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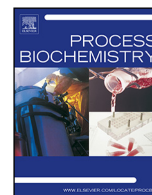

Targeted engineering and scale up of lycopene overproduction in *Escherichia coli*

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ABSTRACT

Engineering microorganisms for production of terpenoids has attracted considerable attention, and initial success was achieved using traditional metabolic engineering strategies or high throughput screening methods. Recently, we used a new targeted engineering strategy to leverage the mevalonate pathway to overproduce farnesene, but it was not clear if this strategy is applicable to production of other terpenoids. Here, we directly extend the information to lycopene production. Only two mutants were constructed, and the titer of lycopene in strain L3 easily reached 1.44 g/L in 2.5 L fed-batch fermentation. When the scale was increased to a 100 L working volume fed-batch fermentation in 150 L fermenter, up to 1.23 g/L (34.3 mg/gDCW) of lycopene was produced at 32 h after induction, the maximum productivity of this process reached up to 74.5 mg/L/h which demonstrates that the L3 fermentation process is easy scalable and that L3 could potentially replace the natural producer *Blakeslea trispora* in industrial production. The information could also be used to develop a highly efficient platform for overproduction of other terpenoids.

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1. Introduction

Lycopene is one of the major precursors to downstream carotenoids and is produced by many plants and microorganisms. It is of interest due to its attractive anti-oxidative, anti-cancer, and anti-inflammatory activities [1–4]. It has been used as a pharmaceutical compound, nutraceutical, functional food, and cosmetics additive, and the demand and market for lycopene has increased dramatically since it was first identified. Lycopene has been produced by extraction from natural materials (primarily tomatoes), microbial fermentation or chemical synthesis. Numerous efforts have focused on the fermentation of the natural lycopene producer *Blakeslea trispora*, and a semi-industrial fermentation was also reported [5–7]. Although 0.93 g/L lycopene was obtained from five days culture after these outstanding works, the industrial application of *B. trispora* is still hindered by its low growth rate and lycopene titer [8]. At the same time, engineering microorganisms to produce lycopene to meet the increasing demand has attracted much attention, with a particular focus on *Escherichia coli* [9,10]. It is not only because lycopene has potential benefits for human health as an antioxidant [2], but more importantly because lycopene has been extensively investigated as a model target for metabolic engineering [11–13].

The initial efforts focused on engineering the methylerythritol phosphate (MEP) pathway for lycopene production in *E. coli* [9,11,12,14–19]. Although a very high specific lycopene production up to 45 mg/g dry cell weight (gDCW) was achieved [20], however, only ∼260 mg/L of lycopene accumulated after 60 h in a high cell density fermentation of lycopene overproducing strains based on the MEP pathway [21]. This titer and productivity are too low to compete with those obtained from fermentation of the carotenogenic fungus *B. trispora* [8]. Beginning in 2006, attention focused on the mevalonate (MVA) pathway for lycopene overproduction, and ∼102 mg/L of lycopene was produced with mevalonate supplementation [22]. The titer was

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Table 1
Plasmids used in this study.

Plasmids	Backbone	Origin	Description	Source
pMH1	pBBR1MCS-1	p15A	plac: AtoB, ERG13 and N-terminal His-tagged tHMG1, Cam^+	[24]
pFZ81	pBBR1MCS-2	pBBR1MCS	plac: ERG12, ERG8, MVD1 and N-terminal His-tagged Idi, Kan^+	[24]
pFZ21	pET28a(+)	pBR322	pT7: N-terminal His-tagged CrtE, Kan^+	This study
pFZ22	pET28a(+)	pBR322	pT7: N-terminal His-tagged CrtB, Kan^+	This study
pFZ23	pET28a(+)	pBR322	pT7: N-terminal His-tagged CrtI, Kan^+	This study
pFZ27	pET28a(+)	pBR322	pT7: N-terminal His-tagged CrtE, CrtB and CrtI, Kan^+	This study
pFZ110	pET21a(+)	pBR322	pT7: N-terminal His-tagged CrtE, CrtB and CrtI, Amp^+	This study
pFZ111	pET21a(+)	pBR322	pT7: N-terminal His-tagged CrtE, CrtB, CrtI and Idi, Amp^+	This study

further improved with systematic optimization of the carbon sources, which achieved up to 1.35 g/L in 2 L fed-batch fermentation with a productivity of 39.7 mg/L/h and specific production of ~32.1 mg/gDCW. This indicates that the engineered *E. coli* may be used for commercial production of lycopene [23].

However, these achievements are not yet scalable to the levels needed for industrial production. Recently, we optimized production of farnesene in *E. coli* through manipulation of the MVA pathway, an approach that may be more broadly applicable to production of other molecules of interest which was designated as targeted engineering. To accomplish this, we first reconstituted the MVA pathway *in vitro* and systematically analyzed the steady-state kinetic and biochemical parameters. Isopentenyl diphosphate isomerase (Idi) was identified as one of the key enzymes of MVA pathway. The detailed information gained from the optimization of this *in vitro* reconstituted system was then used to directly guide the optimization of *in vivo* farnesene production in *E. coli*. Through relatively few steps of targeted engineering, up to 1.1 g/L farnesene was produced at a shake-flask scale. The results of targeted proteomic and intermediate assays indicated that protein expression levels in MVA pathway, substrates, and cofactor supply need to be further engineered [24]. We were eager to determine whether the information gained from the *in vitro* reconstituted system can be used to develop a universal, highly efficient platform to provide the precursors IPP and DMAPP for terpenoid overproduction. To address this, we transferred the information and platform from farnesene production to lycopene overproduction. Guided by information gained from farnesene engineering, a highly efficient lycopene overproducing strain was easily constructed. The fed-batch fermentation process was successfully scaled up to 100 L, and a very high productivity and titer was achieved, demonstrating the potential of this approach to replace the natural producer *B. trispora* for industrial production.

2. Materials and methods

2.1. Plasmids for lycopene overproduction

To produce lycopene in *E. coli*, *crtE*, *crtB* and *crtI* from *Pantoea ananatis* [25] were codon optimized, synthesized by Genescript (DNA sequence is shown in Suppl. Table I), and inserted into pET28a(+) to give pFZ21, pFZ22 and pFZ23. Subsequently, the *Xba*I-*Xho*I fragments of *crtB* and *crtI* were inserted into pFZ21 sequentially to produce pFZ27. The *Xba*I-*Xho*I fragment of pFZ27 was inserted into pET21a(+) to produce pFZ110. An additional copy of *idi* was introduced into pFZ110 by inserting the *Xba*I-*Xho*I fragment of *idi* into *Spe*I-*Xho*I fragment of pFZ110 to produce pFZ111 (Table 1).

2.2. Shake-flask fermentation and analysis of lycopene titer in engineered strains

To compare the lycopene production characteristics in different engineered strains, several colonies of each strain were inoculated into 5 mL 2×TY medium with 2% glycerol and appropriate antibiotics (100 μg/mL ampicillin, 50 μg/mL kanamycin, and 34 μg/mL chloramphenicol), which was cultured overnight at 30 °C. One and a half mLs of each seed culture was in turn used to inoculate 150 mL 2×TY medium with 2% glycerol and appropriate antibiotics in a 500-mL flask, which was grown at 30 °C. When the OD_{600} reached 0.6 to 0.8, IPTG was added to a final concentration of 0.1 mM for induction. Cells of each engineered strain in 1 mL culture were harvested for analysis by periodic withdrawal and stored at −40 °C until further analysis. The lycopene accumulated in cell pellets were extracted with acetone at 55 °C for 15 min in dark and measured at OD_{474}. The titer of lycopene was calculated using a standard curve with appropriate dilution factor and averaged for three replicate analyses as described previously [11]. The cell mass was counted by OD_{600} with a coefficient of 0.3 gDCW/OD_{600}.

2.3. Determination of the metabolic status of each mutant

Mass spectrometry (MS)-based targeted proteomic analysis and intermediate analysis were performed to determine the metabolic status of each mutant as previously described [24]. Although these two methods were just semi-quantitative, we were able to compare the amount of same component (exact protein or intermediate) in different strains. Cells were cultured as described previously for shake-flask fermentation and harvested 20 h after induction. For each mutant, four copies of 200 OD_{600} cells were sampled from a flask, immediately quenched and extract for relative intermediate analysis, and then the rest cells were harvested for relative protein analysis. For relative protein quantification, the harvested cells were resuspended in 100 mM ammonium bicarbonate and lysed by sonication, after centrifugation at 200,000 × g for 1 h, one hundred μg total proteins of each mutant was first digested with trypsin and analyzed on AB SCIEX Q-Trap 4500 mass spectrometer (AB SCIEX) with four replicates of injection. The process of MS based quantification and data analysis was performed as previously described [24].

2.4. Fed-batch fermentation of strain L3 (2.5 L)

To create the seed culture, several single colonies of strain L3 were grown in 10 mL LB medium with 2% glycerol overnight at 30 °C in a rotary shaker at 220 rpm, and then subcultured in 2 × 100 mL M9 medium (10 g/L $(NH_4)_2SO_4$, 8.5 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L sodium citrate, 40 g/L glycerol, 0.063 g/L $CaCl_2$, 10 mL/L thiamine solution, 4.17 mL/L vitamins, and 4.17 mL/L metals solutions) [26] (or 2×TY medium) with 2% glycerol at 30 °C until OD_{600} reached 3. Two bottles of the seed culture (2 × 100 mL) were inoculated into 2.3 L M9 medium with 2% glycerol in 7.5 L New Brunswick 310 fermenter for fed-batch fermentation. Fed-batch fermentation was maintained at 30 °C and pH was maintained at 7.2 using 9.9 N NH_4OH (NH_4OH:H_2O = 2:1, v/v). The initial agitation was set to 200 rpm and foam was controlled by adding antifoam 204. When OD_{600} reached ~15, the culture was fed a sterile glycerol (feed solution: 500 g/L glycerol, 2 g/L $MgSO_4{\cdot}7H_2O$) at a constant rate

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