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Laboratory-Scale Production of ^{13}C -Labeled Lycopene and Phytoene by Bioengineered *Escherichia coli*

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Abstract

Consumption of tomato products has been associated with decreased risks of chronic diseases such as cardiovascular disease and cancer, and therefore the biological functions of tomato carotenoids like lycopene, phytoene and phytofluene are being investigated. In order to study the absorption, distribution, metabolism and excretion of these carotenoids, a bioengineered *Escherichia coli* model was evaluated for laboratory-scale production of stable isotope-labeled carotenoids. Carotenoid biosynthetic genes from *Enterobacter agglomerans* were introduced into the BL21Star(DE3) strain to yield lycopene. Over 96% of accumulated lycopene was in the all-*trans* form, and the molecules were highly enriched with ^{13}C by ^{13}C -glucose dosing. In addition, error-prone PCR of phytoene desaturase (*crtI*) was used to create a phytoene-accumulating strain, which maintained the transcription of phytoene synthase (*crtB*), whereas *crtI* does not encode a functional phytoene desaturase because of introduced mutations. Phytoene molecules were also highly enriched with ^{13}C when the ^{13}C -glucose was the only carbon source. The development of this production model will provide carotenoid researchers a source of labeled tracer materials to further investigate the metabolism and biological functions of these carotenoids.

Keywords

Tomato; Lycopene; Phytoene; ^{13}C ; *E. coli*

Introduction

Tomato consumption has been correlated with decreased risks of chronic diseases including prostate cancer and cardiovascular disease ¹. Among many nutrients and phytochemicals in fresh tomatoes and tomato products, carotenoids are thought to be the bioactive components responsible for disease prevention. Many epidemiological studies, clinical trials, animal studies, and *in vitro* studies have focused on the most abundant tomato carotenoid, lycopene ². However, the absorption, distribution, metabolism and excretion (ADME) of phytoene and phytofluene, the precursors of lycopene and the 2nd- and the 3rd-most abundant tomato carotenoids, have not been fully studied.

As it is difficult to completely deplete the fat-soluble carotenoids from humans, stable isotope-labeled carotenoid tracers allow a single carotenoid dose to be followed within pre-

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existing, endogenous pools. Previously, labeled lutein, β -carotene or vitamin have been utilized to provide information regarding 1) the fraction of the ingested amount that is absorbed (bioavailability), 2) the fraction of that which is converted to vitamin A in the body (bioconversion), 3) the estimated body stores of vitamin A, and 4) the interaction between retinal and rhodopsin^{3–8}. To date, no tracer studies utilizing isotope-labeled phytoene or phytofluene have been conducted, either in animals or humans, and therefore very little is known regarding the ADME. Producing labeled phytoene and phytofluene has been a challenge to answer these important biomedical questions.

Currently, most of the commercially pure carotenoids are produced by chemical synthesis, whereas a few biotechnological production systems including microalgae, fungi, tomato cell culture and *E. coli* culture are being developed and optimized. Several concepts for the phototrophic mass culture of algae have been proposed and compared^{9–11}. Among many fungal strains, *Blakeslea trispora* has been employed for the production of natural lycopene, commercialized by Vitatene. In addition, cell suspension cultures of tomato (*Lycopersicon esculentum* cv. VFNT cherry) have also been developed for laboratory scale production of phytoene, phytofluene and lycopene in our laboratory¹². This system was further optimized by treating cells with two bleaching herbicides, 2-(4-chlorophenyl-thio)triethylamine (CPTA) and norflurazon and utilization of high carotenoid producing cell lines^{13,14}.

It has been well known that *Escherichia coli* harboring carotenogenic genes from a Gram-negative, non-photosynthetic bacterium, *Enterobacter agglomerans* (or *Pantoea agglomerans*, formally *Erwinia herbicola*), can accumulate carotenoids (Figure 1). This carotenogenic gene cluster was first cloned from *E. agglomerans* in 1986¹⁵, and the DNA sequence was reported in 1990¹⁶. Since then, novel carotenoids were produced in *E. coli* by combining different carotenoid biosynthetic genes from *E. agglomerans* and other species^{17–20}. Additionally, carotenoid production was tremendously improved by introducing the mevalonate pathway²¹, increasing precursor supply²² or by knocking out unnecessary genes in *E. coli*^{23–25}. Since *E. coli* can utilize glucose as the sole carbon source, and ¹³C-glucose is a readily available labeled source material, this system could be used to produce isotopically-labeled carotenoids. Therefore, the goal of this research was to utilize carotenoid-producing *E. coli* for producing stable isotope-labeled tomato carotenoids, lycopene and phytofluene, for future biomedical research.

Materials and Methods

Chemicals and reagents

All bacterial strains and culture media were purchased from Invitrogen (Carlsbad, CA), and primers for PCR were synthesized by Integrated DNA Technologies (Coralsville, IA). All restriction enzymes were purchased from New England Biolabs (NEB, Ipswich, MA), and molecular biology kits were purchased from Qiagen (Valencia, CA). All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO), and all organic solvents were of HPLC grade and purchased from Fisher Scientific (Pittsburg, PA).

Construction of truncated plasmid pAC-PTE-TR

The plasmid pAC-LYC (Cunningham et al.²⁶), which contains GGPP synthase (*crtE*), phytoene synthase (*crtB*) and phytoene desaturase (*crtI*) from *E. agglomerans*, was digested by restriction enzymes Sbf I and Bgl II (NEB), and the digested products were separated on a semi-preparative 0.8% agarose gel. The larger DNA fragment, containing the pACYC-184 backbone, was retrieved with a Qiagen Gel Extraction Kit, blunted by a NEB Quick Blunting Kit and ligated by a NEB Quick Ligation Kit. This truncated pAC-LYC, named pAC-PTE-TR hereafter, was amplified by *E. coli* strain DH5 α and confirmed with

electrophoresis and DNA sequencing by an ABI 3730XL capillary sequencer at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

Construction of mutated plasmid pAC-PTE-MT

GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to create the mutant library. The gene *crtI* was amplified by sense primer 5'-TGACATATGAAAAAACC GTTGTGATTGGCGCAGGC -3' and anti-sense primer 5'-ATTCTCGAGTTATCAGGCTGGCGGTGGCTTTTCG -3' per the manufacturer's instructions for high-error-rate PCR. The PCR products were then purified by a Qiagen PCR Purification Kit and mixed with pAC-LYC. The mixture was digested by restriction enzymes SbfI and BglII, purified by Qiagen PCR Purification Kit, ligated by NEB Quick Ligation Kit, and transformed into *E. coli* strain DH5 α . The transformants were grown on LB agar plates with the antibiotic, chloramphenicol (34 μ g/mL), and white colonies were amplified and screened by HPLC for phytoene production. Plasmid pAC-PTE-MT was isolated by a Qiagen Miniprep Kit and the selected mutated-*crtI* was sequenced.

Bacterial culture in LB broth

Escherichia coli strain BL21Star(DE3) and the transformants were grown and maintained in LB broth supplemented with 34 μ g/mL of chloramphenicol at 31°C, 200 rpm. Overnight cultures were used to inoculate 5 mL cultures with a ratio of 1:100, and the cells were grown at 31°C, 200 rpm for 12, 18, 24 and 48 h. Cells were harvested by 3,220 $\times$ g centrifugation at 4°C for 10 min, washed with 1X cold phosphate buffered saline (PBS), weighed and frozen at -80°C until further analysis.

Bacterial culture in 2XM9 minimal salts media

For carotenoid accumulation and labeling experiments, BL21Star(DE3) and the transformants were grown in 2XM9 media, which contained 5 g/L unlabeled or [U]-¹³C-glucose, 34 μ g/mL of chloramphenicol and doubled amount of salts in 1X M9 media, except for CaCl₂ and MgSO₄. Overnight cultures from LB were used to inoculate 5 mL cultures with a ratio of 1:100, and the cells were grown at 31°C, 200 rpm for 12, 18, 24 and 48 h. LB broth was removed by centrifugation before cells were inoculated in 2XM9. Cells were harvested by 3,220 $\times$ g centrifugation at 4°C for 10 min, washed with 1X cold PBS, weighed and frozen at -80 °C until further analysis.

Analysis of glucose and acetate by HPLC

Glucose and acetate concentrations of culture media were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) kept at 50°C. The mobile phase was 0.005 N of H₂SO₄ with a flow rate of 0.6 mL/min. The culture media was injected into the system without further purification and the quantification of glucose was calculated based on a standard curve.

Carotenoid extraction

Bacterial cell pellets were thawed on ice, suspended in 50 μ L of deionized water and briefly vortexed to achieve homogeneous suspensions. Acetone (500 μ L) was then added and followed immediately by a 10-s vortex period and 5-min water-bath sonication. A total of 200 ng of all-*trans* beta-carotene (DSM, Basel, Switzerland) was added as an internal standard, since the system did not contain any of this cyclic carotenoid. The liquid and solid phases were then separated by 4 °C centrifugation at 16,000 $\times$ g for 10 minutes. The acetone/water phase was transferred to clean centrifuge tubes, evaporated by argon and partitioned by 500 μ L of hexanes:methyl *tert*-butyl ether (MTBE) (1:1, v/v) two times. The

extracts were combined and dried down under argon. The samples were stored at -20°C for no more than 24 hours before HPLC analysis, with the entire extraction process performed under yellow light.

Analysis of carotenoids by HPLC

The HPLC system is composed of two pumps (Model SD-200, Rainin Dynamax, Walnut Creek, CA), a YMC Carotenoid C30 column (4.6×250 mm, 3 μm) with a guard column, an 18°C column cooler and a photodiode array detector (Model 2996, Waters, Milford, MA). The mobile phases A (methanol: 15% ammonium acetate aqueous solution= 98:2, v/v) and B (methanol: MTBE: 15% ammonium acetate aqueous solution= 8:90:2, v/v) were used, with the following gradient profile: 0 min, 0% B; 30 min, 100% B; 40 min, 100% B; 45 min, 0% B; 50 min, 0% B. Samples were reconstituted in MTBE for injection and phytoene was measured at 286 nm. Phytoene standards (BASF, Ludwigshafen, Germany) were prepared in petroleum ether using extinction coefficient $A^{1\%}_{1\text{cm}}$ 1250 at 286 nm. Lycopene standards (DSM, Basel, Switzerland) were prepared in hexane using extinction coefficient $A^{1\%}_{1\text{cm}}$ 3450 at 472 nm, and all-*trans* beta-carotene standards (DSM, Basel, Switzerland) were prepared in n-hexane using extinction coefficient $A^{1\%}_{1\text{cm}}$ 2592 at 452 nm.

Mass spectrometry

Mass spectrometry was performed by the Mass Spectrometry Center in the School of Chemical Sciences at the University of Illinois at Urbana-Champaign. Unlabeled and labeled samples were purified by HPLC separation and completely dried before sample submission. The lycopene samples were dissolved in acetone and analyzed by Micromass Q-ToF Ultra (Waters, Milford, MA) with electrospray ionization positive mode (ESI+) and high-resolution scanning. The phytoene samples were dissolved in anisole²⁷ and analyzed by ThermoFinnigan LCQ Deca XP (Thermo Scientific, West Palm Beach, FL) with atmospheric pressure chemical ionization positive mode (APCI+) and low-resolution scanning.

RNA extraction and quantitative real-time PCR of *crtE* and *crtB*

Total RNA was extracted from cells using Trizol (Invitrogen) per the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix (Applied Biosystems). Reactions were monitored by an ABI Prism 7900HT. Primer pairs were designed to measure GGDP synthase (*crtE*) (sense: 5'-GTCAGCCCACTACCCACAAAA-3'; anti-sense: 5'-GCGGCGATCAGACCAAAG-3'), phytoene synthase (*crtB*) (sense: 5'-GATGAGGGGGTGGTGGAT-3'; anti-sense: 5'-CAATAGCCGCATCGTCAATAATAT-3'), and 16s (*rrsB*) (sense: 5'-GCATAACGTCGCAAGACCAA-3'; anti-sense: 5'-GCCGTTACCCACCTACTAGCT-3'). A validation experiment was performed on each set of primers to confirm efficiency and product specificity. A serial dilution was used to create a standard curve for quantification, and 16s was used as a housekeeping gene.

Statistical analysis

Microsoft Excel for Mac 2011 was used to perform Student's t-test. Figures were produced by SigmaPlot 2001 (Systat Software Inc., St. Jose, CA).

Results and Discussion

Lycopene accumulation in BL21Star(DE3) harboring pAC-LYC

The *E. coli* strain BL21Star(DE3) harboring the plasmid pAC-LYC, created by Cunningham *et al.*²⁶, was used for lycopene production. Prior to the labeling run, *E. coli* was maintained in LB agar or LB broth. For the labeling run, 2X modified M9 minimal medium was used, which contained doubled nitrogen, and [U]-¹³C-glucose as the only carbon source to maintain growth characteristics of *E. coli* that were similar to the culture in LB broth. Initially, ¹²C-glucose was provided in the 2X M9 minimal medium at the 5 g/L concentration to observe lycopene accumulation; glucose was completely depleted by 24 h (Figure 2A). Over 60% of glucose was used between 12 h and 24 h, which suggested that the cells were in the logarithmic growth phase, which was further confirmed by measuring the OD600 (data not shown) and the wet cell weight (Figure 2A). Wet cell weight reached its maximum at 24 h, and a trace amount of acetate accumulated in the media at 24 h and depleted by 48 h.

Lycopene concentration was also measured during the 48 h culturing period (Figure 2B). From a 5 mL culture, a total of 1.4 µg lycopene was obtained, 14%, 71% and 15% of which was produced during 0–12 h, 12–24 h and 24–36 h, respectively. Compared with the glucose concentration in the medium, *E. coli* seemed to continue producing lycopene even though the glucose had been depleted. The metabolic intermediates, acetate or pyruvate, which supported the lycopene production, would potentially be utilized by *E. coli* during 24–36 h. Interestingly, during the course of culture, the percentage of all-*trans* lycopene increased over time, and reached over 96% by 24 h (data not shown), which is representative of its level of abundance in tomatoes. Less than 4% of total lycopene was 9-*cis* and 13-*cis*, and no other lycopene isomers were detected. Trace amounts of phytoene were observable only from the 12 h samples, indicating that the phytoene desaturase from *E. agglomerans* was highly efficient in catalyzing the desaturation reaction, which led to the accumulation of the major product, lycopene.

Isotopomer distribution of ¹³C-labeled lycopene

High-resolution mass spectrometry was used to observe the isotopomer distributions of unlabeled and labeled lycopene (Figure 3). HPLC-purified material was ionized by electrospray ionization and analyzed by a quadrupole mass sector and a time-of-flight mass sector. Under the positive mode of ionization, it was clear that the ion m/z 536.4 [M⁺] was the majority of the unlabeled lycopene, followed by the ions m/z 537.4, 538.4 and a few other ions ranging between m/z 546.6 and m/z 568.5. Since the natural abundance of ¹³C is 1.109%, it is expected that a few lycopene molecules would naturally contain some ¹³C, which resulted in the observation of ions with a higher m/z ratio. Comparing the mass spectra of labeled lycopene with unlabeled lycopene, interestingly, the majority of ions are m/z 576.6 [M+40⁺], followed by the ions m/z 575.6 [M+39⁺], and 574.6 [M+38⁺], which indicates that the biosynthesized lycopene was mostly built from the [U]-¹³C-glucose and is highly enriched with ¹³C (Figure 3).

Error-prone PCR rescued phytoene production by maintaining *crtB* transcription

In the *E. agglomerans* carotenogenic gene cluster, there are two promoter regions reported by To *et al.*²⁸. One of the promoters was located within the gene *crtE*, and another was located within the gene *crtI*. Therefore, deletion of *crtI* could result in the disruption of *crtB* transcription derived by the promoter within *crtI*. Therefore, two strategies were applied to create phytoene-accumulating strains: enzymatic digestion and error-prone PCR. Both methodologies were applied to knockout the gene *crtI* on pAC-LYC and to dysfunction phytoene desaturase, resulting in two plasmids, pAC-PTE-TR (representing “truncated”) and

pAC-PTE-MT (representing “mutated”), respectively (Figure 4A). A total of 948 base pairs of *crtI* open reading frame (ORF) (65% of *crtI*) were removed from pAC-LYC to create pAC-PTE-TR, whereas the pAC-PTE-MT maintained the same length of ORF as pAC-LYC, but had mutations in *crtI*. The mutated *crtI* ORF in the pAC-PTE-MT was sequenced, and the result showed that 101 amino acids were deleted from the protein, and 3 amino acids were inserted to the protein by mutations (Figure 4B).

Both plasmids were introduced into BL21Star(DE3) and the phytoene accumulation patterns were evaluated. Both plasmids resulted in phytoene accumulation, and both strains, which were cultured in LB broth, accumulated higher concentrations of carotenoids than in minimal medium. Interestingly, *E. coli* harboring pAC-PTE-MT accumulated more phytoene than the one harboring pAC-PTE-TR (Figure 5). The transcription of either GGDP synthase (*crtE*) or phytoene synthase (*crtB*) was potentially affected by the enzyme digestion of *crtI*, and therefore the real-time PCR was used to evaluate total RNA level of *crtE* or *crtB* among tested strains. The RNA expression of *crtE* was slightly higher in pAC-PTE-TR compared with pAC-LYC or pAC-PTE-MT (Figure 6A), but the expression of *crtB* was significantly decreased (Figure 6B).

In this study, the deletion of *crtI* ORF reduced the mRNA transcription of *crtB* and further resulted in decreased phytoene accumulation, suggesting that the promoter within the *crtI* ORF is involved in the transcription of *crtB*. Using error-prone PCR methodology, a mutated *crtI* that potentially maintained the promoter region within *crtI* was generated; this promoter maintained the transcription of *crtB* and resulted in a higher phytoene accumulation compared with truncated *crtI*. In addition, there may be a polar effect between *crtI* and *crtB* in which the mRNA is more stable if the two genes are transcribed together. Therefore, error-prone PCR rescued phytoene production by maintaining *crtB* transcription, and the *E. coli* harboring pAC-PTE-MT was selected as the model for further ¹³C-labeling.

In addition, hundreds of mutant colonies harboring mutated *crtI* as well as functional *crtB* and *crtE* were screened by UV lamp to locate a phytofluene-accumulating colony, but none of the colonies was identified to accumulate phytofluene. All screened mutants accumulated phytoene. Many studies have characterized diverse products by combining *crtI*-type phytoene desaturases from different species in *E. coli*²⁹. Although the active site of phytoene desaturase from *E. agglomerans* has not been thoroughly studied, based on our error-prone PCR results, we suspect that once the active site is disabled, the enzyme entirely loses its function and is unable to catalyze any desaturation reaction. Error-prone PCR could not generate a mutated phytoene desaturase that only catalyzes 1, 2 or 3 steps of the desaturation cascade.

Phytoene accumulation in BL21Star(DE3)

The *E. coli* strain BL21Star(DE3) harboring pAC-PTE-MT was grown in 2XM9 minimal medium containing 5 g/L ¹²C-glucose, and its growth characteristics were similar to the strain harboring pAC-LYC (Figure 7A). Over 60% of glucose was used between 12 h and 24 h, and the wet cell weight reached its maximum at 24 h. The total phytoene accumulation per 5-mL-culture reached 836 ng by 24 h and further reached to 896 ng by 36 h (Figure 7B). Accumulated phytoene were mainly 15-*cis* and all-*trans* phytoene, and no other phytoene isomers were detected. Interestingly, the phytoene accumulation per gram of wet cells reached its steady state at 12 h and maintained at a similar level, indicating that phytoene accumulation was taking place while *E. coli* was growing. After *E. coli* reached its maximum cell mass, the synthesis of phytoene stopped, which is different from the pattern observed with lycopene accumulation. A trace amount of acetate was also accumulated in the media at 24 h and depleted by 48 h.

Phytoene synthase catalyzes a condensation reaction and yields phytoene, whereas phytoene desaturase catalyzes desaturation reactions and yields lycopene. In general, a desaturation reaction is not spontaneous but requires an energizing component. Unlike fatty acid desaturation, which yields an isolated double bond by NADH-dependent hydroxylation and water abstraction, desaturation of phytoene and other carotenes is thermodynamically favored due to the resulting extension of the conjugated double bond system, which is an exergonic reaction²⁹. Therefore, the desaturation reaction might be continued even though the glucose was depleted in the media, whereas the condensation reaction was stopped.

Isotopomer distribution of ¹³C-labeled phytoene

Mass spectrometry showed that the ion m/z 544.3 was the major ion for the unlabeled phytoene, followed by m/z 545.2 and m/z 546.3 (Figure 8), which was similar to what we observed with unlabeled lycopene sample. Comparing mass spectra of labeled phytoene with unlabeled phytoene, we observed the majority of ions shifted to m/z 584.4 [M+40], followed by the ions m/z 583.4 [M+39], which indicates that the biosynthesized phytoene is enriched with ¹³C. Mass spectrometry of phytoene was performed using APCI under positive mode, which led to a detectable signal, while ESI did not. A similar finding was reported by Rivera *et al.*²⁷.

Advantages and disadvantages of using *E. coli* to produce isotopically labeled carotenoids

Bioengineered *E. coli* have the potential to generate a wide variety of carotenoids by combining different carotenogenic genes from different bacteria. For example, combining phytoene synthase from *E. agglomerans* and phytoene desaturase from a *Rhodobacter* species that catalyzes only 3 steps of desaturation would generate molecules like beta-zeacarotene and hydroxyneurosporene, instead of lycopene³⁰. Furthermore, molecular breeding or *in vitro* evolution techniques have also been applied to create novel carotenoids in *E. coli*. Schmidt-Dannert *et al.* expressed a shuffled phytoene desaturase in *E. coli* and created a fully conjugated carotenoid, 3, 4, 3', 4'-tetrahydrolycopene¹⁹. The C30 pathway was also tested and a novel cyclic carotenoid, C₃₀-diapotorulene, was produced³¹. Therefore, by utilizing an *E. coli* system, labeled carotenoids with novel or rare structures may be easily biosynthesized. In addition, since [U]-¹³C-glucose is the only carbon source in the minimal medium, ¹²C carry-over could be low, resulting in highly-uniformly labeled products.

E. coli is relatively easy to maintain and culture for a biosafety level-2 laboratory and the operation can be managed by an entry-level scientist. In addition, laboratory-scale production can be performed on site, which can provide freshly biosynthesized carotenoids to avoid oxidized or degraded contaminants. In contrast, chemical synthesis of carotenoids is lengthy and it requires experienced organic chemists and a sophisticated organic chemistry laboratory. For mass spectrometry internal standard purposes, *E. coli* produces customized labeled carotenoids in 36 h, and the purification process is relatively simple compared with the extractions from other biotechnological production systems, such as tomato cell culture³² or microalgae³³.

However, like every biotechnological production system, by-products can be produced and extracted during the process, requiring further purification with liquid chromatography. Also, unlike chemical synthesis, it is challenging to label specific atoms in the biosynthesized molecules, since special labeled precursors could be costly. For this model system, one 5 mL culture produced 1.4 µg of ¹³C-labeled lycopene, which costs an estimated \$1.25. Although this model system may not be cost-effective, research has been devoted to improve carotenoid production. For examples, the supply of carotenoid synthesis

precursor IPP had been improved by Kim *et al.* ³⁴, which doubled the lycopene production. Introducing the mevalonate pathway resulted in a 10-fold increase in lycopene production ³⁵. Gene knockout strategies have also been applied to improve lycopene production, resulting in a strain that can accumulate 16 mg of lycopene /g dry cell mass ^{23–25}. These metabolic-engineered strains could potentially make the *E. coli* system cost-effective for labeled carotenoid production.

Summary

This research demonstrated that bioengineered *E. coli* could utilize [U]-¹³C-glucose to produce highly ¹³C-enriched tomato carotenoids, lycopene and phytoene, and that error-prone PCR maintained phytoene production by maintaining the transcription of phytoene synthase. This production system could be further optimized and provide sufficient labeled carotenoids for future absorption, distribution, metabolism and excretion studies of carotenoids.

Acknowledgments

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Figure 2. Characteristics of *E. coli* BL21Star(DE3) harboring pAC-LYC (A) cell growth and the corresponding glucose concentration in 2XM9 media (B) accumulation of total lycopene. Data were presented as mean $\pm$ SE with $n = 3$.

Figure 3. Isotopomer distribution of (A) ^{12}C -all-*trans* lycopene ($^{12}\text{C}_{40}\text{H}_{56}$, MW=536.4) and (B) ^{13}C -labeled all-*trans* lycopene ($^{13}\text{C}_{40}\text{H}_{56}$, MW=576.4). Measured by ESI positive mode in a Q-ToF mass spectrometer.

A **B**

<i>crtI</i>	1	MKKTVVIGAGFGGLALAIRLQAAGIPTVLLQORDKPGGRAYVWHDQGFTEFDAGPTVITDP
<i>mt-crtI</i>	1	M-----L-----T-----T--A-----P
<i>crtI</i>	61	TALEALFTLAGRRMEDYVRLLPVKPFYRLCWESGKTLDYANDSAELEAQITQFNPRDVEG
<i>mt-crtI</i>	7	-----S-----L-----R-----AQITQFNPRDVEG
<i>crtI</i>	121	YRRFLAYSQAVFQEGYLRGSPVFLSFRDMLRAGPQLLKLQAWQSVYQSVSRFIEDEHLR
<i>mt-crtI</i>	23	YRRFLAYSQAVFQEGYLRGSPVFLSFRDMLRAGPQLLKLQAWQSVYQSVSRFIEDEHLR
<i>crtI</i>	181	QAFSFHSLLVGGNPFTTSSIIYTLIHALEREWGVWFPEGGTGALVNGMVKLFTDLGGEIEL
<i>mt-crtI</i>	83	QAFSFHSLLVGGNPFT--SSIIYTLIHALEREWGVWFPEGGTGALVNGMVKLFTDLGGEIEL
<i>crtI</i>	241	NARVEELVV-ADNRVSQVRLADGRIFDTDAVASNADVNTYKKLLG-HPVQGKRAAALE
<i>mt-crtI</i>	142	NARVEELVVV-DNRVSQVRLADGRIFDTDAVASNADVNTYKKLLGLH-PVGQKRAAALE
<i>crtI</i>	299	RKSMNSLSFVLYFGLNQPHSQLAHHTICFGPRYRELIDEIFTGSALADDFSLYLHSPCVT
<i>mt-crtI</i>	200	RKSMNSLSFVLYFGLNQPHSQLAHHTICFGPRYRELIDEIFTGSALADDFSLYLHSPCVT
<i>crtI</i>	359	DPSLAPPGCASFYVLAPVPHLGNAPLDWAQEGP-LRDRIFDYLEERYMPGLRSQVLTQRI
<i>mt-crtI</i>	260	DPSLAPPGCASFYVLAPVPHLGNAPLDWAQEGPKLRDRIFDYLEERYMPGLRSQVLTQRI
<i>crtI</i>	418	FTPADFHTLDAHLGSAFSIEPLLTQSAWFRPHNRDSIANLYLVGAGTHPGAGIPGVVA
<i>mt-crtI</i>	320	FTPADFHTLDAHLGSAFSIEPLLTQSAWFRPHNRDSIANLYLVGAGTHPGAGIPGVVA
<i>crtI</i>	478	SAKATASL
<i>mt-crtI</i>	380	SAKATASL

Figure 4. Comparison of plasmid constructs used in this study (A) phytoene desaturase *crtI* operons were modified by truncation or mutation (B) alignment of protein sequences of wild-type *crtI* and mutated *crtI*.

Figure 5. Phytoene accumulation in *E. coli* BL21Star(DE3) harboring pAC-PTE-TR and pAC-PTE-MT, cultured in LB broth. Data were presented as mean $\pm$ SD with $n = 2$.

Figure 6.

RNA expression patterns of *E. coli* harboring pAC-LYC, pAC-PTE-TR, and pAC-PTE-MT in LB broth (A) GGPP synthase (*crtE*) (B) phytoene synthase (*crtB*). Data were presented as mean $\pm$ SE with $n = 3$. * $p < 0.05$

Figure 7. Characteristics of *E. coli* BL21Star(DE3) harboring pAC-PTE-MT (A) cell growth and corresponding glucose concentration of 2XM9 media (B) accumulation of total phytoene. Data were presented as mean $\pm$ SE with $n = 3$.

Figure 8. Isotopomer distribution of (A) ^{12}C - phytoene ($^{12}\text{C}_{40}\text{H}_{64}$, MW=544.3) (B) ^{13}C -labeled phytoene ($^{13}\text{C}_{40}\text{H}_{64}$, MW=584.4). Measured by ThermoFinnigan LCQ Deca XP with APCI + interface.