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Engineering *Yarrowia lipolytica* for the production of β -carotene by carbon and redox rebalancing

Hojun Lee¹, Jinwoo Song² and Sang Woo Seo^{1,2,3,4,5*}

Abstract

Background β -Carotene is a natural product that has garnered significant commercial interest. Considerable efforts have been made to meet such demand through the metabolic engineering of microorganisms, yet there is still potential for improvement. In this study, engineering approaches including carbon and redox rebalancing were used to maximize β -carotene production in *Yarrowia lipolytica*.

Results The initial production level was increased by iterative overexpression of pathway genes with lycopene inhibition removal. For further improvement, two approaches that redirect the central carbon pathway were evaluated to increase NADPH regeneration and reduce ATP expenditure. Pushing flux through the pentose phosphate pathway and introducing NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase were found to be more effective than the phosphoketolase-phosphotransacetylase (PK-PTA) pathway. Furthermore, flux to the lipid biosynthesis pathway was moderately increased to better accommodate the increased β -carotene pool, resulting in the production level of 809.2 mg/L.

Conclusions The *Y. lipolytica*-based β -carotene production chassis was successfully developed through iterative overexpression of multiple pathways, central carbon pathway engineering and lipid pathway flux adjustment. The approach presented here provides insights into future endeavors to improve microbial terpenoid production capability.

Keywords β -carotene, *Yarrowia lipolytica*, Central carbon pathway engineering

Background

β -Carotene is one of the commonly known carotenoids, the hydrophobic organic pigments consisting of 40-carbon atoms. It has antioxidant [1], anti-inflammatory activities [2] and a natural orange color, which makes it a desirable food-grade additive and colorant. Chemical synthesis and natural extraction are possible but with limitations, and much research on microbial metabolic engineering is ongoing to meet the industrial production demand. Much efforts to increase β -carotene production began with natural microbial producers such as *Rhodotorula glutinis* [3] and recombinant producers based on general chassis microorganisms such as *E. coli* [4]. Not long after, academic attention shifted to microbial chassis

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that are both genetically tractable and fat-tolerant, one of which is *Yarrowia lipolytica* [5]. *Y. lipolytica* is a natural oleaginous yeast with substantial lipid accumulation capabilities [6], which has been considered an essential prerequisite for high-level carotenoid production due to its lipid-associated storage in vivo.

The first β -carotene metabolic engineering attempt in *Y. lipolytica* [7], which began with the overexpression of a single gene *ggs1* yielded 2.22 mg β -carotene/g DCW. Since then, the research in this field has become more comprehensive, with new strategies beyond pathway flux engineering. For example, high cell density and subsequently increased β -carotene titer of 2.01 g/L were achieved through dissolved-oxygen feedback fed-batch fermentation [8], and transition to metabolically less active mycelial morphology was prevented to increase β -carotene production up to 7.6 g/L in fed-batch fermentation [9]. Many of the strategies implemented for other terpenoids and lipids can also be effective. The synthetic two-step isopentenol utilization pathway (IUP) developed and validated for lycopene [10] has significantly increased β -carotene titer during a follow-up study to yield final strain production of 39.5 g/L in fed-batch fermentation [11]. Thus, in order to advance microbial β -carotene production any further, it is equally important to integrate past insights and investigate unconventional strategies.

In this study, metabolic pathway engineering, protein engineering and morphological engineering strategies primarily from past literature were applied to improve β -carotene production capability. In the process, a previously discovered mutation that eliminates substrate inhibition also reduced expression level, but this was restored through further engineering. Also, for the first time within the field of carotenoid production from *Y. lipolytica*, the central carbon pathway was engineered to increase the β -carotene production level. Increasing NADPH and ATP supply is a valid strategy in model microbes, yet it has not been proven for the production of carotenoids in *Y. lipolytica*. Lastly, the flux balancing of the lipid biosynthesis pathway further promotes β -carotene synthesis.

Methods

Culture conditions and mediums

Y. lipolytica strains were grown at 30°C in YPD or YSC-LEU media. YPD medium containing 10 g/L yeast extract (Conda), 20 g/L peptone (BD Difco), and 20 g/L glucose (Alfa Aesar) was used for carotenoid production from genome-integrated strains. YSC-LEU media containing 6.7 g/L Yeast nitrogen base without amino acids (Sigma-Aldrich), 0.69 g/L CSM-LEU (MP Biomedicals), 20 g/L glucose (Alfa Aesar) was used during *Y. lipolytica*

transformant selection in general. For glycolytic gene knockout strains, YSC-LEU-EtOH-Glycerol was used for transformant selection instead [12, 13], which contained 2% ethanol (w/v) and 3% glycerol (w/v) in addition to the standard YSC-LEU components. For vector construction, *Escherichia coli* Mach1™ was grown at 37°C in Luria-Broth (LB) liquid medium and solid medium plate supplemented with 20 g/L agar. LB was supplemented with ampicillin at 75 mg/L when needed.

Construction of strains

Y. lipolytica Po1g *ku70* Δ strain (MATa, *leu2*-270, *ura3*-302::URA3, *xpr2*-332, *xpr2*-332, *xpr2*-332) was used as base strain for this study. All other strains were derived by transformation of Cas9-sgRNA expression plasmid and XmaI-linearized donor template plasmid consisting of ~1-kb homology arms flanking expression cassettes. The one-step lithium-acetate transformation method [14] was used, with a minor difference in spreading transformation cocktail in YSC-LEU instead. After colony PCR screening, the LEU2 marker containing Cas9-sgRNA expression plasmid was cured by overnight culture in YPD. The culture was streaked and incubated until single colony formation, which was patched onto YPD and YSC-LEU to confirm the absence of the plasmid. The actual sequence of the integration locus was verified by Sanger sequencing of PCR amplicons derived from genomic DNA (gDNA), which was prepared using Exgene Cell SV kit (GeneAll, Seoul, Korea) and Zymolyase (Zymo Research, Irvine, CA, USA). The strains constructed and used in this study are listed in Table S1.

Construction of plasmids

The cas9-sgRNA expression plasmid pCRISPRyl-AXP was constructed by the ligation of AvrII-digested fragment obtained from pCRISPRyl [15] and NdeI-digested fragment obtained from PCR amplification using AvrII-AXPsgRNA-FR2-F and NdeI-FR2-R. The rest of the Cas9-sgRNA expression plasmids were constructed by Gibson assembly of a single-strand oligo consisting of 20-bp sgRNA and AvrII-digested pCRISPRyl using Gibson Assembly HiFi master mix (Codex DNA). The plasmids used in this study are listed in Table S2, and the primers used to clone them are listed in Table S3. The sgRNA targeting sequences for all the integration or deletion loci are listed in Table S4.

The coding sequences of CarP (AJ250827.1) and CarB (AJ238028.1) from *Mucor circinelloides* were codon optimized using OPTIMIZER [16] for *Y. lipolytica* and synthesized by Integrated DNA Technologies (IDT). The coding sequences of GGPPxd (DQ016502.1) from *Xanthophyllomyces dendrorhous*, GapC (AF043386.1), GapN (AAK81579.1) and PK (NP_347971.1) from *Clostridium*

acetobutylicum, PTA (AFK86750.1) from *Thermoanaerobacterium saccharolyticum* were codon optimized using IDT codon optimization tool for *Y. lipolytica* and synthesized by Twist Bioscience. The codon-optimized heterologous gene sequences used in this study are listed in Table S5. All other genes used in this study were amplified from gDNA of *Y. lipolytica*.

The donor template plasmids, in general, were constructed using the gibbon assembly method. AmpR-pUC, the fragment consisting of an Ampicillin resistant marker and bacterial origin, was PCR amplified from pHR-AXP-hrGFP using AmpR-pUC_F and AmpR-pUC-R was used in all plasmids for cloning in *E. coli*. In order to construct pDonor-AXP-carRP-carB, the upper homology AXPup and the lower homology AXPdw were PCR amplified using NB-AXPup-F, NB-AXPup-R and NB_AXPdw-F, NB_AXPdw-R. The following fragments were additionally prepared by PCR amplification: P_{TEFin} by SalI-yITeFp-F and TEFin-R4; *carRP* by TEFin-CarRP-F and Terminator-CarRP-R2; T_{CYC1} in reverse orientation by RV and ClaI-NheI-terminator-R; P_{ILV5} by AvrII-ILV5p-F and SpeI-ILV5p-R; *carB* by ILV5p-SpeI-carB-F and PEX16t-carB-R; T_{PEX16} by PEX16t-F and Bl-PEX16t-R. P_{TEFin}-carRP-T_{CYC1(rev)} and P_{ILV5}-carB-T_{PEX16} were assembled through overlap-extension PCR. NheI-digested P_{TEFin}-carRP-T_{CYC1(rev)} and AvrII-digested P_{ILV5}-carB-T_{PEX16} were ligated, then PCR amplified using SalI-yITeFp-F and Bl-PEX16t-R. After gibbon assembly of AXPup, AXPdw, AmpR-pUC, and P_{TEFin}-carRP-T_{CYC1(rev)}-P_{ILV5}-carB-T_{PEX16}, it was PCR amplified using lower_homology-F and Bl-PEX16t-R, followed by T4 polynucleotide kinase reaction and ligation. For pDonor-AXP-carRP-hrGFP construction, the following fragments were additionally prepared by PCR amplification: P_{TEFin}-carRP by SalI-yITeFin-F and carRP-R; hrGFP by carRP-linker-hrGFP-F and CYC1t-hrGFP-R; T_{CYC1(rev)} by RV and CYC1t-R; AXPdw2 by CYC1t-AXPdw-F and NB-AXPdw-R. These fragments, along with AXPup and AmpR-pUC described above, were assembled through gibbon assembly method. For pDonor-AXP-carRPy27r-hrGFP, site-directed mutagenesis was performed using SDM_Y27R-F5 and SDM_Y27R-R5 to apply Y27R mutation. pDonor-AXP-carRPy27r(AGA)-hrGFP was constructed by site-directed mutagenesis using Bl-CarRP-27-F and Bl-CarRP-27-R. pDonor-AXP-hrGFP was cloned by first PCR amplification of hrGFP using TEFin-hrGFP-F and CYC1t-hrGFP-R, followed by assembly with AXPup, P_{TEFin}, T_{CYC1(rev)}, and AXPdw2 through the gibbon assembly method.

For A08 locus integration, the upper homology A08up and the lower homology A08dw were PCR amplified using NB-A08up-F2, NB-A08up-R and NB-A08dw-F, NB-A08dw-R2. For pDonor-A08-HMG1-GGS1

preparation, the following fragments were additionally prepared by PCR amplification: P_{GPM1} by GPM1p-F and GPM1p(full)-R; *hmg1* by GPM1p(full)-HMG1-F and XPR2t-HMG1-R; T_{XPR2} by XPR2t-F and EXP1p-Bsu36I-XPR2t-R; P_{EXP1} by XPR2t-Bsu36I-EXP1p-F and EXP1p-R; *ggs1* by EXP1p-GGS1-F and LIP2t-GGS1-R; T_{LIP2} by LIP2t-F and LIP2t-R. These fragments were assembled through gibbon assembly method. pDonor-A08-HMG1-GGPPxd was constructed in a similar manner, with a difference in using codon-optimized *ggppxd* instead of *ggs1*.

For XDH locus integration, the upper homology XDHup and the lower homology XDHdw were PCR amplified using GA-XDHup-F, NB-XDHup-R2 and NB-XDHdw-F2, GA-XDHdw-R. For pDonor-XDH-HMG1-GGPPxd preparation, the P_{GPM1}-hmg1-T_{XPR2}-P_{EXP1}-ggppxd-T_{LIP2} fragment was obtained from XbaI and AflII enzyme digestion of pDonor-A08-HMG1-GGPPxd. These fragments were assembled through gibbon assembly method.

For MHY1 locus integration, the upper homology MHY1up and the lower homology MHY1dw were PCR amplified using NB-MHY1up-F, NB-MHY1up-R2 and MHY1_dw-F, NB-MHY1dw-R. For pDonor-MHY1-carRPy27r-carB preparation, site-directed mutagenesis was performed on pDonor-AXP-carRP-carB using SDM_Y27R-F5 and SDM_Y27R-R5 to apply Y27R mutation, followed by SalI and NheI enzyme digestion. These fragments were assembled through gibbon assembly method. For MHY1 locus deletion, the lower homology MHY1up2 was additionally PCR amplified using NB-MHY1up-F and NB-MHY1up-R3, followed by gibbon assembly with MHY1dw and AmpR-pUC.

For F1 locus integration, the upper homology F1up and the lower homology F1dw were PCR amplified using GA-F1up-F, GA-F1up-R and GA-F1dw-F, GA-F1dw-R. For pDonor-F1-carRPy27r-carB construction, fragment P_{TEFin}-CarRP^{Y27R}-T_{CYC1(rev)}-P_{ILV5}-CarB-T_{PEX16} was PCR amplified from pDonor-MHY1-carRPy27r-carB using TEFin-F3 and PEX16t-R. These fragments were assembled through gibbon assembly method.

For A1 locus integration, the upper homology A1up and the lower homology A1dw were PCR amplified using GA-A1up-F, GA-A1up-R2 and GA-A1dw-F2, GA-A1dw-R2. For pDonor-A1-ERG20-ERG12 preparation, *erg20* and *erg12* were PCR amplified using GA-ERG20-F, GA-ERG20-R and GA-ERG12-F, GA-ERG12-R. These fragments, along with P_{GPM1}, T_{XPR2}, P_{EXP1}, and T_{LIP2} described above, were assembled through gibbon assembly method. pDonor-LEU2-ZWF1-GND1 was prepared using gibbon assembly after PCR amplification of the following fragments: LEU2up by GA-LEU2up-F and GA-LEU2up-R; LEU2dw by GA-LEU2dw-F and GA-LEU2dw-R; P_{TEFin} by TEFin-F3 and TEFin-R4; *zwf1* by GA-ZWF1-F and GA-ZWF1-R;

T_{LIP2} by LIP2t-F and LIP2t-R; P_{EXP1} by GA-EXP1p-F2 and EXP1p-R; *gnd1* by GA-GND1-F and GA-GND1-R; T_{XPR2} by XPR2t-F and XPR2t-R. For pDonor-A1-ZWF-GND1 construction, A1up-AmpR-pUC-A1dw fragment was PCR amplified from pDonor-A1-ERG20-ERG12 using AscI-A1up-R and AflIII-A1dw-F, followed by AscI and AflIII enzyme digestion. It was ligated with P_{TEFin} -ZWF1- T_{LIP2} - P_{EXP1} -GND1- T_{XPR2} obtained from AscI and AflIII digested pDonor-LEU2-ZWF1-GND1.

For E2 locus integration, the upper homology E2up and the lower homology E2dw were PCR amplified using GA-E2up-F, GA-E2up-R, and GA-E2dw-F, GA-E2dw-R. For pDonor-E2-TKL1-TAL1 preparation, *tal1* was PCR amplified using XbaI-TAL1-F and SbfI-TAL1-R and was inserted into pDonor-LEU2-ZWF1-GND1 after XbaI and SbfI enzyme digestion. The following fragments were additionally prepared by PCR amplification: P_{TEFin} by TEFin-F3 and TEFin-R4; *tkl1* by GA-TKL1-F and GA-TKL1-R; T_{LIP2} by LIP2t-F and LIP2t-R; P_{EXP1} -TAL1- T_{XPR2} by GA-EXP1p-F2 and XPR2t-R. These fragments, along with E2up and E2dw described above, were assembled through gibbon assembly method. For pDonor-E2-TKL1-TAL1-caGapC construction, the following fragments were PCR amplified: P_{GPD} by GA-GPDp-F2 and GPDp-R; *gapC* by GA-caGapC-F and GA-caGapC-R; T_{MIG1} by MIG1t-F and GA-MIG1t-R2. These fragments, along with BamHI-cut pDonor-E2-TKL1-TAL1, were assembled through the gibbon assembly method. pDonor-E2-TKL1-TAL1-caGapN was cloned in a similar fashion, with a difference in not involving amplification of caGapN.

For pDonor-GPD-caGapC construction, the upper homology THD1p and the lower homology GPDdw were PCR amplified using GA-GPDp-F3, GPDp-R and GPDdw-F, GA-GPDdw-R. Fragment *GapC* was PCR amplified using GA-caGapC-F and GA-caGapC-R2. These fragments were assembled through gibbon assembly method. For pDonor-GPD-caGapN, *gapN* was PCR amplified using GPDp-F2 and NheI-caGapN-R, then digested with SpeI and NheI. The fragment was ligated to the same restriction enzymes-digested pDonor-GPD-caGapC.

For pDonor-PFKdel, the upper homology PFKup was PCR amplified using GA-PFKup-F and GA-PFKup-R, and the lower homology PFKdw using PFKdw-F and GA-PFKdw-R from *Y. lipolytica* gDNA. Then gibbon assembly of PFKup, PFKdw and AmpR-pUC was performed.

For D17 locus integration, the upper homology D17up and the lower homology D17dw were PCR amplified using NB-D17up-F2, NB-D17up-R3 and NB-D17dw-F2, NB-D17dw-R2. pDonor-D17-ScCAT2 was constructed by gibbon assembly of D17up, D17dw, P_{EXP1} , ScCAT2, and T_{XPR2} . For pDonor-D17-PK-PTA construction, the

pta was cloned into pDonor-D17-ScCAT2 after Sall and SbfI digestion to yield pDonor-D17-PTA. The following fragments were additionally PCR amplified: P_{TEFin} by D17-TEFin-F2 and TEFin-R4; *pk* by TEFin-F2 and caXPK2-R; T_{LIP2} by LIP2t-F and hg-check-14. These fragments were assembled with AatII digested pDonor-D17-PTA through the gibbon assembly method.

For P_{DGA1} locus integration, the upper homologies DGA1pup and DGA1pup2 were PCR amplified using GA-DGA1pup-F, GA-DGA1pup-R and GA-DGA1pup-F, GA-DGA1pup-R2. The lower homologies DGA1pdw and DGA1pdw2 were PCR amplified using GA-DGA1pdw-F, GA-DGA1pdw-R and GA-DGA1pdw-F2, GA-DGA1pdw-R. For pDonor-DGA1p-TEFin construction, P_{TEFin} was PCR amplified using TEFin-F3 and TEFin-R4 to be assembled with DGA1pup, DGA1pdw, AmpR-pUC through the gibbon assembly method. pDonor-DGA1p-FBA1p was constructed in a similar fashion, with a difference in using DGA1pup2, DGA1pdw2 and P_{FBA1} , which was PCR amplified using FBA1p-F and FBA1p-R. For pDonor-AXP-hrGFP-FBA1p construction, the fragment PCR amplified from pDonor-AXP-hrGFP using AatII-hrGFP-F and SpeI-AXPup-R, and the fragment PCR amplified from gDNA using SpeI-FBA1p-F and AatII-FBA1p-R were SpeI- and AatII-digested and ligated. pDonor-AXP-hrGFP-Dga1p was constructed in a similar fashion, with a difference in the second fragment PCR amplified using SpeI-Dga1p-F and AatII-Dga1p-R.

Quantification of carotenoids

Seed culture was inoculated with fresh colonies in triplicate, and was cultivated overnight. If needed, it was diluted into another round of seed culture to reach mid-exponential phase. The culture in exponential phase was diluted into 12.5 mL YPD in 125 mL baffled flask and cultivated at 30 °C with shaking 250 rpm until 48 h. Carotenoids were extracted from harvested cells as previously described [17]. The standards of lycopene and β -carotene were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). The analytical standards were prepared using the same sample extraction methods as the samples, with 0.1% BHT (w/v) addition. The standards and extracted carotenoids were analyzed using HPLC equipped with an InfinityLab Poroshell 120 EC-C18 column (150 mm $\times$ 4.6 mm, 4 μ m, Agilent) and a VWD detector. Signals were detected using conditions described previously [18].

Fluorescence assay

Seed cultures of hrGFP-expressing strains were diluted to 5 mL YPD in test tubes (30 mm $\times$ 200 mm, 100 mL) in triplicate and cultured for 24 or 48 h. 1 mL of each broth was centrifuged, washed and resuspended in PBS. 200 μ L of the resultant sample was then applied to a 96-well

plate, which was analyzed by a HIDEX sense microplate reader (HIDEX, Turku, Finland) under an excitation-emission filter of 485–535 nm to quantify fluorescence.

RNA extraction and RT-qPCR

Freshly streaked single colonies were inoculated to 3 mL YPD in triplicate and were cultured overnight. The seed cultures were diluted to 12.5 mL YPD in a 125 mL baffled flask at initial $OD_{600} \sim 0.1$, then cultured for 15-h upon which OD_{600} reached 9–12. 50 μ L of the cultured cell were harvested by 1000 $\times$ g, 4 $^{\circ}$ C, 5-min centrifugation, followed by cell lysis using zymolyase and RNA extraction using RNeasy Mini Plus kit (Qiagen). The RNA integrity was checked by using an RNA Pico Sensitivity assay (PerkinElmer) with LabChip GX Touch prior to quantitative reverse transcription polymerase chain reaction (RT-qPCR). After confirmation of intact high-quality RNA samples, RT-qPCR was performed using the Luna Universal One-Step RT-qPCR kit (New England Biolabs, Ipswich, MA, USA). In order to specifically quantitate processed (i.e., spliced) mRNA, forward primers were designed to span the first and second exons of CDS.

Results and discussions

Construction of the synthetic β -carotene pathway

The typical β -carotene biosynthesis pathway construction in yeast involves the conversion of acetyl coenzyme A (acetyl-CoA) to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through mevalonate pathway, followed by consecutive condensation of such 5-carbon molecules into 20-carbon geranylgeranyl

diphosphate (GGPP) via prenyl phosphate metabolism. This is then converted into carotenoids through the synthetic pathway (Fig. 1A). Previous studies on microbial β -carotene production frequently utilized bifunctional phytoene synthase/lycopene cyclase (CarRP) and phytoene dehydrogenase (CarB) from *Mucor circinelloides* [17, 19, 20]. These enzymes are currently the best-performing homologs in *Y. lipolytica*, as they have shown better production capabilities than an alternative enzyme pair CrtI-CrtYB from *X. dendrorhous* [7, 11]. As such, codon-optimized genes encoding the enzymes were expressed in the Po1g *ku70 Δ* strain for the initial β -carotene production. The resulting YB1 strain produced β -carotene, but with a minuscule titer of 6.9 ± 0.1 mg/L (Fig. 1B). To further increase the β -carotene level, two enzymes upstream of the synthetic β -carotene pathway, namely HMGR and GGPPS, were overexpressed (YB2). Next, usage of the GGPPS enzyme from *X. dendrorhous* (i.e., GGPPxd) was considered, as it has higher catalytic productivity than the endogenous homolog [11]. A further increase in production titer was achieved by the introduction of a single copy (YB3) of *ggppxd* or a double copy, including *hmg1* (YB4). The highest β -carotene titer was 493.9 ± 16.4 mg/L with 28.7 ± 16.5 mg/L lycopene.

Lycopene accumulation removal to obtain high-purity β -carotene

Time course analysis of YB4 showed constant lycopene accumulation to ~ 40 mg/L until the end of the 96-h culture, inferring a bottleneck at the lycopene cyclization step (Fig. S1). Despite a relatively low amount, the

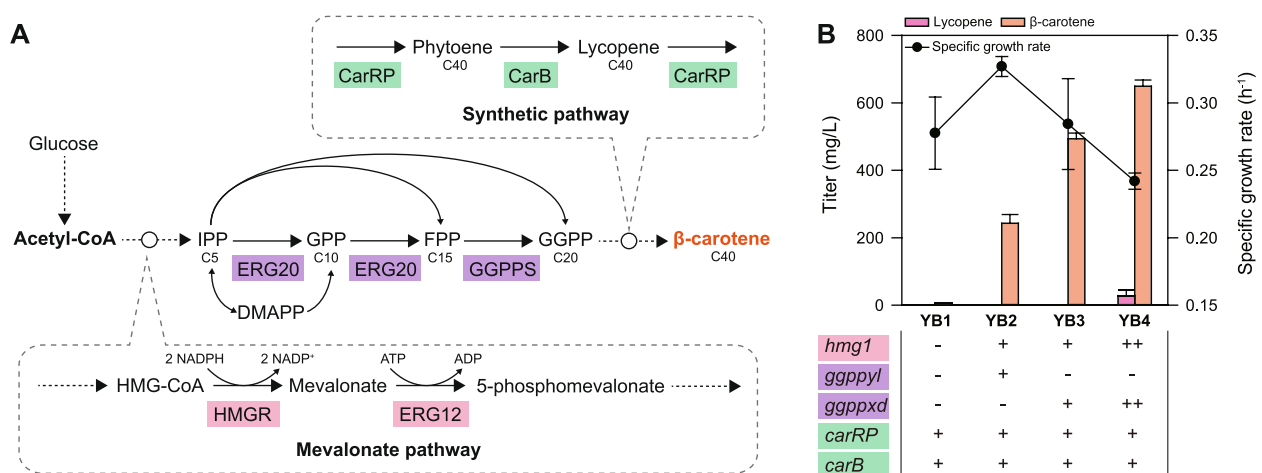


Fig. 1 Construction of the base strain for β -carotene production. **A** Scheme illustrating the engineered metabolic nodes of β -carotene production, including synthetic and mevalonate pathways. Dashed arrows represent multiple metabolic reactions in between. CarRP, phytoene synthase/lycopene cyclase; CarB, phytoene dehydrogenase; ERG20, farnesyl pyrophosphate synthetase; GGPPS, geranyl geranyl diphosphate synthase; HMGR, hydroxymethylglutaryl-CoA reductase; ERG12, mevalonate kinase. **B** Carotenoid production and specific growth rate of derivative strains. Each plus sign represents a single round of integration of corresponding gene expression cassettes into the genome. Error bars represent the standard deviations of three biological replicates

lycopene accumulation problem was addressed antecedently before further engineering. The phenomenon occurs due to substrate inhibition of the CarRP enzyme, where a high concentration of lycopene reduces enzyme activity. In a previous study, the mutation Y27R^{TACCGA} that eliminates the substrate inhibition was identified in the *carRP* gene through engineering guided by structural and evolutionary information [11]. The same mutation was applied to the YB4 strain using the CRISPR-Cas9 system to construct the YB5 strain (Fig. 2A). The β -carotene titer of the YB5 strain surprisingly decreased to 194.0 ± 3.6 mg/L (Fig. S2).

In order to confirm whether this unexpected phenomenon is genuinely based on expression level change, the strain expressing CarRP^{Y27R} mutant-hrGFP fusion protein was constructed (YB7). Its fluorescence was compared to that of the WT-hrGFP fusion protein-expressing strain YB6 (Fig. 2B). Fluorescence normalized by OD₆₀₀ of the mutant strain was nearly a third of the WT strain, inferring decrease of its expression level. One possible reason is codon-specific RNA secondary structure formation or wobble decoding, which may affect translation efficiency [21]. However, this phenomenon doesn't seem to be codon-specific because the CGA codon is the most frequently used arginine codon in *Y. lipolytica* and the AGA codon in YB8 strain also resulted in a similar level of diminished expression level. The reduced expression in the Y27R mutant may stem from increased RNA secondary structure stability, which impairs translation efficiency (Fig. S3). The unique sequence context of the 5'

end of mRNA, consisting of promoter, codon-optimized CDS, and the additional sequence in between, may have created a synergistic effect with Y27R mutation in the formation of structure within mRNA transcript.

To restore β -carotene titer, second copies of WT *carRP* and *carB* were integrated into the *mhy1* locus (YB11). Such integration locus was chosen since *mhy1* deletion is known to not only prevent hyphae formation but also significantly promote β -carotene titer [9, 22]. Indeed, the production level was nearly as much as YB4 without lycopene accumulation (Fig. 2C). This increase was solely due to the introduction of additional copies, as *mhy1* deletion alone did not increase β -carotene titer despite an increase in the specific growth rate (YB10). Another copy of mutant *carRP* and *carB* integration, resulting in YB12, was marginally effective, indicating flux through the carotenoid synthesis pathway has saturated. The strain YB13 with ERG20 and ERG12 overexpression, which were previously reported to increase production level of various terpenoids [23–25], had no significant change in the titer. Thus, the mevalonate pathway also seemed to be no longer limiting, which called for a different approach to increase β -carotene production further.

Central carbon redistribution to enhance NADPH and ATP supply

Flux through the mevalonate pathway consumes two mols of NADPH and three mols of ATP to synthesize one mol of isopentenyl diphosphate (IPP), of which eight mols are required to synthesize one mol of β -carotene.

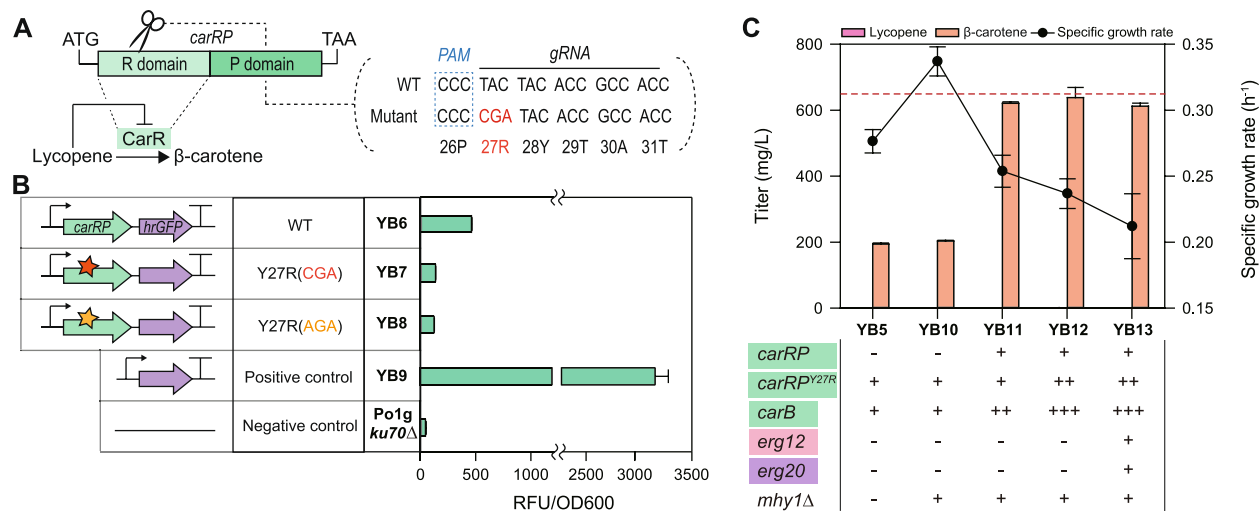


Fig. 2 Elimination of lycopene inhibition and recovery of production level. **A** The CRISPR-cas9 system by which Y27R mutation was introduced into the YB4 strain. **B** Normalized fluorescence intensity of *carRP*-hrGFP fusion protein expressing strains. The star, with its distinct colors, indicates different codons for the Y27R mutation. The double tilde signs represent the discretization of the x-axis for clear comparison between weak fluorescent strains. **C** Carotenoid production and specific growth rate of derivative strains. Each plus sign represents a single round of integration of corresponding gene expression cassettes into the genome or deletion of complete CDS from the genome. Error bars represent the standard deviations of three biological replicates. The red dashed line indicates the β -carotene titer (mg/L) of YB4 from Fig. 1B

Therefore, it is reasonable to presume that regeneration or reduced expenditure of the cofactors may increase carotenoid production. Previous studies on lipid production revealed that the implementation of the phosphoketolase-phosphotransacetylase (PK-PTA) pathway is effective for increasing the cofactor pools [26]. The PK-PTA pathway combined with the overexpression of glucose-6-phosphate dehydrogenase (ZWF1) and 6-phosphogluconate dehydrogenase (GND1) can reduce ATP consumption by bypassing ATP-consuming phosphofructokinase (PFK) reaction step, while increasing NADPH regeneration through pentose phosphate (PP) pathway (Fig. 3A). The PP pathway is the primary source of NADPH during lipogenesis [27], consisting of ZWF1 and GND1, which catalyze the oxidation of glucose-6-P and its derivative, as well as the reduction of NADP⁺ to NADPH. Overexpression of *zwf1* and/or *gnd1* has successfully increased the production of lipids [28], erythritol [29], and scutellarin [30], likely by improving NADPH supply. Expecting similar results for β-carotene, *zwf1* and

gnd1 were overexpressed in the strain YB8. However, the resulting YB14 failed to outperform YB12 in β-carotene titer (Fig. 3B). Nonetheless, the PK-PTA pathway was introduced into YB14 by expression of PK from *C. acetobutylicum* and PTA from *Thermoanaerobacterium saccharolyticum*, both of which were known to have relatively higher activities when expressed in *Y. lipolytica* [12]. The resulting strain YB15 produced 682.8 ± 33.0 mg/L β-carotene, which was not a very significant increase from YB12 (655.8 ± 21.9 mg/L). Alternatively, redirection of carbon flux to the pentose phosphate pathway along with the modification of the cofactor preference of the glyceraldehyde-3-phosphate dehydrogenase (GPD) reaction from NAD⁺ to NADP⁺, and phosphofructokinase (PFK) deletion may increase NADPH regeneration and decrease ATP consumption (Fig. 3A). This alternative approach entails shifting xylulose-5-phosphate and erythrose-5-phosphate pool from the PP pathway to fructose-6-phosphate and glyceraldehyde-3-phosphate, both of which could be reassimilated

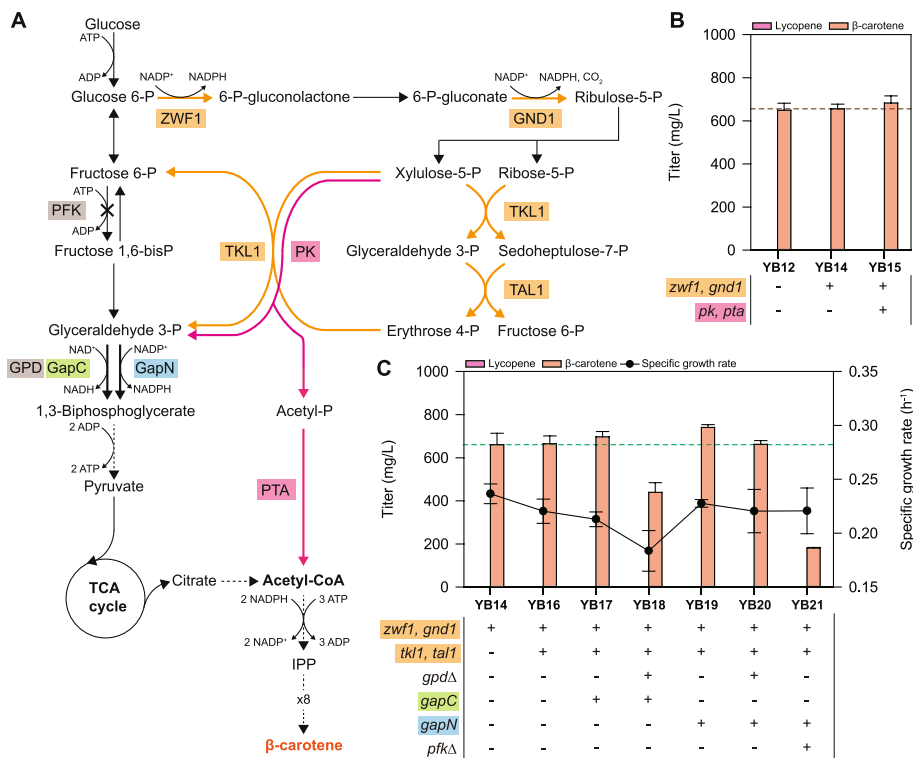


Fig. 3 Central carbon redistribution to regenerate NADPH and reduce ATP expenditure. **A** Scheme illustrating central carbon metabolic engineering strategies. ZWF1, glucose-6-phosphate dehydrogenase; GND1, 6-phosphogluconate dehydrogenase; TKL1, transketolase; TAL1, transaldolase; PFK, phosphofructokinase; GPD, glycerolglyceraldehyde-3-phosphate dehydrogenase; GapC, glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*; GapN, glyceraldehyde-3-phosphate dehydrogenase from *C. acetobutylicum*; PK, phosphoketolase from *C. acetobutylicum*; PTA, phosphotransacetylase from *Thermoanaerobacterium saccharolyticum*; IPP, isopentenyl diphosphate. **B** Carotenoid production of PK-PTA pathway strains. The brown dashed line indicates the β-carotene titer (mg/L) of YB14 from Fig. 3B. **C** Carotenoid production and specific growth rate of PP pathway and glycolysis engineered strains. Each plus sign represents a single round of integration of corresponding gene expression cassettes into the genome or deletion of complete CDS from the genome. The green dashed line indicates the β-carotene titer (mg/L) of YB14 from Fig. 3C. Error bars indicate standard deviations of biological triplicates

to glycolysis. To achieve this, transketolase (TKL1) and transaldolase (TAL1) were additionally overexpressed to yield strain YB16. Yet there was no change in β -carotene production (Fig. 3C). RT-qPCR analysis of YB16 showed that ZWF1's expression level increased nearly fivefold, but the rest of the genes' expression increased only slightly (Fig. S4). Possibly unknown regulatory mechanisms may exist to limit the flux increase in the pentose phosphate pathway.

GapC from *C. acetobutylicum* has been heterologously introduced to *Y. lipolytica* to successfully improve lipid yield, as cytosolic NADPH is a precursor for lipid synthesis as well [31]. Heterologous *gapC* expression (YB17) increased β -carotene titer to 697.0 ± 24.8 mg/L. But replacement of native GPD with GapC (YB18) significantly decreased β -carotene titer to 439.4 ± 44.8 mg/L and specific growth rate to 0.18 ± 0.02 h⁻¹, which suggests decreased glycolytic flux (Fig. 3C). Few literatures report GapN from the same source as another NADP⁺-dependent enzyme equivalent to GapC, which is instead suggested to be NAD⁺-dependent [32, 33]. In such light, GapN was overexpressed in ways corresponding to YB17 and YB18. Heterologous *gapN* expression (YB19) produced 740.7 ± 13.0 mg/L β -carotene, while replacement of the native GPD with GapN (YB20) had neither positive or negative effect on both β -carotene level (662.5 ± 17.4 mg/L) and specific growth rate (0.22 ± 0.02 h⁻¹). This suggests that GapN expression increases β -carotene in growth-independent manner, probably by improving NADPH regeneration. Also there is a possibility of synergistic activity of GPD and GapN such as forming active heterotetramer. PFK deletion (YB21), on the other hand, significantly decreased cell growth, as well as β -carotene production, suggesting that bypass flux through the pentose phosphate pathway was not high enough as glycolysis. Thus, among all the engineered strains, YB19 has shown the highest β -carotene production.

Flux adjustment to balance β -carotene storage and acetyl-CoA pool

Diacylglycerol acyltransferase (DGA), by which diacylglycerol (DAG) is esterified to neutral lipid triacylglycerol (TAG), is one of the widely used overexpression targets for lipid production in metabolic engineering studies [10, 28, 34]. High neutral lipid production can increase carotenoid production by promoting lipid droplet (LD), which is where hydrophobic molecules can be stably accommodated [19]. However, an excessively strong lipid synthesis pathway may not be ideal for carotenoid production, as it could divert precursor acetyl-CoA away from carotenoid biosynthesis, suggesting an optimal balance in expression level between the two pathways' genes (Fig. 4A). In order to find the optimal balance between lipid and β -carotene

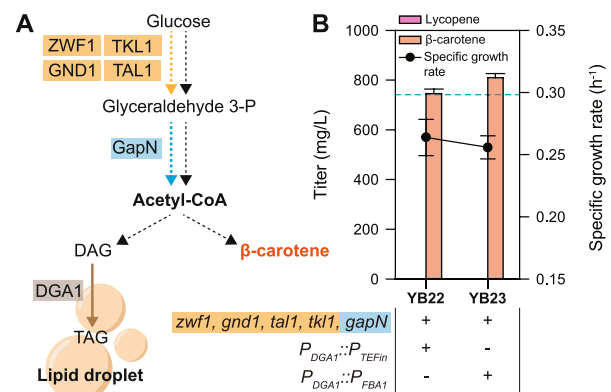


Fig. 4 Flux adjustment of lipid biosynthesis pathway.

A Scheme illustrating flux divergence of lipid biosynthesis and carotenoid biosynthesis pathway from acetyl-CoA (**B**) Carotenoid production and specific growth rate of lipid biosynthesis overexpression strains. Each plus sign represents a single round of integration of corresponding gene expression cassettes or replacement of promoter into the genome. The blue dashed line indicates the β -carotene titer (mg/L) of YB19 from Fig. 3C. Error bars represent the standard deviations of three biological replicates

production, the endogenous DGA1 promoter of YB19 was replaced with TEF_{in} or FBA1 promoter to increase the transcription level of DGA1 to varying levels. As expected, the β -carotene titer and specific growth rate increased (Fig. 4B): YB22 produced 745.7 ± 46.3 mg/L β -carotene, while YB23 produced 809.2 ± 44.1 mg/L. When hrGFP was expressed under these promoters, the TEF_{in} promoter was about 3.5-fold stronger than the FBA1 promoter during the exponential phase (Fig. S5). Thus, the moderate upregulation of DGA1 using FBA1 promoter led to higher β -carotene production than excessive upregulation using TEF_{in} promoter. One possible explanation is that higher lipid content from TEF_{in} promoter expression resulted in a negative feedback regulation of the lipid synthesis pathway. Acetyl-CoA carboxylase (ACC1), which catalyzes acetyl-CoA conversion to malonyl-CoA, is under allosteric inhibition by saturated fatty acids. However, the overexpression of DGA1 'pulls' the lipid synthesis toward DAG, which would limit accumulation of the inhibiting fatty acid [35]. Therefore, the results likely indicate that an optimal lipid synthesis pathway better supports β -carotene production by providing a balance between storage capacity and acetyl-CoA pool [35].

Conclusions

This study describes strategies and their order of implementation for the development of a *Y. lipolytica* strain that can produce β -carotene at 809.2 mg/L titer, 0.017 g/L/h productivity and 0.04 g per g glucose yield at

shake-flask level. The production metrics have not reached the highest reported titers of 7.5 g/L in shake-flask and 39.5 g/L in bioreactor fermentation [11], likely due to expression level imbalance between engineered genes. Fine-tuning of expression level between *carRP*, *carB* and upstream pathway genes is the first aspect to prioritize for further optimization. Nonetheless, this study has demonstrated that redistribution of central carbon flux can be a viable strategy for microbial β -carotene production.

Abbreviations

PK-PTA	Phosphoketolase-phosphotransacetylase
IUP	Isopentenol utilization pathway
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
gDNA	Genomic DNA
Acetyl-CoA	Acetylcoenzyme A
IPP	Isopentenyl diphosphate
DMAPP	Dimethylallyl diphosphate
GGPP	Geranylgeranyl diphosphate
<i>carRP</i>	Bifunctional phytoene synthase/lycopene cyclase
<i>carB</i>	Phytoene dehydrogenase
ZWF1	Glucose-6-phosphate dehydrogenase
GND1	6-Phosphogluconate dehydrogenase
TKL1	Transketolase
TAL1	Transaldolase
GPD	Glyceraldehyde-3-phosphate dehydrogenase
PFK	Phosphofructokinase
DGA	Diacylglycerol acyltransferase
TAG	Triacylglycerol
LD	Lipid droplet

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13036-025-00476-1>.

Additional file 1. Description of data: Table S1. Strains used in this study. Table S2. Plasmids used in this study. Table S3. Primers used in this study. Table S4. Integration or deletion loci used in this study. Table S5. Codon-optimized heterologous genes used in this study. Fig. S1. Time-course growth and carotenoid production of strain YB4. Fig. S2. Significant decrease in β -carotene titer of YB4 after Y27R mutation. Fig. S3. mRNA secondary structure prediction of *carRP* enzymes. Fig. S4. qRT-PCR results of pentose phosphate pathway overexpression strains. Fig. S5. Normalized fluorescence intensity of hrGFP under different promoters.

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Not applicable.

Authors' contributions

HJL and SWS conceived the project. HJL designed and performed the experiments. JWS also performed the experiments. HJL and SWS analyzed and interpreted data collected from the experiments and wrote the manuscript. SWS supervised the project. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare no competing interests.

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