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Enhanced Production of Astaxanthin by Metabolically Engineered Non-mevalonate Pathway in *Escherichia coli*

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Astaxanthin is one of the major carotenoids used in pigment has a great economical value in pharmaceutical markets, feeding, nutraceutical and food industries. This study was to increase the production of astaxanthin by co-expression with transformed Escherichia coli using six genes involved in the non-mevalonate pathway. Involved in the non-mevalonate biosynthetic pathway of the strain *Kocuria gwangalliensis* were cloned *dxs, ispC, ispD, ispE, ispF, ispG, ispH* and *idi* genes in order to increase astaxanthin production from the transformed *E. coli*. And co-expression with the genes to compared the amount of astaxanthin production. This engineered *E. coli*, containing both the non-mevalonate pathway gene and the astaxanthin biosynthesis gene cluster, produced astaxanthin at 1,100 µg/g DCW (dry cell weight), resulting in approximately three times the production of astaxanthin.

Keywords: ***Astaxanthin, isoprenoid biosynthesis gene, production, Paracoccus haeundaensis, Kocuria gwangalliensis, Escherichia coli***

Astaxanthin (3, 3-dihydroxy-β, β-carotene-4, 4-dione) is one of the major carotenoids used is one of the major carotenoids used to enhance the color of aquatic animals. It also has the ability to protect cell membranes from damage by free radicals, which is the cancer causing damage. Astaxanthin, the most valuable ketocarotenoid from the biotechnological and commercial points of view, is found in most crustaceans, such as shrimp, crab, and lobster [1, 2]. Organisms that produce astaxanthin include the basidiomycetous yeast *Phaffia rhodozyma* [2], the green alga *Haematococcus pluvialis* [3], the Gram-negative bacterium *Agrobacterium aurantiacum* [4], *Paracoccus marcusii* [5], *Paracoccus carotinifaciens* [6], *Paracoccus* sp. MBIC1143 [7], and *Paracoccus haeundaensis* (in this study) [8]. Because the quality of synthetically produced astaxanthin is not high enough for commercial production, naturally purified astaxanthin is used only for food additives and coloring. In cosmetology and pharmacology, this pigment is in great demand as a dermal photoprotector. Thus, astaxanthin has high economic value, and various studies have been conducted to increase the production of astaxanthin via metabolic pathway engineering [9, 10].

To improve production of astaxanthin in engineered microbial hosts, the available precursors pool must be optimization of pyruvate 1-deoxy-d-xylulose-5-phosphate and 1-deoxy-d-xylulose-5-phosphate and 4-diphosphocytidyl-2-C-methyl-d-erythritol and 4-diphosphocytidyl-2-C-methyl-d-erythritol and 2-C-methyl-d-erythritol2,4-cyclodiphosphate and 1- hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate and 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate and isopentenyl diphosphate and dimethylallyl diphosphate and balancing the expression of the carotenogenic genes to efficient transform the precursors into the desired carotenoid compounds [10–12]. Although *E. coli* has been used as the most common hosts in the

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metabolic engineering of carotenoid production, but the supply of common precursors such as isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), and farnesyl diphosphate (FPP) is limited. Because the need for such compounds for growth is relatively small [13, 14]. Therefore, it is essential to develop the *E. coli* strains that efficiently produce plentiful supplies of the common precursors.

In order to increase the production of the common precursors—IPP, DMAPP, and FPP—we reconstructed the non-mevalonate pathway in *E. coli* and overexpressed the non-mevalonate pathway genes (*dxs*, *ispC*, *ispD*, *ispE, ispF*, *ispG*, *ispH*, and *idi* genes) from *K gwangalliensis* [15]. This gene was introduced into the *E. coli* strain harboring the astaxanthin biosynthesis gene cluster (*crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtW* genes) of *P. haeundaensis*. The co-expression of both the isoprenoid pathway and astaxanthin biosynthesis genes resulted in approximately three times the production of astaxanthin.

A marine bacterium, *K. gwangalliensis*, was isolated from seawater in the coast of Gwangalli, Busan. This strain was isolated on nutrient agar medium (Difco, Sparks, MD) and maintained on PPES-II medium [containing (L-1) 1 g Bacto tryptone (Difco, Sparks, MD), 1 g Bacto soytone (Difco, Sparks, MD), 1 g Bacto yeast extract (Difco, Sparks, MD), 0.01 g ferric citrate (Fluka, Switzerland), 2 g poly peptone (Difco, Sparks, MD), and 30 g NaCl (Junsei, Japan)] by serial inoculation [15].

E. coli strain XL1-blue [F'::*proA*$^+$*B*$^+$*lacI*^{q} *Δ(lacZ) M15/ Tn10(Tet*^{r}*)/recA1 endA1 gyrA96(Nal*^{r}*) thi hsdR17(*r_K^- m_K^+*) supE44 relA1 lac*] was used for the gene cloning experiments. Astaxanthin was produced using BL21 (DE3) (Stratagene, La Jolla, CA). The transformed cells were cultured at 37℃ in LB medium containing 100 μg/ml of ampicillin and 50 μg/ml of kanamycin until OD_{600} = 0.5, and 0.1 mM of IPTG (isopropyl-β-thiogalactopyranoside) was added and the culture was shaken at 37℃ to overexpression the recombinant protein.

In order to construct the expression plasmid for each *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *idi* gene from *K. gwangalliensis*, PCR was carried out using a pair of oligonucleotides with DXS-F, DXS-R, IspC-F, IspC-R, IspD-F, IspD-R, IspE-F, IspE-R, IspF-F, IspF-R, IspG-F, IspG-R, IspH-F, IspH-R, IDI-F, and IDI-R primers (Table 1) and *K. gwangalliensis* strain SJ2 genomic DNA as a template. The PCR product was ligated to the pGEM-T-easy vector (Promega, Madison, WI), and its nucleotide sequence was confirmed by DNA sequencing. The subcloned plasmid was digested with *NdeI* and *HindIII* restriction enzymes. The excised fragment was ligated into the expression plasmid pET44a(+) vector,

Table 1. Oligonucleotides used for this study.

Primer	Sequence	Remark
DXS-F	5'-CATATGACGCTTCTGGAGACC-3'	Primer for *DXS* (*Nde* I site)
DXS-R	5'-AAGCTTCTACGCGCCGTCCACCCG-3'	Primer for *DXS* (*Hind* III site)
IspC-F	5'-CATATGTCTGACGCACCCCTG-3'	Primer for *IspC* (*Nde* I site)
IspC-R	5'-AAGCTTTCAGCGTGCGCTGAAACG-3'	Primer for *IspC* (*Hind* III site)
IspD-F	5'-CATATGAAGGACGGGCTCGCT-3'	Primer for *IspD* (*Nde* I site)
IspD-R	5'-AAGCTTTCAATCAGCCACGTGGTC-3'	Primer for *IspD* (*Hind* III site)
IspE-F	5'-CATATGAGCCCCGCCGGATTG-3'	Primer for *IspE* (*Nde* I site)
IspE-R	5'-AAGCTTCTAGATTGTGCCCCACTC-3'	Primer for *IspE* (*Hind* III site)
IspF-F	5'-CATATGACCGAACAGCCAGAA-3'	Primer for *IspF* (*Nde* I site)
IspF-R	5'-AAGCTTCTAACGGCGCGGGATCAC-3'	Primer for *IspF* (*Hind* III site)
IspG-F	5'-CATATGCCCGCTGCACCCCAG-3'	Primer for *IspG* (*Nde* I site)
IspG-R	5'-AAGCTTTCATCCGGCCGTCACGAC-3'	Primer for *IspG* (*Hind* III site)
IspH-F	5'-CATATGCCCACCGTTCCCCGC-3'	Primer for *IspH* (*Nde* I site)
IspH-R	5'-AAGCTTTTAGCGCTTGGGGCGCGC-3'	Primer for *IspH* (*Hind* III site)
IDI-F	5'-CATATGACCCAGCAGACCACC-3'	Primer for *IDI* (*Nde* I site)
IDI-R	5'-AAGCTTCTAGCCGAGCTGGGCCTG-3'	Primer for *IDI* (*Hind* III site)

and the resulting plasmid having eight genes of the carotenoid biosynthesis enzymes was inserted into pET44a(+)-DXS, pET44a(+)-IspC, pET44a(+)-IspD, pET44a(+)-IspE, pET44a(+)-IspF, pET44a(+)-IspG, pET44a(+)-IspH, and pET44a(+)-IDI, Fig. 1(A). In a previous study, we constructed pCR-XL-TOPO-Crt-full plasmid, which carried the full-length crotenoid biosynthesis operon gene [16]. The co-expressed pCR-XL-TOPO-Crt-full plasmid, Fig. 1(B) and pET44a(+)-DXS, pET44a(+)-IspC, pET44a(+)-IspD, pET44a(+)-IspE, pET44a(+)-IspF, pET44a(+)-IspG, pET44a(+)-IspH, and pET44a(+)-IDI plasmids were transformed into *E. coli* BL21(DE3) [9].

Ten grams of lyophilized cells of *E. coli* BL21(DE3) carrying the plasmid was resuspended in 10 ml of acetone and incubated overnight at 4℃. The acetone was evaporated and the pellet was dissolved in 10 ml of n-hexane-ethanol (1:1, v/v). Then, the extract was diluted to one-half with distilled water, and two phases were separated with a separatory funnel. The organic phase (*n*-hexane phase) was washed with 30% aqueous ethanol until it was colorless and near neutral pH. After separation, the organic phase was dried under a stream of nitrogen, and the residue was stored in a refrigerator.

The astaxanthin extract was dissolved in 2-propanol and high-performance liquid chromatography (HPLC) was performed using an Agilent 1100 HPLC system (Agilent Technologies, Germany) equipped with a temperature-controlled automatic sampler and a diode array detector. The column was YMC carotenoid C30 column (5 μm steel, 250 mm long × 4.6 mm i.d.; Waters Corp., Milford, MA) and the guard column was a Pelliguard LC-18 cartridge (20 mm; Supelco, Bellefonte, PA). The mobile phase was a methanol/methyl *tert*-butyl ether gradient (A/B) with the following parameters (all percentages expressed as v/v): start, 80% A/20% B; 10 min, 65% A/35% B; 20 min, 10% A/90% B. The flow rate was 1.0 ml/min, the injection volume 10 ml, column temperature was 15℃. Astaxanthin, canthaxanthin, and β-carotene were detected by absorbance at 470 nm (286 nm for phytoene) and purchased from Sigma (USA).

The following equation was used to calculate the amount of the accumulated astaxanthin from the transformed cells [17]:

$$\text{Total carotenoid (g)} = \frac{\text{volume of solvent (ml)} \times A_{\lambda\max}}{E_{1\text{cm}}^{1\%} \times 100}$$

The *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *idi* genes in the non-mevalonate pathway are essential for the biosynthesis of IPP, DMAPP, and FPP. Theses precursors are important compounds for the efficient production of astaxanthin in FPP. However, the *E. coli* cell has a limited supply of these common precursors, because it only needs a small amount of those compounds for growth. The isoprenoid biosynthesis genes were expressed by subcloning *dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*, and *idi* genes into the expression vector, pET44-a(+), which allows expression of a recombinant. The expression plasmids were individually constructed under the control of the T7 promoter and resulted in

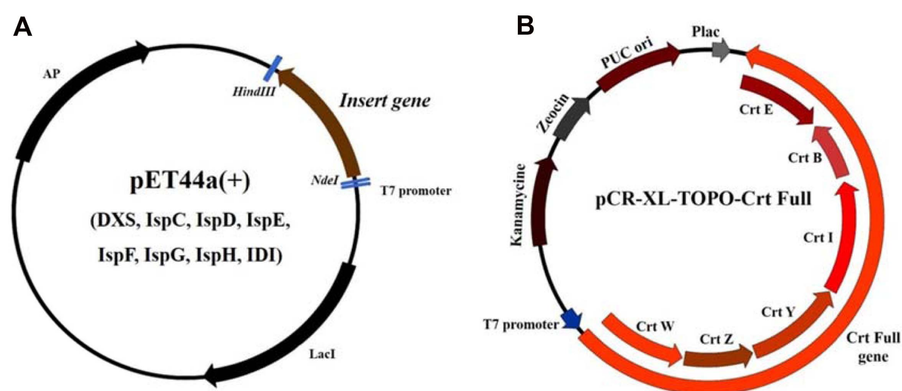

Fig. 1. Recombinant plasmids used for astaxanthin production. (A) Construction of recombinant pET44a(+)-Insert gene plasmids. Each Insert gene (*DXS*, *IspC*, *IspD*, *IspE*, *IspF*, *IspG*, *IspH*, and *IDI)* was subcloned into expression vector pET44a(+). (B) pCR-XL-TOPO-Crt full carrying astaxanthin biosynthesis gene cluster.

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pET44a(+)-DXS, pET44a(+)-IspC, pET44a(+)-IspD, pET44a(+)-IspE, pET44a(+)-IspF, pET44a(+)-IspG, pET44a(+)-IspH, and pET44a(+)-IDI (Fig. 1). These plasmids were transformed into the *E. coli* BL21(DE3) codon plus strain. The recombinant protein was overexpressed by adding IPTG and incubating at 37℃. The expression of the recombinant DXS, IspC, IspD, IspE, IspF, IspG, IspH, and IDI proteins was analyzed by 12% SDS-PAGE. The isoprenoid biosynthesis genes were constructed and expressed in *E. coli*, and the recombinant DXS, IspC, IspD, IspE, IspF, IspG, IspH, and IDI proteins appeared to be approximately 72 kDa, 46 kDa, 26 kDa, 35 kDa, 18 kDa, 40 kDa, 38 kDa, and 20 kDa.

To increase the production of astaxanthin, we used these strains for the co-expression of both the crotenoid biosynthesis operon gene cluster and the *dxs, ispC, ispD, ispE, ispF, ispG, ispH*, and *idi* genes in the non-mevalonate pathway.

The plate with the cells transformed with pCR-XL-TOPO-Crt-full and pET44a(+)-DXS plasmid was shown in red color, indicating that the expressed proteins (DXS, CrtE, CrtB, CrtI, CrtY, CrtZ, and CrtW) mediated the formation of a red-colored astaxanthin metabolite intermediate is produced (canthaxanthin, zeaxanthin, lycopene, β-carotene, phytoene, FPP, GGPP, and IPP). To calculate the amount of carotenoids accumulated from the transformed cells, the equation was introduced [9]. The amounts of accumulated 960 ug/g DCW of astaxanthin, 370 ug/g DCW of canthaxanthin and 180 ug/g DCW of β-carotene were calculated respectively.

The plate with the cells transformed with pCR-XL-TOPO-Crt-full and pET44a(+)-IDI plasmid was also appeared as red astaxanthin. Using the formula, the amounts of astaxanthin, canthaxanthin and β-carotene were calculated as 1,100 ug/g DCW, 380 ug/g DCW, and 190 ug/g DCW, respectively. The accumulated amounts of astaxanthin produced by these strains and analyzed by HPLC are summarized in Table 2.

Lee *et al.* (2007) transformed was into an expression vector (pCR-XL-TOPO-Crt-full) and transformed into *E. coli* to produce astaxanthin harboring a dry weight of 400 μg/g DCW [18]. Comparing the results of that study with the results of the this study, a higher amount of astaxanthin was accumulated by the co-expression system of the the *dxs, ispC, ispD, ispE, ispF, ispG, ispH*, and *idi* genes in the non-mevalonate pathway and the crotenoid biosynthesis operon gene (pCR-XL-TOPO-Crt-full containing *crtW, crtZ, crtY, crtI, crtB,* and *crtE* genes) than an expression system using only astaxanthin biosynthesis genes. This engineered *E. coli* strain containing both non-mevalonate pathway genes and an astaxanthin biosynthesis gene cluster (both pCR-XL-TOPO-Crt-full and pET44a(+)-IDI plasmid) produced 1,100 ug/g DCW of astaxanthin, The production of astaxanthin increased three-fold over *E. coli* transformants harboring only pCR-XL-TOPO-Crt-full plasmid.

Growth patterns and astaxanthin production from recombinant *E. coli* cells harboring pCR-XL-TOPO-Crt-full and pET44a(+)-IDI plasmids are shown in Fig. 2. The production rates in astaxanthin started to increase

Table 2. Effect of astaxanthin production in recombinant *E. coli* BL21(DE3) strain harboring the astaxanthin synthesis genes *crtEBIYZW* and *dxs, ispC, ispD, ispE, ispF, ispG, ispH*, and *idi*.

Plasmids	Genes	β-carotene content (μg/g DCW)	Canthaxanthin content (μg/g DCW)	Astaxanthin content (μg/g DCW)
pCR-XL-Topo-Crt full	crtEBIYZW			400 (±20)
pCR-XL-Topo-Crt full and pET44a(+)-DXS	*crtEBIYZW* and *dxs*	180 (±30)	370 (±20)	960 (±30)
pCR-XL-Topo-Crt full and pET44a(+)-IspC	*crtEBIYZW* and *ispC*	50 (±50)	170 (±40)	380 (±40)
pCR-XL-Topo-Crt full and pET44a(+)-IspD	*crtEBIYZW* and *ispD*	120 (±20)	100 (±30)	240 (±60)
pCR-XL-Topo-Crt full and pET44a(+)-IspE	*crtEBIYZW* and *ispE*	30 (±40)	150 (±20)	350 (±30)
pCR-XL-Topo-Crt full and pET44a(+)-IspF	*crtEBIYZW* and *ispF*	70 (±20)	210 (±30)	480 (±50)
pCR-XL-Topo-Crt full and pET44a(+)-IspG	*crtEBIYZW* and *ispG*	90 (±10)	180 (±10)	520 (±30)
pCR-XL-Topo-Crt full and pET44a(+)-IspH	*crtEBIYZW* and *ispH*	60 (±20)	360 (±10)	680 (±40)
pCR-XL-Topo-Crt full and pET44a(+)-IDI	*crtEBIYZW* and *idi*	190 (±30)	380 (±20)	1,100 (±50)

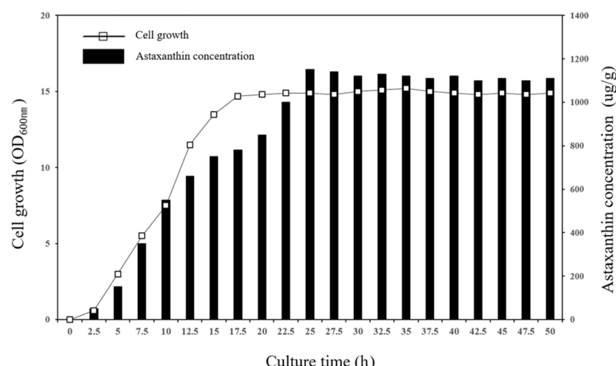

Fig. 2. Time profiles of cell growth and astaxanthin production. BL21(DE3) was used as the host strain with the pCR-XL-TOPO-Crt full and pET44a(+)-IDI plasmids that led to astaxanthin production. Symbol: cell growth; Column: astaxanthin concentration in LB medium.

from 20 h of incubation and it has standardise after 25 h.

The commercial application of natural resources has enormous potential. There is particular value in the practical use of these resources as functional materials in areas such as food, medicine, and cosmetics. One of these natural resources, the natural red pigment astaxanthin, has a broad application area, and thus, high growth potential. Therefore, its application and increased production is extremely important from an industrial and economical perspective.

The combination of the astaxanthin biosynthesis genes of *P. haeundaensis* and the non-mevalonate biosynthetic genes of *K. gwangalliensis* were used to improve the productions of astaxanthin as a co-expression system. Consequently, as a result of these findings, the applications can be used to enhance the production of astaxanthin through co-expression system of carotenoid biosynthetic genes and non-mevalonate biosynthetic genes, which are crucial it is because astaxanthin pigment has a great economical value in pharmaceutical markets, feeding, nutraceuticals and food industries. These results will give a wider knowledge based on the gene expression of astaxanthin biosynthesis gene clusters and biotechnological applications of carotenoids at the molecular level. Futhermore, the possibility of individual control of the expression in levels of *dxs, ispC, ispD, ispE, ispF, ispG, ispH* and *idi* genes in *E. coli* will enable more applications to the *E. coli* co-expression system.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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