Chapter 9

Bioengineering of Oleaginous Yeast *Yarrowia lipolytica* for Lycopene Production

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Abstract

Oleaginous yeast *Yarrowia lipolytica* is capable of accumulating large amount of lipids. There is a growing interest to engineer this organism to produce lipid-derived compounds for a variety of applications. In addition, biosynthesis of value-added products such as carotenoid and its derivatives have been explored. In this chapter, we describe methods to integrate genes involved in lycopene biosynthesis in *Yarrowia*. Each bacterial gene involved in lycopene biosynthesis, *crtE*, *crtB*, and *crtI*, will be assembled with yeast promoters and terminators and subsequently transformed into *Yarrowia* through random integration. The engineered strain can produce lycopene under lipid accumulation conditions.

Key words: Yeast, *Yarrowia*, Lipid accumulation, Carotenoid, Lycopene

1. Introduction

Yarrowia lipolytica has been traditionally used as an industrial organism to produce organic acids (citric acid and succinic acid), enzymes (lipase and esterase), single-cell protein, and single-cell oil (1). Through metabolic engineering, *Yarrowia* is being developed into a versatile production platform to convert lipids, fats, and oils into value-added products (2, 3).

As a value-added product, carotenoids have been widely used commercially (4). Biosynthesis of carotenoids is derived from the isoprenoid pathway (Fig. 1). In non-carotenogenic hosts such as *Escherichia coli* or *Saccharomyces cerevisiae*, introduction of bacterial carotenoid genes leads to production of various carotenoid compounds (5). Lycopene, the first color carotenoid in the pathway, can be made by coordinated expression of three genes, *crtE*, *crtY*, and *crtI*. The *crtE* gene encodes the geranylgeranyl diphosphate (GGPP) synthase, while *crtY* and *crtI* encode the phytoene

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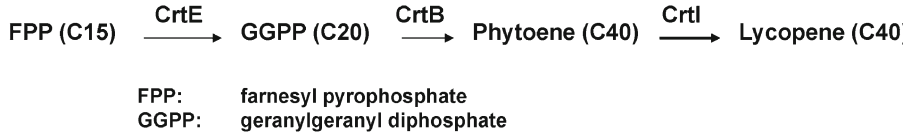

Fig. 1. Lycopene biosynthetic pathway.

synthase and desaturase, respectively. Here in this chapter, we describe a method to produce lycopene in *Y. lipolytica* through expression of *crtE*, *crtY*, and *crtI* genes integrated in chromosomes. Chromosomal integration in *Yarrowia* is predominantly at random (6). Screening of transformants based on color formation is a convenient way to identify strains that produce a large amount of lycopene.

2. Materials

2.1. Strains, Plasmids, Genes, Promoters, and Terminators

1. *Y. lipolytica strain* Y2224 (Ura-, derivative of ATCC20362) (ATCC, Manassas, VA, USA).
2. *E. coli* XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA, USA).
3. Ligase and rapid ligase buffer (Promega, Madison, WI, USA).
4. pZKleuN-6EP (GenBank accession number: GM621387).
5. pEXPGUS1-P (GenBank accession number: GM621389).
6. pZP34R (GenBank accession number: GM621390).
7. *crtE* codon optimized for expression in *Y. lipolytica*, derived from *Pantoea stewartii* DC413 (GenBank accession number: GM621381).
8. *crtB* codon optimized for expression in *Y. lipolytica*, derived from *P. stewartii* DC413 (GenBank accession number: GM621383).
9. *crtI* codon optimized for expression in *Y. lipolytica*, derived from *P. stewartii* DC413 (GenBank accession number: GM621385).
10. FBAIN promoter (2) (see Note 1).
11. *LIP1* terminator (2) (see Note 2).
12. GPDIN promoter (2) (see Note 3).
13. *LIP2* terminator (2) (see Note 4).
14. *EXP1* promoter (2) (see Note 5).
15. *OCT1* terminator (2) (see Note 6).

16. Ligation kit (Promega, Madison, WI, USA).
17. Lycopene (CaroteNature GmbH, Lupsingen, Switzerland).

2.2. Growth Medium

1. LB (Luria Bertani): Add 10 g of bacto-tryptone, 5 g of yeast extract, 10 g of NaCl, and dH_2O up to 1 L. Autoclave at 121°C for 15 min.
2. LB-agar: LB and 15 g/L agar. Autoclave at 121°C for 15 min.
3. Ampicillin stock solution (100 mg/mL).
4. YPD agar medium: Add 10 g of yeast extract, 20 g of peptone, and 20 g of glucose into dH_2O up to 1 L. Autoclave at 121°C for 15 min.
5. Minimal media (MM): Add 20 g of glucose, 1.7 g of yeast nitrogen base (YNB) without amino acids, 1.0 g of proline, and dH_2O up to 1 L, pH 6.1 (do not need to adjust). Autoclave at 121°C for 15 min.
6. Minimal Media + Uracil: Add 20 g of glucose, 1.7 g of YNB without amino acids, 1.0 g of proline, 0.1 g of uracil, 0.1 g of uridine, and dH_2O up to 1 L, pH 6.1 (do not need to adjust). Autoclave at 121°C for 15 min.
7. 5-FOA (5-fluoroorotic acid) (Zymo Research Corp., Orange, CA, USA).
8. Minimal media + 5-fluroorotic acid (MM + 5-FOA): Add 20 g of glucose, 1.7 g of YNB without amino acids, 1.0 g proline, and dH_2O up to 1 L, pH 6.1 (do not need to adjust). Autoclave at 121°C for 15 min. After autoclave, add 300 mg/L of sterile 5-FOA.
9. Fermentation medium (FM): Add 6.7 g of YNB, without amino acids and without ammonium sulfate, 6.0 g of KH_2PO_4, 2.0 g of K_2HPO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 mg of thiamine hydrochloride, 20 g of glucose, 5.0 g of yeast extract, and dH_2O up to 1 L. Autoclave at 121°C for 15 min.
10. FM without yeast extract (YE): Add 6.7 g of YNB, without amino acids and without ammonium sulfate, 6.0 g of KH_2PO_4, 2.0 g of K_2HPO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 mg of thiamine hydrochloride, 20 g of glucose, and dH_2O up to 1 L. Autoclave at 121°C for 15 min.
11. High glucose media (HGM): 80 g of glucose, 2.58 g of KH_2PO_4, 5.36 g of K_2HPO_4, pH 7.5 (do not need to adjust), and dH_2O up to 1 L. Sterilize by filtering through a 0.2-μm filter.
12. Transformation medium: 2.25 mL of 50% PEG (average MW 3350), 0.125 mL of 2 M lithium acetate (pH 6.0), and 0.125 mL of 2 M dithiothreitol (DTT) (see Note 7).

3. Methods

3.1. Cloning of crt Genes in the Integration Vector

1. Follow the specific steps of construction of integration vector for lycopene production outlined in Fig. 2. Set up a four-way ligation mixture containing the promoter, *crt* coding region, terminator, and the vector to clone each *crt* genes to (see Note 8).
2. Digest the plasmid containing codon-optimized *crt*E coding region with *Nco*I and *Not*I, and then gel-purify the fragment (see Note 9).
3. Digest the plasmid pZKleuN-6EP with *Bgl*II and *Nco*I, and then gel-purify the promoter FBAIN fragment.
4. Digest the plasmid pZKleuN-6EP with *Not*I and *Swa*I, and then gel-purify the *LIP1* terminator fragment.
5. Digest the plasmid pZKleuN-6EP with *Swa*I and *Bgl*II, and then gel-purify the vector fragment.
6. Set up a ligation reaction with above four fragments in ligation buffer with T-4 ligase, incubate the ligation reaction for 2 h, and then transform the ligation mixture into *E. coli* competent cells (see Note 10).
7. Name the resulting construct containing the FBAINr::*crtE*::LIP1 chimeric gene pYCRTE.
8. To clone the *crt*B coding region into pYCRTE, digest the plasmid containing synthetic *crt*B coding region with *Nco*I and *Not*I, and then gel-purify the fragment.

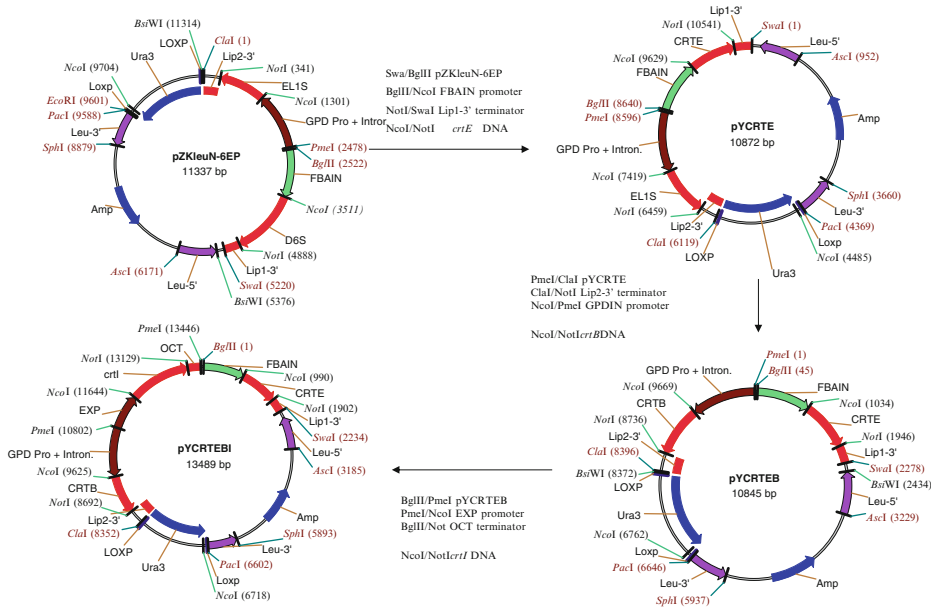

Fig. 2. Construction of integration vector for the expression of carotenoid genes in the chromosome of *Yarrowia lipolytica.*

9. Digest pZKleuN-6EP with *Pme*I and *Nco*I, and then gel-purify GPDIN promoter fragment.
10. Digest pZKleuN-6EP with *Cla*I and *Not*I, and then gel-purify the *LIP2* terminator fragment.
11. Digest pYCRTE with *Pme*I and *Cla*I, and then gel-purify the vector fragment.
12. Set up a ligation reaction with these four purified fragments in ligation buffer with T-4 ligase, incubate the ligation reaction for 2 h, and then transform the ligation mixture into *E. coli* competent cells (see Note 10).
13. Name the resulting construct containing FBAINr::*crtE*::LIP1 and GPDIN::CrtB::LIP2 chimeric genes pYCRTEB, which will be used to clone the *crtI* gene.
14. Digest the plasmid containing the synthetic *crtI* coding region with *Nco*I and *Not*I, and then gel-purify the fragment.
15. Digest the pEXPGUS1-P vector with *Pme*I and *Nco*I, and then gel-purify the EXP1 promoter fragment.
16. Digest pZP34R with *Bgl*II and *Not*I, and then gel-purify the OCT terminator fragment.
17. Digest pYCRTEB with *Bgl*II and *Pme*I, and then gel-purify the vector fragment.
18. Set up a ligation with all four fragments in ligation buffer with T-4 ligase, incubate the ligation reaction for 2 h, and transform the ligation mixture into *E. coli* competent cells (see Note 10).
19. Name the resulting construct containing FBAINr::crtE::LIP1, GPDIN::crtB::LIP2 and EXP::crtI::OCT three chimeric, pYCRTEBI.
20. Use pYCRTEBI digested with *Asc*I and *Sph*I to linearize the integration region from the vector backbone in the DNA transformations (see Note 11).

3.2. Integration of Three Chimeric crt Genes into Yarrowia Genome and Analysis of Lycopene Production

1. Streak *Y. lipolytica* Y2224 (Ura-) cells onto YPD agar plates 1 day prior to transformation.
2. Incubate the cultures at 30°C.
3. Transfer three large loops of cells from the YPD plates to 1 mL of transformation medium.
4. Mix 100–500 ng of digested plasmid DNA with 100 μL of the resuspended *Y. lipolytica* Y2224 (Ura-) cells.
5. Incubate the cell mixture at 39°C for 1 h. Mix the cell every 15 min using a Vortex mixer.
6. Spread the transformation mixture onto MM agar plates and incubate the plates at 30°C for 3–5 days (see Note 12).
7. Pick pink to red colonies from the plates.

8. Glow lycopene producing strain in 50 mL FM medium in a 250-mL flask and place the flask at 30°C in at shaker set at 250 rpm.
9. After 2 days of incubation, harvest 1 mL of culture by centrifugation at 12,000 × *g*.
10. Extract lycopene and analyze lycopene by HPLC (7). Commercial lycopene compound is used as standard.

3.3. Production of Lycopene Under Oil Accumulation Conditions

Oleaginous *Y. lipolytica* strain accumulates significant amounts of oil when grown in high glucose under nitrogen-limiting conditions.

1. Grow the lycopene producing *Yarrowia* strain in 100 mL FM medium in a 500-mL flask in 30°C shaker set at 225 rpm for 2 days.
2. Harvest 30 mL culture by centrifugation at 4,000 × *g*.
3. Resuspend the culture with 30 mL with HGM medium in a 125-mL flask.
4. Incubate the flask at a 30°C shaker (set at 225 rpm) for up to 4 days.
5. Analyze 1 mL of *Yarrowia* culture using HPLC analysis to determine the amount of lycopene produced (7).
6. Use rest of the cells for dry weight and oil analysis (8, 9).

4. Notes

1. *Bgl*II/*Nco*I fragment from pZKleuN-6EP; promoter region plus a portion of 5′ coding region that has an intron of the *FBA1* gene encoding for a fructose-bisphosphate aldolase enzyme (E.C. 4.1.2.13) of *Y. lipolytica*.
2. *Not*I/*Swa*I fragment from pZKleuN-6EP; terminator region of Lipase 1 gene of *Y. lipolytica*.
3. *Pme*I/*Nco*I fragment from pZKleuN-6EP; promoter region plus a portion of 5′ coding region that has an intron of the *GPD* gene encoding glyceraldehyde-3-phosphate-dehydrogenase of *Y. lipolytica*.
4. *Cla*I/*Not*I fragment from pZKleuN-6EP; terminator region of Lipase 2 gene of *Y. lipolytica*.
5. *Pme*I/*Nco*I fragment from pEXGUS1-P; promoter of the *EXP1* gene encoding EXP1 protein.
6. *Bgl*II/*Not*I fragment from pZP34R; terminator region of *OCT* gene encoding for 3-oxoacyl-coA thiolase of *Y. lipolytica*.
7. Prepare the DTT solution fresh prior to each use.
8. Instead of cloning, the each cassette containing the *crt* gene, promoter and terminator (Fig. 2) can be synthesized before

cloning into the integration vector. Alternatively, the entire integration region with all three genes and the necessary promoters and terminators can be synthesized to void multiple cloning steps.

9. All three synthetic *crt* genes contain an *Nco*I site around the start codon ATG and an *Not*I site after the translational stop codon.
10. We use the ligation kit from Promega. The ligation reaction has a final volume of 11 μL that contained 5.25 μL of 2× rapid ligation buffer, 0.75 μL of vector, 1.33 μL of each insert, and 1 μL of ligase. The reaction is allowed to proceed for 2 h at room temperature before transformation. After ligation, 2 μL of the reaction mixture is transformed into *E. coli* XL2-Blue ultracompetent cells. The transformed *E. coli* is spread on LB plates with ampicillin (100 μg/mL). Positive clones from each cloning step should be confirmed by restriction enzyme digestion and sequencing.
11. When transforming with integration plasmids, the DNA is linearized by digestion with appropriate restriction enzymes. There is no need to purify the fragment.
12. The integration fragment contains the *URA3* marker which confers growth selection for the integrated strain on MM plates.

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