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Iterative integration of multiple-copy pathway genes in *Yarrowia lipolytica* for heterologous β-carotene production

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ABSTRACT

β-Carotene is a terpenoid molecule with high hydrophobicity that is often used as an additive in foods and feed. Previous work has demonstrated the heterologous biosynthesis of β-carotene from an intrinsic high flux of acetyl-CoA in 12 steps through 11 genes in *Yarrowia lipolytica*. Here, an efficient biosynthetic pathway capable of producing 100-fold more β-carotene than the baseline construct was generated using strong promoters and multiple gene copies for each of the 12 steps. Using fed-batch fermentation with an optimized medium, the engineered pathway could produce 4 g/L β-carotene, which was stored in lipid droplets within engineered *Y. lipolytica* cells. Expansion of these cells for squalene production also demonstrated that *Y. lipolytica* could be an industrially relevant platform for hydrophobic terpenoid production.

1. Introduction

β-Carotene is used as a color additive or nutritional supplement for humans and animals, and is commercially produced by chemical synthesis or through *Blakeslea trispora* fermentation (Chandi and Gill, 2011; Mehta et al., 2003). Metabolic engineering is considered to be a promising strategy to optimize β-carotene biosynthesis, as quite a few compounds are now produced by engineered microbial cells on an industrial scale, including 1,3-propanediol (1,3-PDO), omega-3 eicosapentaenoic acid (EPA), 2,3-butanediol (2,3-BDO), and farnesene (Meadows et al., 2016; Nakamura and Whited, 2003; Sabra et al., 2016; Xue et al., 2013a).

Previous studies have focused on engineered *Escherichia coli* and *Saccharomyces cerevisiae* for heterologous β-carotene production (Kim et al., 2009; Lange and Steinbüchel, 2011; Li et al., 2015; Nam et al., 2013; Olson et al., 2016; Verwaal et al., 2007; Xie et al., 2014, 2015b; Yan et al., 2011, 2012; Yoon et al., 2007, 2009b; Zhao et al., 2013). For example, one strategy reported was to optimize β-carotene production in *E. coli* by strengthening the precursor pool of methylerythritol-phosphate (MEP) or mevalonic acid (MVA) (Nam et al., 2013; Yoon et al., 2009b; Zhao et al., 2013). The highest observed titer and yield reached 2.47 g/L with a content of 72 mg/g dry cell weight (DCW) (Nam et al., 2013). For *S. cerevisiae*, various strategies, including increasing the farnesyl pyrophosphate (FPP) supply and branch sterol synthesis pathway inhibition, have successfully been applied to enhance production up to 18 mg/g DCW (Lange and Steinbüchel, 2011; Miura et al., 1998; Verwaal et al., 2007; Xie et al., 2014; Yan et al., 2011, 2012).

As a hydrophobic carotenoid, β-carotene accumulates in the lipid bodies of *Dunaliella* algae to > 100 mg/g DCW (Katz et al., 1995; Wayama et al., 2013). As such, the oleaginous yeast *Yarrowia lipolytica* is considered to be better-suited than non-oleaginous *S. cerevisiae* or *E. coli* for the production of highly hydrophobic compounds (Sabirova et al., 2011; Xu et al., 2016). Moreover, the β-carotene precursor lycopene was also found to accumulate in the lipid bodies of *Y. lipolytica* (Matthäus et al., 2014). A sufficient supply of precursor compounds and

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energy sources—such as acetyl-coA, cofactor NADPH, and ATP (Blank et al., 2005; Wasylenko et al., 2015)—further demonstrates that *Y. lipolytica* could serve as a better natural host for β-carotene synthesis, as *S. cerevisiae* lacks acetyl-CoA (Chen et al., 2013; Lian et al., 2014).

As a generally-recognized-as-safe (GRAS) microorganism, *Y. lipolytica* has gained the attention of researchers in recent years (Beopoulos et al., 2009; Ledesma-Amaro and Nicaud, 2016; Xie et al., 2015a; Zhu and Jackson, 2015) and has been metabolically engineered to produce several compounds such as oleochemicals and terpenoids (Cao et al., 2016; Ledesma-Amaro and Nicaud, 2016; Matthäus et al., 2013; Rutter and Rao, 2016; Xu et al., 2016; Yang et al., 2016b). Famously, *Y. lipolytica* was engineered to produce EPA at an industrial scale by introducing 41 copies of 19 different genes followed by extensive random mutagenesis, high-throughput screening, and fermentation optimization (Xie et al., 2015a; Xue et al., 2013b). The typical genome engineering strategies of *Y. lipolytica* involve nonhomologous end-joining (NHEJ) and homologous recombination (HR). HR in *Y. lipolytica* is limited with respect to both the efficiency and the length of DNA integration because of the existence of non-specific NHEJ. As a result, subsequent high-throughput screening is required for every round of genome modification, which can generate unexpected genotypes (Xie et al., 2015a).

In this study, we evaluated whether *Y. lipolytica* can be a suitable host for heterologous β-carotene production. An efficient biosynthetic pathway capable of producing 100-fold more β-carotene than the initial baseline pathway was constructed using 11 β-carotene synthetic genes with strong promoters and sequential multiple-copy integration (Fig. 1).

2. Materials and methods

2.1. Medium and culture conditions

DH5α *E. coli* was grown at 37 °C with constant shaking in Luria–Bertani broth (Teknova) supplemented with 100 mg/mL ampicillin for plasmid propagation. All *Y. lipolytica* strains were cultivated at 30 °C with shaking at 240 rpm. Yeast extract peptone dextrose (YPD) medium consisted of 20g/L glucose, 20g/L peptone, and 10g/L yeast extract. SC-ura or SC-leu medium contained 20g/L glucose, 5g/L $(NH_4)_2SO_4$, 1.7g/L yeast nitrogen base with ammonium sulfate and without amino acids, and 50 mg/L uracil or 50 mg/L L-leucine, respectively. Modified YPD medium (YPDm) consisted of 30g/L glucose, 10g/L peptone, and 5g/L yeast extract.

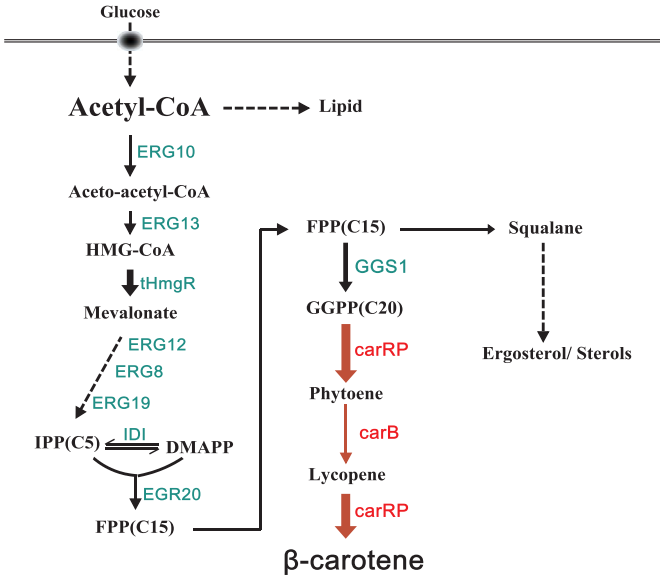

Fig. 1. β-carotene biosynthesis pathway in engineered *Y. lipolytica*. Exogenous and endogenous genes are indicated in red and green, respectively. Dashed lines represent multiple steps. DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HMG-CoA, hydroxymethylglutaryl-CoA. ERG8, phosphomevalonate kinase; ERG9, squalene synthase; ERG10, acetoacetyl-CoA, thiolase; ERG12, mevalonate kinase; ERG13, hydroxymethylglutaryl-CoA synthase; ERG19, mevalonate diphosphate decarboxylase; ERG20, geranyl/farnesyl diphosphate synthase; IDI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; tHmgR, truncated hydroxymethylglutaryl-CoA reductase; GGS1, GGPP synthase.

2.2. Construction of plasmids and strains

The primers used to construct the episomal expression cassettes and plasmids are shown in Supplementary Table S1. Plasmids are listed in Table 1. P_{TEF}-*ggs1-xpr2t*, P_{TEF}-*carRP-xpr2t*, P_{TEF}-*tHmgR-xpr2t*, and P_{TEF}-*carB-xpr2t* expression cassettes were digested with *Hind*III and *Sma*I after amplification with Hind3-tgx-F/Sma1-tgx-R primers for pTA-TGX, pTA-TRPX, pTA-THX, and pTA-TBX, respectively. The amplified fragments were then inserted into pMCSCen1 linearized with *Hind*III and *Sma*I. Four plasmids were constructed: pMCSCen1-ggs1, pMCSCen1-carRP, pMCSCen1-tHmgR, and pMCSCen1-carB. Detailed information on integrative plasmid construction is provided in the Supplementary Information.

DH5α *E. coli* was used for cloning and plasmid propagation. The *Y. lipolytica* strain ATCC MYA2613 served as the host strain, and all derivatives constructed in the present study are listed in Table 2. Fig. 2 and Fig. S1 shows the workflow used to generate the yeast strains. All strain genotypes were confirmed by colony polymerase chain reaction with KOD FX DNA polymerase (Toyobo Co., Ltd.; Shanghai, China) using the primers listed in Table S2. The products were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). Strains carrying episomal expression plasmids were cultivated in SC-leu medium for 5 days to produce carotenoids. Positive transformants with integrative cassettes were cultivated in YPD medium for 4 days to monitor carotenoids or squalene production. A Ura3 selection marker was recycled by incubating the transformants on YPD solid medium containing 1.2 mg/mL 5-fluoroorotic acid for 2 days. Larger colonies were then streaked onto SC and SC-ura plates, and incubated at 30 °C for 2–3 days.

2.3. Transformation

Y. lipolytica transformation with integrative fragments or episomal expression plasmids was performed with Zymogen Frozen EZ Yeast Transformation Kit II (Zymo Research Corporation). For episomal plasmid transformations, the cells were transformed with 1 μg plasmid and then 100 μL of the transformation mixture was plated onto SC-leu solid media. For integrative fragment transformations, approximately 2 μg of linearized DNA was used in the transformation reaction and then the cells were harvested by centrifugation at 13,000 × *g* for 2 min and plated on SC-ura agar. Selection plates were incubated at 30 °C for 2 days.

2.4. Analysis of β-carotene, lycopene, squalene, and mevalonate contents

DCW was measured with an analytical balance after the samples were dried at 85 °C for 24 h. In brief, cultured cells were harvested by centrifugation, re-suspended in 0.7 mL dimethyl sulfoxide, and then incubated for 10 min at 55 °C followed by 45 °C for 15 min after an equal volume of acetone was added. The samples were then centrifuged at 13,000*g* for 5 min. Supernatants containing β-carotene or lycopene were transferred to a new tube and the cell extracts were analyzed by high-pressure liquid chromatography (HPLC; Agilent Technologies 1200 Infinity Series system, Agilent, USA) with a variable-wavelength detector at 450 nm and an XDB-C18 column (5 μm, 4.6 × 150 mm, Eclipse, USA). Methanol, acetonitrile, and dichloromethane (42:42:16) were used in the mobile phase at a flow rate of 1.0 mL/min at 30 °C. Squalene was extracted using hot hydrochloric acid-acetone. In brief, the cells were harvested and treated with 3 M HCl at 95 °C for 5 min,