Electronic Supplementary Material (ESI) for RSC Advances.

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Supporting information

Directed evolution of Mevalonate Kinase in *Escherichia coli* by random mutagenesis for improved lycopene production

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Experimental details

Protein expression and purification

MK was expressed and purified from *E. coli* BL21(DE3) cells harboring the plasmid pET–MK. The bacterial strain with MK mutations were used to inoculate LB medium which was maintained at 37°C until the optical density at 600 nm (OD_{600}) reached between 0.6 and 0.8 units. 0.2 mM IPTG was used to induce recombinant protein expression at 20°C. Cells were pelleted after 20 h by centrifugation at 10,000*g* and resuspended in 50 mM phosphate buffer (pH 7.4) containing 4 mM β-mercaptoethanol. Sonication was used to lyse the cells (3 s pulses with 3 s intervals between cycles at 60% output) at 4°C for a total of 40 min. Lysed cells were then clarified by centrifugation at 18,000*g* for 10 min, with samples kept at 4°C. A 0.22 μm PALL filter was used to further clarify the supernatant then loaded onto a nickel affinity chromatography column, which was pre-washed with 10 ml water and equilibrated with 10 ml binding buffer. Recombinant protein labelled with a 6 His-tag was able to bind to the nickel ions within the column. Sequential washing with 10 ml washing buffer 1 removed unbound protein from the column, while sashing Buffer 2 was used to remove nonspecific or weakly interacting, contaminating proteins. 10 ml elution fractions were collected and the concentration of each fraction was determined with the BCA protein assay quantification kit as per the manufacturer's instructions. Recombinant protein was stored at −20°C after being flash frozen in liquid nitrogen.

Lycopene quantification assay

Lycopene was extracted from cellular fractions of each bacterial culture immediately after total glucose exhaustion. After washing, the pellet was extracted using acetone (1 ml) at 55°C and under intermittent vortexing for 15 min. The lycopene content in the supernatant was quantified by determination of the absorbance at 475 nm and calculated according to a

standard curve. Extractions were completed in the dark to prevent photo-bleaching and degradation.

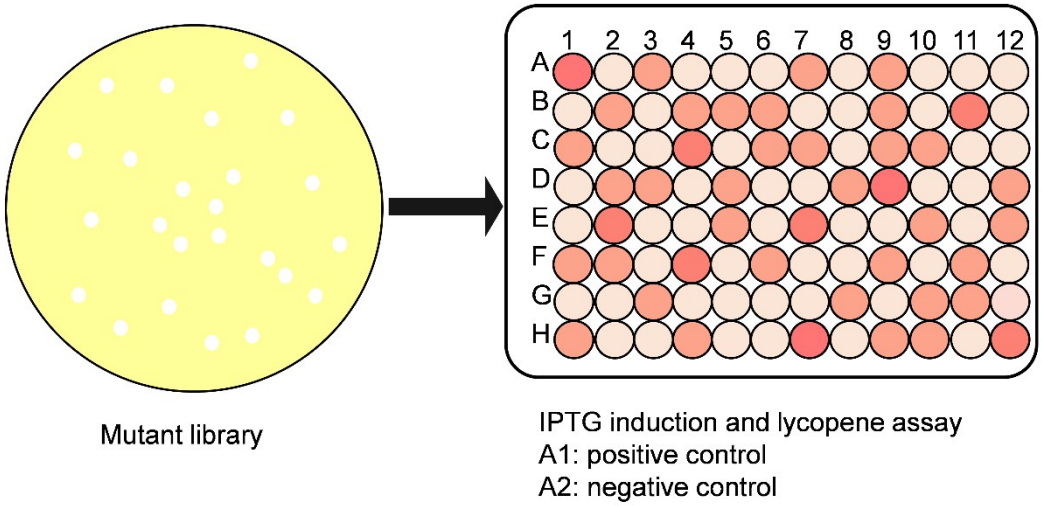

Figure S1. A two-step procedure for screening the MK with improved lycopene production. Negative control: colonies with deactivated MK; positive control: colonies with activated MK.

Table S1. Strains and plasmids used in this study.

Strain/plasmid /primer	**Descriptions**	**Reference**
Strains		
E. coli BL21(DE3)	*E. coli* str. B F⁻ *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3[*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S)	Invitrogen
E. coli DH5α	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ⁻	Invitrogen
CHL-1	BL21(DE3)/pET-CHL/pAC-LYC/ pCLpTrcUpper	This work
CHL-2	BL21(DE3)/pET-CHL-MK (V13D/S148I/V301E)/pAC-LYC/ pCLpTrcUpper	This work
plasmids		
pETDeu-1	Ampicillin resistant; T7 promoter; has encoded N-terminal His6 tag	Invitrogen
pET-CHL1	pETDeu-1 derivative carryinggenes gene *ERG8, T7* promoter, Ap ^{R}	This work
pET-CHL2	pETDeu-1 derivative carryinggenes gene *ERG8* and *ERG19* , T7 promoter, Ap ^{R}	This work
pET-CHL3	pETDeu-1 derivative carryinggenes gene *ERG8*, *ERG19* and *ERG12, T7* promoter, Ap ^{R}	This work
pET-CHL	pETDeu-1 derivative carryinggenes gene *ERG8*, *ERG19, ERG12* and *IDI, T7* promoter, Ap ^{R}	This work
pET-MK	pET28a(+) derivative carryinggenes gene ERG12, T7 promoter, Kan^R	This work

pCLpTrcUpper	pETDeu-1 derivative carryinggenes gene *mvaE* and *mvaS, T7* promoter, Spc ^{R}
pAC-LYC	pACYCDuet-1 derivative carryinggenes gene *crtE, crtI, crtB, T7* promoter, Cm ^{R}
Primers	
ERG12_F	5′-ACGCGTCGACTCATTACCGTTCTTAACTTC-3′
ERG12_R	5′-ATTTGCGGCCGCTTATGAAGTCCATGGTAAAT-3′
ERG8_F	5′-GGAAGATCTCTCAGAGTTGAGAGCCTTCAG-3′
ERG8_R	5′-GGGCCGACGTCTTATTTATCAAGATAAGTTT-3′
ERG19_F	5′-TCGCGACGTCACCGTTTACACAGCATCCGT-3′
ERG19_R	5′-CCGCTCGAGTTATTCCTTTGGTAGACCAG-3′
IDI_F	5′-CGAGCTCGACTGCCGACAACAATAGTAT-3′
IDI_R	5′-ACGCGTCGACTTATAGCATTCTATGAATTT-3′
V13-F	5'-GCACCGGGAAAGNNKATTATTTTTGGTG-3'
V13-R	5'-CACCAAAAATAATKNNCTTTCCCGGTGC-3'
S148-F	5'-GGTGCTGGGTTGGGCNNKAGCGCCTCTATTTC-3'
S148-R	5'-GAAATAGAGGCGCTKNNGCCCAACCCAGCACC-3'
V301-F	5'-CGATGACGAGGCTGNNKAAACTAATAATGAAC-3'
V301-R	5'-GTTCATTATTAGTTTKNNCAGCCTCGTCATCG-3'
V13D-F	5'-CTTCTGCACCGGGAAAGGATATTATTTTTGGTGAAC-3'
V13D-R	5'-GTTCACCAAAAATAATATCCTTTCCCGGTGCAGAAG-3'
S148I-F	5'-GTGCTGGGTTGGGCATCAGCGCCTCTATTTC-3'
S148I-R	5'-GAAATAGAGGCGCTGATGCCCAACCCAGCAC-3'
V301E-F	5'-CACCGATGACGAGGCTGAAGAAACTAATAATGAAC-3'
V301E-R	5'-GTTCATTATTAGTTTCTTCAGCCTCGTCATCGGTG-3'

Table S2 Sequencing results of mutants.

Cycles	Mutant nucleotide bases	Mutant amino acids
1	T38A	V13D
2	T38A/T442G	V13D/S148A
3	T38A/T442G/T902A	V13D/S148A/V301E

Table S3. The results of three-cycles of random mutagenesis

First cycle		Second cycle		Third cycle	
No.	OD_{475}	No.	OD_{475}	No.	OD_{475}
1	0.56	1	0.81	1	1.28
2	0.56	2	0.81	2	1.28
3	0.56	3	0.81	3	1.28
4	0.56	4	0.81	4	1.28
5	0.56	5	0.81	5	1.28
6	0.56	6	0.81	6	1.28
7	0.56	7	0.81	7	1.28
8	0.56	8	0.81	8	1.28
9	0.56	9	0.81	9	1.28
10	0.56	10	0.81	10	1.28
11	0.58	11	0.81	11	1.28
12	0.59	12	0.81	12	1.28
13	0.61	13	0.81	13	1.28
14	0.62	14	0.81	14	1.28
15	0.81	15	0.86	15	1.28
16	0.83	16	1.08	16	1.28

17	0.84	17	1.13	17	1.28
18	0.84	18	1.19	18	1.33
19	0.84	19	1.19	19	1.45
20	0.84	20	1.19	20	1.45
Control1	0.55	Control1	0.55	Control1	0.55
Control2	0.49	Control2	0.57	Control2	0.60
Control3	0.64	Control3	0.50	Control3	0.59

Table S4. The results of saturation mutagenesis at residues V13, S148 and V301

V13X		**S148X**		**V301X**	
No.	OD_{475}	No.	OD_{475}	No.	OD_{475}
1	0.48	1	0.54	1	0.34
2	0.48	2	0.54	2	0.34
3	0.48	3	0.59	3	0.34
4	0.54	4	0.59	4	0.34
5	0.54	5	0.59	5	0.46
6	0.54	6	0.62	6	0.46
7	0.6	7	0.62	7	0.46
8	0.6	8	0.65	8	0.57
9	0.6	9	0.65	9	0.57
10	0.6	10	0.68	10	0.57
11	0.63	11	0.68	11	0.57
12	0.63	12	0.70	12	0.63
13	0.63	13	0.70	13	0.63
14	0.66	14	0.76	14	0.63

15	0.66	15	0.76	15	0.68
16	0.78	16	0.81	16	0.70
17	0.83	17	0.81	17	0.71
18	0.81	18	0.92	18	0.74
19	0.96	19	0.95	19	0.78
20	0.99	20	0.97	20	0.80
Control1	0.58	Control1	0.51	Control1	0.58
Control2	0.61	Control2	0.56	Control2	0.53
Control3	0.61	Control3	0.55	Control3	0.60

Table S5. The result of combinatorial site-specific saturation

No.	OD_{475}
V13D	0.93
S148I	1.22
V301E	0.79
V13D/S148I	1.34
V13D/V301E	1.07
S148I/V301E	1.22
V13D/S148I/V301E	1.59
Control1	0.65
Control2	0.58
Control3	0.60

Table S6. Effect of temperature on triple-mutant MK activity

MK (V13D/S148I/V301E)

Temperature(°C)	Absolute activity (U/mg)			Relative activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
25	96.1	86.1	93	87.1	87.3	89.9	88.1	1.6
30	102.7	90.1	89.5	93.1	91.2	86.6	90.3	3.4
35	110.3	98.6	103.4	100.0	100	100.0	100	0.0
40	96.6	82.0	79.1	87.6	83.1	76.5	82.4	5.6
45	82.8	67.2	70.5	75.1	68.2	68.2	70.5	4.0
50	60.8	61.0	60.3	55.1	61.8	58.1	58.3	3.4
55	47.4	35.9	42.4	43.2	36.4	41.0	39.8	3.4

Table S7. Effect of temperature on wild-type MK activity

	MK							
Temperature(°C)	Absolute activity (U/mg)			Relative activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
25	43.1	41.1	44.2	81.2	84.1	75.9	80.4	4.2
30	48.7	41.7	46.7	91.7	85.3	80.1	85.7	5.8
35	53.1	48.9	58.3	100.0	100.0	100.0	100.0	0.0
40	42.5	35.3	47.8	80.1	72.2	82.0	78.1	5.2
45	36.1	31.8	40.6	68.0	65.2	68.9	67.3	2.1
50	19.5	20.1	21.9	36.8	41.1	37.6	38.5	2.3
55	10.6	8.3	11.0	20.2	16.9	18.9	18.6	1.6

Table S8. Effect of temperature on triple-mutant MK stability

	MK (V13D/S148I/V301E)	
Temperature(°C	Absolute activity	Residual activity (%)

)	(U/mg)							
	#1	#2	#3	#1	#2	#3	mean	SD
25	117.7	112.8	125.7	100.0	99.5	100.0	99.8	0.3
30	116.2	113.4	125.1	98.7	100.0	99.5	99.4	0.7
35	115.7	112.4	124.1	98.3	99.1	98.6	98.7	0.4
40	100.0	98.7	104.1	84.9	87.0	82.8	84.9	2.1
45	77.2	72.7	78.7	65.6	64.1	62.6	64.1	1.5
50	44.3	38.2	44.9	37.6	33.8	35.7	35.7	1.9
55	26.1	28.6	35.4	22.2	25.2	28.2	25.2	3.0

Table S9. Effect of temperature on wild-type MK stability

Temperature(°C)	MK							
	Absolute activity (U/mg)			Residual activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
25	50.1	57.6	45.9	98.1	100.0	100.0	99.3	1.1
30	50.9	57.2	45.2	100.0	99.3	98.5	99.3	0.8
35	50.6	57.0	45.7	99.5	99.1	99.6	100.0	0.3
40	40.5	45.1	35.1	79.6	78.6	76.4	78.2	1.6
45	18.7	23.1	18.3	36.7	40.1	39.9	38.9	1.9
50	13.9	17.6	13.5	27.3	30.5	29.5	29.1	1.6
55	8.7	8.4	6.6	17.0	14.5	14.3	15.3	1.5

Table S10. Effect of pH on triple-mutant MK activity

pH	MK (V13D/S148I/V301E)	
	Absolute activity (U/mg)	Relative activity (%)

	#1	#2	#3	#1	#2	#3	mean	SD
5	17.5	18.2	15.5	17.3	20.1	17.3	18.2	1.6
5.5	33.0	23.2	28.6	33.4	25.6	31.9	30.3	4.1
6	49.8	51.4	41.1	50.5	56.7	45.8	51.0	5.5
6.5	59.7	57.9	61.2	60.5	63.9	68.2	64.2	3.9
7	87.5	77.6	85.8	88.7	85.7	95.6	90.0	5.1
7.5	98.7	90.6	89.7	100.0	100.0	100.0	100.0	0.0
8	91.9	85.9	78.6	93.1	94.8	87.6	91.8	3.8
8.5	65.6	61.0	64.7	66.5	67.3	72.1	68.6	3.0
9	59.7	48.2	46.6	60.5	53.2	52.0	55.2	4.6
9.5	43.2	38.1	39.9	43.8	42.2	44.5	43.4	1.3
10	28.5	33.3	27.4	28.9	36.7	30.5	32.0	4.1

Table S11. Effect of pH on wild-type MK activity

	MK							
pH	Absolute activity (U/mg)			Relative activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
5	5.9	6.0	6.4	14.4	15.1	13.9	14.5	0.6
5.5	8.2	6.6	8.7	20.1	16.7	19.0	18.6	1.7
6	13.4	10.7	14.4	32.7	26.9	31.5	30.4	3.1
6.5	20.0	14.7	17.7	49.0	36.9	38.7	41.5	6.5
7	32.3	31.9	35.6	78.9	80.1	77.8	78.9	1.2
7.5	40.9	39.8	45.7	100.0	100.0	100.0	100.0	0.0
8	36.2	33.2	41.2	88.5	83.4	90.1	87.3	3.5
8.5	28.0	27.7	30.4	68.4	69.5	66.6	68.2	1.5

9	20.5	17.8	25.1	50.1	44.7	54.9	49.9	5.1
9.5	18.2	19.9	20.9	44.5	50.1	45.7	46.8	2.9
10	9.6	11.8	11.7	23.4	29.7	25.6	26.2	3.2

Table S12. Effect of pH on triple-mutant MK stability

MK (V13D/S148I/V301E)								
pH	Absolute activity (U/mg)			Residual activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
5	13.0	16.6	20.8	12.3	16.5	20.1	16.3	3.9
5.5	20.9	20.6	24.1	19.8	20.5	23.2	21.2	1.8
6	42.7	38.9	31.2	40.5	38.6	30.1	36.4	5.5
6.5	60.2	53.8	67.8	57.1	53.4	65.4	58.6	6.1
7	92.6	89.6	93.4	87.9	89.0	90.1	89.0	1.1
7.5	105.4	100.7	103.7	100.0	100.0	100.0	100.0	0.0
8	99.4	98.8	94.6	94.3	98.1	91.2	94.5	3.5
8.5	94.3	84.0	96.3	89.5	83.4	92.9	88.6	4.8
9	71.1	73.2	66.4	67.5	72.7	64.0	68.1	4.4
9.5	48.0	35.8	42.2	45.5	35.6	40.7	40.6	5.0
10	18.7	23.6	21.3	17.7	23.4	20.5	20.5	2.9

Table S13. Effect of pH on wild-type MK stability

MK								
pH	Absolute activity (U/mg)			Residual activity (%)				
	#1	#2	#3	#1	#2	#3	mean	SD
5	6.8	5.3	7.6	13.5	10.9	16.7	13.7	2.9

5.5	10.7	9.2	8.6	21.1	19.0	18.9	19.7	1.2
6	16.9	12.9	16.1	33.4	26.7	35.4	31.8	4.6
6.5	18.8	16.8	16.2	37.1	34.7	35.5	35.8	1.2
7	40.5	36.3	34.4	79.9	75.2	75.4	76.8	2.7
7.5	50.7	48.3	45.6	100.0	100.0	100.0	100.0	0.0
8	45.2	42.7	38.3	89.1	88.5	84.1	87.2	2.7
8.5	43.4	41.7	36.5	85.6	86.3	80.1	84.0	3.4
9	30.7	30.6	28.3	60.5	63.4	62.0	62.0	1.5
9.5	23.2	17.2	17.6	45.8	35.7	38.7	40.1	5.2
10	6.7	7.6	8.8	13.2	15.8	19.4	16.1	3.1

Table S14. The result of cell growth rates of CHL-1 and CHL-2

Time (h)	OD_{600} of CHL-1					OD_{600} of CHL-2				
	#1	#2	#3	mean	SD	#1	#2	#3	mean	SD
0	0	0	0	0.0	0.0	0	0	0	0.0	0.0
15	19	23	21	21.0	2.0	18	19	20	19.0	1.0
20	41	40	43	41.3	1.5	39	44	38	40.3	3.2
25	48	50	45	47.7	2.5	54	52	48	51.3	3.1
30	51	54	51	52.0	1.7	58	65	61	61.3	3.5
35	58	61	59	59.3	1.5	61	62	63	62.0	1.0
40	68	63	64	65.0	2.6	67	70	65	67.3	2.5
45	70	71	67	69.3	2.1	70	71	70	70.3	0.6
50	70	71	70	70.3	0.6	72	71	73	72.0	1.0
55	73	72	75	73.3	1.5	72	74	73	73.0	1.0
60	75	78	74	75.7	2.1	75	75	76	75.3	0.6

65	79	80	78	79.0	1.0	73	73	75	73.7	1.2
70	81	81	80	80.7	0.6	74	72	76	74.0	2.0
75	84	80	79	81.0	2.6	75	76	76	75.7	0.6
80	82	81	83	82.0	1.0	75	74	77	75.3	1.5
85	80	79	83	80.7	2.1	76	77	74	75.7	1.5
90	82	84	83	83.0	1.0	79	78	76	77.7	1.5

Table S15. Lycopene titer during aerobic fed-batch fermentation of CHL-1 and CHL-2

Time (h)	Lycopene titer of CHL-1(mg/L)					Lycopene titer of CHL-2(mg/L)				
	#1	#2	#3	mean	SD	#1	#2	#3	mean	SD
0	0	0	0	0.0	0.0	0	0	0	0	0.0
15	0	0	0	0.0	0.0	0	0	0	0	0.0
20	32.5	37.5	27.5	32.5	5.0	78.5	80.3	81.1	80.1	1.3
25	106.3	109.5	101.3	105.7	4.1	210.3	205.6	207.1	207.2	2.4
30	274.8	275.5	269.8	273.4	3.1	386.6	381.9	394.3	386.6	6.3
35	412.4	422.3	407.4	414.0	7.6	556.4	553.9	560.8	556.4	3.5
40	540.2	544.1	535.2	539.8	4.5	755.8	760.2	751.7	755.8	4.3
45	623.4	637.8	618.4	626.5	10.1	885.9	891.1	890.2	890.2	2.8
50	656.7	665.1	651.7	657.8	6.8	1017.5	1015.7	1020.1	1017.5	2.2
55	691	699.2	686	692.1	6.7	1152.3	1148.7	1144.5	1144.1	3.9
60	714.6	710.6	709.6	711.6	2.6	1224.5	1229.6	1225.5	1224.8	2.7
65	735.6	739.1	730.6	735.1	4.3	1271.4	1270.9	1275.9	1272.3	2.8
70	736	739.8	731	735.6	4.4	1339.7	1338.4	1331.4	1335.1	4.5
75	731.4	734.1	726.4	730.6	3.9	1340.2	1355.8	1351.2	1351	8.0
80	728.4	737.7	723.4	729.8	7.3	1394.5	1390.2	1393.8	1391.6	2.3
85	726.6	728.8	721.6	725.7	3.7	1431.5	1435.2	1438.2	1431	3.4

90	720.8	725.1	715.8	720.6	4.7	1431.2	1435.9	1436.2	1431.2	2.8

Table S16. Lycopene production during aerobic fed-batch fermentation of CHL-1 and CHL-2

Time (h)	Lycopene production of CHL-1 (mg/g DCW)					Lycopene production of CHL-2 (mg/g DCW)				
	#1	#2	#3	mean	SD	#1	#2	#3	mean	SD
0	0	0	0	0.00	0.00	0	0	0	0.00	0.00
15	0.45	0.43	0.41	0.43	0.03	0.35	0.36	0.4	0.37	0.03
20	0.83	0.79	0.81	0.81	0.02	0.73	1.72	1.24	1.23	0.50
25	2.19	1.73	1.25	1.72	0.47	2.7	3.64	3.21	3.18	0.47
30	4.59	4.21	3.65	4.15	0.47	5.66	6.52	6.09	6.09	0.43
35	7.06	6.61	6.03	6.57	0.52	8.72	9.64	9.02	9.13	0.47
40	7.15	6.57	6.11	6.61	0.52	9.48	10.64	10.03	10.05	0.58
45	7.23	6.69	6.25	6.72	0.49	10.74	11.6	11.02	11.12	0.44
50	7.35	6.9	6.29	6.85	0.53	12.06	13.02	12.61	12.56	0.48
55	7.52	7.05	6.51	7.03	0.51	12.96	13.8	13.51	13.42	0.43
60	7.84	7.38	6.83	7.35	0.51	13.54	14.71	14.02	14.09	0.59
65	8.05	7.52	7.01	7.53	0.52	15.49	16.24	15.81	15.85	0.38
70	8.65	8.08	7.69	8.14	0.48	17.2	18.37	17.91	17.83	0.59
75	8.51	8.13	7.71	8.12	0.40	18.62	19.84	19.17	19.21	0.61
80	8.69	8.31	7.75	8.25	0.47	18.86	19.72	19.48	19.35	0.44
85	8.81	8.33	7.81	8.32	0.50	18.9	20.03	19.65	19.53	0.58
90	8.91	7.89	8.15	8.32	0.53	19.34	20.21	19.91	19.83	0.44