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Construction of a β-Carotene-Producing *Pichia pastoris* Strain

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Abstract:
In this study, a high-efficiency β-carotene-producing strain was developed using Pichia pastoris GS115 as the chassis organism. Key genes from Xanthophyllomyces dendrorhous—geranylgeranyl pyrophosphate synthase (GGPPxd), phytoene desaturase (CarB), and a bifunctional lycopene cyclase (CarRP^{Y72R}) with substrate inhibition relieved—were codon-optimized and integrated into the yeast genome through synthetic biology strategies. Expression of GGPPxd, CarB, and CarRP^{Y72R} was driven by the strong constitutive promoter P_{GAP}, enabling simultaneous multi-gene integration. Positive transformants were confirmed via genomic PCR, and engineered strains displayed visually distinct orange colonies on solid medium, reflecting characteristic β-carotene accumulation and allowing direct phenotypic screening. This work demonstrates the feasibility of using P. pastoris as a microbial cell factory for β-carotene production and lays a foundation for its industrial-scale application.
Key Word*: β-carotene; Pichia pastoris; microbial cell factory; synthetic biology; metabolic engineering*

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I. Introduction

β-Carotene is an orange-colored carotenoid widely present in fruits, vegetables, and algae. It is a fat-soluble isoprenoid compound found in both plants and microorganisms, with the chemical formula $C_{40}H_{56}$, a molecular weight of 536.88, and a melting point of approximately 178 °C. As a member of the tetraterpene family, β-carotene exhibits significant biological value, functioning as an antioxidant, enhancing immune responses, and possessing potential anticancer properties [1]. Structurally, β-carotene consists solely of carbon and hydrogen atoms, and its conjugated double bonds and central symmetry confer strong hydrophobicity and lipid solubility [2]. However, β-carotene is highly sensitive to light and heat, prone to degradation, and therefore requires storage under low-temperature and light-protected conditions [3]. Due to its unique structure and cleavage properties, β-carotene is the most effective provitamin A carotenoid, with extensive applications in pharmaceuticals, nutraceuticals, cosmetics, and the food industry [4]. As a potent antioxidant, it can quench singlet oxygen and scavenge reactive oxygen species, particularly peroxyl radicals, providing additional photoprotective effects [5,6].

Driven by increasing health awareness, the global nutraceutical market has expanded substantially, highlighting carotenoids as high-value bioactive compounds and prompting the urgent development of efficient biotechnological production platforms. In recent years, biotechnological approaches have demonstrated clear advantages in production efficiency, sustainability, product quality, and industrial adaptability, gradually becoming the preferred method for carotenoid synthesis. Advances in synthetic biology have enabled remarkable progress in β-carotene production, including innovative metabolic engineering strategies, fermentation optimization, and the diversification of host strains. Despite these achievements, current biotechnological approaches still face challenges in achieving economically viable industrial-scale production.

Previous studies have demonstrated that introducing carotenoid biosynthetic genes from *Xanthophyllomyces dendrorhous* into *Saccharomyces cerevisiae*, with overexpression of *crtYB* (encoding a bifunctional phytoene synthase and lycopene cyclase) and *crtI* (phytoene desaturase), can enable β-carotene biosynthesis [7]. Jing et al. [8] engineered *Yarrowia lipolytica* by expressing heterologous genes, relieving rate-limiting steps in the MVA pathway, enhancing cellular lipid synthesis, and increasing the copy number of key pathway genes. Using fed-batch fermentation, the engineered strain achieved β-carotene titers of 2.7 g/L. These findings indicate that increased intracellular lipid content promotes β-carotene accumulation and that multi-copy expression of key genes is an effective strategy to enhance metabolic flux. Similarly, expression of *CarB* and *CarRP* from *Mucor circinelloides* in *Y. lipolytica* significantly increased β-carotene production [9-11]. Notably, substrate inhibition of lycopene cyclase (*CarRP*) has been identified as a critical bottleneck. Through model-

guided protein engineering, a mutant enzyme, $CarRP^{Y72R}$, was obtained, which completely relieved substrate inhibition without compromising catalytic activity, leading to enhanced β-carotene yield.

Pichia pastoris has emerged as a robust heterologous protein expression platform due to its unique physiological characteristics and genetic engineering advantages. Its highly efficient non-homologous end joining (NHEJ) repair pathway allows direct genomic integration of donor DNA without the need for homologous arms. As a Crabtree-negative yeast, *P. pastoris* accumulates minimal by-products, channeling more carbon toward target metabolites. Moreover, it exhibits high tolerance under diverse environmental conditions and holds GRAS status, making it a safe and reliable industrial host. In this study, we aim to construct a β-carotene-producing microbial cell factory in *P. pastoris*, leveraging the NHEJ pathway for genomic integration and exploring its potential as a platform for industrial carotenoid production.

II. Material And Methods

2.1 Strains and Media

The yeast strain used in this study was *Pichia pastoris* GS115, maintained in our laboratory. YPD medium (per 100 mL) consisted of 2 g glucose, 0.5 g peptone, and 0.5 g yeast extract. During strain activation, Bleomycin was added to a final concentration of 100 μg/mL.

2.2 Acquisition of β-Carotene Biosynthetic Genes

Key enzyme-encoding genes with reported high β-carotene yields were introduced into *P. pastoris*. These include geranylgeranyl diphosphate synthase (*GGPPxd*), phytoene desaturase (*CarB*), and bifunctional phytoene synthase/lycopene cyclase ($CarRP^{Y72R}$). All genes were codon-optimized for *P. pastoris* and synthesized accordingly.

For genomic DNA extraction, the lysis buffer consisted of 0.2 M LiCl and 1% SDS. PCR amplification of target fragments was performed using high-fidelity DNA polymerase (Super Pfx Master Mix) in a 50 μL reaction system, containing 1 μL each of 10 mM forward and reverse primers, 1 μL plasmid template (~1 ng), and ultrapure water to 50 μL. PCR products were analyzed by agarose gel electrophoresis and the donor DNA fragments were purified for subsequent transformation.

2.3 Electroporation of *Pichia pastoris*

Single colonies from YPD plates were inoculated into 5 mL YPD medium and incubated at 30 °C, 180 rpm for 16–18 h. Seed cultures (1–2% inoculum) were transferred to 50 mL fresh YPD medium and grown to an OD_{600} of 0.6–0.8. Cells were harvested at 4 °C by centrifugation at 1,500 × g for 5 min, washed twice with ultrapure water, and resuspended in a pre-prepared transformation mixture. Cells were then washed twice with 1 M sorbitol and finally resuspended in 700 μL 1 M sorbitol to generate competent yeast cells. During electroporation, the suspension buffer contained 0.06 M DTT and 0.25 M LiCl. All procedures were performed at 4 °C, and cells were handled gently by tapping instead of pipetting to avoid damaging the competent cells and reduce loss of transformation efficiency.

2.4 Verification of Transformants

Single colonies from electroporated cells were inoculated into 5 mL YPD medium and cultured at 30 °C, 180 rpm for 16–18 h. Genomic DNA was extracted as follows: 200 μL culture was centrifuged and washed with ultrapure water. Cells were resuspended in 100 μL lysis buffer and incubated at 75 °C for 10 min. Subsequently, 300 μL anhydrous ethanol was added and mixed thoroughly, followed by centrifugation at 12,000 × g for 5 min. The pellet was washed with 500 μL 70% ethanol, centrifuged again, and residual ethanol was allowed to evaporate for 15 min. Finally, the pellet was resuspended in 30 μL ultrapure water, centrifuged briefly, and 1 μL of supernatant was used as template for PCR verification.

III. Result

3.1 Preparation of Donor DNA for β-Carotene Biosynthetic Genes

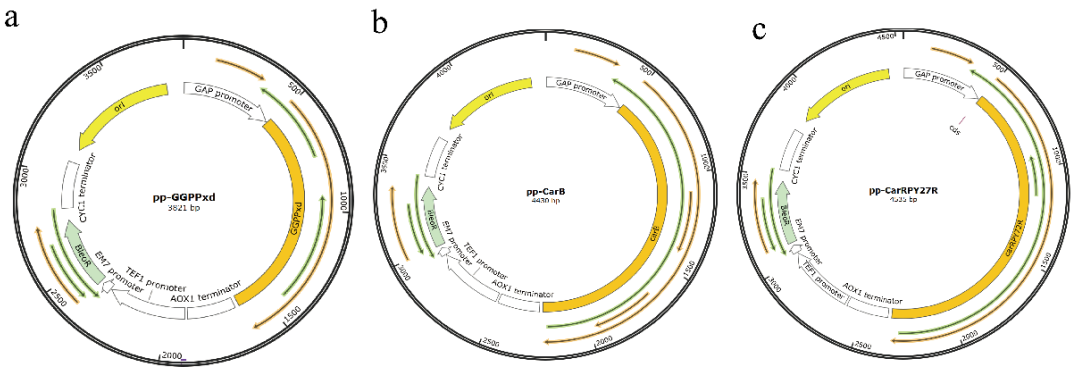

Figure 1: Schematic maps of the β-carotene biosynthetic gene expression plasmids: (a) pp-GGPPxd; (b) pp-CarB; (c) pp-CarRPY72R.

Codon-optimized expression cassettes were constructed using P_{GAP} as the promoter and T_{AOX1} as the terminator, resulting in plasmids pp-GGPPxd, pp-CarB, and pp-CarRPY72R (Fig. 1a–c).

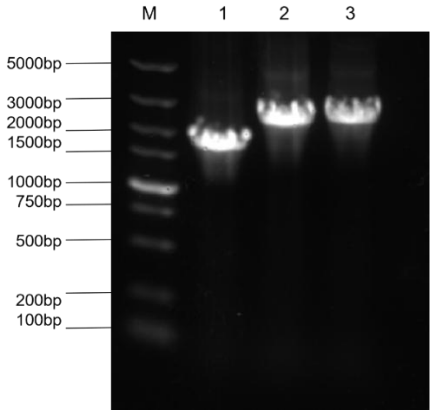

Figure 2: PCR amplification of full-length donor DNA fragments for β-carotene biosynthetic genes: Lane M, DL 5000 Marker; Lane 1, *GGPPxd*; Lane 2, *CarB*; Lane 3, *CarRP*^{Y72R}.

High-fidelity PCR amplification using Super Pfx Master Mix successfully generated the full-length donor DNA fragments for *GGPPxd*, *CarB*, and *CarRP*^{Y72R} (Fig. 2). The amplified cassettes were 1882 bp, 2491 bp, and 2596 bp, respectively, consistent with the expected sizes.

3.2 Transformation and Screening of Positive Clones

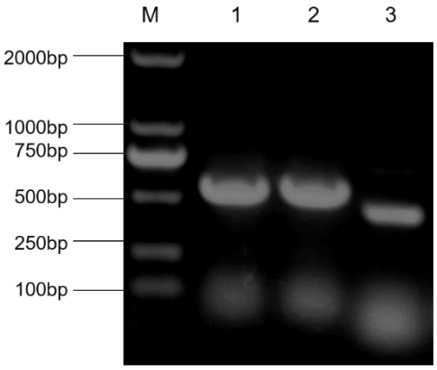

Figure 3: PCR verification of genomic integration of β-carotene biosynthetic genes in *P. pastoris* Lane M, DL 2000 Marker; Lane 1, *GGPPxd*; Lane 2, *CarB*; Lane 3, *CarRP*^{Y72R}.

Competent GS115 cells were prepared as described.Two micrograms of each donor DNA were added to 80 μL competent cells and electroporated at 2,500 V using a Bio-Rad system. Immediately after pulsing, 1 mL 1 M sorbitol was added for recovery. Cells were incubated at 30 °C for 2 h and plated on YPD plates containing Bleomycin.

Figure 4: Screening of positive transformants on YPD plates showing orange-colored colonies indicative of β-carotene accumulation.

Genomic DNA from transformants was verified by PCR (Fig. 3), producing expected bands of 541 bp (*GGPPxd*), 526 bp (*CarB*), and 387 bp ($CarRP^{Y72R}$). Positive clones carrying all three genes were streaked on YPD plates, producing visibly orange colonies. When cultured in liquid YPD medium at 30 °C, 180 rpm for 16 h and subsequently plated on YPD solid medium, descendant colonies retained the orange color (Fig. 4), confirming stable genetic integration.

IV. Discussion

β-Carotene is extensively used in the food, nutraceutical, pharmaceutical, and cosmetic industries, and its market demand has been steadily increasing in recent years. Therefore, developing an efficient, environmentally friendly, and sustainable method for β-carotene production is of paramount importance. With advances in metabolic engineering and synthetic biology, coupled with a deeper understanding of its biosynthetic pathways, microbial cell factories have emerged as one of the most promising platforms for β-carotene production.

In this study, a mutant cyclase, $CarRP^{Y72R}$, was introduced into *Pichia pastoris*. This enzyme completely eliminates substrate inhibition, effectively alleviating the rate-limiting bottleneck in lycopene-to-β-carotene conversion, providing a robust foundation for constructing high-yielding strains. Unlike conventional cyclases, $CarRP^{Y72R}$ maintains high catalytic activity while preventing metabolic flux blockage caused by the accumulation of intermediates.

V. Conclusion

The simultaneous integration of three genes into the genome via a single electroporation significantly shortens the engineering cycle. Furthermore, the visibly orange-colored colonies enable rapid, high-throughput screening, establishing a practical and efficient workflow for the development of β-carotene-overproducing microbial cell factories.

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