A high-throughput screening method for identifying lycopene-overproducing *E. coli* strain based on an antioxidant capacity assay

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Abbreviations:

antioxidant capacity: AOA, reactive oxygen species: ROS, 2,2-diphenyl-1-picrylhydrazyl: DPPH, 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt: ABTS, ferric reducing antioxidant power: FRAP, hydroxyl free radical: •OH, Shine-Dalgarno: SD, aligned spacing: AS, phosphate buffered saline: PBS, dry cell weight: DCW, high-performance liquid chromatography: HPLC, ribosome bind site: RBS.

ABSTRACT

A straightforward, high-throughput screening method for identifying recombinant *Escherichia coli* strains that yield high levels of lycopene was developed based on the antioxidative properties of lycopene. Twenty-four recombinant *E. coli* strains were constructed using plasmids with various orderings of lycopene biosynthesis genes, Shine-Dalgarno regions, and aligned spacing regions. The high-throughput screening was based on antioxidant capacity measurements; we assessed the 2,2-diphenyl-1-picrylhydrazyl method, reducing activity method, and hydroxyl free radical scavenging method; the hydroxyl free radical scavenging method was determined to be the most suitable for use with our lycopene-overproducing system. The accuracy of this method was comparable to that of assay by high-performance liquid chromatography. The strain *E. coli* IEB11, which had the strongest antioxidant capacity and the highest lycopene-accumulating capacity, was selected and investigated for its ability to produce lycopene at different temperatures, glycerol concentrations, and fermentation times. In the optimal conditions in a 5-L fermenter, lycopene content of 1,288 mg/L and a mean productivity of 53.7 mg/L/h were obtained after 24 h.

Keywords: Biosynthesis, Recombinant DNA, Fermentation, Optimization, High-throughput screening, Lycopene

1. Introduction

Lycopene is a C₄₀ carotenoid with strong antioxidative activity that is widely used as a pharmaceutical compound, nutraceutical, functional food, and cosmetics additive [1]. Commercial lycopene is usually produced by multistep chemical synthesis or by chemical solvent-based extraction from tomatoes and tomato products [2]. However, the complexity of the chemical synthesis as well as the hazardous waste produced and the seasonal and geographic variability of the tomato raw materials restrict the use of lycopene in further industrial applications. Since the development of metabolic engineering and synthetic biology, there has been increased interest in microbial production of lycopene. *Erwinia uredovora*, *E. herbicola*, *Blakeslea trispora*, *Xanthophyllomyces dendrorhous*, *Dunaliella salina* and engineered yeasts and bacteria are used for lycopene production [3–6]. Genetic modifications have been

used to overexpress, inhibit, or knockout genes to control lycopene production [7, 8]. Additionally, temperature, pH, dissolved oxygen, and medium components have been investigated as means to control the fermentation process and develop an efficient platform for industrial lycopene production [9, 10].

The construction and screening of high-yield strains are also important for lycopene production. However, the extraction of lycopene is time-consuming and inconvenient when many samples must be processed. Moreover, color-based screening methods are subjective, the results are not quantitative, and there is not a universal method for screening strains for lycopene or any other carotenoid that might have antioxidant capacity (AOA). Therefore, it is important to find an effective and quantitative high-throughput screening method for identifying strains with high yield of lycopene that will allow the cultivation and evaluation of a large number of samples simultaneously with acceptable accuracy and reproducibility.

Reactive oxygen species (ROS) are produced by electron transport chains, enzymes, and redox cycling. Oxidative stress, which occurs when ROS production overwhelms cellular defenses, causes damage to membrane integrity, denaturation of proteins, and DNA strand breakage [11]. Antioxidants relieve oxidative stress and minimize pathological conditions caused by oxidants. A variety of methods allow quick and reliable quantification of AOA, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) bleaching method, ferric reducing antioxidant power (FRAP) method, hydroxyl free radical ($\bullet\text{OH}$) scavenging method, and reducing activity method [12–14]. Results from the FRAP method, ABTS bleaching method, and DPPH method indicate that lycopene is the most potent carotenoid antioxidant *in vitro* [15]. Moreover, the reaction rate constant of lycopene for scavenging single linear oxygen is 100-fold higher than that of Vitamin E and 2-fold higher than that of β -carotene [16]. Oxidative stress has been demonstrated as a selective pressure to enhance carotenoid production when screening high-carotenoid producing strains [17]. Therefore, we hypothesized that microorganisms with high lycopene yield would have strong AOA, and could be screened out by AOA measurements, allowing them to be accurately identified in microtiter plates instead of conventional screening from shaken flasks or tubes.

In this study, we aimed to develop a high-throughput screening method based on antioxidative activity to identify strains yielding high levels of lycopene. To this end, 24 *Escherichia coli* strains were

constructed using recombinant plasmids with various orderings of lycopene biosynthesis genes, Shine-Dalgarno (SD) regions, and aligned spacing (AS) between SD sequences and translation initiation codons. The DPPH method, reducing activity method, and $\bullet\text{OH}$ scavenging method were used to determine AOA. The strain IEB11 with the strongest AOA and the highest lycopene production was selected for further study. To further improve lycopene production, culture conditions including temperature, glycerol concentration, and fermentation time were optimized.

2. Materials and Methods

2.1 DNA manipulation, plasmid construction, and bacterial strains

All bacterial strains and plasmids used in this study are listed in Table 1 and Table S1. The genes *crtE* (encoding geranylgeranyl diphosphate synthase), *crtB* (encoding phytoene synthase), and *crtI* (encoding phytoene desaturase) were amplified from the genome of *Deinococcus wulumiqiensis* R12 [18]. The *crtE* PCR fragment was digested with *NdeI* and *EcoRI*, purified, and ligated into plasmid pET-22b to construct plasmid pET-E. The plasmids pET-B and pET-I were respectively constructed using the *crtB* and *crtI* PCR fragments with the same method. To construct plasmid pET-EB111, (*E*)*crtB*1(*I*1) and (*B*)*crtI*1 PCR fragments were amplified using primers shown in Table S2 with respective 5' and 3' homologous arms and then ligated into pET-E using the ClonExpress® MultiS One Step Cloning Kit (Vazyme, China) by homologous recombination (Fig. 1). Twenty-four polycistronic plasmids that differed in the order of *crtE*, *crtB*, and *crtI* with different SD and AS regions (SD+AS I and SD+AS II) were constructed by the same homologous recombination method. These 24 plasmids were transformed into *E. coli* BL21 (DE3) to form six series of recombinant strains, namely EBI (with the lycopene synthesis genes in the order *crtE*, *crtB*, *crtI*), EIB (with the lycopene synthesis genes in the order *crtE*, *crtI*, *crtB*), BEI (with the lycopene synthesis genes in the order *crtB*, *crtE*, *crtI*), BIE (with the lycopene synthesis genes in the order *crtB*, *crtI*, *crtE*), IEB (with the lycopene synthesis genes in the order *crtI*, *crtE*, *crtB*), and IBE (with the lycopene synthesis genes in the order *crtI*, *crtB*, *crtE*). The strain EDWe and EDW contained the plasmid pET-22b and pET-EBI (without optimized SD+AS regions), respectively.

2.2 Cultivation conditions for shaken flasks, high-throughput screening, and high-density fermentation

D. wulumuqiensis R12 was grown at 30°C with shaking at 200 rpm for 72 h in TGY medium (10 g/L tryptone, 1 g/L glucose, and 5 g/L yeast extract). Recombinant *E. coli* cells were grown at 37°C with shaking at 200 rpm for 48 h in Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), 2×YT medium (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl), or 2×YT+G medium (2×YT medium with 10, 20, 40, 60, 80, or 100 g/L glycerol).

E. coli colonies were inoculated into 50-mL centrifuge tubes containing 5 mL of 2× YT+G medium (10 g/L glycerol) and cultivated at 37°C with shaking at 200 rpm for 16 h. For high-throughput screening cultivation, 24-well microtiter plates with lids were used to prevent cross-contamination. A preculture sample (300 µL) was inoculated into each well containing 2.4 mL of fresh 2× YT+G medium (10 g/L glycerol). The 24-well microtiter plates were cultivated at 30°C with shaking at 200 rpm for 48 h in a dark room. Media were supplemented with 100 mg/L ampicillin for plasmid maintenance. Bacterial cultures were individually cultivated in three microtiter plates to produce three replicates. For high-density fermentation, a 5-L fermenter was used and cells were cultivated in the optimal conditions. As IPTG was shown to decrease lycopene production in our previous study [19], it was not used in this study.

2.3 High-throughput screening by AOA measurement

After cultivation, 2 mL of broth was collected and centrifuged (13,000 × g, 4°C, 5 min). Cell pellets were washed twice with phosphate-buffered saline (PBS, 0.01 M) and resuspended in PBS. Different cell sample concentrations were studied for the DPPH method, reducing activity method, and the •OH scavenging method, and two sample concentrations were finally used: 1 mL of cell pellets resuspended in 0.5 mL of PBS and 2 mL of cell pellets resuspended in 0.5 mL of PBS.

Three methods were used for AOA measurement. A modified DPPH method was used in this study [12]. DPPH was added to suspended cell pellet (1 mL of cell pellets resuspended in 0.5 mL of PBS) and incubated in a dark room for 30 min at room temperature. The supernatant was analyzed at 517 nm using a microplate reader (M3, Thermo). The DPPH clearance rate was calculated according to the formula [12]:

$$\text{DPPH clearance rate (\%)} = \frac{A_{\text{negative}} - A_{\text{sample}}}{A_{\text{negative}}} \times 100\% \quad (1)$$

where A_{negative} is the absorption of 0.5 mL of PBS plus 0.5 mL of 0.2 mM DPPH and A_{sample} is the absorption of 0.5 mL of sample plus 0.5 mL of 0.2 mM DPPH.

The reducing activity method used potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), trichloroacetic acid ($\text{C}_2\text{HCl}_3\text{O}_2$) and ferric chloride (FeCl_3) reacting with the cell pellet (1 mL of cell pellet resuspended in 0.5 mL of PBS) [20]. Ascorbic acid (0.01%) was used as the positive control and the supernatant was analyzed at 700 nm using a microplate reader. Cysteine (100 $\mu\text{mol/L}$) was used as a reference standard for reducing activity.

The $\bullet\text{OH}$ scavenging method for AOA measurement used cell pellet (2 mL of cell pellet resuspended in 0.5 mL of PBS) and 1% hydrogen peroxide (H_2O_2) added to a mixture consisting of 1,10-phenanthroline monohydrate, Tris-HCl (pH 7.4) and ferrous sulfate (FeSO_4) for reaction [12]. The supernatant was analyzed at 536 nm using a microplate reader. The $\bullet\text{OH}$ scavenging rate was calculated according to the formula [12]:

$$\bullet\text{OH scavenging rate (\%)} = \frac{A_{\text{sample}} - A_{\text{negative}}}{A_{\text{blank}} - A_{\text{negative}}} \times 100\% \quad (2)$$

where A_{sample} is the absorption of 0.5 mL of sample plus 0.1 mL of H_2O_2 , A_{negative} is the absorption of 0.5 mL of PBS buffer plus 0.1 mL of H_2O_2 , and A_{blank} is the absorption of 0.6 mL of PBS buffer.

Each of the 24 strains was cultivated in four centrifuge tubes to obtain a total of 96 samples. After 1 h of the $\bullet\text{OH}$ scavenging reaction, these 96 samples were centrifuged ($13,000 \times g$, 5 min), and 200 μL of supernatant was transferred into each well of a 96-well plate by multi-channel pipette and analyzed at 536 nm using a microplate reader to obtain parameter for the high-throughput screening method.

2.4 Genetic stability of the strain IEB11

The strain IEB11 was inoculated into 50-mL centrifuge tubes containing 5 mL of 2 $\times$ YT+G medium (10 g/L glycerol) and cultivated at 37°C with shaking at 200 rpm for 16 h. The culture was subcultured in 2 $\times$ YT+G medium (10 g/L glycerol) every 48 h for 10 cycles. Lycopene was extracted to evaluate the genetic stability of the strain IEB11. The culture was collected and diluted 10⁴-fold with 2 $\times$ YT+G medium (10 g/L glycerol) and then plated on 2 $\times$ YT+G medium (10 g/L glycerol) plates at 37°C for 16 h with and without ampicillin to determine the numbers of colony-forming units (CFUs). The plasmid stability was calculated according to the formula:

$$\text{Plasmid stability of strain IEB11 (\%)} = \frac{\text{CFU with ampicillin}}{\text{CFU without ampicillin}} \times 100\% \quad (3)$$

2.5 Biomass measurement

Biomass was measured by the dry cell weight (DCW) method. Briefly, 1 mL of broth was collected in a weighted centrifuge tube and centrifuged ($13,000 \times g$, 4°C , 5 min). The resulting cell pellets were collected and washed twice with PBS. Then, the cell pellets were incubated in a dry oven (100°C) overnight until a constant weight was achieved.

2.6 Lycopene extraction and analysis

After fermentation, broth was collected and centrifuged ($13,000 \times g$, 4°C , 5 min). The resulting cell pellets were collected and washed once with double-distilled water. Cell pellets were resuspended in acetone and incubated at 55°C for 15 min, followed by centrifugation ($13,000 \times g$, 25°C , 10 min). The supernatant was filtered through a $0.45\text{-}\mu\text{m}$ filter, and transferred to amber screw vials for high-performance liquid chromatography (HPLC) analysis. All extraction procedures were conducted in the dark [21].

For HPLC analysis, 20 μL of each supernatant was analyzed using a Venusil XBP C18 column ($4.6 \times 150\text{ mm}$, 5 μm , Agela Technologies) with monitoring at 472 nm and eluted with a solvent solution of 80% acetonitrile, 15% methanol, and 5% isopropanol at a flow rate of 1 mL/min for 40 min. Commercial lycopene (Sigma, USA) was dissolved in acetone and used as a control to generate a standard curve using the method above. The results represent the mean $\pm$ standard deviation of three independent experiments.

2.7 Strain number and accession numbers of nucleotide sequences

D. wulumuqiensis R12 (= CGMCC 1.8884^T = NBRC 105665^T) was deposited in the China General Microbiological Culture Collection Center. The nucleotide sequences of the three genes studied in this work were deposited in GenBank as follows: orf01490 (*crtE*), GenBank accession no. KP319019; orf00123 (*crtB*), GenBank accession no. KP319020; and orf00124 (*crtI*), GenBank accession no. KP319021.

3. Results

3.1 Construction of the 24 polycistronic plasmids and recombinant *E. coli* strains

The lycopene biosynthesis genes *crtE*, *crtB*, and *crtI* from a red-pigmented strain, *D. wulumuqiensis* R12, were integrated into plasmid pET-22b and sequenced to confirm they were present in the right direction and order. All possible combinations of ordering of the lycopene biosynthesis genes and SD+AS regions were explored (Table S1). Gene 1 was regulated by the SD+AS region of the T7 promoter of the plasmid pET-22b and genes 2 and 3 were under the regulation of the manipulated SD+AS I (taaggaggatataca) or SD+AS II (aggaggattacaaa) region (Fig. 1). Then, the 24 polycistronic plasmids were transformed into *E. coli* BL21 (DE3), generating the 24 recombinant strains.

3.2 Three AOA measurements for the 24 *E. coli* strains

A culture volume of 2.7 mL (including 300 μ L of preculture) and silica gel lids were established as the basic conditions for microtiter plates (Table 2). Different cell sample concentrations were studied in AOA measurements (Fig. S1). The sample concentration of 1 mL of cell pellet resuspended in 0.5 mL of PBS showed the most distinct differences between the strain EDW and the strain EDWe (the control strain) in the DPPH method and reducing activity method. The sample concentration of 2 mL of cell pellet resuspended in 0.5 mL of PBS was the most effective concentration for the $\bullet$ OH scavenging method. In the condition of 2.7 mL culture volume with silica gel lids, the 24 engineered *E. coli* strains were each cultivated in wells of 24-well plates. Obvious differences in color shading, signifying different lycopene accumulation, were observed for these strains (Fig. 2).

Fig. 3a shows the DPPH clearance rates of the 24 recombinant strains, the EDWe control strain, and 0.01% ascorbic acid. The EDWe control strain and 0.01% ascorbic acid had clearance rates of 25.31% and 86.63%, respectively. The 24 recombinant strains showed clearance rates ranging from 29.03% to 51.89%. The strain IEB11 had the highest rate (51.89%), while the strain EIB12 had the lowest (29.03%). Clearance rates of the IEB series of strains were higher than any of the other strain series, indicating they had the strongest AOA.

The reducing abilities of the 24 recombinant strains, the EDWe control strain, and 0.01% ascorbic acid were measured by comparison with the reference standard cysteine. The EDWe control strain and

0.01% ascorbic acid had reducing activities equivalent to 8.14 $\mu\text{mol/L}$ and 42.15 $\mu\text{mol/L}$ of cysteine, respectively. The 24 recombinant strains showed reducing activities equivalent to 11.13–20.40 $\mu\text{mol/L}$ of cysteine (Fig. 3b). The reducing activity of the strain IEB11 was equivalent to 20.40 $\mu\text{mol/L}$ of cysteine, 1.8 times higher than the activity of the strain EIB12, which had the lowest reducing activity.

The $\bullet\text{OH}$ scavenging capacities of the 24 recombinant strains, the EDWe control strain, and 0.01% ascorbic acid were measured (Fig. 3c). The EDWe control strain and 0.01% ascorbic acid had clearance rates of 5.70% and 94.02%, respectively. The 24 recombinant strains showed clearance rates ranging from 8.05% to 87.34%. The strain IEB11 displayed the highest clearance rate (87.34%), over 10.8 times that of strain BEI11 (8.05%) and 15.3 times that of the EDWe control strain (5.70%), demonstrating that it had the highest AOA. The IEB series of strains showed significantly higher $\bullet\text{OH}$ clearance rates than the other series, indicating high levels of AOA. The BEI series of strains had the lowest $\bullet\text{OH}$ scavenging capacities.

3.3 High-throughput screening of strains yielding high levels of lycopene

Among the three investigated methods for AOA measurement, the $\bullet\text{OH}$ scavenging method showed the most significant results and involved a simple procedure for high-throughput screening of the strains. Therefore, we chose the $\bullet\text{OH}$ scavenging method as the most suitable method to screen the strains with high AOA in our lycopene-overproducing system. To further verify the reliability of high-throughput screening by the $\bullet\text{OH}$ scavenging method, lycopene was extracted from the 24 strains for HPLC analysis (Table 3). The highest lycopene content (1,020 mg/L) was obtained from the strain IEB11, and the lowest lycopene content (178 mg/L) was obtained from the strain EIB12. Comparison of the results obtained by the $\bullet\text{OH}$ scavenging method and HPLC methods showed adequate agreement, suggesting that the $\bullet\text{OH}$ scavenging method is reliable for screening high lycopene-overproducing strains.

In the high-throughput screening method, it took about 2 h to obtain the final $\bullet\text{OH}$ scavenging data for 96 samples (24×4 replicates) from the time of adding reagents. The data showed a good repeatability of the $\bullet\text{OH}$ scavenging method (Table S3).

3.4 The genetic stability of strain IEB11

The strain IEB11 was selected out by the high-throughput screening method as having the highest

lycopene content. Fig. 4 shows that the lycopene content of the strain IEB11 was between 901 and 980 mg/L. The plasmid stability slowly decreased from 95.6% to 80.3% after 10 rounds of subcultivation. Thus, overall, the recombinant strain IEB11 had stable capacity for lycopene production and good plasmid stability. As a result, the strain IEB11 was selected for further optimization of fermentation.

3.5 The effect of temperature on cell growth and lycopene production

Three temperatures (25°C, 30°C, and 37°C) were assessed. As Fig. 5a shows, according to the DCW results, 37°C was best for the growth of the strain IEB11. The biomass increased more quickly at 37°C than at 25°C or 30°C in the first 12 h of cultivation. Lycopene content was much higher at 30°C and 37°C than at 25°C. The highest DCW and lycopene contents were 7.1 g/L and 818 mg/L, respectively, at 37°C after cultivation for 24 h. The high biomass obtained at 37°C may explain the high lycopene content in the recombinant cells. The lycopene content decreased after 24 h of cultivation, suggesting that cultivation at 37°C for 24 h is optimal for biomass accumulation and lycopene production in our system.

3.6 The effect of glycerol concentration on cell growth and lycopene production

Glycerol (0–100 g/L) was used as an extra carbon source for the strain IEB11 to produce lycopene (Fig. 5b). Without glycerol, we observed a maximum biomass of 7.75 g/L at 24 h. The biomass decreased sharply after 24 h in other glycerol conditions (10–100 g/L). Lycopene content increased rapidly during the first 12 h of culture and then slowly accumulated when there was no glycerol in the medium. With 20 g/L glycerol, the maximum lycopene concentration reached 1,017 mg/L, nearly 1.56-fold higher than that without glycerol. Therefore, we decided to use 2× YT medium for seed cultivation and 2× YT+G medium (with 20 g/L glycerol) for fermentation.

3.7 Optimal conditions for lycopene production in shaken flasks and in a 5-L fermenter

Based on our fermentation optimization results, the temperature was fixed at 37°C during the entire cultivation process, and 2× YT medium was used for seed cultivation for 12 h. The preculture was then inoculated into 50 mL of fresh 2× YT+G medium (with 20 g/L glycerol) in 250-mL shaken flasks. Cultivation was conducted in the dark. As Fig. 5c shows, biomass and lycopene content varied over time.

Lycopene content increased quickly in the first 12 h and then fluctuated slightly before reaching a maximum at 24 h (1,137 mg/L). The biomass increased quickly in the first 6 h of cultivation, increased slowly from 6 h to 24 h, and then reached a maximum of 7.67 g/L DCW at 24 h. After 24 h, both cell growth and lycopene content decreased, suggesting that the optimal fermentation period for the strain IEB11 is 24 h. In a 5-L fermenter, the highest lycopene content (1,288 mg/L) was achieved after 24 h of fermentation and the cell growth was 8.2 g/L at that time. The mean lycopene productivity was 53.7 mg/L/h.

4. Discussion

In this work, we have described a screening strategy for lycopene-overproducing *E. coli* strains based on AOA measurements. Comparing the DPPH method, reducing activity method, and •OH scavenging method, the DPPH method was the easiest to perform, but it gave unclear results. The reducing activity method was dangerous, with some toxic reagents, and complicated, leading to obscure results. The •OH scavenging method gave the most significant results with a simple procedure for high-throughput screening of the strains. The number of double bonds is closely related to the scavenging capability of carotenoids. Lycopene is an open chain hydrocarbon containing 11 conjugated and two non-conjugated double bonds arranged in a linear array, which has the strongest capability for scavenging •OH and single linear oxygen among all the other carotenoids [16]. This may explain why the results of the •OH scavenging method were the most distinct.

The feasibility and accuracy of our method were verified by HPLC measurements, it was then used to screen the recombinant *E. coli* strains with high AOA generated in this study. The data in Table 3 demonstrated that there was a correlation between AOA and lycopene content in our recombinant *E. coli* strains. The clearance rates of the IEB series of strains were the highest among all the strains, and they showed the highest lycopene content as well. The BEI series of strains with the lowest clearance rates showed the lowest lycopene contents. These results clearly indicated that the strains with high lycopene content have high AOA, so they can be screened out using data from the •OH scavenging method. Many traditional screening methods are performed in flasks, requiring a large amount of material. However, these methods have some shortcomings for application in complex screening systems; for example, they

can be tedious and time-consuming. In contrast, our high-throughput screening method using AOA measurement to screen lycopene-overproducing strains could be carried out in many 24-well microtiter plates with a universal microplate reader requiring only a simple testing apparatus. This enabled us to screen about 96 of samples in one screening cycle in only 2 h.

The 24 recombinant strains had different color shadings, signifying different lycopene accumulation capacities. These results mean that the ordering of the lycopene biosynthesis genes and the SD+AS regions had an influence on the level of lycopene production. Multiple gene expression is mainly modulated by regulatory components at the transcriptional, post-transcriptional and translational levels, for example by promoters and ribosome binding sites (RBSs) [22, 23]. Altering the order of the *BmGDH* and *PsCR* genes in the recombinant *E. coli* led to obviously different enzyme activities and expressions and thus varying ethyl(S)-4-chloro-3-hydroxybutanoate production [24]. In succinate production, modulating *dcuB* and *dcuC* genes in combination with RBS libraries led to a 34% increase in succinate titer [25]. Libraries of artificial regulatory parts were used to modulate *galP* and *glk* gene expression to improve glucose utilization by *E. Coli* [26]. These strategies suggest that multiple genes need to be arranged appropriately to achieve balanced expression and regulatory elements upstream of each gene must be compatible with each other for gene expression. Lycopene is synthesized from farnesyl pyrophosphate by three key enzymes: geranylgeranyl diphosphate synthase, phytoene synthase, and phytoene desaturase; this is a complex metabolic process. This kind of multienzyme reaction needs balanced gene expression to avoid over-accumulation of intermediates or bottlenecks. Because of this unpredictability, it is of great importance to properly regulate the expression of the three genes in lycopene synthesis. In this work, a lycopene-overproducing strain IEB11 with the gene order *crtI*, *crtE*, and *crtB* and with manipulated SD+AS I sequences upstream of the *crtE* and *crtB* genes was selected by the high-throughput screening method; this strain had a lycopene content of 1,136.97 mg/L after 24 h of fermentation in a shaken flask, which was 2.7-fold higher than the strain EDW ((417 mg/L)) in our previous study [19].

The synthesis of lycopene is affected by many factors such as light, temperature, carbon source, and fermentation time. We studied the lycopene synthesis process with one LED and without light (data not shown). The lycopene content exposed to the LED was lower than that cultivated in the dark. Lycopene is

rather unstable and decomposed by light irradiation [27]. However, the role of light in lycopene synthesis is still unclear, and it is important to investigate its effects in the future. Temperature is considered the main physical element directly controlling bacterial growth rate and thus plays an important role in the biosynthesis of carotenoids. In our lycopene-overproducing system, biomass and lycopene content were higher at 37°C than at 30°C or 25°C, indicating that the optimal temperature for lycopene accumulation was 37°C. The growth rate and biomass of the recombinant *E. coli* strains were improved at 37°C, which resulted in increased lycopene accumulation.

Our previous work demonstrated that 2×YT+G medium was the optimal culture medium among the three media tested (LB, 2×YT+G, and synthetic medium) [19]. However, the best glycerol concentration, which is of great importance, was not determined. If the glycerol content in the medium is insufficient, it will lead to a decrease in lycopene production; if it is in excess, this would increase the industrial cost. In our experiments, when the glycerol concentration was over 20 g/L, the lycopene content decreased, suggesting that high glycerol concentration was unnecessary for lycopene production. A low cell concentration was observed when glycerol was used as the sole carbon source [9]. Biomass apparently decreased with the increase of the initial glycerol concentration and the low cell concentration constituted a limit to the production of lycopene (Fig. 5b). We concluded that medium without added glycerol was beneficial for cell growth, while medium with 20 g/L glycerol was optimal for lycopene accumulation.

Lycopene is a secondary metabolite and its synthesis is very complex and time-consuming. Prior studies have shown that it reaches its maximum level (228–3520 mg/L) after 20 h–100 h of fermentation [8, 9, 19, 21, 28, 29] (Table S4). However, we found that lycopene production in the strain IEB11 reached the peak level after 24 h of fermentation (Fig. 5). The mean productivity of the strain IEB11 was 53.7 mg/L/h, which was 1.2-fold the level after controlling the mevalonate pathway in *E. coli* in 2.5-L fed-batch fermentation (43.6 mg/L/h) [8] and 1.4-fold the level under a synergistic effect of glucose and L-arabinose in fed-batch culture (39.7 mg/L/h) [9]. Scaling up cultivation from temperature optimization in flasks to a 5-L fermenter, lycopene content was increased from 818 mg/L to 1,288mg/L, an increase of 57% (Table 4). This great improvement in lycopene production indicated a significantly shorter fermentation time and dramatically reduced industrial costs.

5. Conclusions

A high-throughput screening method, based on antioxidant capacity, was developed for identifying *E. coli* strains that yield high levels of lycopene. The •OH scavenging method was determined to be the most valid and effective method for screening the lycopene-overproducing strains. The strain IEB11 was selected as the most effective lycopene producer. The effects of temperature, glycerol concentration, and fermentation time on biomass and lycopene content were investigated for this strain. High lycopene production and productivity were achieved in a short fermentation time in the optimized conditions, which is promising for applications and commercial value on the industrial scale.

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Figure captions

Fig. 1 Schematic representation of the polycistronic plasmids construction. Gene1, gene2, and gene3 are indicated by closed arrows in blue, green, and yellow colors, respectively. SD+AS regions upstream of the gene2 and gene3 are indicated by closed boxes in purple. Homologous arms are indicated by closed boxes in red color. The plasmid pET-gene1 was first digested by *EcoRI* and *XhoI* to obtain linear fragment gene1-E-X. Gene2 with SD+AS region has 5'homologous arm and 3'homologous arms which are overlap with 3'end of gene1-E-X and 5'end of gene3. Gene3 with SD+AS region has 3'homologous arm which is overlap with 5'end of gene1-E-X. The three parts, linear fragment gene1-E-X, gene2 (with SD+AS region and homologous arms), gene3 (with SD+AS region and homologous arm), were ligated by homologous recombination to form the recombinant plasmid pET-gene1-gene2-gene3.

Fig. 2 Microtiter plate cultivation for the 24 recombinant *E. coli* strains. These strains contained 6 series of strains and each series had 4 different strains. The 6 series of strains were inoculated in vertical lines and 4 different strains in a series were inoculated in horizon lines.

Fig. 3 AOA determined by the DPPH method, reducing activity method, and •OH scavenging method. a AOA determined by the DPPH method. b AOA determined by the reducing activity method. c AOA determined by the •OH scavenging method. The EBI, EIB, BEI, BIE, IEB, IBE series of strains, and 0.01% ascorbic acid were showed in different shades of red and the EDWe control strain was displayed in light blue. Values are the average $\pm$ standard deviations of three experiments.

Fig. 4 Lycopene content and plasmid stability of the strain IEB11 after 10 rounds of subcultivation.

Fig. 5 Effects of temperatures and glycerol concentrations on biomass and lycopene content of the strain IEB11. a effects of different temperatures, 25 °C (filled squares), 30 °C (filled circles), and 37 °C (filled triangles) on biomass and lycopene content. b effects of different glycerol concentrations, 0 g/L (filled squares), 10 g/L (filled circles), 20 g/L (filled triangles), 40 g/L (filled diamonds), 60 g/L (open squares), 80 g/L (open circles), and 100 g/L (open triangles) on biomass and lycopene content. c DCW (filled

circles) and lycopene content (filled squares) of the strain IEB11 under the optimal conditions in a shake flask. Values are the average $\pm$ standard deviations of three experiments.

Tables

Table 1

Bacterial strains and plasmids.

Plasmids	Relevant properties	Source
pMD T19 Simple Vector	Cloning vector, Amp ^R	Takara
pET-22b	Amp ^R , inducibleT7 promoter	Invitrogen
pET-E	Amp ^R , carrying <i>crtE</i> gene of <i>Deinococcus wulumiqiensis</i> R12	This study
pET-B	Amp ^R , carrying <i>crtB</i> gene of <i>D. wulumiqiensis</i> R12	This study
pET-I	Amp ^R , carrying <i>crtI</i> gene of <i>D. wulumiqiensis</i> R12	This study
pET-EBI	Amp ^R , carrying <i>crtE</i> , <i>crtB</i> , and <i>crtI</i> gene of <i>D. wulumiqiensis</i> R12	This study
Strains		
<i>D. wulumuqiensis</i> R12	Aerobic, gram-stain-positive, non-spore-forming, non-motile	[18]
<i>E.coli</i> DH _{5α}	deoR endA1 gyrA96 hsdR17 (rK ⁻ -mK ⁺) recA1 relA1 supE44 thi-1 Δ(lacZYA-argF)U169 Φ80lacZ ΔM15 F ⁻ λ ⁻	Vazyme
<i>E.coli</i> BL21(DE3)	F - ompThsdS (rB- mB-) gal dcm (DE3)	Vazyme
EDWe	Amp ^R , <i>E.coli</i> BL21(DE3) containing the plasmid pET-22b	This study
EDW-E	Amp ^R , <i>E.coli</i> BL21(DE3) containing the plasmid pET-E	This study
EDW-B	Amp ^R , <i>E.coli</i> BL21(DE3) containing the plasmid pET-B	This study
EDW-I	Amp ^R , <i>E.coli</i> BL21(DE3) containing the plasmid pET-I	This study
EDW	Amp ^R , <i>E.coli</i> BL21(DE3) containing the plasmid pET-EBI	This study

1 **Table 2**

2 Contaminations of the five conditions for microtiter plate cultivation.

Conditions	Numbers of contamination well ^a
Culture volume	
2.4 mL 2YT+G medium and 300 µL pre-culture	3± 0.6
4.5 mL 2YT+G medium and 500 µL pre-culture	8± 0.6
Microtiter sealing	
Parafilm	2± 0.6
Silica gel lids	0± 0.6
Simple plastic lids	7± 1.0

3 ^a 2YT+G medium was added in the 24 wells and 12/24 wells were interval inoculated.

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5 **Table 3**

6 •OH clearance rates and lycopene contents of the 24 recombinant strains.

Strain names	•OH clearance rate (%) ^a	Lycopene content (mg/L) ^a	Strain names	•OH clearance rate (%) ^a	Lycopene content (mg/L) ^a	Strain names	•OH clearance rate (%) ^a	Lycopene content (mg/L) ^a
EBI11	15.6 ± 1.3	338 ± 20	BEI11	8.8 ± 0.4	220 ± 12	IEB11	87.3 ± 2.2	1020 ± 15
EBI12	26.0 ± 6.3	455 ± 14	BEI12	9.3 ± 0.1	227 ± 10	IEB12	82.5 ± 2.5	820 ± 8
EBI21	13.0 ± 1.1	321 ± 19	BEI21	8.7 ± 1.0	218 ± 21	IEB21	85.4 ± 2.3	950 ± 9
EBI22	15.7 ± 4.5	339 ± 15	BEI22	8.6 ± 0.7	210 ± 23	IEB22	81.5 ± 6.0	790 ± 12
EIB11	12.6 ± 0.6	316 ± 23	BIE11	20.6 ± 1.3	410 ± 18	IBE11	22.4 ± 1.8	426 ± 14
EIB12	8.0 ± 0.7	178 ± 19	BIE12	21.9 ± 2.8	424 ± 14	IBE12	20.0 ± 0.3	392 ± 15
EIB21	10.8 ± 1.1	308 ± 17	BIE21	25.1 ± 0.4	451 ± 17	IBE21	34.0 ± 3.4	575 ± 14
EIB22	12.2 ± 0.4	310 ± 14	BIE22	35.2 ± 3.1	585 ± 12	IBE22	28.0 ± 3.0	478 ± 7

7 ^a Values are the average ± standard deviations of three experiments.

8 **Table 4**

9 Lycopene contents of the strain IEB11.

Strain IEB11	Lycopene content (mg/L) ^a
Temperature optimization	818 mg/L
Glycerol concentration optimization	1,017 mg/L
Optimized condition (shake flask)	1,137 mg/L
Optimized condition (5-L fermenter)	1,288 mg/L

^a Values are the average $\pm$ standard deviations of three experiments.

Figure1

Figure2

	Series of strains					
	EBI	EIB	BEI	BIE	IEB	IBE
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12
21
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Figure3

Figure4

Figure5