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Metabolic engineering of *Escherichia coli* for high-level astaxanthin production with high productivity

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ABSTRACT

Astaxanthin is a reddish keto-carotenoid classified as a xanthophyll found in various microbes and marine organisms. As a powerful antioxidant having up to 100 times more potency than other carotenoids such as β-carotene, lutein, and lycopene, astaxanthin is a versatile compound utilized in animal feed, food pigment, health promotion and cosmetic industry. Here, we report development of metabolically engineered *Escherichia coli* capable of producing astaxanthin to a high concentration with high productivity. First, the heterologous *crt* genes (*crtE*, *crtY*, *crtI*, *crtB*, and *crtZ*) from *Pantoea ananatis* and the truncated *BKT* gene (*trCrBKT*) from *Chlamydomonas reinhardtii* were introduced to construct the astaxanthin biosynthetic pathway. Then, eight different fusion tags were examined by attaching them to the N- or C-terminus of the trCrBKT membrane protein to allow stable expression and to efficiently guide trCrBKT to the *E. coli* membrane. When the signal peptide of OmpF and TrxA were tagged to the N-terminus and C-terminus of trCrBKT, respectively, astaxanthin production reached 12.90 mg/L (equivalent to 3.84 mg/gDCW), which was 2.08-fold higher than that obtained without tagging. Upon optimization of culture conditions, this engineered strain WLGB-RPP harboring pAX15 produced 332.23 mg/L (5.38 mg/gDCW) of astaxanthin with the productivity of 3.79 mg/L/h by fed-batch fermentation. In order to further increase astaxanthin production, *in silico* flux variability scanning based on enforced objective flux (FVSEOF) was performed to identify gene overexpression targets. The engineered strain WLGB-RPP (pAX15, pTrc-ispDF) which simultaneously overexpressing the *ispD* and *ispF* genes identified by FVSEOF produced astaxanthin to a higher concentration of 377.10 mg/L (6.26 mg/gDCW) with a productivity of 9.20 mg/L/h upon induction with 1 mM IPTG. When cells were induced with 0.5 mM IPTG to reduce the metabolic burden, astaxanthin concentration further increased to 432.82 mg/L (7.12 mg/gDCW) with a productivity of 9.62 mg/L/h. To more stably maintain plasmid during the fed-batch fermentation of WLGB-RPP (pAX15, pTrc-ispDF), the post-segregational killing *hok/sok* system was introduced. This strain produced 385.04 mg/L (6.98 mg/gDCW) of astaxanthin with a productivity of 7.86 mg/L/h upon induction with 0.5 mM IPTG. The strategies reported here will be useful for the enhanced production of astaxanthin and related carotenoid products by engineered *E. coli* strains.

1. Introduction

Carotenoids (or tetraterpenoids) biosynthesized by fruits, vegetables, algae and bacteria are important class of organic pigments that have been widely used as food ingredients and nutritional supplements. Among more than 600 known natural carotenoids, β-carotene, lutein, lycopene, zeaxanthin and astaxanthin are the best studied compounds. Astaxanthin is the strongest antioxidant known to be 100 times more potent than the other carotenoids (Higuera-Ciapara et al., 2006; Naguib, 2000). Astaxanthin is a reddish keto-carotenoid classified as a xanthophyll, which is found in various microbes and marine organisms (Ambati et al., 2014). It has diverse biological activities such as anti-tumor (Jyonouchi et al., 2000), anti-inflammatory (Miyachi et al., 2015) and immune system enhancing (Higuera-Ciapara et al., 2006) properties. It is also a promising candidate as a therapeutic agent against cardiovascular diseases (Pashkow et al., 2008), neurological diseases (Wu et al., 2015) and diabetes (Yuan et al., 2011). Due to these versatile properties, demand for astaxanthin has continuously grown

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and its global market size is predicted to be reaching $2.57 billion worldwide by 2025 (Grand View Research Inc., 2017).

In the current commercial market, most astaxanthin products are manufactured through chemical synthesis. The synthetic astaxanthin products comprising a racemic mixture of stereoisomers have predominantly been used for animal and aquaculture feeds (Schmidt et al., 2011). On the other hand, biologically produced astaxanthin is much more stereoselective than chemical synthesis and is approved for human consumption (Ambati et al., 2014). However, its low production efficiency has been a major hurdle to make the process economically competitive. Thus, there have been many attempts to overproduce astaxanthin using genetically modified plants (Huang et al., 2013; Mann et al., 2000), yeasts (Rodriguez-Saiz et al., 2010), microalgae (Ip et al., 2004; Park et al., 2014) and other microorganisms (Ide et al., 2012; Lemuth et al., 2011). Among them, microalgae have been the most common astaxanthin production host employed. In this study, we chose *Escherichia coli* as a host strain for the efficient production of astaxanthin to a high concentration with high productivity because it can be grown to a high density with well-established fermentation techniques (Lee, 1996); this was thought to be necessary as astaxanthin is produced intracellularly and associated with cell membrane. Although there have been several studies on astaxanthin production by engineered *E. coli* strains, the cultivation scales were often limited to rather small volumes ranging from several tens to several hundreds of milliliters (Li and Huang, 2018; Lu et al., 2017; Ma et al., 2016; Zhang et al., 2018).

Here, we report development of metabolically engineered *E. coli* strain capable of efficiently producing astaxanthin in a lab-scale fermenter. First, gene expression levels of the upper astaxanthin biosynthetic pathway were optimized by testing different promoters and genetic configurations to prevent accumulation of toxic precursors such as lycopene. In addition, various fusion tags were attached to the truncated *BKT* gene (*trCrBKT*) from *Chlamydomonas reinhardtii*, a key heterologous membrane protein, to improve its stability and to assist its membrane binding. Then, culture conditions were optimized for fed-batch fermentative production of astaxanthin. Gene overexpression targets identified by *in silico* flux variability analysis as well as the *hok/sok* system to stabilize the engineered strain were also examined. Consequently, we were able to achieve the highest astaxanthin titer of 432.82 mg/L (7.12 mg/gDCW) with a productivity of 9.62 mg/L/h by simultaneously overexpressing the *ispD* and *ispF* genes, and a reasonably high astaxanthin titer of 385.04 mg/L (6.98 mg/gDCW) with a productivity of 7.86 mg/L/h by introducing *hok/sok* system.

2. Materials and methods

2.1. Strains and media

All strains used in this study are listed in Table S1. *E. coli* DH5α was used for gene cloning works. It was aerobically cultivated in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per liter) at 37 °C in a rotary shaker at 220 rpm. WLGB-RPP which was constructed in the previous research (Choi et al., 2010) was used as a host strain for astaxanthin production. Strains for production were first incubated at 37 °C in 25 mL test tube containing 10 mL of LB medium. After 12 h, the cells were transferred to a 250 mL volume baffled flask containing 50 mL of 2xYT medium (16 g tryptone, 10 g yeast extract and 5 g NaCl per liter) (Choi et al., 2010) and cultured at 37 °C shaken at 220 rpm for 24 h. After optimization of culture conditions, the culture scheme was changed. Cells were first incubated at 30 °C in a 250 mL baffled flask containing 20 mL of TB medium until the optical density at 600 nm (OD_{600}) reached 1–2. After that, they were transferred to a 250 mL baffled flask containing R/2 medium with 3 g/L of yeast extract and 20 g/L of glycerol, and incubated at 30 °C shaken at 220 rpm for 36 h. The TB medium contains 24 g yeast extract, 20 g tryptone, 4 mL of glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 per liter. The R/2 medium (pH 6.8) contains 2 g $(NH_4)_2HPO_4$, 6.75 g KH_2PO_4, 0.85 g citric acid, 0.7 g $MgSO_4{\cdot}7H_2O$, and 5 mL of a trace metal solution (TMS) [10 g $FeSO_4{\cdot}7H_2O$, 2.25 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}5H_2O$, 0.23 g $Na_2B_4O_7{\cdot}10H_2O$, 2 g $CaCl_2{\cdot}2H_2O$ and 0.1 g $(NH_4)_6Mo_7O_{24}$ per liter of 5 M HCl] per liter (Jeong and Lee, 2002). For strains harboring β-carotene producing plasmids or pTrc99a-based plasmids, 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction when OD_{600} reached 0.4–0.6. When required, 50 mg/L of kanamycin (Km) and/or 100 mg/L of ampicillin (Ap) was added into the medium. The cell growth was measured by spectrophotometer observing optical density at 600 nm (Ultraspec3000 spectrophotometer; Pharmacia Biotech). The dry cell weight (DCW) was measured after drying the cell pellets at 75 °C over 48 h.

2.2. Construction of plasmids

All DNA manipulation works were carried out using standard procedures (Sambrook and Russel, 2001). Restriction enzyme digestion and Gibson assembly methods (Gibson et al., 2009) were performed to construct designed plasmids. Routine cultures for the construction of plasmids were performed in LB broth or LB plates (1.5%, w/v, agar) containing appropriate antibiotics. All plasmids and primers used in this study are listed in Table S1 and Table S2, respectively. To construct β-carotene producing plasmids (pBC1 to pBC5), the *crt* genes originated from *P. anantis* were amplified from plasmid pCar184 (Choi et al., 2010) using corresponding primers. To construct plasmid pZea, the *crtEYIB* operon was relocated at *EcoRI/SacI* site of plasmid pTac15k after PCR-amplification from plasmid pBC1, then *crtZ* gene was inserted at the downstream of the *crt* operon after PCR-amplification from genomic DNA of *Pantoea ananatis*. The *E. coli*-codon optimized version of the *trCrBKT* gene originated from *Chlamydomonas reinhardtii* was synthesized (Genescript, USA) which sequence is shown in Table S3. The synthetic *trCrBKT* gene was PCR-amplified using corresponding primers and inserted into plasmid pZX to construct plasmid pAX1. Before constructing the fusion *trCrBKT* genes, the *Fh8* gene which is originated from *Fasciola hepatica* was synthesized (Mbiotech, Korea), the *malE*, *trxA*, *glpF* and *ompF* genes were took from genomic DNA of *E. coli* K12 W3110 and the *mstX* gene was amplified from genomic DNA of *Bacillus subtilis* 168. The sequence encoding signal peptide of OmpF was synthesized by extension PCR using corresponding primers. The amplified fusion tags were linked to the *trCrBKT* gene by overlapping PCR using corresponding primers. The *crtZ* genes originated from *Alcaligenes* sp. (PC-1) and *Agrobacterium aurantiacum* were synthesized (Mbiotech, Korea) and PCR-amplified using corresponding primers to construct plasmids pAX16 and pAX17. To construct target gene overexpressing pTrc99a-based plasmids, the *ispD, ispE, ispF, ispG, ispH, dxs, dxr, ribB, purC, purK, purF, purl, purM* and *glpF* genes were PCR-amplified from genomic DNA of *E. coli,* the *gapB* gene was PCR-amplified from genomic DNA of *B. subtilis* and the *idi2* gene was PCR-amplified from genomic DNA of *Bacillus licheniformis* using corresponding primers. The *hok/sok* system originated from R1 plasmid of *E. coli* was synthesized (Mbiotech, Korea) and PCR-amplified to be inserted into 3 different sites of plasmid pAX15 using corresponding primers.

2.3. Fed-batch fermentation for astaxanthin production

Fed-batch fermentations were carried out at 30 °C in a 5 L MARADO-05D-PS fermenter (BioCNS, Daejeon, Republic of Korea) containing 1.6 L of modified R/2 medium which consists of 7.5 mL of TMS per liter and unchanged other components supplemented with 5 g/L yeast extract and 30 g/L glycerol. Cells were grown in 20 mL TB medium in a 250 mL baffled flask at 30 °C shaken at 220 rpm until OD_{600} value reached 1–2 and transferred into a new 250 mL baffled flask with 50 mL R/2 medium supplemented with 3 g/L yeast extract and 20 g/L glycerol. After incubation at 30 °C shaken at 220 rpm until OD_{600} value reached 3–4, 40 mL of the seed culture was inoculated into the bioreactor containing the medium saturated by filtered air. The