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Combinatorial expression of bacterial whole mevalonate pathway for the production of β-carotene in *E. coli*

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ABSTRACT

The increased synthesis of building blocks of IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate) through metabolic engineering is a way to enhance the production of carotenoids. Using *E. coli* as a host, IPP and DMAPP supply can be increased significantly through the introduction of foreign MVA (mevalonate) pathway into it. The MVA pathway is split into two parts with the top and bottom portions supplying mevalonate from acetyl-CoA, and IPP and DMAPP from mevalonate, respectively. The bottom portions of MVA pathway from *Streptococcus pneumonia*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Saccharomyces cerevisiae* were compared with exogenous mevalonate supplementation for β-carotene production in recombinant *Escherichia coli* harboring β-carotene synthesis genes. The *E. coli* harboring the bottom MVA pathway of *S. pneumoniae* produced the highest amount of β-carotene. The top portions of MVA pathway were also compared and the top MVA pathway of *E. faecalis* was found out to be the most efficient for mevalonate production in *E. coli*. The whole MVA pathway was constructed by combining the bottom and top portions of MVA pathway of *S. pneumoniae* and *E. faecalis*, respectively. The recombinant *E. coli* harboring the whole MVA pathway and β-carotene synthesis genes produced high amount of β-carotene even without exogenous mevalonate supplementation. When comparing various *E. coli* strains – MG1655, DH5α, S17-1, XL1-Blue and BL21 – the DH5α was found to be the best β-carotene producer. Using glycerol as the carbon source for β-carotene production was found to be superior to glucose, galactose, xylose and maltose. The recombinant *E. coli* DH5α harboring the whole MVA pathway and β-carotene synthesis genes produced β-carotene of 465 mg/L at glycerol concentration of 2% (w/v).

1. Introduction

Carotenoids are a family of yellow to orange-red terpenoid pigments synthesized by photosynthetic organisms including some bacteria and fungi (Britton et al., 1998). Industrially, carotenoids are used in pharmaceuticals, nutraceuticals, and animal feed additives as well as colorants in cosmetics and food. β-Carotene has provitamin A activity, and it is responsible for the synthesis of retinoids. Currently, more than 90% of commercialized β-carotene is produced through chemical synthesis. In recent years, interest in production of natural carotenoids by microbial fermentation has increased, and carotenogenic microbes such as *Xanthophyllomyces dendrorhous*, *Haematococcus* alga, and *Blakeslea trispora* have been investigated for large-scale production (An and Choi, 2003; Jacobson et al., 2000; Mehta et al., 2003; Olaizola, 2000). The increasing interest of microbial carotenoid is due to the consumer preferences for natural additives, as well as the potential cost effectiveness of mass production via microbial biotechnology (Dufosse et al., 2005). The availability of carotenoid genes from carotenogenic microbes has made possible the synthesis of carotenoids in non-carotenogenic microbes, e.g. *Escherichia coli*, *Zymomonas mobilis*, *Candida utilis*, and *Saccharomyces cerevisiae* (Misawa and Shimada, 1997). In particular, *E. coli* is an excellent host for carotenoid production because it has powerful genetic tools for metabolic engineering, and it readily expresses many carotenogenic genes that is able to make various carotenoids, such as lycopene, zeaxanthin, and astaxanthin (Cheng, 2006; Das et al., 2007; Ruther et al., 1997; Sandmann, 2002). However, until now, there have been only few attempts to produce recombinant β-carotene in non-carotenogenic microbes using metabolic engineering approach (Rodriguez-Saiz et al., 2007; Verwaal et al., 2007; Yoon et al., 2007).

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Carotenoids are derived from two common building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized through the MVA pathway in eukaryotes or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in prokaryotes (Fig. 1) (Lange et al., 2000; Lee and Schmidt-Dannert, 2002; Rohmer, 1999). The MEP pathway has been engineered to increase the supply of IPP and DMAPP building blocks in *E. coli* for increased synthesis of carotenoids (Farmer and Liao, 2001; Kajiwara et al., 1997; Kim and Keasling, 2001). As an alternative to using the MEP pathway, a foreign MVA pathway was introduced into *E. coli*, and the production of carotenoids was increased further by using this pathway compared to the endogenous MEP pathway. Vadali et al. (2005) reported 4.3 mg/L of lycopene production from recombinant *E. coli* harboring the whole MVA pathway of *Streptomyces* sp. CL190, which was two-fold higher than the lycopene production using the native MEP pathway. Previously, we obtained 102 mg/L of lycopene and 503 mg/L of β-carotene from recombinant *E. coli* using the bottom portion of MVA pathway of *Streptococcus pneumoniae* with exogenous supplementation of MVA (Yoon et al., 2006, 2007). Although we did not use the entire MVA pathway but only the bottom portion of the MVA pathway with the addition of mevalonate, our carotenoids production was much higher than that obtained with the whole MVA pathway of *Streptomyces* sp. CL190. The most probable reason for the huge difference in lycopene production could be due to differences in culture conditions, host strain, expression level of carotenoids genes, etc. However, we suspect that the different efficiency of the MVA pathways used for the lycopene production may be the principal reason. As *Streptomyces* sp. is a high GC% strain and therefore its codon preference is different from that of *E. coli*, the genes encoding the MVA pathway of *Streptomyces* sp. CL190 may not be well expressed in *E. coli*. In this study, we compared the MVA pathway of different microorganisms in *E. coli*. The MVA pathway was divided into two portions: top (acetyl-CoA to mevalonate) and bottom (mevalonate to IPP and DMAPP) for comparison and to simplify the construction of foreign whole MVA pathway in *E. coli* (Fig. 1). The MVA pathway was optimized in *E. coli* by combining the most efficient top and bottom portions of the MVA pathways from different organisms. β-Carotene was used as a model carotenoid here to investigate the effect of the optimized MVA pathway.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli DH5α was used for gene cloning and the reference β-carotene production, and *E. coli* MG1655, BL21 (DE3), XL1-Blue and S17-1 were used to compare effect of different host strains (Table 1). The top portions of MVA pathway were obtained from *E. faecalis* (ATCC 14508), *Staphylococcus aureus* (ATCC 35556) and *S. pneumoniae* (ATCC 6314, purchased from American Type Culture Collection (ATCC, Manassas, USA), and *Ralstonia eutropha* (KCTC 1006), purchased from Korean Collection for Type Cultures (KCTC, Daejeon, Korea) (Table 1). Cultivation of *E. coli* strains was carried out in 2YT medium (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) at 37 °C for gene cloning and at 29 °C for β-carotene production on a rotary shaker at 250 rpm. Recombinant *E. coli* harboring the top portion of MVA pathway was inoculated and grown in TT medium (20 g glucose, 16 g K_2HPO_4, 14 g KH_2PO_4, 5 g peptone, 1 g citric acid, 25 mg $MgSO_4{\cdot}7H_2O$, 50 mg $FeSO_4{\cdot}7H_2O$, 8 mg thiamine·HCl for 1 L, pH 7.2) containing 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) initially to induce mevalonate synthesis (Tabata and Hashimoto, 2004). Ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) (Sigma Chemical Corp., St. Louis, MO, USA) were added to the culture medium as required. Carbon sources such as glucose, galactose, xylose, maltose, and glycerol were added in concentrations of 0.5% (w/v) to 2.0% (w/v), and 0.2% (w/v) arabinose was included as an additional carbon source. Mevalonate, prepared as previously described (Kim et al., 1992) from mevalonolactone (Sigma Chemical Corp.), was supplied in concentrations of 3.3 mM for IPP synthesis via the bottom MVA pathway. Cell growth was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}).

2.2. Construction of plasmids for expression of MVA pathway in E. coli

Common procedures, including genomic DNA preparation, restriction digestions, transformations, and other standard molecular biological techniques, were carried out as described in the Molecular Cloning (Sambrook and Russell, 2001). PCR was per-

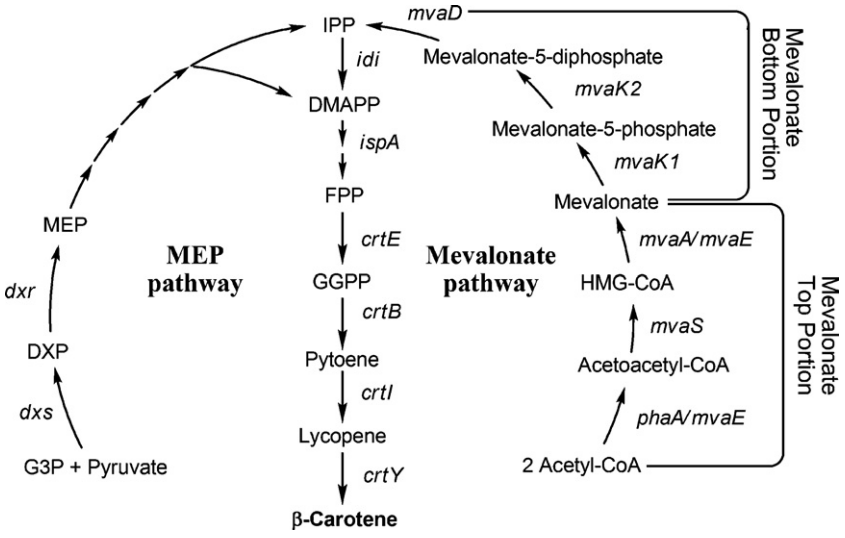

Fig. 1. Biosynthesis of β-carotene in recombinant *E. coli*. IPP, precursor for β-carotene biosynthesis is synthesized via both MEP and MVA pathways. The MVA pathway was divided into two portions of top (acetyl-CoA to MVA) and bottom (MVA to IPP and DMAPP). The top portion is composed of *phaA* (acetyl-CoA acetyltransferase), *mvaS* (HMG-CoA synthase) and *mvaA* (HMG-CoA reductase) genes, while the bottom is made up of *mvaK1* (mevalonate kinase), *mvaK2* (phosphomevalonate kinase), *mvaD* (Diphosphomevalonate decarboxylase) and *idi* (IPP isomerase). IPP is converted to β-carotene via foreign carotenoid synthesis pathway which includes *crtE*, *crtB*, *crtI*, and *crtY* genes. Abbreviations: G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

Table 1
Strains, plasmids and primers used in this study.

Strains, plasmids and primers	Description	Reference or source
E. coli strains		
MG1655	Wild type	
DH5α	F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1 *endA*1, *hsdR*17(r_K^- m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, gyrA96, *rel*A1	
BL21(DE3)	F^-, *ompT*, *hsdS*$_B$($r_B^-m_B^-$), *gal* (c *I*857, *ind*1, *Sam*7, *nin*5, *lac*UV5-T7*gene*1), *dcm* (DE3)	
XL1-Blue	*hsdR*17, *supE*44, *recA*1, *endA*1, *gyrA*46, *thi*, *relA*1, *lac*/F′[*proAB*$^+$, *lacI*^{q}, *lacZ* M15::Tn10(*tet*^{r})]	
S17-1	*recA*, *pro*, *hsdR*, RP4-2-Tc::Mu-Km::Tn7	
Plasmids		
pBluescript	P_{lac} cloning vector, ColE1 origin, *lacZ*, Amp^r	Stratagene
pSTV28	P_{lac} expression vector, pACYC184 origin, *lacZ*, Cm^r	Takara Co., Ltd.
pTrc99A	P_{trc} expression vector, pBR322 origin, lacI^q, Amp^r	Amersham Biosci.
pSEF12Didi	pSTV28 containing *mvaK1, mvaK2* and *mvaD* of *E. faecalis*, and *idi* of *E. coli*	Zahiri et al. (2006)
pSSA12Didi	pSTV28 containing *mvaK1, mvaK2* and *mvaD* of *S. aureus*, and *idi* of *E. coli*	Zahiri et al. (2006)
pSSC12Didi	pSTV28 containing *mvaK1, mvaK2* and *mvaD* of *S. cerevisiae*, and *idi* of *E. coli*	Zahiri et al. (2006)
pSSN12Didi	pSTV28 containing *mvaK1, mvaK2* and *mvaD* of *S. pneumoniae*, and *idi* of *E. coli*	Yoon et al. (2006)
pSSY12Didi	pSTV28 containing *mvaK1, mvaK2* and *mvaD* of *S. pyogenes*, and *idi* of *E. coli*	Zahiri et al. (2006)
pTSNASP	pTrc99A containing *mvaA* and *mvaS* of *S. pneumoniae*, and *phaA* of *R. eutropha*	This study
pTSAASP	pTrc99A containing *mvaA* and *mvaS* of *S. aureus*, and *phaA* of *R. eutropha*	This study
pTEFAES	pTrc99A containing *mvaE* and *mvaS* of *E. faecalis*	This study
pS-NA	pSTV28 containing *mvaE* and *mvaS* of *E. faecalis*, *mvaK1*, *mvaK2*, and *mvaD* of *S. pneumoniae*, and *idi* of *E. coli*	This study
pT-DHB	pTrc99A containing *crtE*, *crtB*, and *crtI* of *P. agglomerans*, *ipiHP1* of *H. pluvialis*, *crtY* of *P. ananatis*, and *dxs* of *E. coli*	Yoon et al. (2007)
Primers[a]		
SNmvaA-5	5′-GGAATTC**ATAATGAAGATAAGTTGGAATGG**-3′	
SNmvaA-3	5′-GGGTACC**TTATGATCTTAAATTTTCGAG**-3′	
SNmvaS-5	5′-CGGTACC**AGAAGGATTTTTTACATGAATG**-3′	
SNmvaS-3	5′-CTCTAG***ATTA***TTTTTCAACCTTGCTATAAC-3′	
SAmvaA-5	5′-C**ACATGTCCATGCAAAGTTTAGATAAG**-3′	
SAmvaA-3	5′-CGAGCTC**TTATTGTTGTCTAATTTCTTG**-3′	
SAmvaS-5	5′-CGAGCTCAGGAG**AAACCTTATGACAATAGGTATC**-3′	
SAmvaS-3	5′-CGGTACC**TTACTCTGGTCTGTGATATTC**-3′	
phaA-5	5′-GCTGCAG**AGGACTACACAATGACTGACGTTG**-3′	
phaA-3	5′- CCTGCAG**TTATTTGCGCTCGACTGCCAGCG**-3′	
EFmvaE-F	5′-CGAGCTC**AGGAGCATTTA**GATG**TTGAAAACAGTAGTTATTATTG**-3′	
EFmvaE-R	5′- GCCCGGG**GTGGCCTGAAACGGCTACC**-3′	
EFmvaS-F	5′- CGGATCC**CTTAGTTTCGATAAGAGCGAAC**-3′	
EFmvaS-R	5′-CGGATCC**CTTAGTTTCGATAAGAGCGAAC**-3′	
EFES-F	5′-GCTCGAG**GAAACAGACCATGGAATTC**-3′	
EFES-R	5′-GCTCGAGGCTGCAG**TTAGTTTCGATAAGAGCGAAC**-3′	

[a] Template binding regions are indicated by bold letters, start and stop codons are underlined, and restriction sites are double underlined.

formed using *pfx* DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA) and standard protocols. PCR products were cloned into pBluescript for DNA sequence analysis. The plasmids of pBluescript, pTrc99A, and pSTV28 were used as vectors for gene cloning and gene expression studies (Table 1).

Plasmids and PCR primers used in this study are listed in Table 1. Plasmids for the bottom portion of MVA pathway, pSEF12Didi, pSSA12Didi, pSSC12Didi, pSSN12Didi, and pSSY12Didi, were used as described previously (Yoon et al., 2006; Zahiri et al., 2006). To construct the top portion of MVA pathway, *mvaA* and *mvaS* genes of *S. pneumoniae* were amplified from its genomic DNA by PCR using primers of SNmvaA-5, SNmvaA-3, SNmvaS-5, and SNmvaS-3. The PCR product of *mvaA* cleaved by EcoRI and KpnI was ligated into the same site of pTrc99A resulting in pTSNA. The plasmid of pTSNAS was constructed by inserting the PCR product of *mvaS* digested with KpnI and XbaI into the corresponding sites of pTSNA. The gene of *phaA* amplified by PCR using primers of phaA-5 and phaA-3 was digested with PstI and inserted into the same site of pTSNAS, resulted in pTSNASP (Supplementary Fig. 1). The *S. aureus mvaA* gene amplified by using primers of SAmvaA-5 and SAmvaA-3 was restricted with PciI and SacI site, and cloned into NcoI and SacI sites of pTrc99A to construct pTSAA. The PCR product of *S. aureus mvaS* obtained with SAmvaS-5, and SAmvaS-3 was inserted into pTSAA using SacI and KpnI site to make pTSAAS. The blunt-end PCR product of *phaA* amplified by using primers of phaA-5 and phaA-3 was cloned into SmaI site of pTSAAS to construct pTSAASP (Supplementary Fig. 1). The bifunctional *mvaE* gene of *E. faecalis* was amplified with primers of EFmvaE-F and EFmvaE-R, followed by digesting with *Sac*I and SmaI for its insertion into the same sites of pTrc99A resulting in pTEFAE. The *E. faecalis mvaS* gene amplified with EFmvaS-F and EFmvaS-R was cloned using SmaI and BamHI sites on pTEFAE to construct pTEFAES (Supplementary Fig. 1). The whole MVA pathway operon of pS-NA plasmid consists of the top portion of *E. faecalis* MVA pathway and the bottom portion of *S. pneumoniae* MVA pathway. To construct the pS-NA plasmid, the top portion of MVA pathway was amplified from pTEFAES by using primers of EFES-F and EFES-R. The PCR product was digested with XhoI, and inserted into SalI site of pSSN12Didi resulting into pS-NA (Supplementary Fig. 1).

2.3. Measurement of β-carotene, mevalonate, and acetate production

To determine β-carotene concentration, cells were harvested by centrifugation at 14,000 rpm for 40 s and washed once with water. The cell pellet was then resuspended in 1 mL of acetone and incubated at 55 °C for 15 min in the dark. The acetone extracted supernatant was obtained by centrifuging at 14,000 rpm for 10 min. The β-carotene content of the cell extracts was analyzed using high performance liquid chromatography (LC-20A, Shimadzu, Kyoto, Japan) with a UV/VIS detector at 454 nm using a Symmetry C18 column (250 mm × 4.6 mm, 5 μm, Waters, Milford, USA) equipped

with Sentry Guard C18 column (15 mm × 4.6 mm, 5 μm, Waters, Milford, USA). Methanol and acetonitrile (70:30) were used as the mobile phase at a flow rate of 1.5 mL/min at 40 °C. β-Carotene (Cat. No. C4582, Sigma, USA) was used as the standard. The results represent the means ± SD from three independent experiments.

The concentration of mevalonate was determined by gas chromatography analysis (Otsuka et al., 1973; Tabata and Hashimoto, 2004). Culture filtrate was adjusted to pH 2 with 3 M HCl, incubated at 45 °C for 1 h, and saturated with anhydrous Na_2SO_4, followed by extraction of mevalonate with ethylacetate. The solvent layer was analyzed for mevalonate by gas chromatography (6890N, Agilent Technologies, Santa Clara, CA, USA).

Acetate in culture broth was measured using Megazyme Acetic Acid Assay Kit (Megazyme, Co., Wicklow, Ireland) as described by the manufacturer.

3. Results

3.1. Comparison of various bottom portions of MVA pathway on β-carotene production

Several gram-positive cocci such as *Enterococcus faecalis*, *S. aureus*, *S. pneumoniae* and *Streptococcus pyogens*, and yeast such as *S. cerevisiae* are well known to have the MVA pathway (Wilding et al., 2000). Differential production of Coenzyme Q_{10} was observed in *E. coli* using the bottom MVA pathway of different origin (Zahiri et al., 2006). Therefore, we compared the bottom MVA pathway of the microorganisms in *E. coli* for β-carotene production. The bottom MVA pathway genes of *mvaK1*, *mvaK2*, and *mvaD* were cloned from four gram-positive cocci of *S. pneumoniae*, *S. pyogenes*, *S. aureus*, and *E. faecalis*, and from *S. cerevisiae*. The IPP isomerase encoding *idi* was isolated from *E. coli*. The four genes *mvaK1*, *mvaK2*, *mvaD*, and *idi* were combined to make the artificial operon of bottom MVA pathway on the pSTV28 vector. The plasmids containing the bottom MVA pathway operon were named according to the name of microorganism from which the operon was cloned, such as pSSN12Didi (*S. pneumoniae*), pSSY12Didi (*S. pyogenes*), pSSA12Didi (*S. aureus*), pSEF12Didi (*E. faecalis*) and pSSC12Didi (*S. cerevisiae*). In order to compare the efficiency of the bottom MVA pathway on β-carotene production, the bottom MVA pathway plasmids were transformed into the β-carotene producing *E. coli* harboring pT-DHB. The pT-DHB plasmid is composed of *crtE*, *crtB* and *crtI* of *P. agglomerans*, *crtY* of *P. ananatis*, *ipi* of *H. pluvialis*, and *dxs* of *E. coli* (Yoon et al., 2007). IPP isomerase (*ipi*) and DXP (deoxyxylulose phosphate) synthase (*dxs*) are rate-limiting enzymes in carotenoid biosynthesis and the MEP pathway, respectively. The recombinant *E. coli* harboring pT-DHB and the bottom MVA pathway plasmids was cultivated at 29 °C for 48 h in 2YT medium containing 3.3 mM mevalonate, and 0.5% (w/v) glycerol and 0.2% (w/v) arabinose as carbon sources. As shown in Fig. 2, β-carotene production was significantly increased in *E. coli* harboring the bottom portions of MVA pathway, especially from *S. pneumoniae* (pSSN12Didi) and *S. cerevisiae* (pSSC12Didi) which produced β-carotene of 141 mg/L and 140 mg/L, respectively in 48 h of culture, a more than three-fold increase compared to the β-carotene production from the empty vector pSTV28 acting as a control. However, there was no significant difference in the extent of cell growth of pSSN12Didi and pSSC12Didi when compared with the other MVA bottom portion plasmids and empty vector. Thus, the results showed that the bottom portions of MVA pathway of *S. pneumoniae* and *S. cerevisiae* were sufficient for IPP synthesis which is essential for β-carotene production. In all recombinant *E. coli* harboring the bottom MVA pathway, the β-carotene productions at 48 h of culture were approximately three-fold higher than those at 24 h of culture, whereas the cell growths were increased slightly from 24 h to 48 h of culture. This indicated that the major amount of β-carotene production took place during the stationary phase of cell growth, and the bottom mevalonate pathway could drive β-carotene production well even after cessation of cell growth.

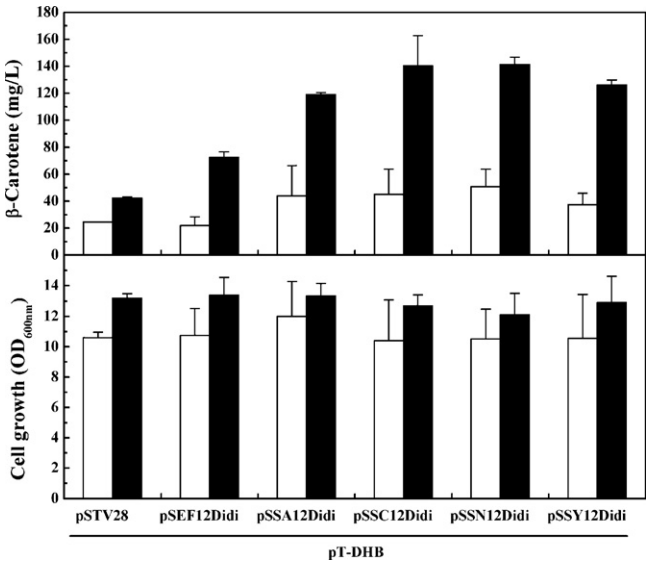

Fig. 2. β-Carotene production and cell growth of recombinant *E. coli* harboring the β-carotene plasmid of pT-DHB and the bottom MVA pathway plasmids containing *mvaK1*, *mvaK2*, and *mvaD* and *idi* genes. The culture was carried out in 2YT medium containing 0.5% (w/v) glycerol, 0.2% (w/v) arabinose, and 3.3 mM mevalonate at 29 °C for 48 h. Open bars and solid bars represent the results obtained at 24 and 48 h, respectively.

3.2. Comparison of various top portions of MVA pathway on mevalonate production

For the construction of top portion of MVA pathway, *mvaS* and *mvaA* encoding HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthase and reductase, respectively were cloned from *S. pneumoniae* and *S. aureus*, and combined with *phaA* encoding acetyl-CoA acetyl transferase of *R. eutropha*. The top portion genes of *S. pyogenes* were not studied here because *S. pyogenes* and *S. pneumoniae* belonged to the same genus and the bottom portion of *S. pyogenes*'s MVA pathway was found to be less efficient than that of *S. pneumoniae* in Fig. 2. The top portion genes of *S. cerevisiae* were also not used here because the optimization of MVA production using those genes had been well studied in *E. coli* (Pfleger et al., 2006). The *phaA* gene of *R. eutropha* was used for the construction of the top MVA operon instead of the endogenous *atoB* gene of *E. coli* while both PhaA and AtoB have the acetyl-CoA acetyltransferase activity. The *E. coli* harboring *pha* genes (*phaA*, *phaB* and *phaC*) of *R. eutropha* was proved to be highly active to produce more than 80% of PHB in dry cell weight (Resch et al., 1998). Also, PhaA rather than AtoB was found to be responsible for most of the acetyl-CoA acetyltransferase activity in *Paracoccus zeaxanthinifaciens* (Humbelin et al., 2002).

E. faecalis was also used as a source of top portion genes of the MVA pathway. It has *mvaE* encoding a bifunctional protein which possesses both activities of HMG-CoA reductase and acetyl-CoA acetyltransferase. The top portion composed of *mvaE* and *mvaS* of *E. faecalis* does not require *phaA* gene. The pTrc99A vector was inserted with the top MVA operons and named as pTSNASP, pTSAASP, and pTEFAES which were originated from *S. pneumoniae*, *S. aureus*, and *E. faecalis*, respectively. Culture was carried out using the recombinant *E. coli* harboring the plasmids of the top MVA operons in TT medium for 48 h at 30 °C (Fig. 3). In order to induce expression of the operon, 0.1 mM IPTG was initially added to the culture. Mevalonate was produced from the recombinant *E. coli* harboring the top MVA pathway whereas there was no production of meval-

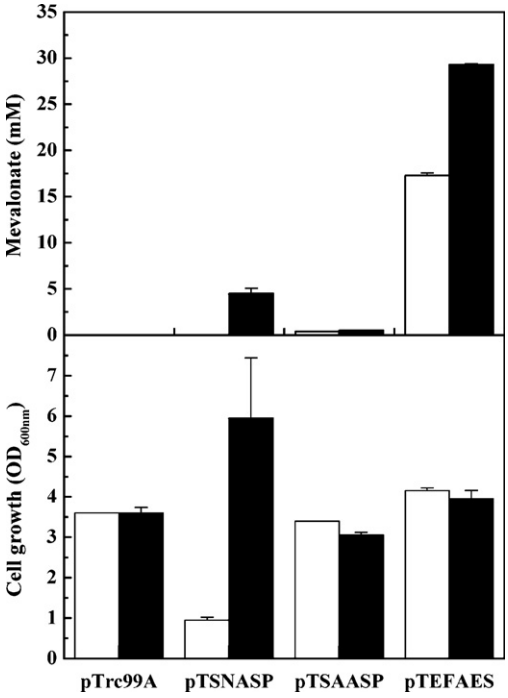

Fig. 3. Mevalonate production and cell growth of recombinant *E. coli* harboring pTrc99A-based plasmids containing top portion genes of MVA pathway. The recombinant *E. coli* was grown in TT medium containing 0.1 mM IPTG at 29 °C for 48 h. Open bars and solid bars represent the results obtained at 24 and 48 h, respectively.

onate from the *E. coli* with pTrc99A empty vector. It was therefore confirmed that the selected mevalonate pathway enzymes also function in *E. coli* (Martin et al., 2003). The highest mevalonate production of 29.3 mM was obtained with pTEFAES at 48 h of cultivation, which was significantly higher than 4.49 mM of pTSNASP and 0.49 mM of pTSAASP. There was no significant difference in cell growth among pTrc99A, pTSAASP, and pTEFAES. However, the cell growth of *E. coli* harboring pTSNASP increased abruptly after the severe growth inhibition for initial 24 h, by unknown mechanisms. The same result was obtained in repeated experiments. Thus, the results indicated that the recombinant *E. coli* harboring the top MVA portion of *E. faecalis* could produce high amount of mevalonate which had insignificant inhibition on cell growth.

3.3. Effect of host strains on β-carotene production using the whole MVA pathway

The artificial operon of the whole MVA pathway, being able to synthesize IPP and DMAPP from acetyl-CoA, was constructed by combining the bottom and top portions of MVA pathway from pSSN12Didi and pTEFAES, respectively. The supplementation of 3.3 mM mevalonate was found to be sufficient for the production of β-carotene using pSSN12Didi and 0.5% glycerol (Yoon et al., 2007). Therefore, the top MVA portion in pTEFAES (high copy number and strong P_{trc}) was moved to pSSN12Didi (low copy number and P_{lac}), resulting in the whole MVA pathway plasmid of pS-NA (Table 1). It has been observed that there is significant strain dependence in lycopene production from recombinant *E. coli* (Kim and Keasling, 2001). We therefore investigated the production of β-carotene by various *E. coli* host strains of MG1655, DH5α, S17-1, XL1-Blue and BL21 harboring the whole MVA pathways of pS-NA (Fig. 4). BL21 is a B-type strain and the other strains are of K-type. MG1655 is a wild type strain and the rest are genetically modified strains for gene cloning and expression. Culture was carried out in 2YT medium containing 0.5% glycerol and 0.2% arabinose for 48 h without exogenous supplementation of mevalonate. The DH5α strain produced the highest amount of β-carotene, 141 mg/L, which was more than three-fold higher than the least, 45 mg/L of β-carotene obtained with BL21 strain. The highest concentration of mevalonate, 2.5 mM was observed at 48 h in the culture broth of XL1-Blue producing lesser amount (98 mg/L) of β-carotene, while the next highest mevalonate concentration of 1.2 mM was measured in the culture broth of DH5α strain, the highest β-carotene producer. The results suggested that the highest mevalonate producer was not the highest β-carotene producer. Among the strains, DH5α and XL1-Blue only showed considerable lag period of about 10 h in their 48 h of growth (data not shown). However, the amount of cells obtained at 24 h of culture was almost similar for them as well as for all the other strains. Although the growth pattern of DH5α and XL1-Blue was similar, there were significant differences in the amount of mevalonate and β-carotene productions by the strains. In the other three strains of MG1655, S17-1, and BL21, the amount of mevalonate and β-carotene productions were also different as well. Therefore, it seems that there was no linear relationship between the growth of the strains, and the production of β-carotene and mevalonate.

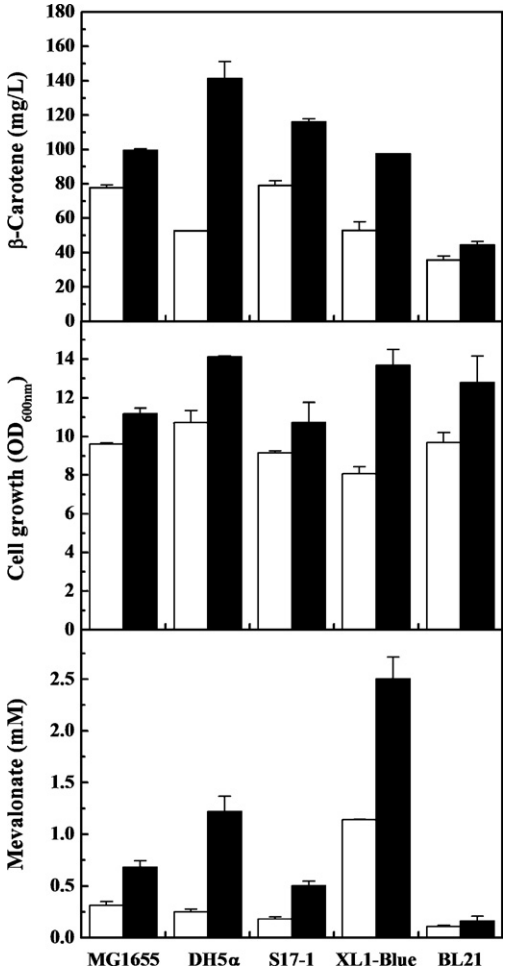

Fig. 4. β-Carotene production and cell growth of different *E. coli* strains harboring pT-DHB (β-carotene pathway) and pS-NA (whole MVA pathway). Mevalonate concentration in culture broth was measured. The culture was carried out in 2YT medium containing 0.5% (w/v) glycerol and 0.2% (w/v) arabinose at 29 °C for 48 h. Open bars and solid bars represent the results obtained at 24 and 48 h, respectively.

The β-carotene production of DH5α (pS-NA, pT-DHB) strain using the whole MVA pathway was the same as that obtained only with the bottom MVA pathway of pSSN12Didi with exogenous supplementation of 3.3 mM mevalonate in Fig. 2. It suggested that the whole MVA pathway of pS-NA could provide enough amount of

IPP and DMAPP for β-carotene production, much like the bottom MVA pathway of pSSN12Didi starting from the mevalonate supplied exogenously. The final cell growth (OD_{600nm}, 14.1) of DH5α (pS-NA, pT-DHB) was slightly higher than 12.1 (OD_{600nm}) observed in DH5α (pSSN12Didi, pT-DHB) in Fig. 2. The cells harboring pS-NA did not seem to have significant metabolic burden, although pS-NA contained three more genes of top MVA portion than pSSN12Didi.

3.4. Effect of carbon sources on β-carotene production using the whole MVA pathway

The acetyl-CoA is the starting metabolite for PHB synthesis. It has been reported that PHB synthesis by recombinant *E. coli* was enhanced when the availability of acetyl-CoA was increased (Lee and Chang, 1995). The level of intracellular acetyl-CoA concentration was reported to be dependent on the type of carbon source added in the media (Takamura and Nomura, 1988). Since acetyl-CoA is the starting metabolite not only for PHB synthesis but also for carotenoid synthesis through the MVA pathway, β-carotene production was expected to be affected by the type of carbon sources. Therefore, the production of β-carotene by the recombinant *E. coli* DH5α harboring pS-NA and pT-DHB was measured using various carbon sources such as glucose, galactose, xylose, maltose and glycerol. The recombinant *E. coli* DH5α was cultivated in 2YT medium containing 0.5% (w/v) of various carbon sources along with 0.2% (w/v) arabinose (Fig. 5). Among the various carbon sources tested, glycerol was found to be the best with regard to both β-carotene production and cell growth. Culture containing 0.5% of glycerol produced 141 mg/L of β-carotene which was almost nine-fold higher than the culture containing 0.5% glucose producing 16 mg/L of β-carotene. Both β-carotene production and cell growth in the presence of glucose or galactose were significantly lower than the other carbon sources and even lower than the control (None) which did not contain 0.5% carbon source. The pH of culture containing glucose and galactose was decreased to 4.8 at 48 h (data not shown), which may have caused growth inhibition and the subsequent low β-carotene production. In the culture containing other carbon sources, there was no decrease of pH below 5.0 (data not shown). Mevalonate concentration was also measured at 24 and 48 h of the culture. The mevalonate concentration of 1.1 mM was observed in the glycerol containing culture, which was significantly higher than 0.1 mM mevalonate of the culture containing glucose or galactose. This suggested that sufficient amount of mevalonate was synthesized in the culture containing glycerol, which contributed to the highest production of β-carotene. In the culture of different strains of Fig. 4, the β-carotene production was not correlated with the amount of mevalonate in culture broth because the efficiency of β-carotene production and mevalonate formation through the MVA pathway could be different among the strains.

3.5. Time course of β-carotene production with increase of glycerol addition

In order to investigate the increase of β-carotene production dependent on glycerol concentration, glycerol of 0.5–2.0% (w/v) was initially added to the culture which was carried out for 168 h (Fig. 6). The highest β-carotene production of 464 mg/L was obtained with culture containing 2% of glycerol at 120 h of cultivation, which was about 3.7-fold and 1.9-fold higher than those of 0.5% and 1.0% of glycerol, respectively. It was observed that the production of β-carotene decreased in culture containing 2% glycerol after 120 h of cultivation. In the case of culture containing 0.5% and 1.0% of glycerol, β-carotene production ceased after 48 and 72 h of culture, respectively. The highest amount of cell growth was 32 (OD_{600nm}), obtained with 2% glycerol at 96 h, which was significantly higher than those of the other glycerol concentrations used. This result suggested that at lower concentration of glycerol the cell growth was limited with the concomitant decrease in the production of β-carotene.

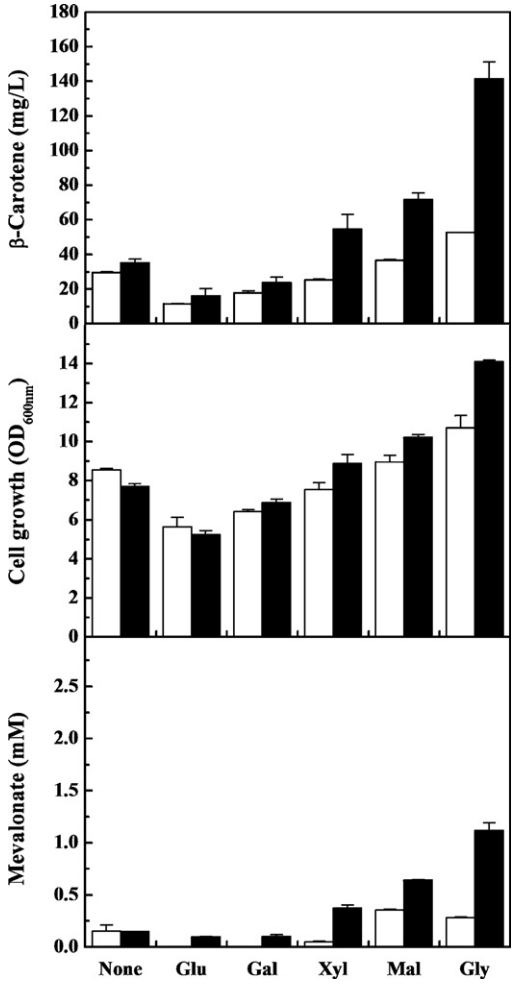

Fig. 5. Effect of carbon sources on β-carotene and mevalonate production, and cell growth of *E. coli* harboring pT-DHB and pS-NA. Carbon sources of glucose, galactose, xylose, maltose, and glycerol were added initially as 0.5% (w/v) in 2YT medium containing 0.2% (w/v) arabinose. The culture was carried out at 29 °C for 48 h. Open bars and solid bars represent the results obtained at 24 and 48 h, respectively. None represents without addition of 0.5% (w/v) carbon source.

4. Discussion

The increased synthesis of building blocks of IPP and DMAPP through metabolic engineering, along with balancing the expression of carotenogenic genes for efficient transformation of the building blocks to the desired carotenoid is a way to enhance the production of carotenoids. Using *E. coli* as a host, IPP and DMAPP supply can be increased significantly through the introduction of foreign MVA pathway into it (Martin et al., 2003; Yoon et al., 2006, 2007). Lycopene production in *E. coli* was increased several-fold using the bottom MVA pathway of *S. pneumoniae* with exogenous supplementation of mevalonate (Yoon et al., 2006). Recently, it has been shown that the highest production of β-carotene could be achieved in *E. coli* using the same bottom MVA pathway of *S. pneumoniae* with exogenous addition of mevalonate (Yoon et al., 2007).

The mass production of mevalonate of 47 g/L (317 mM in molar concentration) was achieved by fed-batch culture from recombinant *E. coli* harboring *mvaE* and *mvaS* genes of *E. faecalis* (Tabata

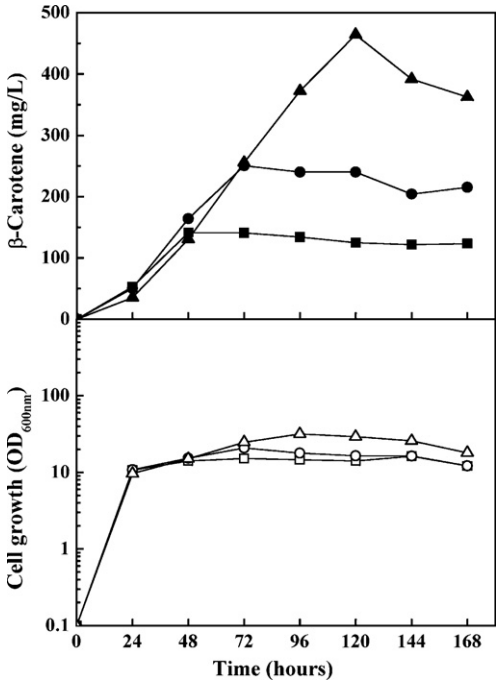

Fig. 6. Time course of β-carotene production and cell growth of recombinant *E. coli* harboring pT-DHB and pS-NA. The culture was carried out in 2YT medium containing glycerol of different concentrations and 0.2% (w/v) arabinose at 29 °C for 168 h. Squares, circles, and triangles represent the addition of 0.5%, 1.0%, and 2.0% (w/v) glycerol, respectively.

and Hashimoto, 2004). Pfleger et al. (2006) obtained mevalonate concentration of 596 μM (normalized to cell growth, OD_{600nm}) in a simple batch culture of *E. coli* harboring top portion of the mevalonate pathway from *S. cerevisiae*. The mevalonate production was obtained by controlling the expression of *HMGS* and *tHMGR* genes (corresponded to *mvaS* and *mvaA*, respectively) by using tunable intergenic region (TIGR) sequences that consist of messenger RNA (mRNA) secondary structures, RNase cleavage sites, and ribosome-binding site (RBS) sequestering sequences. In our study, the *mvaE* and *mvaS* genes of *E. faecalis* were found to be the most efficient in *E. coli* for mevalonate production among the top MVA portions used here. The 29.3 mM of mevalonate was produced from cell growth of OD_{600nm} 4.0 in simple batch flask culture. Therefore, the top MVA pathway composed of the *mvaE* and *mvaS* genes of *E. faecalis* was combined with the bottom MVA pathway of *S. pneumoniae* to generate the whole MVA pathway. The artificial combined whole MVA pathway could produce the same amount of β-carotene even without the supplementation of mevalonate, in comparison with the bottom MVA pathway of *S. pneumoniae* with mevalonate supplementation.

The mevalonate concentration in culture broth of *E. coli* harboring both the whole mevalonate pathway and the β-carotene synthesis pathway reflects the difference between production and consumption of mevalonate through the top MVA pathway and the β-carotene synthesis pathway, respectively. Since mevalonate is a small molecule, the unmetabolized intracellular mevalonate can be diffused out of cells. In Fig. 4, the mevalonate concentration in culture broth of XL1-Blue was the highest whereas the β-carotene production was not. It was suspected in XL1-Blue that the β-carotene synthesis pathway might be less efficient than the top MVA pathway. In the culture of BL21, the amounts of both β-carotene production and mevalonate were the least which could be attributed to the inefficiency of the top MVA pathway in BL21. The BL21 strain has an active glyoxylate shunt as a route for acetyl-CoA consumption (Van de Walle and Shiloach, 1998), which will cause limited use of acetyl-CoA for mevalonate synthesis through the top MVA pathway.

The successful expression of the top MVA portion along with the bottom MVA portion in *E. coli* eliminated the necessity of exogenous addition of mevalonate required in our previous study (Yoon et al., 2007). This is very important for mass production of carotenoids on an industrial scale because mevalonate is not cheap and convenient for separate addition into production medium. In the recent study of Pitera et al. (2007), it has been found that the overexpression of the top MVA portion genes of *S. cerevisiae* in *E. coli* resulted in poor cell growth and low mevalonate production. Normal cell growth and efficient mevalonate production were obtained by balancing the reaction rate of HMGS and tHMGR (corresponding to MvaS and MvaA, respectively) by reducing HMGS expression and increasing tHMGR expression which were accomplished by modulating the intergenic regions of the top MVA portion genes (Pitera et al., 2007). In our study, we observed low mevalonate production in pTSNASP and pTSAASP, and growth inhibition for initial 24 h in pTSNASP. This could be due to the imbalance of reaction rate of HMG-CoA synthase and HMG-CoA reductase encoded by *mvaS* and *mvaA*, respectively. However, the expression of top MVA portion of *E. faecalis* in *E. coli* made a significant production of mevalonate and showed no inhibition on cell growth. This suggested that there was no need to do the sophisticated balancing between reactions of HMG-CoA synthase and HMG-CoA reductase when *mvaE* and *mvaS* of *E. faecalis* were used.

Carotenoids production from metabolically engineered *E. coli* has been studied intensively. However it has been mainly focused on lycopene as a target carotenoid and MEP pathway as the precursor supply of IPP and DMAPP. There are few reports on β-carotene production from *E. coli*. Kim et al. (2006) reported the β-carotene production of 390 mg/L in concentration and 10 mg/g DCW in content by using MEP pathway, which was carried out with fed-batch fermentation using a fermentor. We reported the highest β-carotene production of 503 mg/L and 49.3 mg/g DCW by using bottom portion of MVA pathway in simple batch shake-flask culture (Yoon et al., 2007). Although our previous β-carotene production using the bottom MVA pathway in simple batch shake-flask culture was found to be much more efficient than the β-carotene production using MEP pathway by Kim et al. (2006), it required exogenous supplementation of expensive mevalonate. In the present study, we produced the similar amount of β-carotene to our previous report without exogenous supplementation of the mevalonate.

There are contradictory reports on the use of carbon source for the production of carotenoids. Lee et al. (2004) obtained an enhanced production of torulene using glycerol as a source of carbon whereas its production was significantly repressed in presence of glucose-containing medium. However, Alper et al. (2006) obtained significant amount of lycopene using glucose as a carbon source in high cell density fed-batch fermentations. In the present study using whole MVA pathway, glycerol was found to increase the production of β-carotene while glucose and galactose repressed the β-carotene production. *E. coli* was reported to have the highest specific growth rate of 0.92 h^{-1} when glucose and galactose were used as the carbon source (Lendenmann et al., 2000). Therefore, it can be suspected that the readily metabolizable carbon sources may not be a good carbon source for β-carotene production. The acetate concentration in the cultures containing glucose or galactose was around 3 g/L whereas the acetate concentration below 2 g/L was observed in the cultures containing other carbon sources (data not shown). The higher concentration of acetate, an inhibitory metabolite, may also decrease the β-carotene production. Another reason for the low β-carotene production could be the catabolite repression of both the *lac* promoter for the whole MVA pathway and the *trc* promoter for the β-carotene synthesis pathway by glucose, the readily metabolizable carbon source (Guzman et al., 1995).

Martin et al. (2001) also reported a positive effect of glycerol on a sesquiterpene synthesis in *E. coli* harboring the whole MVA pathway of *S. cerevisiae*. These suggest that in the use of MVA pathway, glycerol is better than glucose as a carbon source. Glycerol could be a cheap carbon source for large-scale production of carotenoids because there is a massive formation of glycerol as by-product in biodiesel production that is in demand due to soaring petroleum price. For every 9 kg of biodiesel produced, about 1 kg of a crude glycerol by-product is formed (Dasari et al., 2005).

Martin et al. (2003) is the first to use the foreign MVA pathway in *E. coli* for production of terpenoids. However, the MVA pathway was obtained from the eukaryote *S. cerevisiae*. Although Vadali et al. (2005) used the prokaryotic MVA pathway of *Streptomyces* sp. CL190 in *E. coli* for the production of lycopene, the prokaryotic MVA pathway was not optimized in *E. coli*. We optimized the prokaryotic whole MVA pathway in *E. coli* by combination of both top and bottom portions of the MVA pathway from different microorganisms. The optimized whole MVA pathway of prokaryotic origin was successfully applied for the production of β-carotene. Since IPP and DMAPP are the universal precursors to all terpenoids, *E. coli* harboring the optimized MVA pathway can serve as the platform host for the production of any terpenoid for which the biosynthetic genes are available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2009.01.008.

References

Alper, H., Miyaoku, K., Stephanopoulos, G., 2006. Characterization of lycopene-overproducing *E. coli* strains in high cell density fermentations. Appl. Microbiol. Biotechnol. 72, 968–974.

An, G.H., Choi, E.S., 2003. Preparation of the red yeast, *Xanthophyllomyces dendrorhous*, as feed additive with increased availability of astaxanthin. Biotechnol. Lett. 25, 767–771.

Britton, G., Liaaen-Jensen, S., Pfander, H., 1998. Carotenoids. Birkhauser Verlag, Basel, Switzerland.

Cheng, Q., 2006. Structural diversity and functional novelty of new carotenoid biosynthesis genes. J. Ind. Microbiol. Biotechnol. 33, 552–559.

Das, A., Yoon, S.H., Lee, S.H., Kim, J.Y., Oh, D.K., Kim, S.W., 2007. An update on microbial carotenoid production: application of recent metabolic engineering tools. Appl. Microbiol. Biotechnol. 77, 505–512.

Dasari, M.A., Kiatsimkul, P.P., Sutterlin, W.R., Suppes, G.J., 2005. Low-pressure hydrogenolysis of glycerol to propylene glycol. Appl. Catal. A: Gen. 281, 225–231.

Dufosse, L., Galaup, P., Yaron, A., Arad, S.M., Blanc, P., Murthy, K.N.C., Ravishankar, G.A., 2005. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? Trends Food Sci. Tech. 16, 389–406.

Farmer, W.R., Liao, J.C., 2001. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. Biotechnol. Prog. 17, 57–61.

Guzman, L.M., Belin, D., Carson, M.J., Beckwith, J., 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. 177, 4121–4130.

Humbelin, M., Thomas, A., Lin, J., Li, J., Jore, J., Berry, A., 2002. Genetics of isoprenoid biosynthesis in *Paracoccus zeaxanthinifaciens*. Gene 297, 129–139.

Jacobson, G.K., Jolly, S.O., Sedmark, J.J., Skatrud, T.J., Wasileski, J.M., 2000. Astaxanthin over-producing strains of *Phaffia rhodozyma*. Method for their cultivation and their use in animal feeds. US Patent 6015684.

Kajiwara, S., Fraser, P.D., Kondo, K., Misawa, N., 1997. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. Biochem. J. 324, 421–426.

Kim, C.M., Goldstein, J.L., Brown, M.S., 1992. cDNA cloning of MEV, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. J. Biol. Chem. 267, 23113–23121.

Kim, S.W., Kim, J.B., Jung, W.H., Kim, J.H., Jung, J.K., 2006. Over-production of β-carotene from metabolically engineered *Escherichia coli*. Biotechnol. Lett. 28, 897–904.

Kim, S.W., Keasling, J.D., 2001. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. Biotechnol. Bioeng. 72, 408–415.

Lange, B.M., Rujan, T., Martin, W., Croteau, R., 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc. Natl. Acad. Sci. USA 97, 13172–13177.

Lee, P.C., Mijts, B.N., Schmidt-Dannert, C., 2004. Investigation of factors influencing production of the monocyclic carotenoid torulene in metabolically engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. 65, 538–546.

Lee, P.C., Schmidt-Dannert, C., 2002. Metabolic engineering towards biotechnological production of carotenoids in microorganisms. Appl. Microbiol. Biotechnol. 60, 1–11.

Lee, S.Y., Chang, H.N., 1995. Production of poly(3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. Can. J. Microbiol. 41 (Suppl. 1), 207–215.

Lendenmann, U., Snozzi, M., Egli, T., 2000. Growth kinetics of *Escherichia coli* with galactose and several other sugars in carbon-limited chemostat culture. Can. J. Microbiol. 46, 72–80.

Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat. Biotechnol. 21, 796–802.

Martin, V.J., Yoshikuni, Y., Keasling, J.D., 2001. The in vivo synthesis of plant sesquiterpenes by *Escherichia coli*. Biotechnol. Bioeng. 75, 497–503.

Mehta, B.J., Obraztsova, I.N., Cerda-Olmedo, E., 2003. Mutants and intersexual heterokaryons of *Blakeslea trispora* for production of β-carotene and lycopene. Appl. Environ. Microbiol. 69, 4043–4048.

Misawa, N., Shimada, H., 1997. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J. Biotechnol. 59, 169–181.

Olaizola, M., 2000. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. J. Appl. Phycol. 12, 499–506.

Otsuka, K., Itoh, M., Yoshizawa, K., 1973. On the determination of Mevalonic acid by thin-layer and gas chromatography. Agr. Biol. Chem. 37, 1013–1017.

Pfleger, B.F., Pitera, D.J., Smolke, C.D., Keasling, J.D., 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat. Biotechnol. 24, 1027–1032.

Pitera, D.J., Paddon, C.J., Newman, J.D., Keasling, J.D., 2007. Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metab. Eng. 9, 193–207.

Resch, S., Gruber, K., Wanner, G., Slater, S., Dennis, D., Lubitz, W., 1998. Aqueous release and purification of poly(β-hydroxybutyrate) from *Escherichia coli*. J. Biotechnol. 65, 173–182.

Rodriguez-Saiz, M., Sanchez-Porro, C., De La Fuente, J.L., Mellado, E., Barredo, J.L., 2007. Engineering the halophilic bacterium *Halomonas elongata* to produce β-carotene. Appl. Microbiol. Biotechnol. 77, 637–643.

Rohmer, M., 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat. Prod. Rep. 16, 565–574.

Ruther, A., Misawa, N., Boger, P., Sandmann, G., 1997. Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmids. Appl. Microbiol. Biotechnol. 48, 162–167.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning, 3rd ed. Cold spring Harbor Laboratory Press, New York.

Sandmann, G., 2002. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. Chembiochem 3, 629–635.

Tabata, K., Hashimoto, S., 2004. Production of mevalonate by a metabolically-engineered *Escherichia coli*. Biotechnol. Lett. 26, 1487–1491.

Takamura, Y., Nomura, G., 1988. Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. J. Gen. Microbiol. 134, 2249–2253.

Vadali, R.V., Fu, Y., Bennett, G.N., San, K.Y., 2005. Enhanced lycopene productivity by manipulation of carbon flow to isopentenyl diphosphate in *Escherichia coli*. Biotechnol. Prog. 21, 1558–1561.

Van de Walle, M., Shiloach, J., 1998. Proposed mechanism of acetate accumulation in two recombinant *Escherichia coli* strains during high density fermentation. Biotechnol. Bioeng. 57, 71–78.

Verwaal, R., Wang, J., Meijnen, J.P., Visser, H., Sandmann, G., van den Berg, J.A., van Ooyen, A.J., 2007. High-level production of β-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl. Environ. Microbiol. 73, 4342–4350.

Wilding, E.I., Brown, J.R., Bryant, A.P., Chalker, A.F., Holmes, D.J., Ingraham, K.A., Iordanescu, S., So, C.Y., Rosenberg, M., Gwynn, M.N., 2000. Identification, evolution, and essentiality of the mevalonate pathway for isopentenyl diphosphate biosynthesis in gram-positive cocci. J. Bacteriol. 182, 4319–4327.

Yoon, S.H., Lee, Y.M., Kim, J.E., Lee, S.H., Lee, J.H., Kim, J.Y., Jung, K.H., Shin, Y.C., Keasling, J.D., Kim, S.W., 2006. Enhanced lycopene production in *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate. Biotechnol. Bioeng. 94, 1025–1032.

Yoon, S.H., Park, H.M., Kim, J.E., Lee, S.H., Choi, M.S., Kim, J.Y., Oh, D.K., Keasling, J.D., Kim, S.W., 2007. Increased β-carotene production in recombinant *Escherichia coli* harboring an engineered isoprenoid precursor pathway with mevalonate addition. Biotechnol. Prog. 23, 599–605.

Zahiri, H.S., Yoon, S.H., Keasling, J.D., Lee, S.H., Won Kim, S., Yoon, S.C., Shin, Y.C., 2006. Coenzyme Q10 production in recombinant *Escherichia coli* strains engineered with a heterologous decaprenyl diphosphate synthase gene and foreign mevalonate pathway. Metab. Eng. 8, 406–416.