공학석사학위논문

Metabolic Engineering of *Yarrowia lipolytica* for the production of carotenoids, ß-carotene, and crocetin

*Yarrowia lipolytica*의 대사공학을 통한 카로티노이드 베타카로틴과 크로세틴 생산

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Abstract

Metabolic Engineering of *Yarrowia lipolytica* for the production of carotenoids, ß-carotene, and crocetin

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Carotenoids and apocarotenoids are unique molecules that can be used for various purposes such as colorants or health care. Their antioxidant and anticancer properties have brought much industrial demands and explain the increasing number of studies involved in their synthesis by microbial species.

In this study, the oleaginous yeast *Yarrowia lipolytica* was used to produce carotenoids by the introduction of heterologous genes phytoene

desaturase (*crtI*) and lycopene cyclase/phytoene synthase (*crtYB*) from the red yeast *Xanthophyllomyces dendrorhous,* in multiple copies in the *Y. lipolytica* genome, mediated by CRISPR/Cas9 system. The carotenoid content was further improved by fine tuning of the cytoplasmic acetyl-CoA pool, involving the overexpression of endogenous and exogenous genes regulating the flux towards acetyl-CoA. This approach improved carotenoid content by 1.63 folds. Further engineering was performed by pulling the acetyl-CoA out from the mitochondria; The *IDP* gene, encoding isocitrate dehydrogenase involved in the TCA cycle, was down regulated by the CRISPRi system and the *ACL* gene, encoding ATP citrate lyase providing the cytosolic acetyl-CoA, was overexpressed. This resulted in as much as 2.63-fold increase in the carotenoid content. The final strain JHYB102 could produce as much as 1.54 mg/g DCW of ß-carotene and 0.17 mg/g DCW of lycopene from 20 g/L of glucose.

Moreover, additional heterologous genes, ß-carotene reductase (*crtZ*) from *Pantoea ananatis* and carotenoid cleavage dioxygenase (*CCD2*) from *Crocus sativus* were introduced to produce zeaxanthin and crocetin, respectively. The final strain JHYB104 with increased acetyl-CoA flux could produce up to 0.874 mg/g DCW of zeaxanthin.

Keywords: Metabolic engineering, Carotenoids, *Y. lipolytica,* rDNA, CRISPR/Cas9, CRISPRi

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Chapter 1.

Introduction

1.1. Carotenoids

Carotenoids are C_{40} compounds of natural colorants, which are diversely utilized and known for their antioxidant and anti-cancer activity. The two common carotenoids often studied are lycopene and ß-carotene, which are also found frequently in human diets. Lycopene is a red pigment found in tomatoes used in food and cosmetic industries, while ß-carotene is an orange pigment found in carrots, found commonly in health care industries [1]. The global market for carotenoids is currently at $1.5 billion in 2017, and is postulated to grow to $2.0 billion by 2022 [2]. Crocetin is a red C_{20} apocarotenoid found in crocus flower, which is recently in increasing demands for its positive physiological effects like anti-atherosclerotic and anti-depressant activities [3]. However, the current methods of producing carotenoids naturally are limited, as natural resources like tomatoes or carrots contain only small amounts of carotenoids and need long growth cycles. Therefore, microbial biosynthesis of carotenoids and apocarotenoids is more advantageous to meet the growing demands due to its feasibility, efficiency and safety.

1.2. Production of carotenoids in *Y. lipolytica*

A promising candidate for such production strategy is *Yarrowia lipolytica.* The oleaginous yeast *Y. lipolytica* is a non-conventional GRAS (generally recognized as safe) microorganism that is receiving much attention in the metabolic engineering fields owing to its large precursor pool for industry-level metabolic processes as well as its ability to withstand harsh

environments like low pH [4]. *Y. lipolytica* not only has a large acetyl-CoA pool but can also produce large lipid droplets that can hold large non-polar molecules like carotenoids. Recently, diverse genetic tools such as the CRISPR/Cas9 or multiple site integration system have been implemented for *Y. lipolytica,* enabling it to become a more feasible metabolic engineering platform.

Y. lipolytica does not produce carotenoids naturally but carries an innate mevalonate pathway that can produce up to farnesyl pyrophosphate, a precursor for carotenoid biosynthesis. Two heterologous enzymes, bifunctional lycopene cyclase/phytoene synthase and phytoene desaturase, are required for lycopene and ß-carotene production. Then, two additional enzymes, ß-carotene hydroxylase and carotenoid cleavage dioxygenase, are required to produce zeaxanthin and crocetin (Figure 1).

1.3. Previous approaches for carotenoid production in *Y. lipolytica*

Many studies have been reported for the biosynthesis of carotenoids lycopene, ß-carotene, and zeaxanthin in a range of different hosts including *Escherichia coli, Saccharomyces cerevisiae* and *Y. lipolytica,* as summarized in Table 1. These studies usually focus on extensive pathway engineering, media composition optimization and disruption of competitive pathways. On the other hand, only one study has been reported for the production of crocetin by *S. cerevisiae,* which also focused on fine-tuning of essential heterologous enzymes and media optimization [3].

Enlargement of the cytoplasmic acetyl-CoA pool is a promising strategy to increase the carotenoid content. This strategy has been used in variety of studies to increase the flux of different biosynthetic products like fatty acids, triacetic lactone, and squalene in *Y. lipolytica*, products which also use cytoplasmic acetyl-CoA as the main precursor [5, 6]. For example, the lipid productivity in *Y. lipolytica* increased by 3.1 folds after rewiring and engineering the acetyl-CoA pathway.

1.3. Objectives

In this study, a carotenoid-producing *Y. lipolytica* strain was constructed by multi-site rDNA integration of essential heterologous genes, mediated by CRISPR/Cas9. Moreover, various push and pull gene manipulations were performed to increase the cytoplasmic acetyl-CoA flux, by overexpressing both endogenous and exogenous genes as well as downregulating *IDP,* a gene regulating acetyl-CoA in the TCA cycle, using the CRISPRi system (Figure 2). The genes beneficial in increasing the carotenoid content was integrated in multiple sites of the genome using NHEJ mediated integration, with the expression of *KU70* gene responsible for the NHEJ repair system [7]. Lastly, zeaxanthin was produced for the first time reported in *Y. lipolytica.*

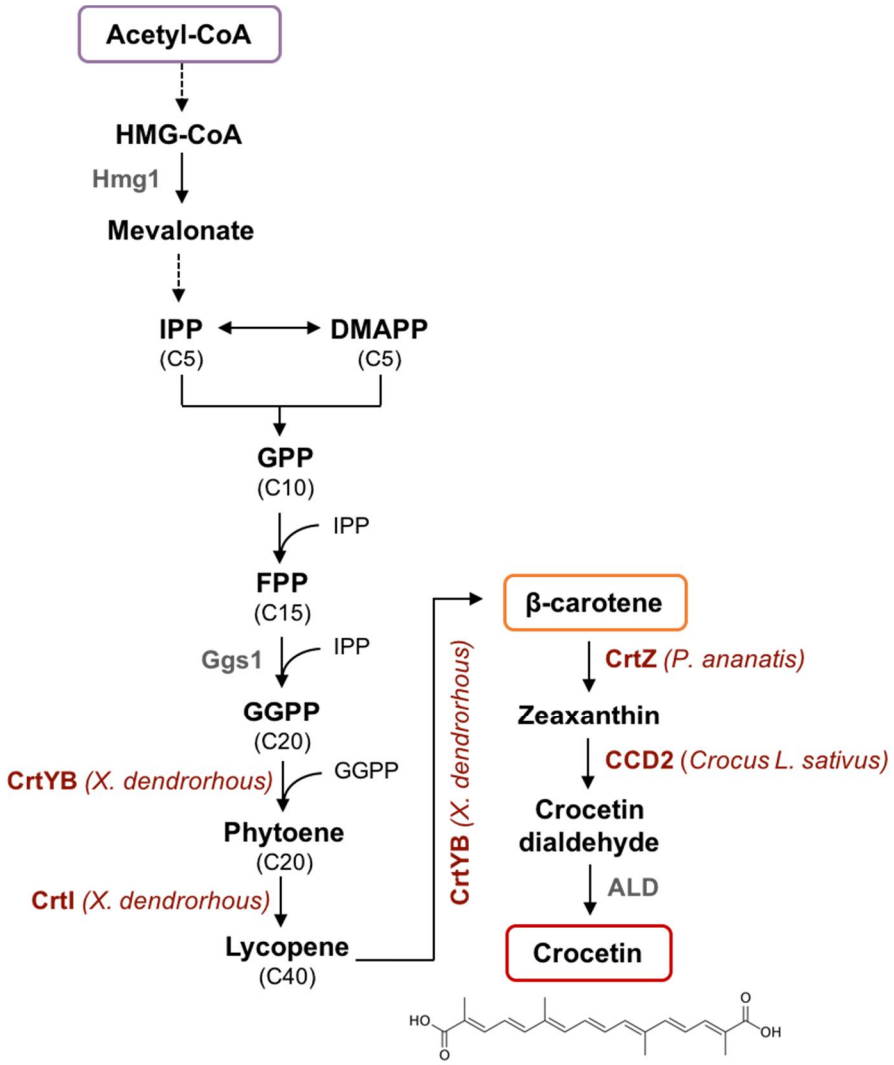

Figure 1. Metabolic pathway for carotenoid production in *Y. lipolytica*

Cytoplasmic acetyl-CoA is utilized through the mevalonate pathway to produce GGPP. HMG-CoA reductase (Hmg1) and geranylgeranyl diphosphate synthase (Ggs1) regulate the rate limiting steps in the pathway. Lycopene cyclase/phytoene synthase (CrtYB) and phytoene desaturase (CrtI) from *X. dendrorhous* convert two molecules of GGPP to one molecule of ß-carotene. ß-carotene reductase (CrtZ) from *P. ananatis* and carotenoid cleavage dioxygenase 2 (CCD2) with the addition of aldehyde dehydrogenase (ALD) reduce ß-carotene into two molecules of crocetin; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA

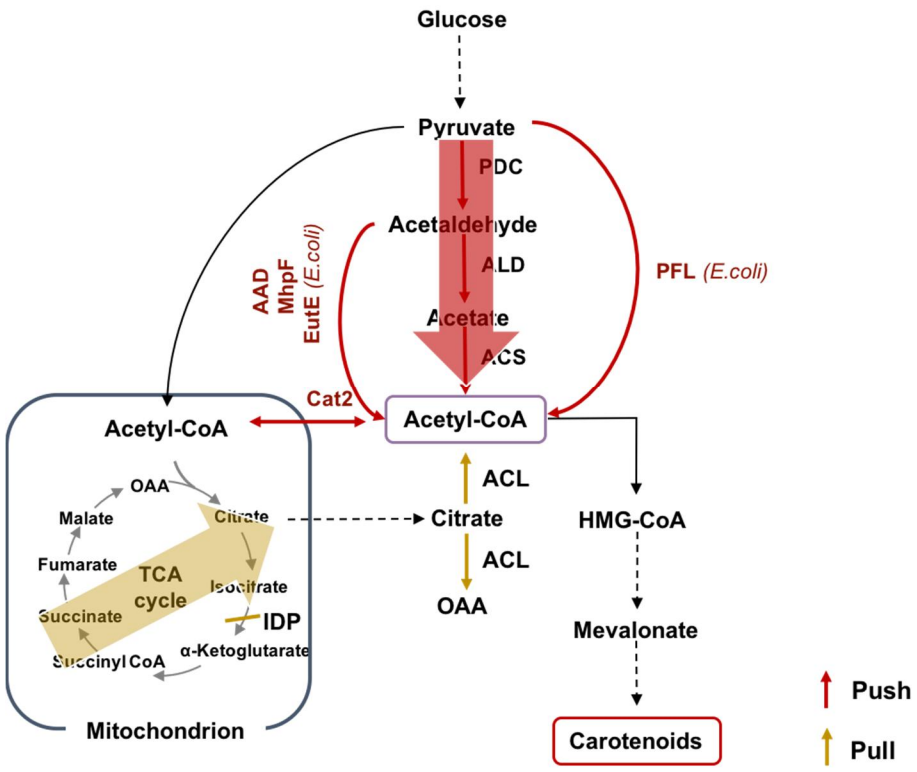

Figure 2. Metabolic pathway for cytoplasmic acetyl-CoA in *Y. lipolytica*.

Red arrows indicate steps involved in "push"ing while yellow arrows indicate "pull"ing cytoplasmic acetyl-CoA. Innately, pyruvate is converted to acetaldehyde through pyruvate decarboxylase (PDC), which is then converted to acetate by acetyldehyde dehydrogenase (ALD), and finally metabolized to cytoplasmic acetyl-CoA by acetyl-CoA synthetase (ACS). Cytoplasmic acetyl-CoA production can also be achieved by the introduction of foreign enzymes like acetyl dehydrogenase (MhpF and EutE) or pyruvate formate lyase from *E. coli.* The cytoplasmic acetyl-CoA pool is also strengthened by carnitine O-acetyltransferase (Cat2) by *S. cerevisiae*. Isocitrate dehydrogenase (IDP) regulates the enzymatic conversion of isocitrate to α-ketoglutarate in the TCA cycle. ATP-citrate lyase (ACL) converts cytoplasmic citrate to acetyl-CoA and oxaloacetate (OAA)

Table 1. Overview of study for carotenoid production

Host strains	Strategies	Product and content	References
B. trispora	Optimization of ratio of (+) and (-) mating type, fermentation	175 mg/g DCW, ß-carotene	[8]
Y. lipolytica	Optimization of promoter-gene pairs , heterologous *crt* pathway, media optimization, flask culture	61.1 mg/g DCW, ß-carotene	[9]
	Iterative multiple-copy integration and competitive pathway deletion, flask culture	33 mg/g DCW, ß-carotene	[10]
X. dendrorhous	Mutagenesis of astaxanthin synthase and overexpression of ß-carotene hydrolase, flask culture	0.5 mg/g DCW, zeaxanthin	[11]
E. coli	MEP pathway engineering for IPP and DMAPP supply, central pathway (TCA, PPP) for carbon flux, fed-batch fermentation	60.0 mg/g DCW, ß-carotene	[12]
	Comparison of *crtZ* hosts, tuning intergenic regions, protein-mediated substrate channeling, flask culture	11.95 mg/g DCW, zeaxanthin	[13]
S. cerevisiae	Rational mutation of *crtI* and *crtYB,* heterologous expression of *mva,* flask culture	0.39 mg/g DCW, ß-carotene	[14]
	Iterative multiple-copy integration of *crtI* and *tHMG1*, flask culture	5.9 mg/g DCW, ß-carotene	[15]
	Optimization of *crtZ, CCD2, ALD5* enzymes, temperature control, flask culture	1.22 mg/L, crocetin	[3]

Chapter 2.

Materials and methods

2.1. Strains and culture conditions

All strains used in this study are listed in Table 2. *E. coli* strain DH5α [F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ^- *th*i-*1 gyrA96 relA1*] was used for genetic manipulations. *E. coli* was cultured in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 50 μg/mL ampicillin. *Y. lipolytica* PO1g *ku70*Δ strain was used as a parental strain of all the engineered strains. The *URA3* deletion mutant was constructed by using the CRISPR/Cas9-mediated genome editing system [16]. Cells were cotransformed with pCRISPRyl-URA3gRNA plasmid, consisting of Cas9 gene and guide RNA (gRNA) targeted to *URA3* gene, and a DNA template for *URA3* deletion obtained by PCR. After confirmation of the *URA3* deletion by PCR, pCRISPRyl-URA3gRNA plasmid was removed by growing cells in YPD (10 g/L yeast extract, 20 g/L bacto-peptone, and 20 g/L glucose) medium overnight. *TRP1* and *IDP* were deleted through targeted nucleotide editing by pCRISPRyl-nCas9-pmCDA1-UGI as was described previously with few modifications [17] [18].

Yeast cells were cultured in YPD or in synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids and 1.4 g/L amino acids dropout mixture lacking His, Trp, Leu, and Ura) supplemented with auxotrophic nutrients and/or hygromycin-B as needed. For shake flask fermentation, yeast cells harboring plasmids were pre-cultured in selective SC medium containing 20 g/L glucose and inoculated to OD_{600} of 0.5 in 10 mL of

selective SC medium containing 20 g/L glucose in 100 mL flask, and then cultured at 30 °C with shaking at 170 rpm.

2.2. Plasmid construction

Plasmids and primers used in this study are described in Table 3 and Table 4. The genes *crtI* and *crtYB* were PCR amplified from coex416-crtI and p414GPD-crtYB, respectively [19]. Native sequence of *crtZ* from *Pantoea ananatis* was synthesized by Mbiotech, Inc. (Hanam, South Korea). *CCD2* from *Crocus sativus* was codon optimized (Thermo Fisher Scientific, Waltham, MA, USA) and synthesized. *PDC2, ALD5, ACS1,* and *KU70* were obtained from *Y. lipolytica* genome. *eutE, mhpF, pflA,* and *pflB* were obtained from *Y. lipolytica* genome. *CAT2* was obtained from *S. cerevisiae* genome. pUAS1B8-TEF-Cas9 plasmid was generated by Gibson cloning of *LEU2*-$P_{UAS1B8\text{-}TEF}$-*Cas9*-T_{CYC1} fragment from pCRISPRyl with Amp^R gene cassette amplified from pCRISPRyl. To generate single-gene-expression plasmids, PCR-amplified gene cassettes were cloned into pYL-LEU, pYL-URA using *Asc*I and *Not*I sites resulting in pYL-LEU-PDC2, pYL-LEU-ku70, pYL-URA-ACS1, and pYL-URA-ALD5. ORFs were cloned into pUAS1B8-TEF plasmids using *Asc*I and *Nhe*I sites, pUAS1B8-TEF-eutE, pUAS1B8-TEF-mhpF, pUAS1B8-TEF-pflA, pUAS1B8-TEF-crtZ and pUAS1B8-TEF-CCD2. For plasmid coexpYL-LEU-TEF-PDC2ALD5 harboring multiple genes the gene expression cassette (promoter-ORF-terminator) flanked by *Mau*BI and *Not*I sites was obtained by PCR from the single-gene-expression vectors, and then cloned between the *Asc*I and *Not*I sites of pYL-LEU-PDC2. For

coexpUAS1B8-TEF-pflA-pflB, coexpUAS1B8-TEF-CCD2-crtZ, the sequential gene expression cassettes were flanked by *Bsp*119I and *Pac*I from the single-gene-expression vectors, and then cloned between the *Cla*I and *Pac*I sites of coexpression plasmids.

For rDNA site integrations, two half DNA fragments of rDNA1 (672 bp) and rDNA2 (630 bp) were PCR-amplified from *Y. lipolytica* genome and Amp^R cassette was PCR-amplified from pCRISPRyl. The DNA fragment containing rDNA1, Amp^R, and rDNA2 was generated by overlap PCR, and then cloned between the *Bsp*120I and *Pac*I sites of pADHintDSN [20], resulting in rDNAintDSN. Gene-expression cassettes (P_{EXP1}-*crtI*-T_{LIP2} and P_{TEF1}-*crtYB*-T_{MIG1}) obtained by overlap PCR were cloned between *Pac*I and *Asc*I sites of rDNAintDSN, resulting in rDNAintcrtI and rDNAintcrtYB, respectively. Additional gene expression cassettes (P_{GPD}-*GGS1*-T_{MIG1} and P_{TEF1}-*tHMG1*-T_{LIP2}) also obtained by overlap PCR (ORF obtained from *Y. lipolytica* genome) were digested by *Mau*BI and *Not*I then cloned into *Asc*I and *Not*I sites of rDNAintcrtI and rDNAintcrtYB vectors resulting in rDNAintcrtIGGS1 and rDNAintcrtYBtHMG1, respectively.

For NHEJ-mediated random integrations, gene fragments *PDC2*, *eutE* and *crtZ* were digested by and cloned into *Bam*HI and *Xho*I sites of rDNAintcrtI. Additional gene cassettes (P_{EXP1}-*ALD5*-T_{CYC1}, P_{EXP1}-*ACS1*-T_{LIP2}, $P_{UAS1B8\text{-}TEF}$-*CAT2*-T_{CYC1}, $P_{UAS1B8\text{-}TEF}$-*CCD2*-T_{CYC1}) or auxotrophic marker gene cassettes (P_{URA3}-*URA3*-T_{URA3}, P_{TRP1}-*TRP1*-T_{TRP1} and P_{EXP1}-*hphMX*-T_{LIP2}) were PCR amplified from *Y. lipolytica* genome or corresponding vector and digested by *Mau*BI and *Not*I and subsequently cloned into *Asc*I and *Not*I sites

of appropriate plasmids to eventually create rDNAintPDC2ALD5ACS1URA3, rDNAinteutECat2TRP1 and rDNAintcrtZCCD2ALD5hphMX.

For *URA3* deletion using the CRISPR/Cas9 system, plasmid pCRISPRyl was purchased from Addgene. For *TRP1* and *IDP* deletion and downregulation using CRISPR/Cas9 system, pCRISPRyl-nCas9-pmCDA1-UGI and pCRISPRyl-dCas9-Mxi1 vectors were constructed from pCRISPRyl. *URA3, TRP1, IDP*-targeting gRNA was designed by CRISPR-RGenTools (http://www.rgenome.net/), and inserted into pCRISPRyl with appropriate gene cassettes (Cas9 or dCas9-Mxi1 or nCas9-pmCDA1-UGI) by *Dpn*I-mediated site directed mutagenesis based on PCR, generating pCRISPRyl-URA3, pCRISPRyl-nCas9-pmCDA1-UGI-TRP1, pCRISPRyl-nCas9-pmCDA1-UGI-IDP and pCRISPRyl-dCas9-Mxi1-IDP.

Strain	Genotype	Reference
E. coli		
DH5α	F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ^- *th*i-*1 gyrA96 relA1*	
Y. lipolytica		
PO1g	*MATa ura3-302::URA3, leu2-270,xpr2-332, axp-2*	[21]
PO1g *ku70*Δ	*PO1g ku70*Δ*::loxP*	[22]
PO1g *ku70*Δ*ura3*Δ	PO1g *ku70*Δ, *URA3*Δ	This study
JHYB100	Selected strain from rDNA integration of P_{EXP1}-*crtI*-T_{LIP2}-P_{GPD}-*GGS1*-T_{MIG1} and P_{TEF1}-*crtYB*-T_{MIG1}-P_{TEF1}-*tHMG1*-T_{LIP2} into PO1g *KU70*Δ*URA3*Δ	This study
JHYB101	JHYB100, *TRP1*Δ	This study
JHYB102	Selected strain from NHEJ-mediated random integration of P_{EXP1}-*PDC2*-T_{LIP2}-P_{EXP1}-*ACS1*-T_{LIP2}-P_{EXP1}-*ALD5*-T_{CYC1} using *URA3* in JHYB101	This study
JHYB103	Selected strain from NHEJ-mediated random integration of P_{EXP1}-*eutE*-T_{LIP2}-$P_{UAS1B8\text{-}TEF}$-*CAT2*-T_{CYC2} using *TRP1* in JHYB102	This study
JHYB104	Selected strain from NHEJ-mediated random integration of P_{EXP1}-*crtZ*-T_{LIP2}-$P_{UAS1B8\text{-}TEF}$-*CCD2*-T_{CYC1}-P_{EXP1}-*ALD5*-T_{CYC1} by hygromycin-B in JHYB102	This study

Table 2. Strains used in this study.

Table 3. Primer sequences used in this study.

Primers	Sequence (5'-3')
CRISPR/Cas9 based gRNA exchange and deletion cassettes	
URA3_gRNA_F	ATTGACGACTTCACCTACGCGTTTTAGAGCTAGAAATAGCAAGT
P_{URA3}_del_F	TTCTTGAGAACCGTGGAGAC
P_{URA3}_del_R	AGTCTAACCTTTTGGTGGTGAAGAGGAGAC
T_{URA3}_del_F	CACCACCAAAAGGTTAGACTATGGATATGTAATTTAACTG
T_{URA3}_del_R	ACCTATGAAGGTGTTTGAGG
IDP_nCas9gRNA_F	CGCAGGTTGACGTCCGAGACCAGACTAACGACCGTTTTAGAGCTAGAAATAGCAAGT
IDP_dCas9gRNA_F1	GTGGAGTATCGGCTCCGAGCGTTTTAGAGCTAGAAATAGCAAGT
IDP_dCas9gRNA_F2	AGCAAGGTTAAGCTTTGGATGTTTTAGAGCTAGAAATAGCAAGT
IDP_dCas9gRNA_F3	AAGCTTTGGATGAAACTTTCGTTTTAGAGCTAGAAATAGCAAGT
IDP_dCas9gRNA_F4	CAGACCCTCCCTTGCCAGCCGTTTTAGAGCTAGAAATAGCAAGT
IDP_dCas9gRNA_F5	GCTACCACCCGGCTGCTAGCGTTTTAGAGCTAGAAATAGCAAGT
pCRISPRyl_Univ_R	ACGTCAACCTGCGCCGACCCGG
rDNA_gRNA_F	TCCCGCCGTTTACCCGCGCTGTTTTAGAGCTAGAAATAGCAAGT
rDNA_gRNA_R	AGCGCGGGTAAACGGCGGGAGTCAACCTGCGCCGACCCGGAAT
PCR, Overlap extension PCR, Cloning	
rDNA1_F	ATATGGGCCCGCCGTTTACCCGCGC
rDNA1_R	CCCGAAAAGTGCATTTAAATTCGATCCTAAGGGGTGG
rDNA2_F	ATTTAAATTGAGAAATGGGTGAATGTTGAGCTTCGGTATGATAGGAAGAGC
rDNA2_R	ATATTTAATTAAGGGAGTAACTATGCTCTCTT
AmpR_F	ATTTAAATGCACTTTTCGGGGAAA
AmpR_R	CTCAACATTCACCCATTTCTCAATTTAAATCGCAGGAAAGAACATGTGAG
crtI_BamHI_F	GCGGATCCAAAAATGGGAAAAGAACAAGATCAGG
crtI_XhoI_R	GCGCTCGAGTCAGAAAGCAAGAACACCAAC
crtYB_overlap_F	GGGGGCCCCCGGGGGGGATCCAAAAATGACGGCTC
crtYB_overlap_R	CCCGGGCCCGGGCCCCTCGAGTTACTGCCCTTCC
crtZ_AscI_F	ATCGGCGCGCCATGTTGTGGATTTGGAATGC

crtZ_NheI_R	ATCGCTAGCTTACTTCCCGGATGCGGGC
ALD5_AscI_F	ATCGGCGCGCCATGCAAGTTACTCTTCCCGAC
ALD5_NheI_R	ATCGCTAGCCTAATCCAGGTTAATGTGGACGG
PDC2_AscI_F	ATCGGCGCGCCATGACTGCCACTTCTGCCA
PDC2_NheI_R	ATCGCTAGCCTAAGGATTCTTCTTCTGGTACTCG
ACS1_AscI_F	ATCGGCGCGCCATGTCTGAAGACCACCCAG
ACS1_NheI_R	ATCGCTAGCTTACTTTTTCAACGAGTGAACAACC
mhpF_AscI_F	ATCGGCGCGCCATGAGTAAGCGTAAAGTCG
mhpF_NheI_R	ATCGCTAGCTCATGCCGCTTCTCCTG
CAT2_AscI_F	ATCGGCGCGCCATGAGGATCTGTCATTCGAGA
CAT2_NheI_R	ATCGCTAGCTCATAACTTTGCTTTTCGTTTATTCT
eutE_AscI_F	ATCGGCGCGCCATGAATCAACAGGATATTGAAC
eutE_SpeI(NheI)_R	ATCACTAGTTTAAACAATGCGAAACGCATC
pflA_AscI_F	ATCGGCGCGCCATGTCAGTTATTGGTCGCATT
pflA_NheI_R	ATCGCTAGCTTAGAACATTACCTTATGACCGTACT
pflB_MauBI(AscI)_F	ATCCGCGCGCGATGTCCGAGCTTAATGAAAAGT
pflB_NheI_R	ATCGCTAGCTTACATAGATTGAGTGAAGGTACGA
ACL1_AscI_F	ATCGGCGCGCCATGTCTGCCAACGAGAACAT
ACL1_NheI_R	ATCGCTAGCCTATGATCGAGTCTTGGCCT
ACL2_AscI_F	ATACGGCGCGCCATGTCAGCGAAATCCATTCAC
ACL2_NheI_R	ATACGCTAGCTTAAACTCCGAGAGGAGTGGA
pUAS1B8_MauBI_F	ATGCGCGCGCGGGTACCCGAATTCCTGAGGT
CYC1t_AscINotI_R	ATCGCGGCCGCGGCGCGCCGCAAATTAAAGCCTTCGAGCGTC
rDNAint_Univ_F	ATCACGCGCGCGCTTAAGAGAGCATAGTTACTCCCTTAATT
rDNAint_Univ_R	ATTCGCGGCCGCTAATGG
qPCR	
qPCR_ACT1_F	CCAAGCGAGGTATCCTGACC
qPCR_ACT1_R	CGGGAGCGTTGAAAGTCTCG
qPCR_GGS1_F	GCGTTCGGAGCGGTTATCAA
qPCR_GGS1_R	CGGAGTTGATGGTTTGGGGG
qPCR_HMG1_F	CCCGTTCTCTGGACGACCTA
qPCR_HMG1_R	CGTAGTGCAGGTAAGGAAGC

Table 4. Plasmids used in this study.

Plasmid	Description	Reference
pIMR53-AUX	*URA3, ARS18, AmpR*	[23]
pIMR53-AUX-EXP1	pIMR53-AUX, P_{EXP1}, T_{CYC1}	This study
pIMR53-crtI-crtYB	pIMR53-AUX-EXP1 with the sequential insertion of *crtI* and *crtYB*	This study
pCRISPRyl	$P_{UAS1B8\text{-}TEF(136)}$-*CAS9*-$T_{CYC1}$, P_{SCR1}-$tRNA^{Gly}$-gRNA, *LEU2*, *CEN*, *Ori1001*, *AmpR*, *ColE1*	[16]
pCRISPRyl-URA3	pCRISPRyl incorporating *URA3* gRNA	This study
pCRISPRyl-rDNA	pCRISPRyl incorporating rDNA gRNA	This study
pCRISPRyl-dCas9-Mxi1	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1*	This study
pCRISPRyl-dCas9-Mxi1-IDP1	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1* and incorporating *IDP* gRNA1	This study
pCRISPRyl-dCas9-Mxi1-IDP2	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1* and incorporating *IDP* gRNA2	This study
pCRISPRyl-dCas9-Mxi1-IDP3	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1* and incorporating *IDP* gRNA3	This study
pCRISPRyl-dCas9-Mxi1-IDP4	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1* and incorporating *IDP* gRNA4	This study
pCRISPRyl-dCas9-Mxi1-IDP5	pCRISPRyl replacing *CAS9* with *dCAS9-Mxi1* and incorporating *IDP* gRNA5	This study
pCRISPRyl-nCas9-pmCDA-UGI-TRP1	pCRISPRyl replacing *CAS9* with *nCAS9-pmCDA1-UGI* and incorporating *TRP1* gRNA	This study
pCRISPRyl-nCas9-pmCDA-UGI-IDP	pCRISPRyl replacing *CAS9* with *nCAS9-pmCDA1-UGI* and incorporating *IDP* gRNA	This study
pUAS1B8-TEF-Cas9	$P_{UAS1B8\text{-}TEF(136)}$-*CAS9*-$T_{CYC1}$, *LEU2, CEN, Ori1001, AmpR, ColE1*	This study
pUAS1B8-TEF-Cat2	pUAS1B8-TEF-Cas9 replacing *CAS9* with *CAT2*	This study
pUAS1B8-TEF-eutE	pUAS1B8-TEF-Cas9 replacing *CAS9* with *eutE*	This study
pUAS1B8-TEF-Cat2-eutE	pUAS1B8-TEF-Cat2 with the insertion of $P_{UAS1B8\text{-}TEF(136)}$-*eutE*-$T_{CYC1}$	This study
pUAS1B8-TEF-mhpF	pUAS1B8-TEF-Cas9 replacing *CAS9* with *mhpF*	This study
pUAS1B8-TEF-Cat2-mhpF	pUAS1B8-TEF-Cat2 with the insertion of $P_{UAS1B8\text{-}TEF(136)}$-*mhpF*-$T_{CYC1}$	This study
pUAS1B8-TEF-pflA	pUAS1B8-TEF-Cas9 replacing *CAS9* with *pflA*	This study
pUAS1B8-TEF-pflA-	pUAS1B8-TEF-pflA with the insertion of	This study

pflB	$P_{UAS1B8\text{-}TEF(136)}$-*pflB*-$T_{CYC1}$	
pUAS1B8-TEF-crtZ	pUAS1B8-TEF-Cas9 replacing *CAS9* with *crtZ*	This study
pUAS1B8-TEF-CCD2	pUAS1B8-TEF-Cas9 replacing *CAS9* with *CCD2*	This study
pUAS1B8-TEF-CCD2-crtZ	pUAS1B8-TEF-CCD2 with the insertion of $P_{UAS1B8\text{-}TEF(136)}$-*crtZ*-$T_{CYC1}$	This study
pYL-LEU	*LEU2, CEN, Ori1001, AmpR* with *Asc*I, *Not*I enzyme sites	This study
pYL-URA	*URA3, CEN, Ori1001, AmpR* with *Asc*I, *Not*I enzyme sites	This study
pYL-LEU-KU70	pYL-LEU with the insertion of P_{KU70}-*KU70*-T_{KU70}	This study
pYL-LEU-PDC2	pYL-LEU with the insertion of P_{EXP1}-*PDC2*-T_{LIP2}	This study
pYL-LEU-PDC2-ALD5	pYL-LEU-PDC2 with the insertion of P_{EXP1}-*ALD5*-T_{CYC1}	This study
pYL-URA-ACS1	pYL-URA with the insertion of P_{EXP1}-*ACS1*-T_{LIP2}	This study
pYL-URA-ALD5	pYL-URA with the insertion of P_{EXP1}-*ALD5*-T_{CYC1}	This study
rDNAintDSN	rDNA1, rDNA2, *AmpR,* P_{TEF1}-*alsD*-T_{GPM1}-P_{TDH3}-*alsS*-T_{CYC1}-P_{FBA1}-*noxE*-T_{FBA1} (DSN)	This study
rDNAintcrtI	rDNAintDSN replacing DSN with P_{EXP1}-*crtI*-T_{LIP2}	This study
rDNAintcrtYB	rDNAintDSN replacing DSN with P_{TEF1}-*crtYB*-T_{MIG1}	This study
rDNAintcrtIGGS1	rDNAintcrtI with the insertion of P_{GPD}-*GGS1*-T_{MIG1}	This study
rDNAintcrtYBtHMG1	rDNAintcrtYB with the insertion of P_{TEF1}-*tHMG1*-T_{LIP2}	This study
rDNAintPDC2	rDNAintcrtI replacing *crtI* with *PDC2*	This study
rDNAintPDC2-ALD5-ACS1-URA3	rDNAintPDC2 with the sequential insertion of P_{EXP1}-*ALD5*-T_{CYC1}, P_{EXP1}-*ACS1*-T_{LIP2}, *URA3*	This study
rDNAinteutE	rDNAintcrtI replacing *crtI* with *eutE*	This study
rDNAinteutECat2TRP1	rDNAinteutE with the sequential insertion of $P_{UAS1B8\text{-}TEF(136)}$-*CAT2*-$T_{CYC1}$, *TRP1*	This study
rDNAintcrtZ	rDNAintcrtI replacing *crtI* with *crtZ*	This study
rDNAintcrtZCCD2ALD5 hphMX	rDNAintcrtZ with the sequential insertion of $P_{UAS1B8\text{-}TEF(136)}$-*CCD2*-$T_{CYC1}$, P_{EXP1}-*ALD5*-T_{CYC1}, *hphMX*	This study

2.3. rDNA-integration and NHEJ-mediated random integration

The rDNA integrated strain was constructed by CRISPR/Cas9-mediated genome editing system. Cells were cotransformed with pCRISRPyl-rDNA plasmid and rDNA integrative vector linearized by *Swa*I. To construct the NHEJ-mediated randomly integrated strains, JHYB101 cells were first transformed with pYL-LEU-KU70. A transformant was cultured overnight in SC-Leu medium with 20 g/L glucose in 30 °C with shaking at 170 rpm. After 24 h, the cells were harvested ($O.D._{600}$=3) and transformed with rDNA integrative vector with genes of interest, linearized by *I-Ppo*I and *Not*I. The strains were screened by color and selected in SC medium supplemented with auxotrophic nutrients as needed and 20 g/L of glucose.

2.4. Quantitative PCR (qPCR)

To determine the copy numbers of the genome-integrated genes, *GGS1* and *HMG1,* qPCR was performed with gene-specific primers and 1xSYBR Green I master mix (Roche Applied Science). qPCR was performed with 45 cycles of 95°C for 40 s, 60°C for 20 s, and 72°C for 20 s on a LightCycler 480 II System (Roche Applied Science). The *ACT1* gene was used as a reference control. The crossing point (Cp) values were processed using LightCycler Software version 1.5 (Roche Applied Science) and expression levels were normalized as target/reference ratios. Primers used for qPCR are listed in Table 3.

2.5. Analytical methods

Cell growth was determined by the measurement of an optical density at 600 (OD_{600}) with spectrophotometer (Varian Cary® 50 UV-Vis). To determine the concentrations of glucose and other secondary metabolites 500 µL of culture supernatants filtered through a 0.22 µm syringe filter were analyzed by High performance liquid chromatography (HPLC) as previously described [24]. Carotenoids were extracted with the customized hot-HCl method from the cultured cells [25]. Same amounts of cultured cells were used for dry cell weight measurement. The sampled cells were resuspended in 3N HCl and broken by boiling for 10 min. After cooling in ice for 10 min, the broken cells were washed with water and collected by centrifugation for 1 min. An appropriate volume of HPLC grade acetone was added and vortexed for 5 min. The cell debris was removed by centrifugation for 1 min. The supernatant was used for the analysis of carotenoids. Carotenoids were quantitated by Surveyor Plus HPLC system (Thermo Fisher Scientific) with Agilent Eclipse XDB-C18 column (5 µm, 4.6 x 250 mm) using a standard curve generated with lycopene, ß-carotene and zeaxanthin obtained from Sigma Aldrich. Analysis was performed with methanol: dichloromethane: acetonitrile (42:16:42) as a mobile phase at a flow rate of 1.2 mL/min and Surveyor PDA Detector at 450 nm. The column temperature was maintained at room temperature. Standard crocetin was purchased from CoreScience Korea. Crocetin analysis was performed with the same column with methanol:water (70:10) as a mobile phase at a flow rate of 1.0 mL/min and UV-Vis detector at

430 nm.

Chapter 3.

Results and discussion

3.1. Construction of ß-carotene biosynthetic pathway in *Y. lipolytica*

To produce carotenoids in *Y. lipolytica*, heterologous ß-carotene biosynthetic pathway genes from carotenoid-producing red yeast *X. dendrorhous*, consisting of genes encoding phytoene desaturase (*crtI*) and bifunctional lycopene cyclase and phytoene synthase (*crtYB*) were introduced into *Y. lipolytica* strain PO1g *ku70*Δ *ura3*Δ (Figure 1). The two ß-carotene producing genes were expressed under the control of P_{EXP1} promoter by using ARS-based low copy plasmid based on pIMR53-AUX. *Y. lipolytica* harboring pIMR53-AUX-crtI-crYB produced a trace amount of ß-carotene, as validated by the yellow color when compared to the control strain. However, the amount of ß-carotene produced here was too low to be determined by HPLC (data not shown). On the other hand, the control strain harboring empty pIMR53-AUX plasmid maintained a white color, confirming that the biosynthetic genes from *X. dendrorhous* are functional in producing ß-carotene in *Y. lipolytica*.

3.2. Construction of a carotenoid-producing *Y. lipolytica* strain by rDNA integration of biosynthetic genes

For a more efficient and stable production of carotenoids, ß-carotene biosynthetic genes were integrated into the chromosome. Homologous recombination using these rDNA sites allows random multiple copy integration of target genes. Therefore, to improve ß-carotene production, we

integrated the two ß-carotene biosynthetic genes together with the native genes, *GGS1* and truncated *HMG1* (*tHMG1*), which regulate the rate-limiting steps in the mevalonate pathway [26]. For a marker-less integration, CRISPR/Cas9 system was implemented to target the rDNA sequence. Two rDNA-integration cassettes containing either *crtI* and *GGS1* genes or *crtYB* and *tHMG1* genes were transformed together with the rDNA targeting plasmid pCRISPRyl-rDNA into *Y. lipolytica* PO1g *ku70*Δ *ura3*Δ, and the transformants were selected based on orange color in SC-Leu medium. Strain JHYB100 with the strongest color was selected for further engineering experiments. The copy numbers of the integrated genes were determined by qPCR. Results indicated that JHYB100 genome contains 2 copies of *crtI* and *GGS1* and 3 copies of *crtYB* and *tHMG1* (Figure 3B). JHYB100 alone could produce around 0.84 mg/g DCW carotenoids consisting of lycopene and ß-carotene. *TRP1* from JHYB100 was deleted to generate JHYB101 for use as an additional auxotrophic marker.

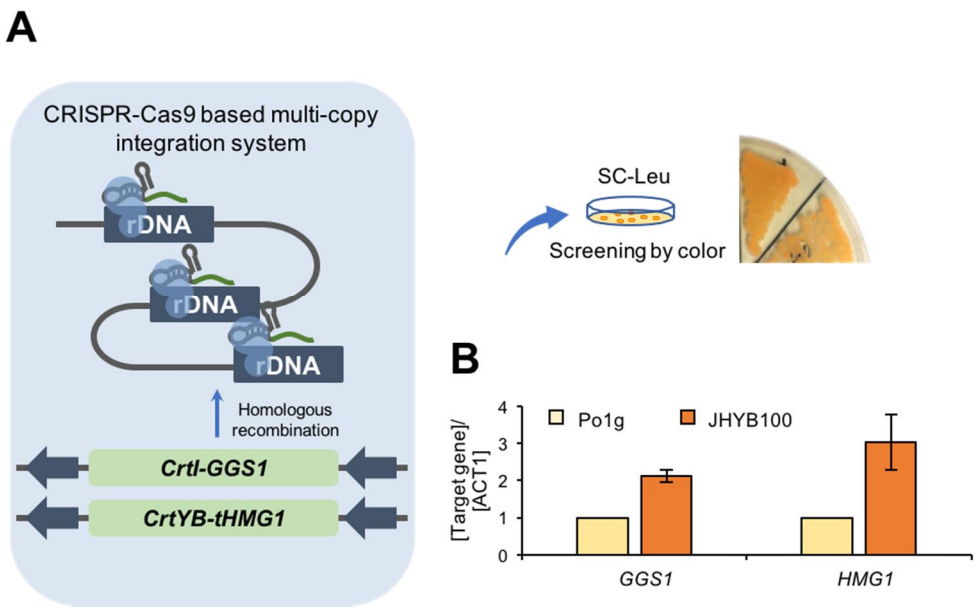

Figure 3. Construction of ß-carotene producing strain by rDNA integration of *X. dendrorhous* genes.

(A) Schematic illustration of the procedure of rDNA integration of *X. dendrorhous* genes into *Y. lipolytica*. rDNA sequence was targeted by CRISPR-Cas9 method and the rDNA integrative vector was linearized and transformed into *Y. lipolytica*. The colonies with integrated genes were screened and selected based on orange color. (B) Copy numbers of the integrated genes in the selected strain was analyzed qPCR. Each value indicates the average ± SD of duplicate experiments.

3.3. “Push” strategy of cytoplasmic acetyl-CoA flux to improve carotenoid production

Although multiple integration of biosynthetic genes demonstrated an improvement in the production of carotenoids, the production levels were still low, which might be due to a limited pool of cytoplasmic acetyl-CoA, the first precursor of carotenoids. Consequently, multiple genes were overexpressed to enlarge the acetyl-CoA pool and their corresponding effects on total carotenoid levels were observed.

Three native genes, *PDC2*, *ALD5*, and *ACS1*, were overexpressed and four genes that allow for alternative pathways to cytoplasmic acetyl-CoA, including *CAT2* from *S. cerevisiae, mhpF*, *eutE*, and *pflA-pflB* from *E. coli* (Figure 2) were introduced by constitutive expression plasmids. The overexpression of endogenous genes mentioned above has successfully improved the production of triacetic acid lactone in *Y. lipolytica* [6], while the expression of exogenous genes from other microbial species has increased lipid production [5], of which both metabolites require acetyl-CoA as the main precursor. The five gene sets were expressed under the control of strong constitutive promoter $P_{UAS1B8\text{-}TEF}$ by expression plasmids. All five gene sets, excluding *pflA-pflB,* showed improvements in carotenoid levels (Figure 4A), which suggests that the expression and overexpression of exogenous and endogenous pathways were helpful in pushing the acetyl-CoA flux and hence allowing for a larger availability of precursor pool for carotenoid synthesis. Among the tested genes, *CAT2* showed the highest improvement in carotenoid

production, increasing it by 1.27 fold. The two genes both encoding acetaldehyde dehydrogenase, *mhpF* and *eutE* showed similar increase in carotenoid level.

To assess the effect of overexpressing multiple exogenous genes that up-regulate the acetyl-CoA flux, *mhpF*, *eutE*, and *pflA-pflB* were overexpressed together with *CAT2*, which showed the largest improvement in carotenoid level. Simultaneous expression of *CAT2* and *eutE* showed the maximum carotenoid level (1.04 mg/g DCW) but the level was not largely different to the expression of *CAT2* alone (Figure 4B). This result may be because of epistatic characteristics of genes which does not always allow for a synergistic effect [27].

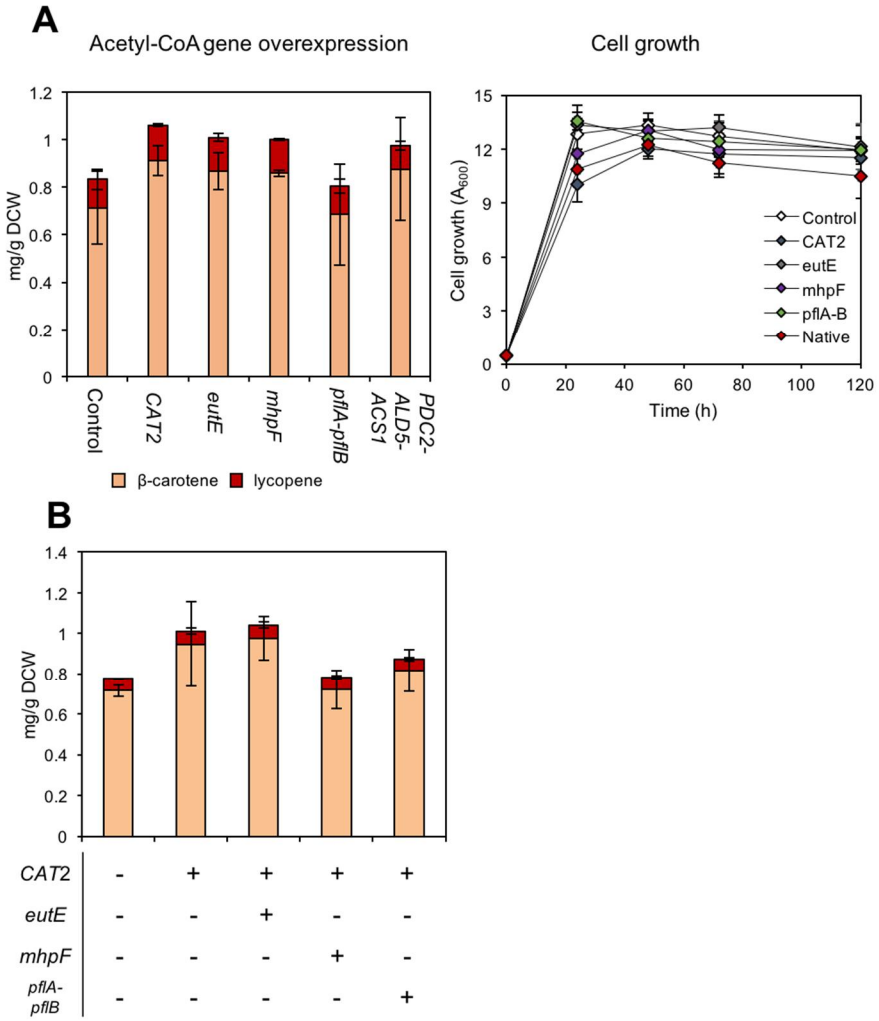

Figure 4. Effects of the overexpression of native and foreign genes involved in cytoplasmic acetyl-CoA pool on carotenoid production.

(A) JHYB101 harboring an empty vector and harboring pUAS1B8-TEF vectors with indicated genes (B) JHYB101 harboring an empty vector and JHYB101 harboring coexpUAS1B8-TEF vectors with indicated genes in combination with *CAT2* were grown in SC-Leu, Ura medium containing 20 g/L glucose for 120 h and ß-carotene production was measured. Each value indicates the average ± SD of triplicate experiments.

3.4. “Pull” strategy of mitochondrial acetyl-CoA flux out to the cytoplasm

An alternative approach to increase the cytoplasmic acetyl-CoA flux available for carotenoid production is to “pull” the acetyl-CoA occupied in the mitochondria via the TCA cycle out to the cytoplasm. Hence, downregulation of $NADP^+$-dependent isocitrate dehydrogenase (*IDP*) was performed. This may lead to citrate accumulation followed by cytosolic transport, which pulls out the acetyl-CoA to the cytoplasm. This approach can impose a similar effect as to when yeast cells are under nitrogen depletion [28] and hence result in amount increase of secondary metabolites like lipid or carotenoids.

Downregulation of *IDP* was mediated by the CRISPRi system [29]. Plasmid pCRISPRyl-dCas9 fused with a Mxi1 repressor was constructed for the targeting and transcriptional repression of the promoter of *IDP*. Five sites of the promoter region within 300 bp of the ORF of *IDP* were randomly selected as the gRNA target sites as illustrated (Figure 5A). The repression of *IDP* showed a significant improvement in carotenoid production in the strain JHYB101. With the expression of pCRISPRyl-dCas9-Mxi1-IDP(2), the carotenoid biosynthesis improved as much as 2.63 folds with carotenoid content reaching up to 1.81 mg/g DCW (Figure 5B). This suggests that much of acetyl-CoA concentration is occupied in the TCA cycle, and pulling it out of the mitochondria is indeed successful in increasing the flux toward the cytoplasm, improving the carotenoid concentration in return. Furthermore, the cell growth was not hindered when *IDP* was downregulated (Figure 5B),

which also indicates that this approach of increasing the flux of secondary metabolites in *Y. lipolytica* may be applicable for various industrial purposes. To confirm the effects of *IDP* downregulation, *IDP* gene was deleted and the results were compared to the strain harboring dCas9-Mxi1-IDP(2), which showed the best result in increasing carotenoid flux. Results showed that its deletion showed similar effects to *IDP* downregulation, with downregulation showing slightly better results (Figure 5C).

An additional method to pull the cytoplasmic acetyl CoA flux out from the mitochondria is to overexpress ATP citrate-lyase (*ACL*) gene, which mediates the conversion of citrate in the cytosol to acetyl-CoA and oxaloacetate. Citrate accumulated in the mitochondria, especially upon inactivation of Idp, can be transported into the cytoplasm, serving as a source of the cytoplasmic acetyl-CoA. Subsequently, *ACL1* and *ACL2* were overexpressed by a plasmid in JHYB101, of which the overexpression of *ACL1* and *ACL2* together increased the carotenoid content by 1.35 fold (Figure 6).

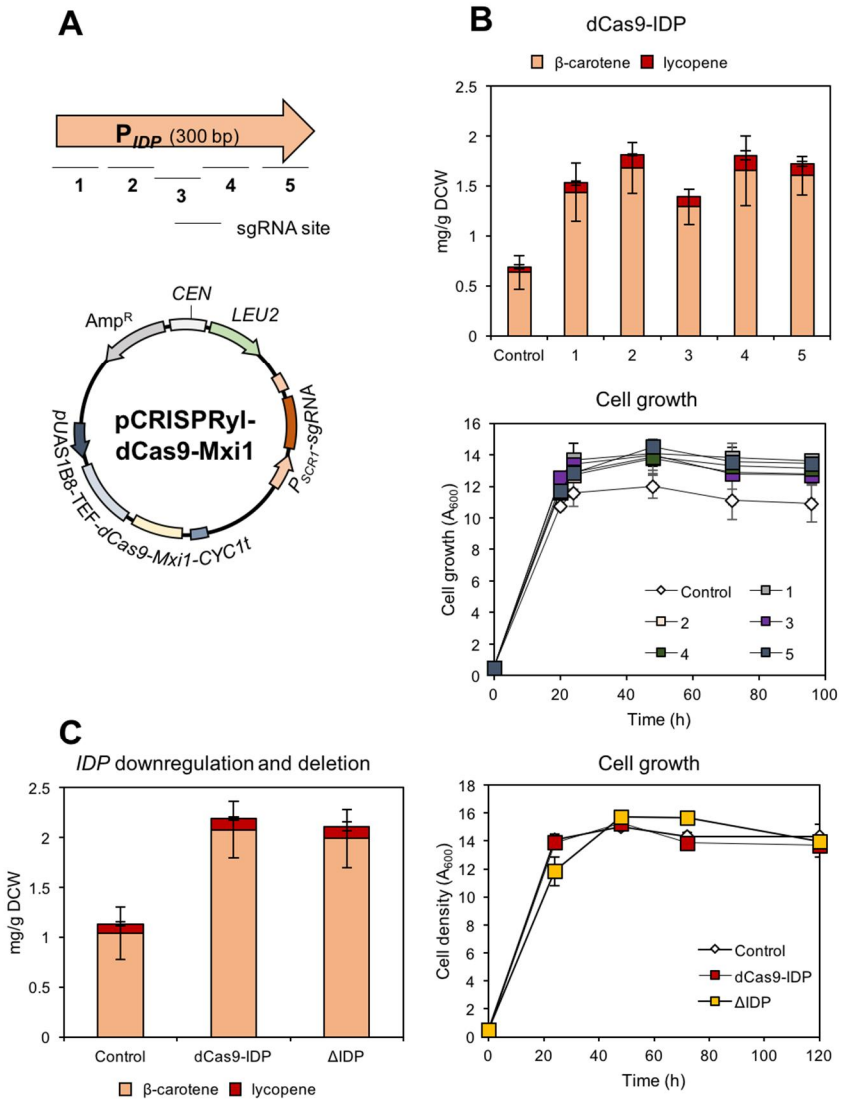

Figure 5. Effects of "pull" of acetyl-CoA from mitochondria to the cytoplasm on carotenoid production.

(A) Schematic illustration of downregulation of P_{IDP} mediated by CRISPRi. Five sites on the promoter sequence (within 300 bp) of *IDP* were randomly selected as the sgRNA target sites. Downregulation was mediated by pCRISPRyl-dCas9-Mxi1. (B) JHYB101 harboring pCRISPRyl-dCas9-Mxi1 and JHYB101 harboring pCRISPRyl-dCas9-Mxi1 targeting five different sits on P_{IDP} (numbered 1 through 5) were grown in SC-Leu medium containing 20 g/L glucose for 96 h and their carotenoid production levels and $O.D._{600}$ were measured. (C) JHYB101 harboring pCRISPRyl-dCas9-Mxi1, JHYB101 harboring pCRISPRyl-dCas9-Mxi1-IDP(2) and JHYB106 were grown in SC-Leu medium containing 20 g/L glucose for 120 h and their carotenoid production levels and $O.D._{600}$ were measured. Each value indicates the average ± SD of triplicate experiments.

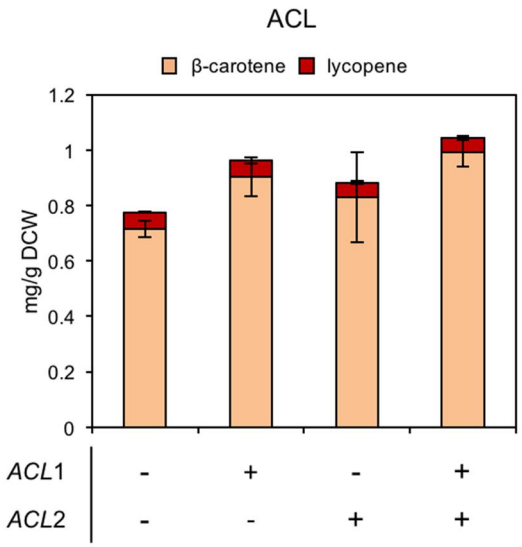

Figure 6. Effects of overexpression of *ACL* on carotenoid production.

JHYB101 harboring empty vector and pUAS1B8-TEF expressing the indicated genes (*ACL1, ACL2* or *ACL1* and *ACL2*) were grown in SC-Leu medium containing 20 g/L glucose for 120 h and their carotenoid production levels were measured. Each value indicates the average ± SD of triplicate experiments.

3.5. Production of further carotenoids, zeaxanthin and crocetin

After establishing a strain (JHYB101) that stably produces ß-carotene, this strain was further engineered to produce the next carotenoid metabolites, zeaxanthin and crocetin, which are carotenoids that have not yet been reported to be produced in *Y. lipolytica*. The *crtZ* gene from *P. ananatis,* encoding ß-carotene hydroxylase, was expressed under a constitutive promoter on a plasmid. HPLC result confirmed the production of zeaxanthin in the strain JHYB101, reaching a content of 70.3 μg/g DCW (Figure 7).

To produce crocetin, three enzymes, CrtZ, CCD2 and ALD, need to be expressed. When all three genes were expressed simultaneously, the cell color turned red, indicating the color of crocetin, while other flasks remained orange or yellow, indicating the color of ß-carotene or zeaxanthin (data not shown). However, the amount of crocetin could not be measured due to the low concentration.

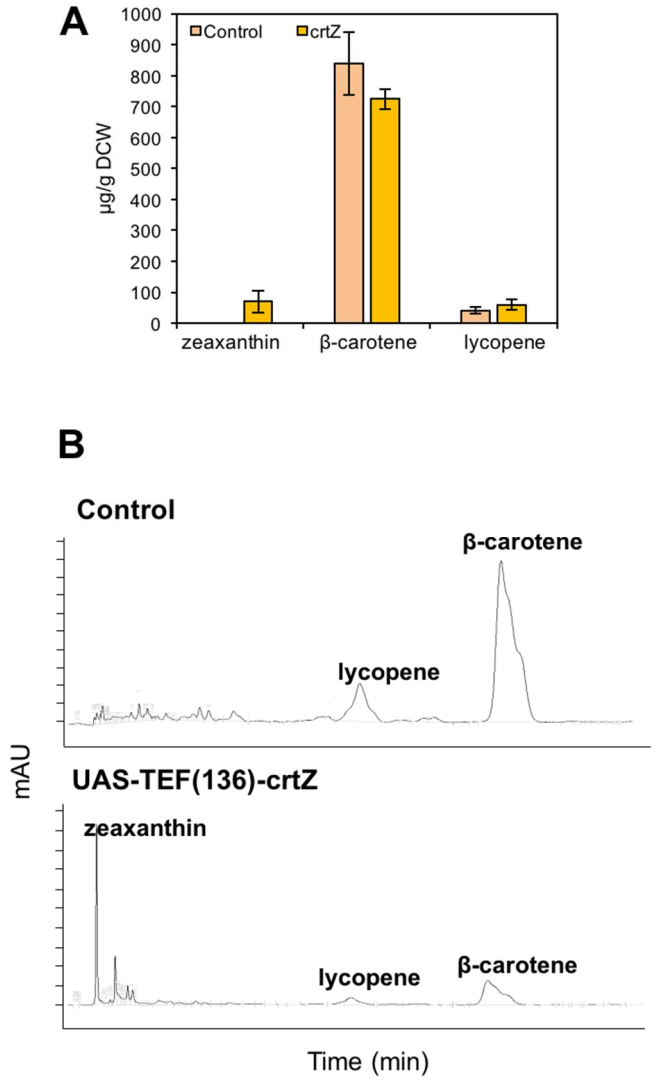

Figure 7. Zeaxanthin production by JHYB101.

(A) JHYB101 harboring empty vector and JHYB101 harboring pUAS1B8-TEF-*crtZ* were grown in SC-Leu medium containing 20 g/L glucose for 120 h and their carotenoid production levels were measured. Each value indicates the average ± SD of triplicate experiments. (B) HPLC spectra of carotenoids for cell extracts of JHYB101 harboring an empty vector and JHYB101 harboring pUAS1B8-TEF-*crtZ* vector.

3.6. Integration of beneficial genes for optimal production of carotenoids

To increase the flux of acetyl-CoA and stably produce carotenoids, several genes were sequentially integrated into the genome. The random integration method was selected for an increase in chances of multiple copy integration. The platform strain JHYB101 has its gene *KU70* deleted. In order to facilitate random integration by non-homologous end joining, *KU70* had to be expressed (data not shown). Therefore, before every occasion of random integration, pYL-LEU-ku70 was transformed into the strain of interest. The transformant was incubated in SC-Leu medium for 24 h before the subsequent transformation of integrative gene cassette.

To construct a strain that optimally and natively produces carotenoids by enlarging the endogenous acetyl-CoA flux in *Y. lipolytica*, the gene cassette *PDC2-ALD5-ACS1* was integrated into the chromosome by NHEJ-mediated random integration using the *URA3* auxotrophic marker. Among 16 strains tested, the strain with the largest carotenoid level (JHYB102) was able to produce 1.71 mg/g DCW of carotenoids, which is 1.63 fold of that of JHYB101 (Figure 8).

Next, *CAT2-eutE* gene cassette, which showed the largest improvement of carotenoids by plasmid, was additionally integrated by random integration using the *TRP1* auxotrophic marker. However, this did not result in an increase in an increase in carotenoid level, which is thought to be due to epistatic characteristics of the genes (data not shown).

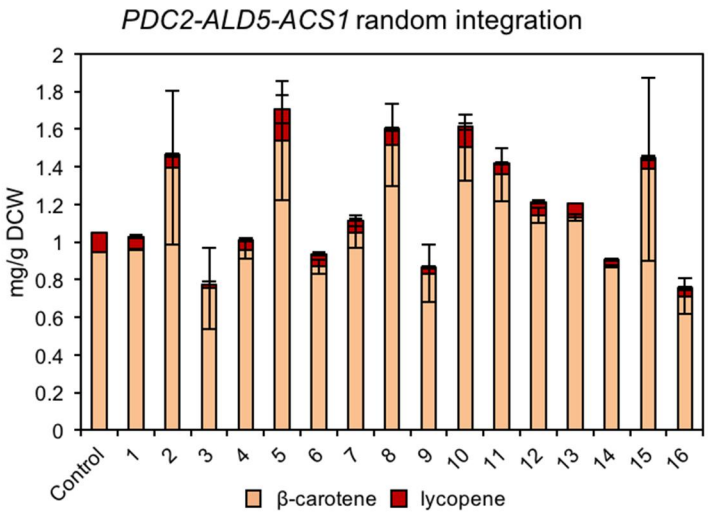

Figure 8. Effect of additional integration of *PDC2-ALD5-ACS1* on carotenoid production.

16 transformants of *PDC2-ALD5-ACS1* integration into JHYB101 by *URA3* were randomly selected, grown in SC-Leu medium containing 20 g/L glucose for 120 h and their carotenoid production levels were measured. Each value indicates the average ± SD of duplicate experiments.

Lastly, in an effort to increase the concentration and pool of zeaxanthin, the gene cassette containing *crtZ*, *CCD2*, and *ALD* was integrated into the genome by hygromycin-B selection. Among 6 colonies tested, the colony with the largest improvement could reach up to 3.50 mg/L in titer and 87.4 µg/g DCW in content of zeaxanthin (Figure 9). Yet, this content was too low to allow for flux toward crocetin.

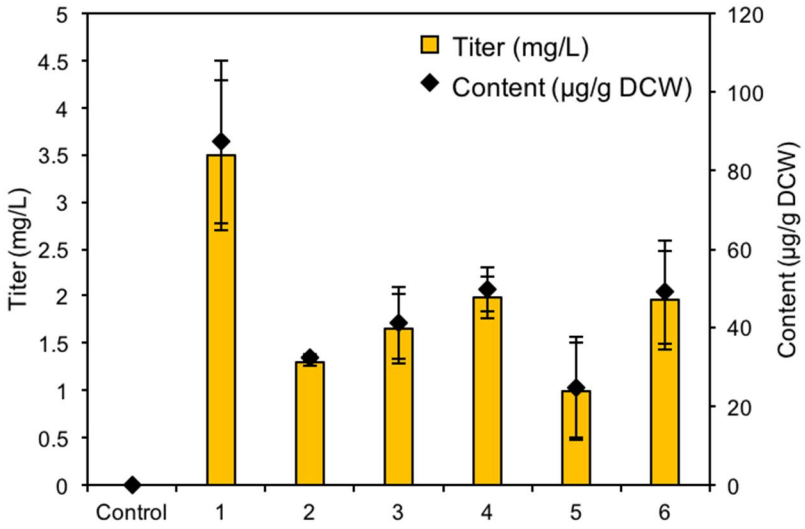

Figure 9. Zeaxanthin production levels after integration of *crtZ-CCD2-ALD* on JHYB102.

6 transformants were selected and tested for zeaxanthin production after random integration of *crtZ-CCD2-ALD* using hygromycin-B marker. Each colony's titer (mg/L) and content (μg/g DCW) were measured. Cells were grown in SC-Leu medium containing 20 g/L glucose for 120 h and their zeaxanthin production levels were measured. Each value indicates the average ± SD of duplicate experiments.

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Chapter 4.

Conclusions

Carotenoids and apocarotenoids, namely lycopene, ß-carotene, zeaxanthin and crocetin, are chemicals in increasing demands for their colorant and antioxidant functions, which can also serve as anticancer agents. They are frequently found in forms of health supplements, cosmetics and animal feeds. However, due to the limited production size and rate of carotenoids from natural resources, a large number studies have focused on producing carotenoids from microbial species including bacteria and yeasts, which allows for a more affordable and efficient production of carotenoids.

The oleaginous yeast *Y. lipolytica* does not naturally produce carotenoids. However, it holds an advantage owing to its large acetyl-CoA pool, the main precursor molecule for carotenoids. Also, the innate mevalonate pathway exists in *Y. lipolytica* producing up to GGPP. This allows for *Y. lipolytica* to have a better tolerance to products in the mevalonate pathway like FPP and GGPP, and environmental stress like pH. *Y. lipolytica* has previously proven its capability of producing large amounts of carotenoids and is increasingly being used as the platform for metabolic engineering of a variety of bioproducts, which also uses Acetyl-CoA as the precursor molecule. In this study, we were able to efficiently produce ß-carotene and up to zeaxanthin, for the first time reported, from *Y. lipolytica* using various strategies to enlarge the acetyl-CoA precursor pool.

Two heterologous genes *crtI* and *crtYB* from *X. dendrorhous* were introduced, together with two native genes *tHMG1* and *GGS1*, into the *Y. lipolytica* genome. This step was successfully performed by the integration

into rDNA sites, which exist in multiple copies in *Y. lipolytica,* mediated marker-less by the CRISPR/Cas9 system. The subsequent strain JHYB100 could around 0.84 mg/g DCW of lycopene and ß-carotene. The marker gene *TRP1* was deleted from JHYB100 to generate JHYB101.

The acetyl-CoA pool was strengthened by the introduction of native genes *PDC2, ALD5* and *ACS1,* which are responsible for the flux from pyruvate to cytoplasmic acetyl-CoA in *Y. lipolytica.* Random integration using the *URA3* marker was performed in JHYB101 by pre-expression of *KU70* to encourage the NHEJ repair system. From many candidates, the strain with the best performance in carotenoid production, JHYB102, was chosen with a 1.63-fold improvement (1.71 mg/g DCW) in carotenoid production than JHYB101. Then, heterologous genes *CAT2* from *S. cerevisiae* and *eutE* from *E. coli,* which also act by "pushing" the flux toward cytoplasmic acetyl-CoA were integrated into the genome by random integration using the *TRP1* marker, but this step did not result in a positive outcome.

To pull the acetyl-CoA out from mitochondria to the cytoplasm, the gene *IDP* was downregulated by using the CRISPRi system targeting the promoter regions of *IDP.* This strategy largely improved the carotenoid production in JHYB101 as much as 2.63 folds, resulting in 1.81 mg/g DCW of carotenoids. Deletion of *IDP* showed a similar effect in carotenoid production levels. Additionally, *ACL1* and *ACL2* were overexpressed, increasing the carotenoid production by 1.35 fold.

Additional genes, *crtZ* from *P. ananatis* was introduced into the *Y.*

lipolytica genome to produce crocetin by random integration using the hygromycin-B resistance marker. The strain JHYB104, with integrated *crtZ*, could successfully produce up to 0.874 mg/g DCW zeaxanthin.

This study focused on improving carotenoid production by a push and pull strategy to increase the cytoplasmic acetyl-CoA pool and producing crocetin for the first time in *Y. lipolytica*. This work showed that a significant amount of acetyl-CoA in *Y. lipolytica* is occupied in the mitochondria by the TCA cycle and this approach is useful in improving the bio-production efficiency of cytoplasmic acetyl-CoA-derived chemicals. Hence, we expect this study approach to become a basic platform not just for metabolic engineering of carotenoids but for numerous acetyl-CoA-derived products in *Y. lipolytica*.

Further genetic manipulations remaining will be downregulating *IDP* in JHYB103 as well as overexpressing *ACL*. Also, more efforts shall be made to increase the zeaxanthin pool to allow for crocetin biosynthesis.

References

1. Giuliano, G., et al., *Metabolic engineering of carotenoid biosynthesis in plants.* Trends Biotechnol, 2008. **26**(3): p. 139-45.
2. Tang, W., et al., *Biosynthetic Pathway of Carotenoids in Rhodotorula and Strategies for Enhanced Their Production.* J Microbiol Biotechnol, 2019. **29**(4): p. 507-517.
3. Chai, F., et al., *Heterologous biosynthesis and manipulation of crocetin in Saccharomyces cerevisiae.* Microb Cell Fact, 2017. **16**(1): p. 54.
4. Abdel-Mawgoud, A.M., et al., *Metabolic engineering in the host Yarrowia lipolytica.* Metab Eng, 2018. **50**: p. 192-208.
5. Xu, P., et al., *Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals.* Proc Natl Acad Sci U S A, 2016. **113**(39): p. 10848-53.
6. Markham, K.A., et al., *Rewiring Yarrowia lipolytica toward triacetic acid lactone for materials generation.* Proc Natl Acad Sci U S A, 2018. **115**(9): p. 2096-2101.
7. Kretzschmar, A., et al., *Increased homologous integration frequency in Yarrowia lipolytica strains defective in non-homologous end-joining.* Curr Genet, 2013. **59**(1-2): p. 63-72.
8. Varzakakou, M., T. Roukas, and P. Kotzekidou, *Effect of the ratio of (+) and (−) mating type of Blakeslea trispora on carotene production from cheese whey in submerged fermentation.* World Journal of Microbiology and Biotechnology, 2010. **26**(12): p. 2151-2156.
9. Larroude, M., et al., *A synthetic biology approach to transform Yarrowia lipolytica into a competitive biotechnological producer of beta-carotene.* Biotechnol Bioeng, 2018. **115**(2): p. 464-472.
10. Gao, S., et al., *Iterative integration of multiple-copy pathway genes in Yarrowia lipolytica for heterologous beta-carotene production.* Metab Eng, 2017. **41**: p. 192-201.
11. Pollmann, H., J. Breitenbach, and G. Sandmann, *Engineering of the carotenoid pathway in Xanthophyllomyces dendrorhous leading to the synthesis of zeaxanthin.* Appl Microbiol Biotechnol, 2017. **101**(1): p. 103-111.
12. Zhao, J., et al., *Engineering central metabolic modules of Escherichia coli*

for improving beta-carotene production. Metab Eng, 2013. **17**: p. 42-50.

13. Li, X.R., et al., *Metabolic engineering of Escherichia coli to produce zeaxanthin.* J Ind Microbiol Biotechnol, 2015. **42**(4): p. 627-36.
14. Li, Q., et al., *Enhancing beta-carotene production in Saccharomyces cerevisiae by metabolic engineering.* FEMS Microbiol Lett, 2013. **345**(2): p. 94-101.
15. Verwaal, R., et al., *High-level production of beta-carotene in Saccharomyces cerevisiae by successive transformation with carotenogenic genes from Xanthophyllomyces dendrorhous.* Appl Environ Microbiol, 2007. **73**(13): p. 4342-50.
16. Schwartz, C., et al., *Standardized Markerless Gene Integration for Pathway Engineering in Yarrowia lipolytica.* ACS Synth Biol, 2017. **6**(3): p. 402-409.
17. Komor, A.C., et al., *Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage.* Nature, 2016. **533**(7603): p. 420-4.
18. Nishida, K., et al., *Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems.* Science, 2016. **353**(6305).
19. Kim, S. and J.S. Hahn, *Efficient production of 2,3-butanediol in Saccharomyces cerevisiae by eliminating ethanol and glycerol production and redox rebalancing.* Metab Eng, 2015. **31**: p. 94-101.
20. Bae, S.J., S. Kim, and J.S. Hahn, *Efficient production of acetoin in Saccharomyces cerevisiae by disruption of 2,3-butanediol dehydrogenase and expression of NADH oxidase.* Sci Rep, 2016. **6**: p. 27667.
21. Madzak, C., B. Treton, and S. Blanchin-Roland, *Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast Yarrowia lipolytica.* J Mol Microbiol Biotechnol, 2000. **2**(2): p. 207-16.
22. Yu, A.Q., et al., *Genetic Engineering of an Unconventional Yeast for Renewable Biofuel and Biochemical Production.* J Vis Exp, 2016(115).
23. Song, Y., et al., *Engineering of the yeast Yarrowia lipolytica for the production of glycoproteins lacking the outer-chain mannose residues of N-glycans.* Appl Environ Microbiol, 2007. **73**(14): p. 4446-54.
24. Park, S.H., S. Kim, and J.S. Hahn, *Metabolic engineering of Saccharomyces cerevisiae for the production of isobutanol and 3-methyl-1-butanol.* Appl Microbiol Biotechnol, 2014. **98**(21): p. 9139-47.
25. Hong, J., et al., *Efficient production of lycopene in Saccharomyces cerevisiae*

by enzyme engineering and increasing membrane flexibility and NAPDH production. Appl Microbiol Biotechnol, 2019. **103**(1): p. 211-223.

26. Polakowski, T., U. Stahl, and C. Lang, *Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast.* Appl Microbiol Biotechnol, 1998. **49**(1): p. 66-71.
27. Yang, D., et al., *Expanded synthetic small regulatory RNA expression platforms for rapid and multiplex gene expression knockdown.* Metab Eng, 2019. **54**: p. 180-190.
28. Tang, W., et al., *The isocitrate dehydrogenase gene of oleaginous yeast Lipomyces starkeyi is linked to lipid accumulation.* Can J Microbiol, 2009. **55**(9): p. 1062-9.
29. Larson, M.H., et al., *CRISPR interference (CRISPRi) for sequence-specific control of gene expression.* Nat Protoc, 2013. **8**(11): p. 2180-96.

국문 초록

카로티노이드 및 아포카로티노이드는 는 이소프레노이드 계열 물질로 항산화 및 발암 억제 작용 기능을 위해 상업적으로 사용되어 각광받고 있다. 현재까지 카로티노이드의 제한적인 정제 방법과 생산 방법은 대사공학적 접근을 통한 대량생산의 연구로 극복되고 있으며, 해당 방법을 통한 수요는 증가하고 있는 추세이다.

본 연구에서는 유지 효모로 분류되는 *Y. lipolyitca*의 유전체에 rDNA 위치에 *X. dendrorhous*의 phytoene desaturase 와 phytoene synthase/lycopene cyclase를 암호화 하는 두가지 유전자 *crtI*와 *crtYB*를 CRISPR/Cas9 시스템을 통해 도입함으로써 ß-carotene을 생산하는 균주를 제작하였다. 또한 카로티노이드의 전구체 물질로 작용하는 acetyl-CoA의 양을 증가시키고자 acetyl-CoA 생산에 관여하는 유전자들을 과발현하였고, 이를 통해 균주 내에에 카로티노이드 양을 약 1.63배 증가할 수 있었다. 또한, 미토콘드리아 내에 존재하는 acetyl-CoA를 세포질로 이동시키고자 CRISPRi 시스템을 통해 TCA cycle에 관여하는 유전자인 *IDP*를 억제하고, citrate로부터 acetyl-CoA를 생산하는 *ACL* 유전자를 과발현 하였을 때 카로티노이드 양이 최대 2.63배 증가하였다. 최종 균주를 통해 20 g/L의 포도당으로부터 1.54 mg/g DCW의 ß-carotene과 0.170 mg/g DCW의 lycopene을 생산하였다.

마지막으로, *P. ananatis*에서 유래된 ß-carotene reductase (*crtZ*)

유전자와 *C. sativus*에서 유래한 carotenoid cleavage dioxygenase 유전자를 추가적으로 *Y. lipolytica* 내로 도입함으로써 zeaxanthin까지 생산하는 최초의 *Y. lipolytica* 균주를 얻었다. 최종 균주는 20 g/L의 포도당을 사용해 0.874 mg/g DCW의 zeaxanthin을 생산하였다.

주요어 : 대사공학, 카로티노이드, *Yarrowia lipolytica,* rDNA, CRISPR/Cas9, CRISPRi

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