Electronic Supplementary Information

Metabolic, redox, and spatial engineering of *Yarrowia lipolytica* for high-level zeaxanthin production

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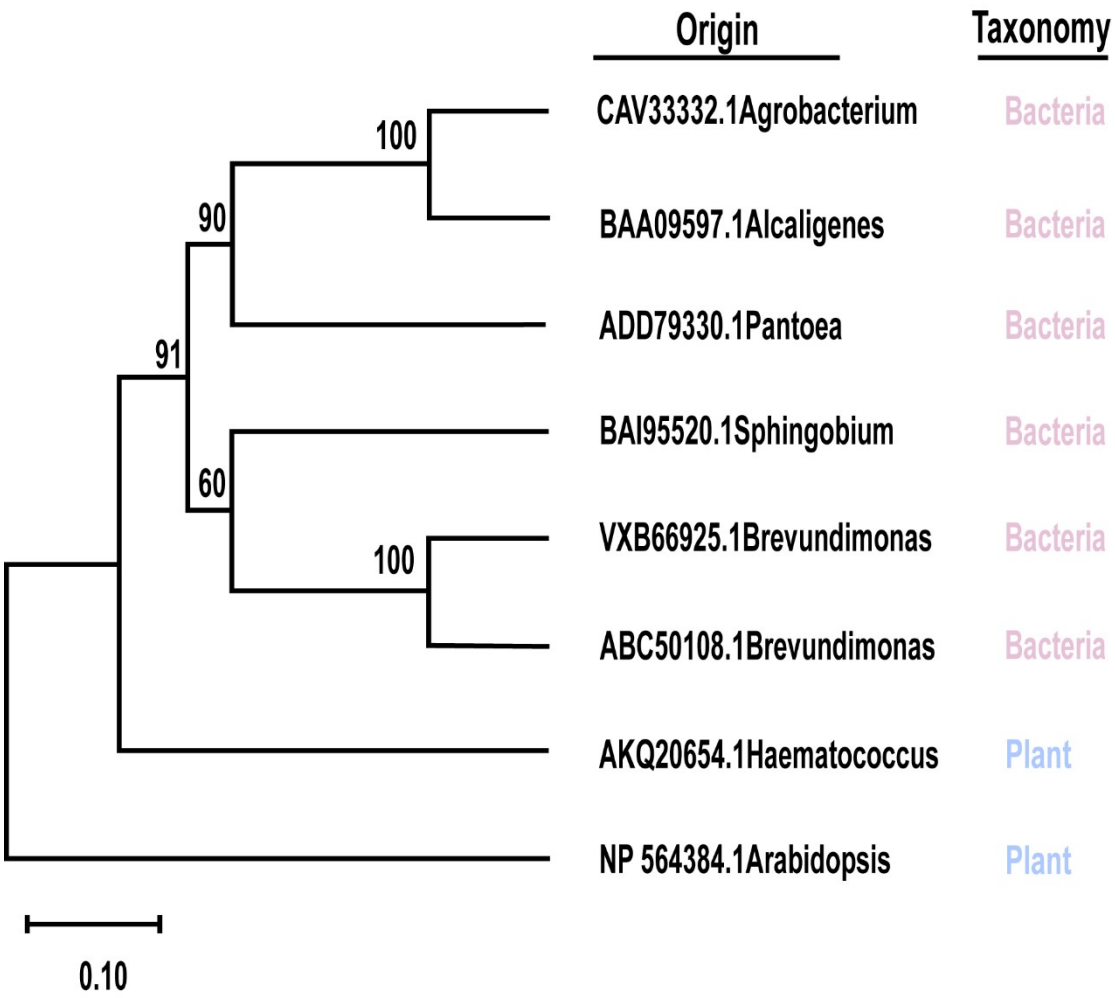

Figure. S1 Evolutionary tree analysis of β-Carotene hydroxylases.

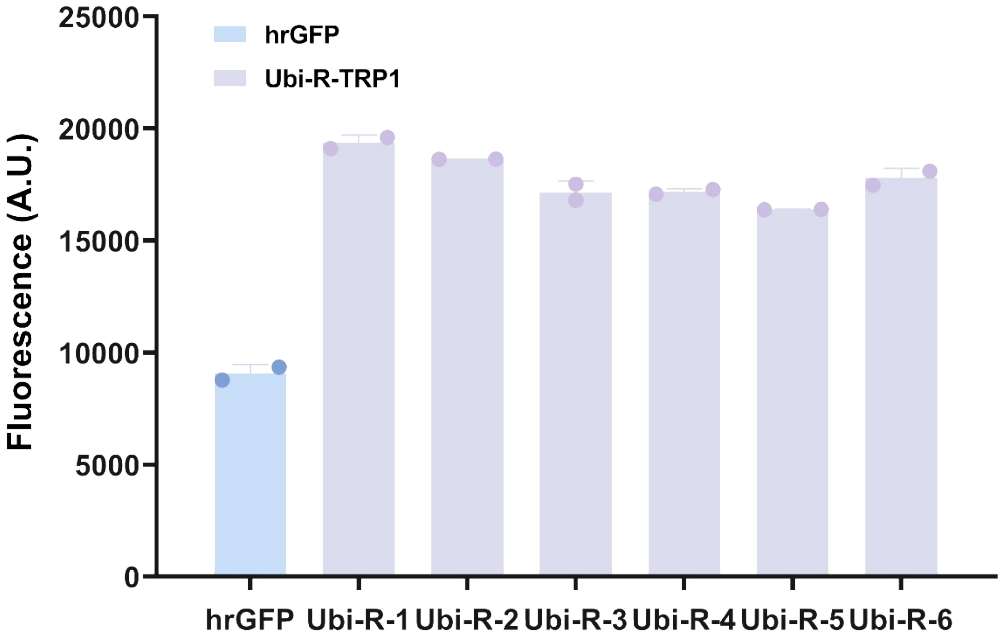

Figure. S2 The fluorescence intensity of the strain with the plasmid containing Ubi-R-TRP1 and hrGFP.

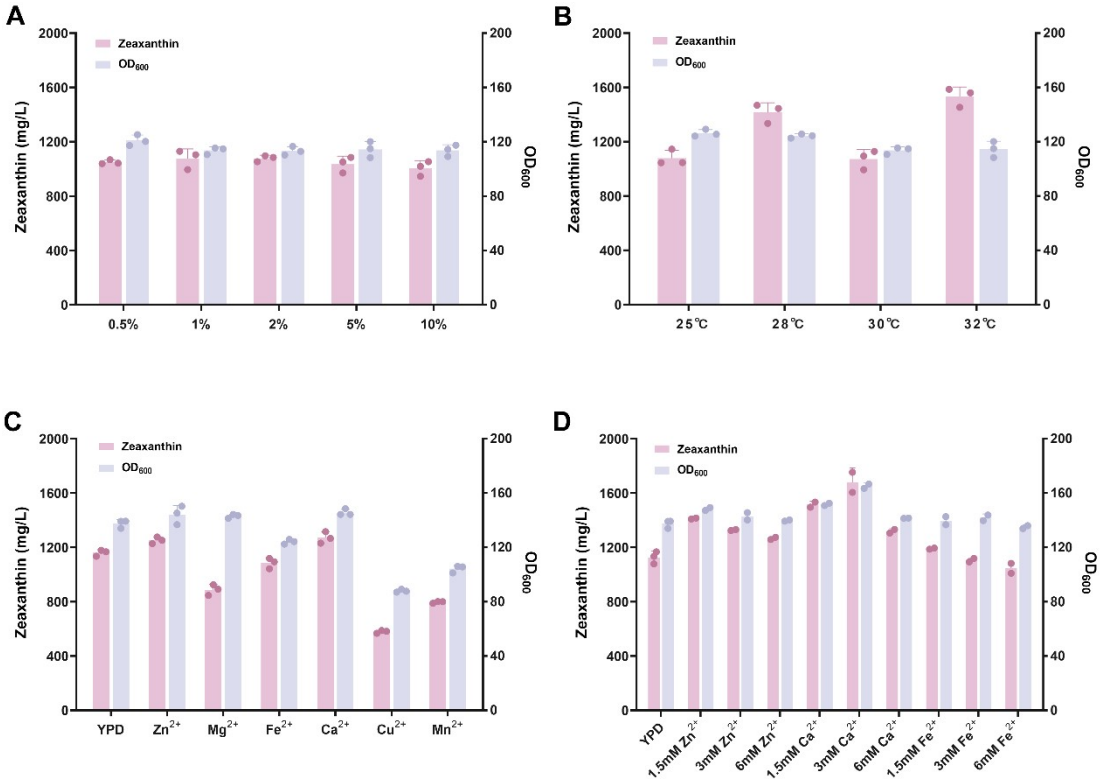

Figure. S3 Zeaxanthin fermentation condition optimization. (A) Zeaxanthin production at different inoculum sizes (0.5%, 1%, 2%, 5%, 10%). (B) Zeaxanthin production at different fermentation temperatures (25 ℃, 28 ℃, 30 ℃, 32 ℃). (C) Zeaxanthin production with various metal ions (Zn^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+} and Mn^{2+}). (D) Zeaxanthin yield in corn at varying metal ion concentrations (1.5 Mm, 3 mM and 6 mM Zn^{2+}; 1.5 Mm, 3 mM and 6 mM Ca^{2+}; 1.5 Mm, 3mM and 6mM Fe^{2+}).

Table S1. The bioproduction cost evaluation of zeaxanthin.

Product	Titer (g/L)	Yield (g/g glucose)	Feedstock	Feedstock price (dollar/ton)	Estimated feedstock cost(dollar/kg)	Market price of zeaxanthin (dollar/kg)
Zeaxanthin	6.9	0.0175	Glucose	500	28.57	270

Table S2. Genes used in this study.

Name	Sources	Reference sequence	Reference
HpGGPPs	*Haematococcus pluvialis*	KX236181	(Huang et al., 2019)
AfGGPPs	*Archaeoglobus fulgidus*	AAD26851	(Wang et al., 1999)
YlGGPPs	*Yarrowia lipolytica*	NC_089381.1	(Kildegaard, 2017)
SaGGPPs	*Sulfolobus acidocaldarius*	D28748.1	(Ohnuma S. et al., 1994)
AaCrtZ	*Agrobacterium aurantiacum*	CAV33332.1	(Misawa et al., 1995)
BsCrtZ	*Brevundimonas sp. G8*	GCF_902506205.1	(Nishida et al., 2005)
HpCrtZ	*Haematococcus pluvialis*	AKQ20654.1	(Linden et al., 2003)
SiCrtZ	*Sphingobium indicum UT26S*	BAI95520.1	(Nagata et al., 2010)
BvCrtZ	*Brevundimonas vesicularis*	ABC50108.1	(Tao et al., 2006)
AsCrtZ	*Alcaligenes sp.*	BAA09597.1	(Misawa et al., 1995)
CYP97A3	*Arabidopsis thaliana*	NP_564384.1	(Theologis et al., 2000)
PaCrtZ	*Pantoea ananatis*	ADD79330.1	(De Maayer et al., 2010)
RFNR1	*Arabidopsis thaliana*	NC_003075.7	(Mayer K, et al., 1999)
FD3	*Arabidopsis thaliana*	NC_003071.7	(Lin X, et al., 1999)
EfMvaE	*Enterococcus faecalis*	GCF_000007785.1	(Matija Hedl et al., 2002)
$EfMvaS^{A110G}$	*Enterococcus faecalis*	AF290092.1	(Matija Hedl et al., 2002)

Table S3. Plasmids used in this study.

Name	Genotype	Reference
JMP-nat-GPD-TEF	Integrative vector harboring the *NAT* marker, *GPD* promoter and *TEF* promoter	(Cui et al., 2023)
pKi1-TRP	Integrative vector harboring the TRP1 selection marker, ut8 promoter, CYC1 terminator,	This study
YLEP-Leu	Integrative vector harboring the Leu selection marker, ut8 promoter, CYC1 terminator,	This study
Pki1-URA	Integrative vector harboring the URA3 selection marker, GPD promoter, tXPR2 terminator, TEF-in promoter, tCYC1 terminator,	This study
pKi1-TRP-HpGGPPs	pKi1-TRP harboring HpGGPPs expression cassette	This study
pKi1-TRP-SaGGPPs	pKi1-TRP harboring SaGGPPs expression cassette	This study
pKi1-TRP-YlGGPPs	pKi1-TRP harboring YlGGPPs expression cassette	This study
pKi1-TRP-AfGGPPs	pKi1-TRP harboring AfGGPPs expression cassette	This study
113-GPD-TEF-URA3	Y. lipolytica integrative vector, GPD promoter with XPR2 terminator, TEF promoter with CYC1 terminator, URA3 selection marker	This study
113-GPD-CarRP-HpGGPPs-URA3	113-GPD-TEF-URA3 vector containing TEFp-HpGGPPs-CYC1t and GPDp-CarRP-XPR2t	This study
113-GPD-CarB-HpGGPPs-URA3	113-GPD-TEF-URA3 vector containing TEFp-HpGGPPs-CYC1t and GPDp-CarB-XPR2t	This study
113-GPD-CarRP-CarB-HpGGPPs- URA3	113-GPD-TEF-URA3 vector containing TEFp-HpGGPPs-CYC1t, GPDp-CarRP-XPR2t and TEFp-CarB-CYC1t	This study
pKi1-TRP-ubi-R-PaCrtZ	pKi1-TRP vector containing ut8p-PaCrtZ-CYC1t	This study
pKi1-URA-FD3	pKi1-URA harboring FD3 expression cassette	This study
pKi1-URA-RFNR1	pKi1-URA harboring RFNR1 expression cassette	This study
pKi1-URA-FD3-RFNR1	pKi1-URA harboring FD3, RFNR1expression cassette	This study
pKi1-LEU-RIDD-EfMvaE-RIDD-EfMvaS-RIAD-A-IDPs	YLEP-Leu vector containing TEFp-EfMvaE-RIDD-CYC1t, GPDp-EfMvaS-RIDD-XPR2t and ut8p-A-IDPs-RIDD-CYC1t	This study

Table S4. Strains used in this study.

Name	Genotype	Reference
PO1f	*MATa, leu2-270, ura3-302, xpr2-322, axp1-2,*	
PO1f△ Trp	*MATa, leu2-270, ura3-302, xpr2-322, axp1-2, ⊿Trp*	This study
Car1	pKi1-TRP harboring HpGGPPs expression cassette	This study
Car11	Car1 with integrated free replicating plasmid pKi1-TRP- HpGGPPs	This study
Car12	Car1 with integrated free replicating plasmid pKi1-TRP- SaGGPPs	This study
Car13	Car1 with integrated free replicating plasmid pKi1-TRP- YlGGPPs	This study
Car14	Car1 with integrated free replicating plasmid pKi1-TRP- AfGGPPs	This study
Car2	Car1 with integrated linearized plasmid 113-GPD-CarRP-HpGGPPs-URA3	This study
Car3	Car1 with integrated linearized plasmid 113-GPD-CarB-HpGGPPs- URA3	This study
Car4	Car1 with integrated linearized plasmid 113-GPD-CarRP-CarB-HpGGPPs-URA3	This study
Car5	Car1 carrying two genomic copies of the linearized plasmid 113-GPD-CarRP-HpGGPPs-URA3 integrated by homologous recombination.	This study
Car6	Car1 carrying two genomic copies of the linearized plasmid 113-GPD-CarRP-CarB-HpGGPPs-URA3 integrated by homologous recombination.	This study
Zea1	Car6 with integrated linearized plasmid pKi1-TRP-ubi-R-PaCrtZ	This study
Zea2	Zea1 with integrated linearized plasmid pKi1-URA-FD3	This study
Zea3	Zea2 with integrated linearized plasmid pKi1-URA-RFNR1	This study
Zea4	Zea2 with integrated linearized plasmid pKi1-URA-FD3-RFNR1	This study
Zea5	Zea4 with integrated linearized plasmid 113-GPD-ScPOS5△17-	This study
Zea6	Zea4 with integrated linearized plasmid 113-GPD-YlPOS5△20	This study
Zea7	Zea4 with integrated linearized plasmid 113-ZWF1-PGL-GND1	This study
Zea8	Zea4 with integrated linearized plasmid 113-ZWF1-PGL-GND1-YlPOS5△17	This study
Zea9	Zea8 with integrated linearized plasmid pKi1-LEU-RIDD-EfMvaE-RIDD-EfMvaS	This study
Zea10	Zea8 with integrated linearized plasmid pKi1-LEU-EfMvaE-EfMvaS	This study
Zea11	Zea8 with integrated linearized plasmid pKi1-LEU-EfMvaE-EfMvaS-RIAD-A-IDPs	This study
Zea12	Zea8 with integrated linearized plasmid pKi1-LEU-RIDD-EfMvaE-RIDD-EfMvaS-RIAD-A-IDPs	This study

Table S5. Main primers used in this study.

Primer	Nucleotide sequence (5′→3′)
ut8-PaCrtZ-F	ATAAGAATCATTCAAAGATTTAAATATGGCTTTCCCGTTGTCCTATAC
tCYC1-PaCrtZ-R	AAGCGTGACATAACTAATTACATGAtcaAGAAAGAGCTGATGACACCTCG
ut8-CYP97A3-F	TGGAGCTCCACCGCGGTGGCGGCCGCGGTACCaaggaagcatgcGGTAC
ut8-HpCHY-F	TGGAGCTCCACCGCGGTGGCGGCCGCGGTACCaaggaagcatgcGGTAC
ut8-BsCrtZ-F	TGGAGCTCCACCGCGGTGGCGGCCGCGGTACCaaggaagcatgcGGTAC
Arg(R)-Trp-R	ATGTCGAAGAGTAGAGAAAGTCCATGCGACCACCTCTCAGTCTCAAC
Met (M)-Trp-R	ATGTCGAAGAGTAGAGAAAGTCCATCATACCACCTCTCAGTCTCAACACC
Gln(Q)-Trp-R	ATGTCGAAGAGTAGAGAAAGTCCATCTGACCACCTCTCAGTCTCAACACC
Glu(E)-Trp-R	ATGTCGAAGAGTAGAGAAAGTCCATCTCACCACCTCTCAGTCTCAACACC
GAP-PaCrtZ1-F	tcgccaagaaggatgcttccgcttagATGCTGTGGATCTGGAACGCTC
GAP-PaCrtZ1-R	TGCATGCCTGCAGGTCGACTCTAGAtcaCTTGCCGGAAGCAGGCTC
pTEF-trFD3-F	tgcagtactaaccgcagATTTAAATATGTCTTCCCTAATTCTCTCTCTCTCG
tCYC1-trFD3-R	TAAGCGTGACATAACTAATTACATGACCTATTTTAACATGTTACTGAATGAT AATGATG
trRFNR1-seqF	AGAGTCTGAAATAACAATTGGTCTTCG
trFD3-seqR	AGATGATTAGCATCTGAATAGCAACG
ylPOS5Δ20-pGPD-F	AATTAAACACACATCAACAGTCGACATGTCCACATGGATTCGGCTTC
scPOS5Δ17-pGPD-F	AATTAAACACACATCAACAGTCGACATGAGTACGTTGGATTCACATTCCCT
EfMvaS-RIDD-F	ACTGTGCGCTCTTACAGAAATGGCGGTGGTGGTTCCCTGCGAGAATGCGAGC TGTACG
RIDD-EfMvaS-R	TCGCATTCTCGCAGGGAACCACCACCGCCATTTCTGTAAGAGCGCACAGTGT TGTTG
pGPD-RIAD-A-IDPs-RIDD-F	ggagcctatggaaaaacgcCATATGGGTTGAAATGAATCGGCCGAC
tCYC1-RIAD-A-IDPs-RIDD-R	TGGCATCCCTAAATTTGATGAAAGCGCTTGCAAATTAAAGCCTTCGAGCGTC
ut8-mCherry-F	AAGAATCATTCAAAGGTTCGAAATGgtgagcaagggcgaggaggataac
RIDD-mCherry-R	TCGCATTCTCGCAGAGAACCACCGCCcttgtacagctcgtccatgccgc
pTEF-RIAD-A-IDPs-F	agcctatggaaaaacgcCATATGAAAgagaccgggttggcggc
CYC1-RIAD-A-IDPs-R	TGGCATCCCTAAATTTGATGAAAGCGCGCAAATTAAAGCCTTCGAGCGTCC

Table S6. Comparison the E-factor of our process with representative chemical synthesis.

Process Type	Strategies	Carbon Efficiency (%)	E-factor (kg waste/kg product)	Key Green Chemistry Features
Chemical Synthesis	**C_{15}–C_{10}–C_{15} double Wittig construction of the C_{40} carotenoid backbone**	**4.2**	**63**	**Fossil-based feedstocks; multiple steps, requiring solvents and hazardous reagents**
Biosynthesis	**This study (Zea12 strain)**	**3.6**	**54**	**Whole-cell aqueous biocatalysis; renewable feedstock; mild operation**