69	YT442	YT443	pAL622	*ATF1*[e]
pAL689	YT087	YT018	pSA69	YT470	YT471	pGR03	*cat*
pAL692	YT468	YT467	pAL633	YT466	YT467	pAL684	*EHT1*
pAL693	YT468	YT467	pAL633	YT466	YT479	pAL689	*cat*
pAL723	YT040	YT018	pSA40	YT442	YT443	pAL622	*ATF1*[e]

a. All plasmids and oligonucleotides are listed in Supplementary Table 1 and 5, respectively.

b. BBa_E1010 sourced from parts.igem.org

c. *Vibrio harveyi* BB120 (ATCC BAA-1116D-5)

d. *Saccharomyces cerevisiae* BY4741 (ATCC 4040004)

e. Codon optimized *ATF1* was synthesized by GenScript USA Inc. (Piscataway, NJ). The sequence is shown in Supplementary Fig. 6.

f. NCBI reference number: *mhpF*, NP_414885; the KDHC genes (*bkdA1-bkdA2-bkdB-lpdV*), YP_001266792.1 YP_001266793.1 YP_001266794.1 YP_001266795.1; *EHT1*, NP_009736.3; *EEB1*, NP_015230.1; *ATF1*, NP_015022.3.

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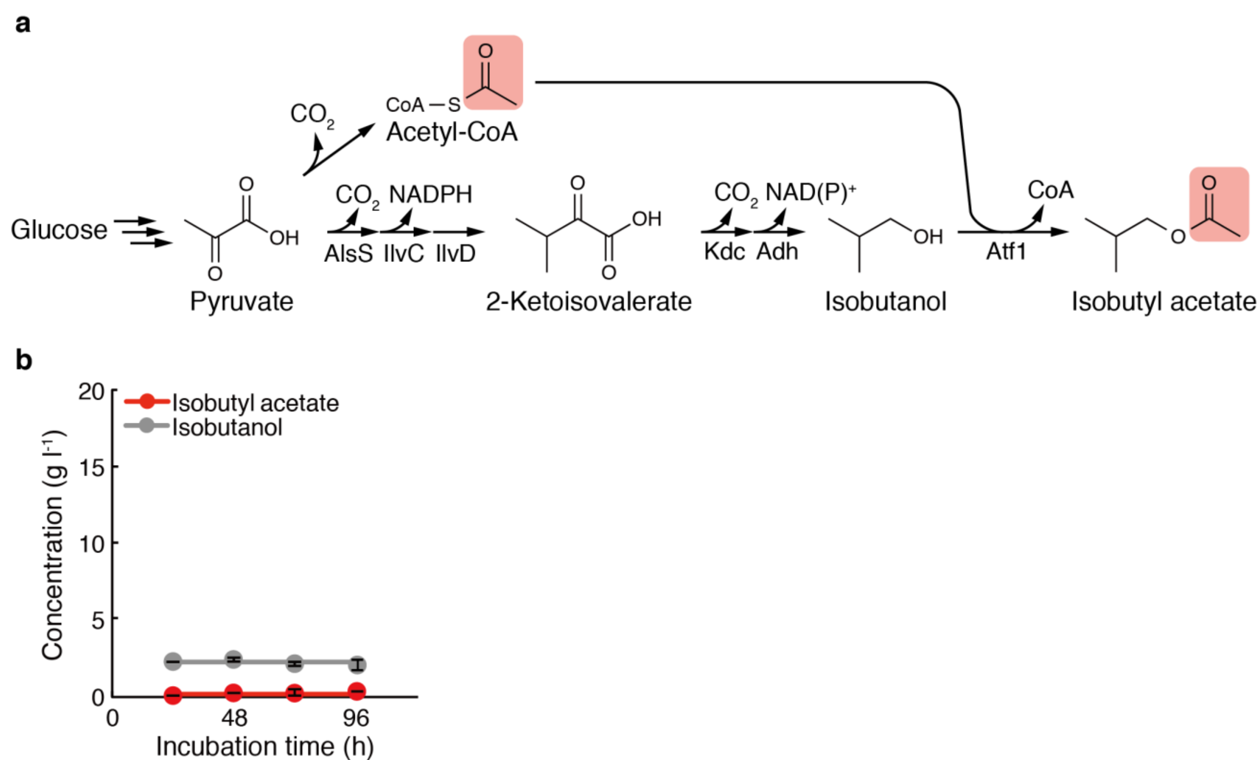

Supplementary Figure 1. Isobutyl acetate from glucose in strain 5. (**a**) Isobutyl acetate synthesis pathway from glucose in *E. coli*. (**b**) Isobutyl acetate and isobutanol concentrations in culture layer during the production with a hexadecane layer by strain 5. Production of isobutyl acetate from strain 5 grown in M9P containing 50 g l^{-1} glucose for 96 h. Cells were grown in 20 ml media in 250 ml screw-cap flasks at 37°C until D_{600} ~0.4, then 1 mM IPTG was added. Hexadecane (20 ml) was added 1 h after induction. Production was performed at 30°C in a rotary shaker at 250 rpm. Errors are s.d. (n = 3).

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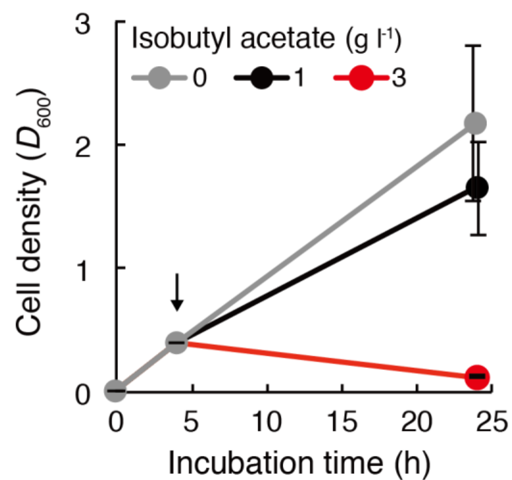

Supplementary Figure 2. Effect of isobutyl acetate on *E. coli* growth. JCL260 was grown at 30°C in 5 ml M9P media containing 50 g l^{-1} glucose in 15 ml-screw-cap tubes. Black Arrow indicates the time point when isobutyl acetate (None (gray), 1 g l^{-1} (black), or 3 g l^{-1} (red)) was added into culture. Error bars are s.d. (n = 3).

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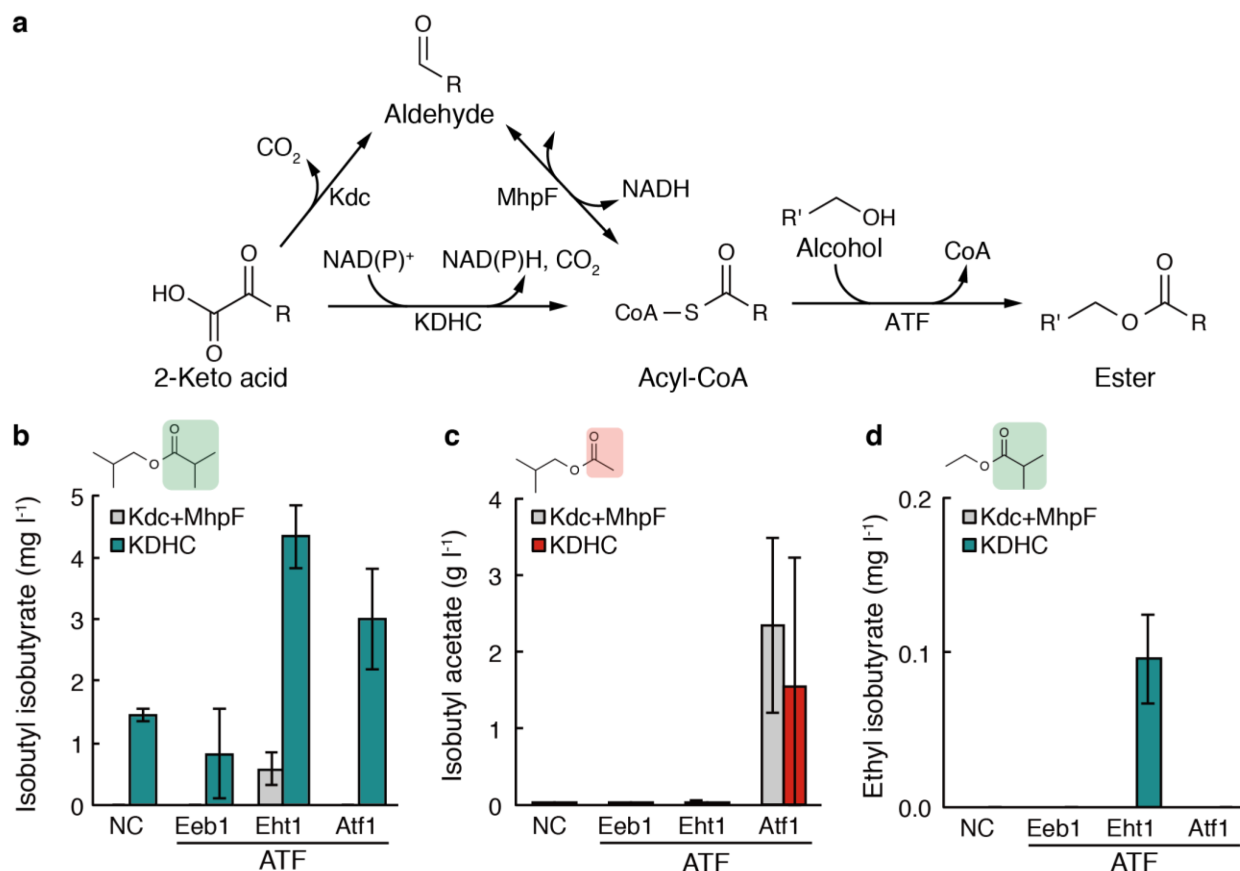

Supplementary Figure 3. Biosynthesis pathway for higher chain ester. (**a**) Higher chain ester pathways from 2-keto acid *via* Kdc-MhpF route and KDHC route. Cells were grown in M9P media containing 10 g l^{-1} glucose in 5 ml M9P media in 15 ml-screw-cap tubes at 37˚C until D_{600} ~0.4. Then, 1 mM IPTG was added and cultures were transferred to 30˚C. After 1 h of induction, 3 g l^{-1} 2-ketoisovalerate and 3 g l^{-1} of isobutanol were added. Production was performed at 30˚C for 24 h. The concentrations of isobutyl isobutyrate (**b**), isobutyl acetate (**c**), ethyl isobutyrate (**d**) in the cultures were measured. Error bars are s.d. (n = 3).

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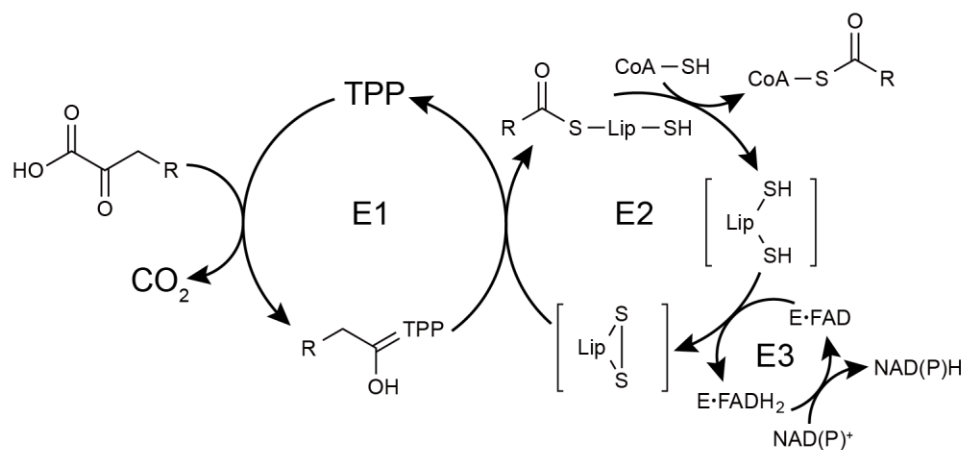

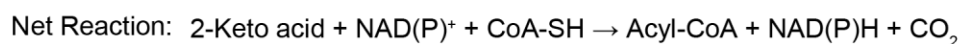

Supplementary Figure 4. Reaction mechanism of KDHC. KDHC consists of four proteins. *bkdA1*, *bkdA2*, *bkdB*, and *lpdV* encode α-subunit (E1), β-subunit (E1), dihydrolipoyl transacylase (E2), and dihydrolipoamide dehydrogenase (E3), respectively. Thiamine diphosphate (TPP), lipoamide-E (Lip), and flavin adenine dinucleotide (FAD) are involved in this reaction as cofactor.

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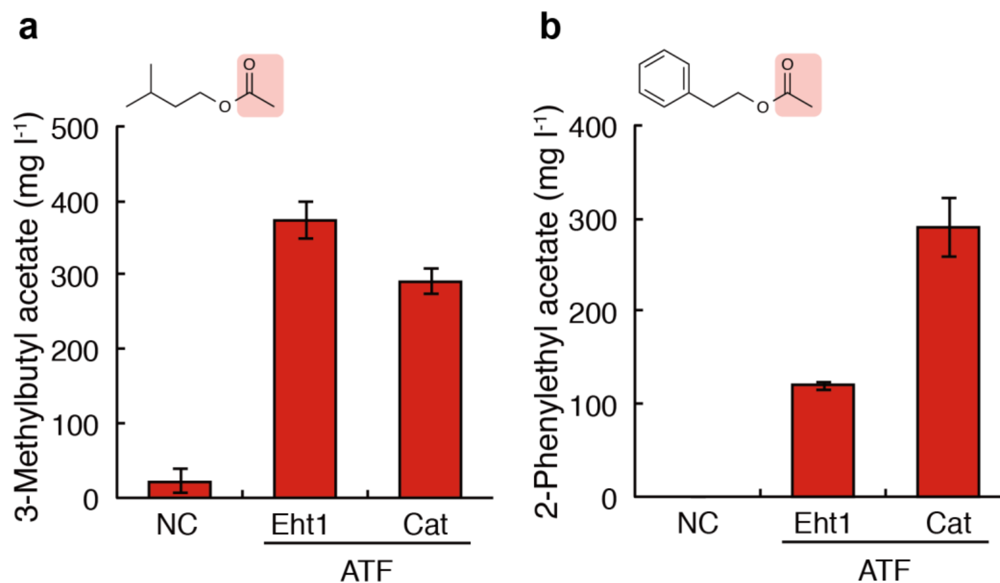

Supplementary Figure 5. Functions of Eth1 and Cat as acetyl-CoA transferase. Cells were grown in 5 ml M9P media containing 10 g l^{-1} glucose in 15 ml-screw-cap tubes at 37˚C until D_{600} ~0.4. Then, 1 mM IPTG was added. After 1 h of induction, 3 g l^{-1} 2-ketoisovalerate and 3 g l^{-1} 3-methyl-1-butanol (**a**) or 3 g l^{-1} 2-phenylethanol (**b**) were added and production was performed at 30˚C for 24 h at 250 rpm in a rotary shaker. Error bars are s.d. (n = 3).

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1 ATGAACGAAA TCGACGAAAA GAATCAAGCC CCGGTCCAAC AAGAATGCCT
51 GAAAGAAATG ATCCAGAATG GTCACGCCCG CCGTATGGGC TCAGTGGAAG
101 ACCTGTATGT TGCACTGAAC CGTCAGAATC TGTACCGCAA TTTTTGCACC
151 TATGGTGAAC TGTCGGACTA CTGTACGCGT GATCAACTGA CCCTGGCTCT
201 GCGCGAAATC TGCCTGAAAA ACCCGACGCT GCTGCATATT GTGCTGCCGA
251 CCCGTTGGCC GAACCACGAA AACTACTACC GTAGCTCTGA ATACTACAGT
301 CGCCCGCATC CGGTTCACGA TTATATTAGT GTCCTGCAAG AACTGAAACT
351 GTCCGGCGTG GTTCTGAATG AACAGCCGGA ATACAGCGCG GTTATGAAGC
401 AAATCCTGGA AGAATTTAAA AACAGCAAGG GTTCTTACAC GGCCAAAATC
451 TTTAAGCTGA CCACGACCCT GACGATTCCG TACTTCGGTC CGACCGGTCC
501 GAGCTGGCGC CTGATCTGCC TGCCGGAAGA ACATACCGAA AAGTGGAAGA
551 AGTTCATCTT CGTGTCAAAC CACTGTATGT CGGATGGCCG TAGTTCCATC
601 CATTTCTTTC ACGACCTGCG CGATGAACTG AACAATATCA AGACCCCGCC
651 GAAAAAGCTG GACTACATCT TCAAGTACGA AGAAGATTAC CAGCTGCTGC
701 GTAAGCTGCC GGAACCGATT GAAAAAGTGA TCGATTTTCG TCCGCCGTAC
751 CTGTTTATCC CGAAAAGTCT GCTGTCCGGC TTTATTTACA ATCATCTGCG
801 TTTCTCATCG AAGGGTGTGT GCATGCGCAT GGATGACGTT GAAAAAACGG
851 ATGACGTCGT GACCGAAATT ATCAACATTA GCCCGACCGA ATTTCAGGCG
901 ATCAAGGCCA ACATCAAGTC TAACATCCAA GGCAAATGCA CGATCACCCC
951 GTTTCTGCAT GTCTGTTGGT TCGTGAGCCT GCACAAATGG GGCAAGTTTT
1001 TCAAACCGCT GAACTTTGAA TGGCTGACGG ACATTTTCAT CCCGGCGGAT
1051 TGTCGTTCTC AGCTGCCGGA TGACGATGAA ATGCGTCAAA TGTATCGCTA
1101 CGGCGCCAAT GTGGGTTTTA TCGATTTCAC CCCGTGGATT AGTGAATTTG
1151 ACATGAACGA TAACAAGGAA AACTTCTGGC CGCTGATCGA ACATTATCAC
1201 GAAGTTATTT CCGAAGCGCT GCGTAACAAA AAGCATCTGC ACGGCCTGGG
1251 TTTCAACATC CAGGGTTTCG TTCAAAAGTA CGTCAACATC GACAAAGTCA
1301 TGTGTGATCG CGCCATTGGC AAACGTCGTG GCGGCACCCT GCTGTCCAAC
1351 GTTGGTCTGT TTAATCAGCT GGAAGAACCG GACGCAAAAT ATTCAATTTG
1401 CGATCTGGCT TTTGGCCAGT TCCAAGGTTC GTGGCATCAG GCATTCAGCC
1451 TGGGCGTCTG TTCTACGAAC GTGAAGGGTA TGAATATTGT TGTCGCTTCT
1501 ACCAAAAATG TGGTTGGTAG CCAAGAATCG CTGGAAGAAC TGTGTAGTAT
1551 CTATAAGGCA CTGCTGCTGG GTCCGTAA

Supplementary Figure 6. DNA sequence of codon optimized *ATF1* gene

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