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

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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 1288 mg/L and a mean productivity of 53.7 mg/L/h were obtained after 24 h.

1. Introduction

Lycopene is a C_{40} 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*, *Blaskeslea 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

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

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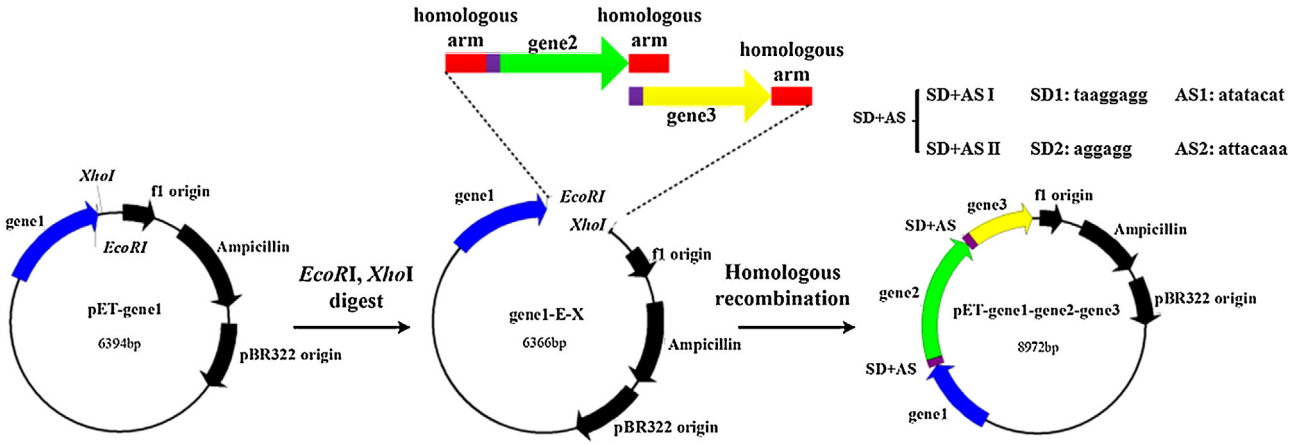

Fig. 1. Schematic representation of the polycistronic plasmids construction. Gene1, gene2, and gene3 are indicated by closed arrows in blue, green, and yellow colors, respectively. SD + AS regions upstream of the gene2 and gene3 are indicated by closed boxes in purple. Homologous arms are indicated by closed boxes in red color. The plasmid pET-gene1 was first digested by *Eco*RI and *Xho*I 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 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

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 (•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 •OH scavenging method was used to determine AOA. The strain IEB11 with the strongest AOA and the highest lycopene production were 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 Tables 1 and 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 *Nde*I and *Eco*RI, 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-EB1I1, (*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)